# Exploring the Oligomerization of $Na_V1.5$ and Its Implication for the Dominant-Negative Effect

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## Abstract

Clusters of the α-subunit of voltage-gated sodium ($Na_v$) channels have been observed in various tissues and are recognized as key regulators of cellular excitability and action potential propagation. In cardiomyocytes, the most abundant $Na_v$ α-subunit, $Na_v1.5$, is expressed at specialized membrane microdomains within the intercalated disc and lateral membrane. While $Na_v1.5$ remodeling within these microdomains could cause abnormal cardiac phenotypes, the molecular mechanisms underlying single-molecule redistribution and biophysical cooperativity of $Na_v1.5$ remain not fully understood.

This review summarizes the current knowledge on the oligomerization of $Na_v1.5$. In particular, direct α-α-subunit interactions and oligomerization through intermediary proteins such as $Na_v$β-subunits and 14-3-3 proteins are discussed. The possible implication of $Na_v1.5$ oligomerization in the coupled gating in *cis* and *trans* conformations as well as in the dominant-negative effect is reviewed.

## Introduction

Cardiac electrical activity serves to excite the contractile myocardium, preceding each heartbeat. Generated by the pacemaker cells, electrical signals travel through the conduction pathway to the atrial and ventricular cardiomyocytes. There, the initial phase of the action potential is mainly provided by the α-subunit of the voltage-gated sodium channel $Na_v1.5$ (encoded by the gene *SCN5A*). Due to the fast $Na^+$ influx, the cardiomyocyte membrane depolarizes. This gives rise to the action potential, which further propagates through the entire contractile myocardium.

Inward sodium current ($I_{Na}$) is finely tuned by the biophysical properties, expression level, and localization of $Na_v1.5$. These properties are not only regulated by genetic alterations of *SCN5A*, but also by protein partners of $Na_v1.5$ that form functional macromolecular complexes in cardiomyocytes together with the α-subunit.[1,2] Native clusters of $Na_v1.5$ macromolecular complexes, mainly found at the intercalated discs and lateral membrane, were shown to differ in their composition.[1,3–7] It suggests that the protein partners play an important role in the targeting of $Na_v1.5$ to the specific sarcolemmal areas and hence regulate its clusterization.[8]

It is highly debated whether, within the native clusters, $Na_v1.5$ channels are in direct contact with each other or are located in close proximity as individual molecules. Recent studies provided evidence on $Na_v1.5$ oligomerization in heterologous expression systems, raising the possibility of direct interaction between α-subunits.[9,10] Many ion channels, including $Na_v1.5$, may open in a cooperative manner within clusters.[10,11] Thus, the specific distribution of $Na_v1.5$ within different clusters could change the cooperative output for cardiomyocyte depolarization leading to abnormal characteristics and propagation of the action potential.[12] For instance, some loss-of-function mutants exerting dominant-negative effect were shown to impact biosynthesis, degradation, cell surface addressment, and functional output of the wild-type $Na_v1.5$ presumably due to oligomerization between the channels.[13,14] Therefore, identification of the sites or domains of $Na_v1.5$ responsible for its oligomerization could provide new insights into the molecular mechanisms underlying cardiac arrhythmias.

This review discusses the current literature describing several proposed mechanisms of $Na_v1.5$ oligomerization: 1) direct *cis*-interactions of α-subunits; 2) indirect *cis*-

interactions mediated by 14-3-3 proteins; and 3) indirect *cis*- and *trans*-interactions mediated by auxiliary subunits.

## *Cis*-oligomerization

$Na_v1.5$ consists of four homologous domains I-IV, each comprising six transmembrane segments S1-S6, of which S4 is positively charged, and the pore-forming region is located between S5-S6 (Fig. 1). The N- and C-termini, as well as linkers between the domains, are intracellular. Fast inactivation of the channel is mediated by the Ile-Phe-Met motif within the linker between domains III and IV (L3) and the C-terminal region.[15] Association of $Na_v1.5$ channels within the same plasma membrane (i.e., *cis*-oligomerization, as opposed to *trans*-oligomerization across two membranes) is proposed to result either from direct α-α-subunit interactions or "bridging" provided by auxiliary proteins.[10,16]

### *Direct α-α interaction sites*

In many studies, co-immunoprecipitation analyses between differently tagged full-length and truncated $Na_v1.5$ proteins revealed the channels interact in heterologous expression systems.[9,13,16–18] Analysis of GFP-$Na_v1.5$ photobleaching steps indicated that the major proportion of α-subunits were monomers (~50%) and dimers (~50%), with the small minority (<5%) being represented by the higher order of oligomers.[10] In addition, $Na_v1.5$-specific dimers (~460 kDa) were detected in the protein lysates by immunoblotting technique after performing native acrylamide gel electrophoresis and disuccinimidyl suberate cross-linking experiments.[10,13]

Further confirming the dimerization of heterologous $Na_v1.5$ occurs in living cells, our group revealed with protein complementation assays that N-N- and C-C-termini arrangement of $Na_v1.5$ α-subunits was more common than N-C- and C-N-termini arrangement.[17] These results revealed a preference of α-subunits orientation within the dimer.[17] In line with these results, interacting C-termini were reported for the asymmetric *cis*-dimerization of $Na_v1.5$ when using Glu1773-Arg1929 peptides (Fig. 1-2).[19] According to this model,[19] the C-terminal portion of one $Na_v1.5$ protein binds to the EF-hand-like motif located at the C-terminus of another $Na_v1.5$. This interaction was regulated by calmodulin binding at the adjacent IQ motif (Fig. 2).[19] Previously, in the somewhat similar manner calmodulin was reported to mediate dimerization of the voltage-gated calcium channels.[20] Specifically, crystal structure of calcium/calmodulin

binding at pre-IQ and IQ motifs at C-termini of $Ca_v1.2$, was shown to bridge two α1-subunits providing for their functional coupling.[20,21]

