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Functional Microdomains in Heart's Pacemaker: A Step Beyond Classical Electrophysiology and Remodeling

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Spontaneous beating of the sinoatrial node (SAN), the primary pacemaker of the heart, is initiated, sustained, and regulated by a complex system that integrates ion channels and transporters on the cell membrane surface (often referred to as "membrane clock") with subcellular calcium handling machinery (by parity of reasoning referred to as an intracellular "Ca^{2+} clock"). Stable, rhythmic beating of the SAN is ensured by a rigorous synchronization between these two clocks highlighted in the coupled-clock system concept of SAN timekeeping. The emerging results demonstrate that such synchronization of the complex pacemaking machinery at the cellular level depends on tightly regulated spatiotemporal signals which are restricted to precise sub-cellular microdomains and associated with discrete clusters of different ion channels, transporters, and regulatory receptors. It has recently become evident that within the microdomains, various proteins form an interacting network and work together as a part of a macromolecular signaling complex. These protein–protein interactions are tightly controlled and regulated by a variety of neurohormonal signaling pathways and the diversity of cellular responses achieved with a limited pool of second messengers is made possible through the organization of essential signal components in particular microdomains. In this review, we highlight the emerging understanding of the functionality of distinct subcellular microdomains in SAN myocytes and their functional role in the accumulation and neurohormonal regulation of proteins involved in cardiac pacemaking. We also demonstrate how changes in scaffolding proteins may lead to microdomain-targeted remodeling and regulation of pacemaker proteins contributing to SAN dysfunction.

Keywords: sinoatrial node, pacemaker, microdomain, ion channel, remodeling, signaling complex

INTRODUCTION

The sinoatrial node (SAN) is the primary pacemaker of the heart. Spontaneous beating of the SAN is initiated, sustained, and regulated by a complex system that integrates ion channels and transporters located on the cell membrane surface (often referred to as "membrane clock") with subcellular calcium handling machinery (by parity of reasoning referred to as an intracellular "Ca-Clock") (Lakatta and DiFrancesco, 2009; Lakatta et al., 2010). Stable, rhythmic beating of the SAN is ensured by a rigorous synchronization between these two clocks highlighted in

the coupled-clock system concept of SAN timekeeping. Following achievement of the maximal diastolic potential, K^+ current (a combination of a rapidly recovering transient outward current I_{to}, and a rapidly, I_{Kr}, and slowly, I_{Ks}, activating delayed rectifier currents) conductance decreases which unmasks several inward background currents. Together with hyperpolarization-activated current (I_f) and low-voltage activated T-type Ca^{2+} current ($I_{Ca,T}$), these start gradual changes of the membrane potential (V_m) (early diastolic depolarization). Then, spontaneous and rhythmic submembrane local Ca^{2+} releases (LCR) from ryanodine receptors (RyRs) occur and activate an inward Na^+/Ca^{2+} exchange (NCX) current (I_{NCX}) to boost diastolic depolarization rate and fire an action potential (AP) via activation of L-type Ca^{2+} current ($I_{Ca,L}$).