However, another independent study demonstrated successful co-immunoprecipitation between α-subunits lacking the C-terminal region, $Na_v1.5$-Arg1860Gfs*12, and their preserved interaction with WT $Na_v1.5$. [22] Thus, suggesting that C-termini of $Na_v1.5$ might be dispensable for its oligomerization. Instead, few years later, the same group reported that $Na_v1.5$ dimerization occurred through interaction via the intracellular loop located between domain I and domain II, hereafter named linker 1 (L1) (Fig. 1-2).[10] In this study, the authors proposed that the direct α-α-subunit interaction site is situated at Arg493-Arg517 in L1 (Fig. 1-2).[10] Interestingly, however, in adult rat and mouse ventricular cardiomyocytes, the native clusters of $Na_v1.5$ were successfully immunolabeled against the human analogue of Asp492-Thr512 epitope.[3,6] In this case, if $Na_v1.5$ channels interacted through the Arg493-Arg517 binding site, the Asp492-Thr512 epitope would become unavailable for immunolabeling. Thus, it is unclear whether a native proportion of $Na_v1.5$ with an unoccupied Arg493-Arg517 site is mainly present in non-diseased cardiomyocytes or whether this suggested α-α-subunit interaction is species specific.

Homo-oligomerization analysis of the single membrane spanning peptides in the living bacterial cells revealed that the transmembrane segments of the human $Na_v1.5$ are prone to homo-dimerize.[23] In particular, *cis* homo-dimerization was observed for S1 of domain I (using peptide Ala123-Ala149) and domain IV (using peptide Ile1521-Glu1548) but not for S1 of domain II and domain III (Fig. 1).[23] Additionally, other single transmembrane segments with cytosolic N-termini, S3 and S5, also demonstrated the lack of ability to homo-dimerize.[23] Since no previously known motifs characteristic for transmembrane dimerization were identified within S1 of domain I and domain IV, random mutagenesis was performed on these regions.[23] It revealed the single amino acid substitutions that led to the decreased homo-dimerization of these segments without affecting their membrane insertion: Met135Lys, Leu136His, Met138Lys, Cys139Pro – for S1 of domain I; and Asp1531Val, Val1532Gly, Thr1533Pro, Ile1534Thr, Phe1536Ser, Glu1548Lys - for S1 of domain IV.[23] Since S1 of domain I and domain IV are facing external protein border, one could raise the plausibility of $Na_v1.5$ *cis*-dimerization due to the transmembrane interactions (Fig. 2).[24]

Nevertheless, further assessment of homo-dimerization of these segments when expressed in mammalian cells as well as within the full-length $Na_v1.5$ is required.

*Intermediary proteins*

Intermediary proteins that could facilitate α-α-subunit interactions should possess more than one binding site for $Na_v1.5$ or form multimer complexes themselves. To date, the protein partners proposed to mediate $Na_v1.5$ oligomerization are sodium channel β-subunits ($Na_v\beta$) and 14-3-3 proteins, both of which are known to homo- and hetero-oligomerize within the same protein family.

$Na_v\beta$-subunits

$Na_v\beta1$, $Na_v\beta1B$, $Na_v\beta2$, $Na_v\beta3$, and $Na_v\beta4$-subunits are encoded by *SCN1B*, *SCN2B*, *SCN3B,* and *SCN4B* genes, respectively. Their N-terminal is extracellular and represents an immunoglobulin (Ig)-like domain. $Na_v\beta1B$ is an alternatively spliced variant of $Na_v\beta1$ with retained intron 3, no transmembrane region and extracellular C-tail. Unlike $Na_v\beta1B$, $Na_v\beta1$-4 contain an α-helical transmembrane domain with an intracellular C-terminal region.

The highest sequence similarity within $Na_v\beta$-subunits is shared between the pairs of $Na_v\beta1/Na_v\beta3$ and $Na_v\beta2/Na_v\beta4$. To date, the macromolecular complex composition of voltage-gated sodium channels has been shown to consist of one α-subunit with two distinct $Na_v\beta$-subunits, suggesting that the complex is restricted to a heterotrimeric structure.[25–27] Notably, most of the interactions between α-subunit and $Na_v\beta$-subunits occurred at the extracellular domains. Furthermore, homo- and heterophilic interactions within different $Na_v\beta$-subunits and with members of other protein families were demonstrated.[28–32]

Although heterologous $Na_v1.5$ proteins were shown to oligomerize even in the absence of $Na_v\beta$-subunits, $Na_v\beta1$ enhanced the interaction between α-subunits [16]. In analogy with the resolved $Na_v1.4$-$Na_v\beta1$ complex, Maroni *et al*., predicted the interaction sites for $Na_v\beta1/Na_v\beta3$ on $Na_v1.5$.[33] These $Na_v1.5$ sites could be important to mediate its oligomerization through binding with $Na_v\beta1/Na_v\beta3$ (Fig. 1-2). However, the recently resolved cryo-EM-based structure of $Na_v1.5$ demonstrated a unique glycosylation site at the extracellular loop of domain I that could impede the binding of $Na_v\beta1$.[34,35] Moreover, although $Na_v\beta3$ can form dimeric and trimeric structures, it did not affect the oligomerization of $Na_v1.5$.[30,36]

$Na_v\beta4$ was shown to form *cis*-homophilic dimers through interactions at its extracellular N-terminus: an intermolecular disulfide bridge at Cys58 and hydrogen bonds at Ser30-Val35.[29] At the same time, Cys58 for $Na_v\beta4$ and Cys55 for $Na_v\beta2$ were shown to form disulfide bonds with α-subunits of voltage-gated sodium channels at the extracellular loop of domain II: Cys919 in $Na_v1.1$, Cys910 in $Na_v1.2$, Cys911 in $Na_v1.3$, Cys904 in $Na_v1.6$, Cys895 in $Na_v1.7$.[25–27,37–39] However, the analogous cysteine is absent in $Na_v1.5$ and is replaced by Leu869.[34,40,41] Therefore, if $Na_v\beta2$ and $Na_v\beta4$ would still interact with $Na_v1.5$ through other yet unidentified sites, such as transmembrane and intracellular regions, it is plausible that $Na_v\beta2$ and $Na_v\beta4$ could indeed mediate oligomerization of $Na_v1.5$ due to the availability of Cys55 and Cys58, respectively, to form *cis*-homophilic disulfide bonds. A similar mechanism could have been proposed for $Na_v\beta1$/$Na_v\beta3$; however, in both cases, this model remains highly hypothetical.[42]

14-3-3 proteins

14-3-3 are ubiquitous phosphoserine and phosphothreonine binding proteins. The seven isoforms β, γ, ε, σ, η, θ, and ζ were shown to homo- and hetero-dimerize.[43–46] 14-3-3 dimers were reported to modulate their targets' activity, subcellular localization and protein-protein interactions.[47] In human muscle tissues, 14-3-3 proteins (except 14-3-3σ, which was not found in myocytes) were proposed to regulate excitation-contraction coupling by binding various membrane transporters and ion channels.[48]

In adult rabbit cardiomyocytes, clusters of 14-3-3η colocalized with clusters of $Na_v1.5$ at the intercalated discs.[49] Furthermore, 14-3-3η co-immunoprecipitated with $Na_v1.5$ in heterologous expression system, while 14-3-3 proteins were pulled down with L1 of $Na_v1.5$ and co-immunoprecipitated with pan-$Na_v$ antibody from mouse heart lysates.[17,49,50] Using yeast two-hybrid assay, Allouis *et al*., revealed the association between L1 of $Na_v1.5$ with 14-3-3η and to a lower extent with 14-3-3θ and 14-3-3ζ.[49] According to their findings, the specific interaction site between 14-3-3η and L1 of $Na_v1.5$ was restricted to Glu417-Ala467, while His445-Glu488, Pro468-Asn507, Arg504-Gln573, Ser560-Leu633 and Glu610-Lys711 fragments of $Na_v1.5$ did not associate with 14-3-3η (Fig. 1-2).[49] However, a different study reported that $Na_v1.5$ interacts with 14-3-3 at Ser460 and Arg517-Glu555 of L1 (Fig. 1-2).[10] Nevertheless, neither overexpression of 14-3-3η nor inhibition of endogenous 14-3-3/ligand interactions did affect $I_{Na}$ density in heterologous expression system.[17,49,51]

In summary, 14-3-3 proteins might interact with $Na_v1.5$ at one or more sites but were not yet demonstrated to directly mediate the oligomerization of $Na_v1.5$.

*Coupled gating of $Na_v1.5$ in cis conformation*

Although $Na_v$ α-subunits were thought to function as monomers, some studies suggested coupled gating between *cis*-interacting/located in proximity $Na_v1.5$ channels.[10,13] In line with these findings, Förster resonance energy transfer studies demonstrated that not only α-α-subunits interaction occurred before the protein trafficking to the plasma membrane, but that $Na_v1.5$ oligomers were still preserved at the cell surface.[10,52]

Clatot *et al*. were the first to observe that $Na_v1.5$ could gate as dimers at the single-channel level.[10] Moreover, the authors reported the importance of 14-3-3 proteins in the functional coupling of $Na_v1.5$. In particular, the inhibition of endogenous 14-3-3/ligand interactions with difopein and mutation of putative 14-3-3 interaction site in L1 at Ser460Ala led to the increase of single-level non-simultaneous openings of $Na_v1.5$ channels.[10] Similarly, the same group showed that even though $Na_v1.5$ WT dimerized with the loss-of-function $Na_v1.5$-Leu325Arg, coupled gating between these α-subunits was nearly negligible.[13]

Overall, $Na_v1.5$ was proposed to gate cooperatively in a 14-3-3-dependent manner. Further studies with single-channel recordings should elucidate the details of this mechanism, and especially reveal the debated effects of single-channel coupled gating on macroscopic $I_{Na}$.[10,53]

### ***Trans*-oligomerization**

Action potentials are transmitted to neighbouring cardiomyocytes through gap junctions at the intercalated discs of cardiomyocytes. $Na_v1.5$ clusters around the gap junctions have been proposed to form *trans*-oligomers through binding *trans*-interacting cell adhesion molecules (CAM) such as $Na_v$β-subunits and cadherins (Fig. 3). [29,42,54]

$Na_v$β-subunits

Various *trans*-homophilic and *trans*-heterophilic interactions between $Na_v$β-subunits have been described.[28,55] $Na_v$β1, $Na_v$β2, and $Na_v$β4 were found to be capable of forming *trans*-homophilic associations.[29,55–58] The putative binding site mediating

*trans*-homophilic interactions between $Na_v\beta1$-subunits was proposed to lay within Phe67–Phe86.[54] Initially, $Na_v\beta3$ was reported to lack *trans*-homophilic adhesive properties.[56] However, a subsequent study demonstrated the ability of $Na_v\beta3$ to self-associate presumably via the disulfide bond at the extracellular N-terminals between Cys2 of one subunit and Cys24 of another.[58]

Furthermore, $Na_v\beta1$ was reported to *trans*-associate with $Na_v\beta2$, $Na_v\beta3$, and $Na_v\beta4$.[28,58,59] Interestingly, the amount of secreted free extracellular domain of $Na_v\beta3$ did not change upon co-expression with $Na_v\beta2$, but was significantly retained in the presence of $Na_v\beta1$ and $Na_v\beta3$.[58] These data suggest that, despite sharing 57% sequence homology, $Na_v\beta3$ did not *trans*-associate with $Na_v\beta2$, unlike $Na_v\beta1$.[58] The alternatively spliced $Na_v\beta1$ isoform $Na_v\beta1B$ is however secreted extracellularly as it lacks a transmembrane segment.[60] $Na_v\beta1B$ could thus compete for extracellular targets of $Na_v\beta1$ and impede its binding with trans-associating CAMs. Therefore, the presence of $Na_v\beta1B$ could regulate $Na_v\beta1$-mediated *trans*-oligomerization of $Na_v1.5$.

In cardiomyocytes, $Na_v\beta1$ with phosphorylated Tyr181 at the intracellular C-terminus was shown to localize at the intercalated discs, while non-phosphorylated $Na_v\beta1$ was specific to the transverse tubules.[31] Similarly, *trans*-association between $Na_v\beta1$ and $Na_v\beta2$ was shown to be determined by the intracellular C-terminal tail of $Na_v\beta2$ at Thr169-Ala181 residues, which contain a putative phosphorylation site.[28] $Na_v\beta3$ homophilic interactions were not observed in protein extracts from cells that were separately transfected with different constructs, lysed, and then mixed.[58] The authors concluded that $Na_v\beta3$ homophilic interactions required cellular integrity, suggesting the involvement of the phosphorylation site at the intracellular C-terminus of $Na_v\beta3$.[58] Overall, the cytosolic C-terminal phosphorylation state could play an important role in controlling *trans*-interactions of $Na_v\beta$-subunits and should be addressed further.

$Na_v\beta$-subunits could also mediate *trans*-oligomerization of $Na_v1.5$ through their *cis*- and *trans*-heterophilic interactions with other CAMs expressed in cardiomyocytes (Fig. 3). $Na_v\beta1$ was shown to associate with contactin, in *cis* (through its extracellular domain) but not in *trans* configuration with neurofascins, *N*-cadherin, and immunoglobulin (Ig) superfamily CAM (Ig-CAM).[28,31,32] $Na_v\beta2$ was shown to associate in *trans* configuration with laminin.[61] $Na_v\beta3$ was shown to associate in *trans* with neurofascins.[32]

Interestingly, the presence of $Na_v1.5$ was demonstrated to directly correlate with cell-to-cell adhesion: knock-down of endogenous *SCN5A* in mouse cardiomyocyte cell line HL-1 decreased the intercellular adhesion, whereas overexpression of *SCN5A* potentiated cell-to-cell adhesion of human embryonic kidney cell line HEK293.[7] The mechanism of the observed *SCN5A*-dependent adhesion was not elucidated. The interactions of $Na_v1.5$ with endogenously expressed CAMs may however promote the formation of *trans*-adhesive macromolecular complexes. In line with this hypothesis, some $Na_v\beta$-subunits are endogenously present in HL-1 and HEK293 cell lines.[62–66]

*Coupled gating of $Na_v1.5$ in trans conformation or ephaptic coupling*

The intercellular distance between two interacting cardiomyocytes at the intercalated discs varies greatly: 0-4 nm at the gap junction plaque; 5-15 nm at the region surrounding gap junctions named perinexus; and 60-65 nm at the area of mechanical adhesion junctions, that is, desmosomes and fascia adherens junctions.[67–70] The perinexus extends up to 200 nm from the gap junction edge and is rich in connexin 43 and $Na_v1.5$ (Fig. 3).[67,69–72] Theoretical studies indicated that a membrane spacing of less than 30 nm could enable $I_{Na}$ and extracellular potential in the intercalated disc to contribute to cardiac conduction when gap junction conductance is reduced.[73–75] This ability of $Na_v$ channels to communicate in *trans* conformation was named ephaptic coupling. Since the narrow spacing (<30 nm) between opposing $Na_v1.5$ channels is a prerequisite for ephaptic coupling, it would likely occur in the perinexus.[42,54,74,76] In contrast, the $Na_v1.5$ channels colocalizing with N-cadherin, named the plicate pool, would be further apart (>30 nm), which does not suffice for ephaptic coupling.[7,54,72]

Importantly, enlarged perinexal width was shown to correlate with slowed conduction and cardiac arrhythmias in animal models as well as in human patients.[54,72,77–80] In particular, inhibition of *trans* cell adhesion with a specific peptide mimicking the extracellular binding domain of $Na_v\beta1$ at Phe67–Phe86 (FVKILRYENEVLQLEEDERF, named as βadp1) enlarged $Na_v\beta1$-enriched perinexal nanodomains and induced proarrhythmic conduction slowing.[54] Interestingly, βadp1 did not affect the whole-cell $I_{Na}$ density and action potentials of isolated cardiomyocytes, although it significantly reduced junctional $I_{Na}$ density of contacting myocytes.[54] Overall, these data suggested the importance of $Na_v\beta1$ in facilitating ephaptic conduction, possibly due to its *trans*-adhesive properties.[54] Potentially, $Na_v\beta1B$ could also exert similar effects, but this has not yet been shown experimentally.[81]

## $Na_v1.5$ oligomerization as a prerequisite for the dominant-negative effect

Brugada syndrome (BrS) is a life-threatening cardiac arrhythmia that can be difficult to diagnose due to the relative lack of symptoms in the affected population [82]. Fever, pharmacological agents, age, and biological sex are among the risk factors for the disease manifestation.[82,83] Up to 30% of all mutations found in BrS are attributed to variants of the *SCN5A* gene, and are expressed in a heterozygous manner.[82] It means that one allele encodes the mutated $Na_v1.5$, while the other allele encodes for the "healthy" wild-type (WT) $Na_v1.5$. Most of the mutants found in BrS patients are loss-of-function.[82,84]

Interestingly, when the heterozygous state was initially mimicked *in vitro* by co-expressing 50% *SCN5A* WT and 50% of a BrS mutant, $I_{Na}$ was significantly lower when compared to cells co-expressing 50% WT and 50% of an empty vector.[85] In other words, the presence of the BrS mutant inhibited $I_{Na}$ conducted through the WT $Na_v1.5$ channel (Fig. 4). This phenomenon is referred to as the dominant-negative effect (DNE). One of the proposed hypotheses explaining the underlying mechanism is the $Na_v1.5$ oligomerization.[10,13] For example, the BrS mutant could oligomerize with WT channels, affecting protein turnover, channel trafficking to the cell surface, and functional coupling (Fig. 4).[9,13] Interestingly, loss-of-function *SCN5A* mutants exerting DNE were linked with elevated BrS risk.[84] In line with this analysis, *SCN5A*-mediated DNE was demonstrated *in vivo* in mouse hearts that were transduced with the DNE-potent mutant.[86]

Transcomplementation, the process in which truncated proteins were able to rescue the loss-of-function mutants by forming bimolecular complex, has been reported for cystic fibrosis transmembrane conductance regulator,[87] and analogous observations have been published for $I_{Na}$. In particular, whole-cell $I_{Na}$ was partially recovered once one loss-of-function $Na_v1.5$ variant was co-expressed with another interacting loss-of-function mutant.[9,14] Furthermore, the sole expression of Leu567Gln variant led to $I_{Na}$ density comparable with WT, while their heterologous co-expression resulted in co-immunoprecipitation and DNE.[88] The presence of the single-nucleotide polymorphism at His558Arg alleviated DNE mediated by the loss-of-function mutant with the interacting $Na_v1.5$ variant.[18,52] Coupled and uncoupled cooperative gating was also reported for the interacting α-subunits.[10,13]

Thus, on the one hand, it is tempting to speculate that disrupting $Na_v1.5$ oligomers could represent a potential therapeutic strategy to rescue the WT properties of the channel for patients carrying heterozygous *SCN5A* mutations and suffering from cardiac arrhythmias like BrS (Fig. 4). On the other hand, the current body of experimental evidence does not yet suffice to reliably link biochemical interaction between $Na_v1.5$ channels with the causation of DNE. For example, the truncated mutant Arg535X did not exhibit DNE when co-expressed with the WT channel,[85] but still co-immunoprecipitated with full-length $Na_v1.5$.[10] To our current knowledge, non-oligomerizing variants of *SCN5A* have not been described thus far. Also, it was not yet shown whether the disrupted biochemical interaction within WT α-subunits affects the macroscopic $I_{Na}$ and the properties of the single-channel $I_{Na}$.

Alternatively, oligomerization of $Na_v1.5$ might represent the underlying basis for an additional mechanism leading to DNE, but not the cause of it *per se*. To confirm this hypothesis (or to disprove it), one would have to demonstrate that the disrupted interaction between the DNE-potent variant and WT channel leads to the rescue of $I_{Na}$ (or not) (Fig. 4). Intriguingly, only half of the heterologously expressed WT $Na_v1.5$ were shown to be dimers, while the other half were monomers.[10] Thus, it would be interesting to elucidate whether certain benefits to retaining $Na_v1.5$ monomers exist, and to see whether these $Na_v1.5$ monomers could be somehow stimulated to dimerize.

**Current Limitations and Future Perspectives**

One of the biggest limitations of the data available at this point regarding the oligomerization of $Na_v1.5$ - it originates from protein overexpression experiments. Growing number of evidence indicate that transiently and constitutively overexpressed proteins may carry a multitude of artifacts including exhaustion of cellular resources with subsequent protein misfolding, mislocalization and aggregation. This limitation could be partially alleviated by using gene constructs with low strength promoters, leading to the decreased quantity of overexpressed protein. However, in turn it would signify the requirement of a larger amount of sample for analysis.

Another critical aspect is that currently all oligomers of $Na_v1.5$, both full-length and truncated regions, were obtained in heterologous expression systems. Non-native environment could also affect intrinsic protein properties and lead to the artifactual

aggregation. Therefore, $Na_v1.5$ oligomerization should be re-examined in native cardiomyocytes.

Furthermore, *in vivo* relevance of $Na_v1.5$ oligomerization should be extensively addressed, for example, in animal models and in differentiated cardiomyocytes from human induced pluripotent stem cells. However, these models are more challenging in handling, require ethical approval and are time/cost-consuming.

Interestingly, homo-oligomerization of α-subunit was demonstrated not being exclusive for $Na_v1.5$, and to occur also for $Na_v1.1$, $Na_v1.2$ and $Na_v1.7$.[10,89] But to date, most of the protein structures of α-subunits obtained by using X-ray crystallography and cryogenic electron microscopy were resolved as monomers with and without $Na_v\beta$-subunits.[25–27,37–40,90,91] However, it is not clear whether only monomeric α-subunits were observed during the process of protein resolution, or the previously unexpected homo-oligomers of α-subunits were considered artifactual and hence being disregarded during the analysis. In any case, it highlights the importance of this review that raises the awareness for the future studies about the emerging phenomenon of homo-oligomerization of the voltage-gated sodium channel α-subunit.

## Conclusions

Many different sites on $Na_v1.5$ were suggested to mediate its oligomerization in *cis* and *trans* conformations, whether due to direct α-α interactions or through binding with its protein partners such as $Na_v\beta$-subunits and 14-3-3 proteins. Since $Na_v1.5$ were shown to oligomerize in the absence of $Na_v\beta$-subunits and 14-3-3 proteins were not yet proven to directly mediate the oligomerization, one could speculate that sites mediating direct α-α interactions would be more important for this process.

*Cis*-coupled gating was reported for *cis*-dimers, while ephaptic coupling for the *trans* clusters of $Na_v1.5$ across two membranes.

While oligomerization of $Na_v1.5$ is unlikely the main cause of the DNE, it is still an interesting phenomenon and could help understanding the mechanisms underlying the $Na_v$ clusterization. Nevertheless, so far, all reported $Na_v1.5$ oligomers have been shown exclusively in overexpression systems. Thus, to demonstrate the physiological relevance of $Na_v1.5$ *cis*- and/or *trans*-oligomerization, it is crucial to develop genetic

animal models with differently tagged alleles of *SCN5A* and to test their protein ability to interact *in vivo*, for instance by co-immunoprecipitation.

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**Authorship contribution statement**

**O.I.**: conceptualization (lead); writing – original draft (lead); writing – review & editing (equal). **J.-S. R.**: writing – original draft (supporting); writing – review & editing (equal). **H. A.**: funding acquisition (lead); writing – review & editing (equal).

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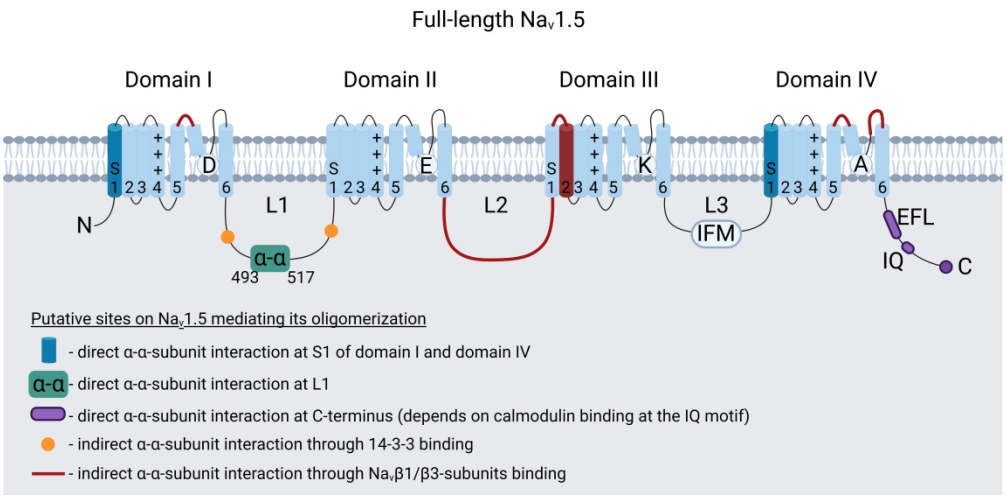
**Figure 1. Graphic representation of sites that may mediate $Na_v1.5$ oligomerization.** Direct α-α-subunit interaction was suggested to occur at the intracellular loop between domain I and domain II (L1) at positions Arg493-Arg517. The binding of 14-3-3 proteins around this region was proposed to stabilize $Na_v1.5$ oligomers. Also, transmembrane α-α-subunit interactions could have occurred due to the homo-dimerization of S1 in domain I and S1 in domain IV. Additionally, it was proposed that the C-terminus of one $Na_v1.5$ interacts with the EF-hand-like (EFL) motif upwards of the calmodulin-binding motif (IQ motif) of another $Na_v1.5$ protein. $Na_v\beta$-subunits were suggested to potentiate interaction within $Na_v1.5$ proteins. Putative sites of $Na_v\beta1$/$Na_v\beta3$ are indicated as suggested previously. The figure was created with BioRender.

**Figure 2. Graphic representation of the putative *cis*-oligomerization of $Na_v1.5$.** This figure depicts a dimeric structure since $Na_v1.5$ is suggested to dimerize rather than to form higher-order oligomers. L1, L2 and L3 are intracellular linkers connecting domain I with domain II, domain II with domain III, and domain III with domain IV, respectively. Transmembrane S1 of domain I and S1 of domain IV have been reported to homo-dimerize. $Na_v\beta$-subunits and 14-3-3 proteins are known to homo- and heterodimerize within the same protein family; hence, they are depicted as dimers. C-C-dimers of $Na_v1.5$ have been described as asymmetric and proposed to depend on the binding of calmodulin (CaM) downwards the interaction site. The figure was created with BioRender.

**Figure 3. Graphic representation of the putative *trans*-oligomerization of $Na_v1.5$.** Typical distances between membranes of two non-diseased cardiomyocytes as reported for the intercalated discs: from 0-4 nm in gap junction to 60-65 nm in the area of mechanical adhesion junctions. The perinexus surrounds the gap junctions and was

shown to be 5-15 nm wide and rich in connexin 43 and $Na_v1.5$. *Trans*-oligomerization was suggested to occur due to $Na_v1.5$ binding with *trans* cell adhesive molecules (CAMs) such as $Na_v\beta$-subunits and cadherins. Due to the narrow spacing, ephaptic coupling has been suggested to occur within the perinexal pool of $Na_v1.5$ channels, while the plicate pool in the area of mechanical adhesion junctions would be unlikely to participate in the *trans* functional coupling between opposing $Na_v1.5$. For clarity, plicate and perinexal pools are shown side-by-side rather than perpendicularly, and *cis*-oligomerization of $Na_v1.5$ is not depicted. The figure was created with BioRender.

**Figure 4. Disrupting $Na_v1.5$ oligomers could be a potential therapeutic strategy to abolish *SCN5A* dominant-negative effect (DNE).** In non-diseased *SCN5A*$^{WT/WT}$ cardiomyocytes, functional $Na_v1.5$ channels were proposed to exist in monomeric as well as in dimeric form and to provide for a physiological macroscopic inward sodium current ($I_{Na}$). In patients with Brugada syndrome (BrS), heterozygous mutations encode for one WT and one mutant $Na_v1.5$. If the mutated allele is not used to produce $Na_v1.5$ protein, the expected $I_{Na}$ would be approximately twice reduced. However, if the mutated protein is produced and the interaction between the proteins is preserved, the mutant may inhibit $I_{Na}$ conducted through the WT channel, constituting the DNE and leading to cardiac arrhythmia detected by the alterations in electrocardiogram (ECG) morphology. If the DNE depends on the oligomerization state of $Na_v1.5$, then disruption of protein interaction may rescue $I_{Na}$ and hence decrease the disease burden. This highly speculative hypothesis needs to be meticulously investigated. The figure was created with BioRender.

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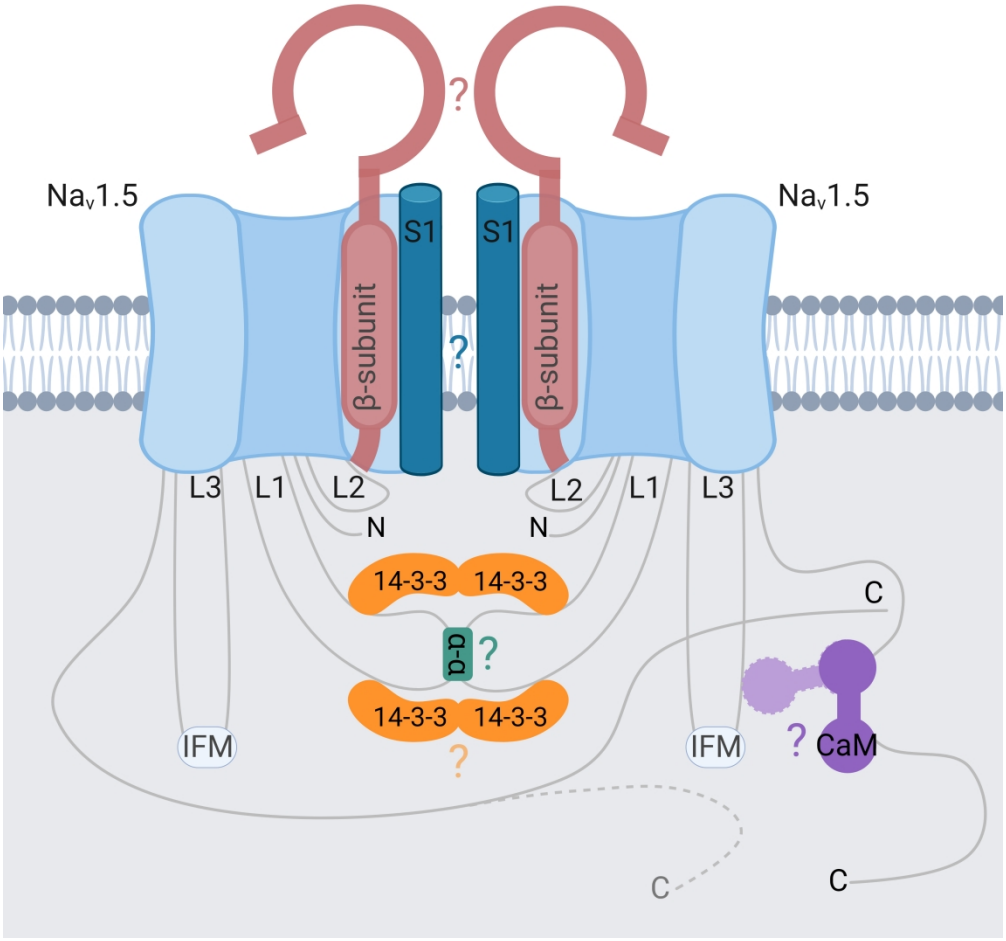


**Figure 2. Graphic representation of the putative *cis*-oligomerization of Na$_v$1.5.** This figure depicts a dimeric structure since Na$_v$1.5 is suggested to dimerize rather than to form higher-order oligomers. L1, L2 and L3 are intracellular linkers connecting domain I with domain II, domain II with domain III, and domain III with domain IV, respectively. Transmembrane S1 of domain I and S1 of domain IV have been reported to homo-dimerize. Na$_v$β-subunits and 14-3-3 proteins are known to homo- and heterodimerize within the same protein family; hence, they are depicted as dimers. C-C-dimers of Na$_v$1.5 have been described as asymmetric and proposed to depend on the binding of calmodulin (CaM) downwards the interaction site. The figure was created with BioRender.

469x447mm (236 x 236 DPI)

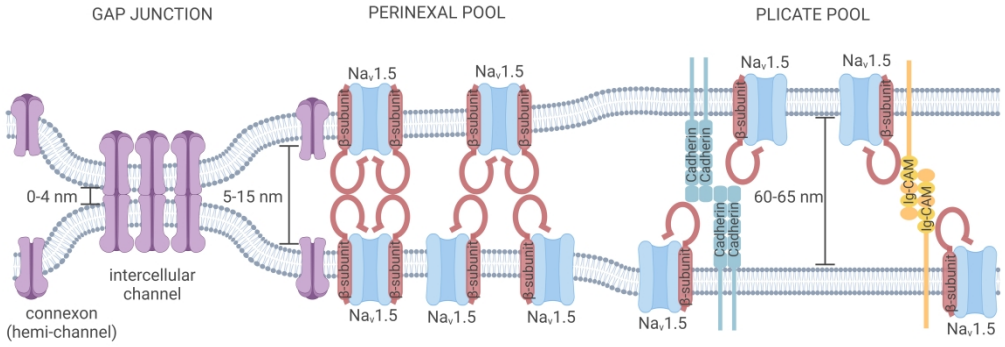


**Figure 3. Graphic representation of the putative *trans*-oligomerization of Na$_v$1.5.** Typical distances between membranes of two non-diseased cardiomyocytes as reported for the intercalated discs: from 0-4 nm in gap junction to 60-65 nm in the area of mechanical adhesion junctions. The perinexus surrounds the gap junctions and was shown to be 5-15 nm wide and rich in connexin 43 and Na$_v$1.5. Trans-oligomerization was suggested to occur due to Na$_v$1.5 binding with trans cell adhesive molecules (CAMs) such as Na$_v$β-subunits and cadherins. Due to the narrow spacing, ephaptic coupling has been suggested to occur within the perinexal pool of Na$_v$1.5 channels, while the plicate pool in the area of mechanical adhesion junctions would be unlikely to participate in the *trans* functional coupling between opposing Na$_v$1.5. For clarity, plicate and perinexal pools are shown side-by-side rather than perpendicularly, and *cis*-oligomerization of Na$_v$1.5 is not depicted. The figure was created with BioRender.

645x252mm (236 x 236 DPI)

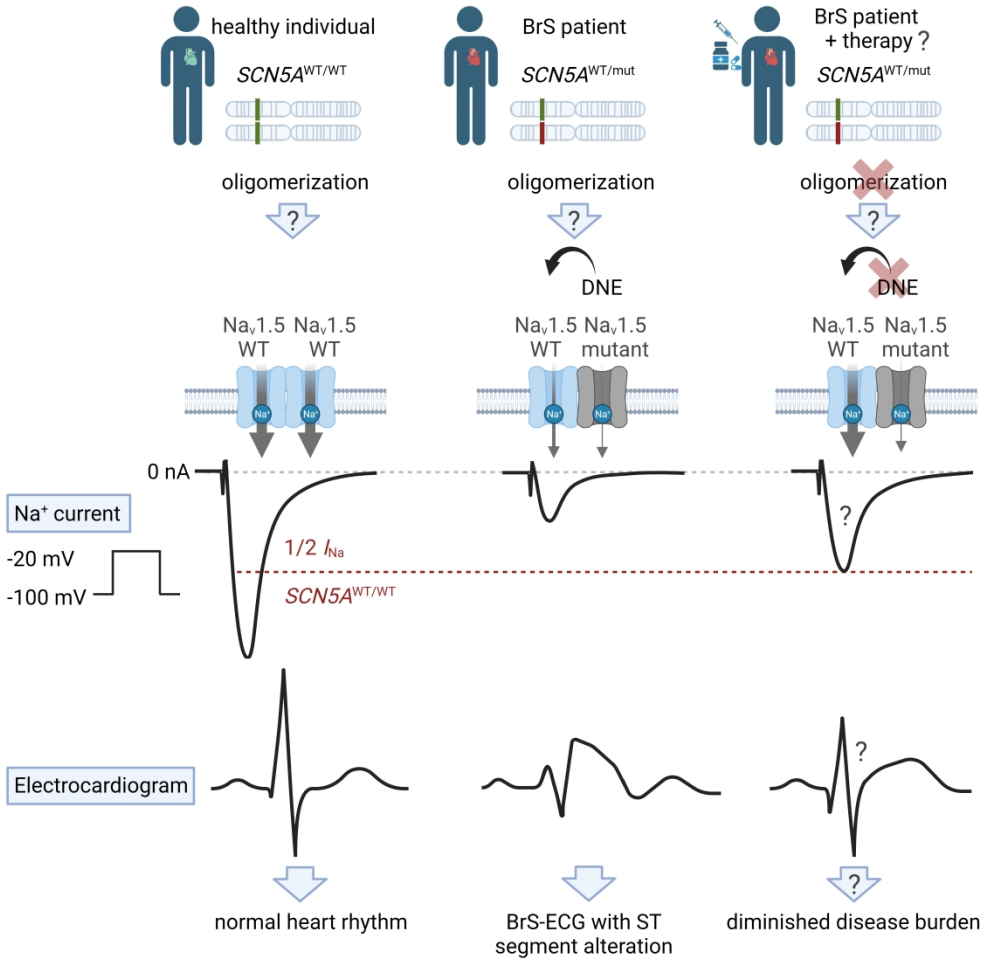


**Figure 4. Disrupting $Na_v1.5$ oligomers could be a potential therapeutic strategy to abolish *SCN5A* dominant-negative effect (DNE).** In non-diseased *SCN5A*$^{WT/WT}$ cardiomyocytes, functional $Na_v1.5$ channels were proposed to exist in monomeric as well as in dimeric form and to provide for a physiological macroscopic inward sodium current ($I_{Na}$). In patients with Brugada syndrome (BrS), heterozygous mutations encode for one WT and one mutant $Na_v1.5$. If the mutated allele is not used to produce $Na_v1.5$ protein, the expected $I_{Na}$ would be approximately twice reduced. However, if the mutated protein is produced and the interaction between the proteins is preserved, the mutant may inhibit $I_{Na}$ conducted through the WT channel, constituting the DNE and leading to cardiac arrhythmia detected by the alterations in electrocardiogram (ECG) morphology. If the DNE depends on the oligomerization state of $Na_v1.5$, then disruption of protein interaction may rescue $I_{Na}$ and hence decrease the disease burden. This highly speculative hypothesis needs to be meticulously investigated. The figure was created with BioRender.

587x572mm (236 x 236 DPI)