Until recently, the prevailing concept of cardiac electrophysiology has been that ion channels and receptors are freely mobile in the plasma membrane providing uniform activity through the sarcolemma. Though such simplification was beneficial for computational modeling and enabled the development of relatively straightforward biophysical models based on non-linear dynamics and oscillatory theory, it recently became evident that this simple "random collision model" is inadequate to explain the emerging experimental results which highlight microdomain-specific regulation of cardiomyocyte physiology (reviewed in details elsewhere, Zaccolo and Pozzan, 2002; Warrier et al., 2007; Best and Kamp, 2012; Balycheva et al., 2015; Vinogradova et al., 2018). In the SAN, these include findings on a complex spatial-temporal coupling between the membrane- and Ca^{2+} clocks confirmed in various species, including human (Kim et al., 2018; Tsutsui et al., 2018), synchronization of spontaneous LCRs between discrete RyR clusters (Stern et al., 2014; Torrente et al., 2016), compartmentalized autonomic regulation of pacemaker ion channels which relies on tightly confined cAMP signaling (Barbuti et al., 2004; St Clair et al., 2017; Vinogradova et al., 2018), as well as microdomain-specific remodeling of ion channels secondary to structural alterations including changes in scaffolding proteins (Le Scouarnec et al., 2008; Alcalay et al., 2013; Bryant et al., 2018). The emerging results demonstrate that the functioning of the complex pacemaking machinery at the cellular level depends on tightly regulated spatiotemporal signals which are restricted to precise subcellular microdomains and associated with discrete clusters of different ion channels, transporters and regulatory receptors. Within different subcellular compartments, various proteins form an interacting network and work together as a part of a macromolecular signaling complex. These protein–protein interactions are tightly controlled and regulated by a variety of neurohumoral signaling pathways, and the diversity of cellular responses achieved with a limited pool of second messengers is made possible through the organization of essential signal components in particular microdomains. Importantly, on a tissue level, these are manifested by a dynamic pattern of beat-to-beat migration of leading pacemaker location within the SAN at baseline and during autonomic stimulation, a complex interaction between discrete pacemaker clusters, and the development of SAN arrhythmias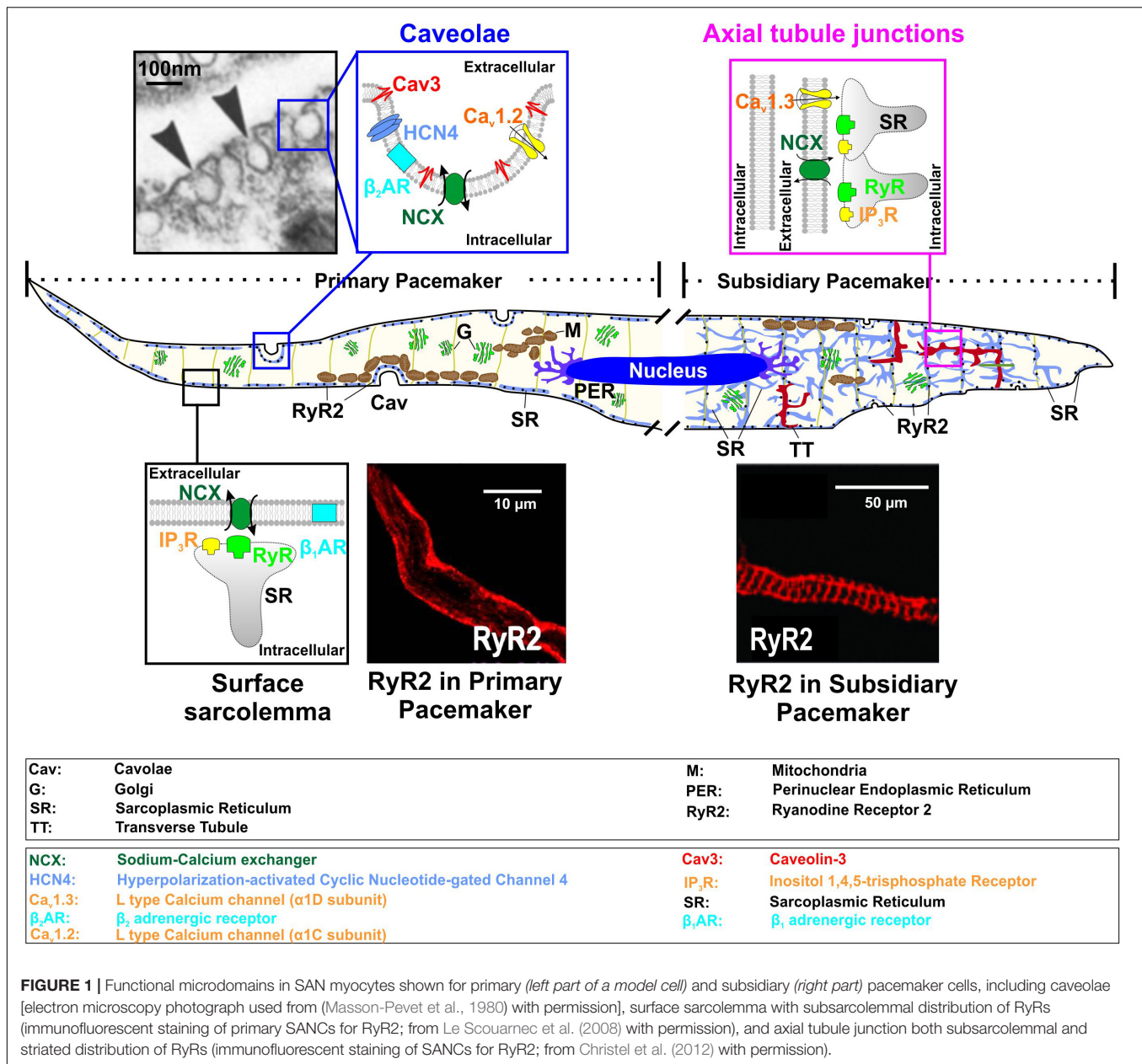 associated with pathological remodeling which could not be described in simplified oscillatory models of cardiac pacemaking.

In this review, we highlight the emerging understanding of the functionality of distinct subcellular microdomains in SAN cardiomyocytes (SANCs) (**Figure 1**) and their role in autonomic regulation of cardiac pacemaking. We also demonstrate how changes in scaffolding proteins may lead to microdomain-targeted remodeling and regulation of pacemaker proteins and contribute to SAN dysfunction (SND).

FUNCTIONAL MACRO- AND MICRO-ARCHITECTURE OF THE SAN

The SAN has a highly complex and heterogeneous structure (reviewed in detail elsewhere, Boyett et al., 2000; Fedorov et al., 2012; Csepe et al., 2016). It was shown in humans (Boineau et al., 1988; Li et al., 2017) and dogs (Glukhov et al., 2013) that the SAN consist of several intranodal pacemaker clusters which have different electrophysiological properties, including automaticity robustness and varying response to autonomic stimulation. These were thought to underlie the dynamic pattern of a beat-to-beat shift of the leading pacemaker location within the SAN pacemaker complex during heart rate change under various conditions (Boyett et al., 2000), the development of intranodal conduction blocks, temporally unexcitable areas, SAN micro-reentry and exit block (Glukhov et al., 2013; Li et al., 2017). On the cellular level, the difference between intranodal pacemaker clusters was linked to distinct pacemaker protein expression profiles as well as cellular microarchitecture (Boyett et al., 2000). The latter is critical for pacemaker proteins' distribution as well as their communication with each other and with subcellular Ca^{2+} clock. Transmission electron microscopy studies by Ayettey and Navaratnam demonstrated that specialized transversal (T)-tubular system is either absent or far less developed in rat SAN than atrial or ventricular myocytes (Ayettey and Navaratnam, 1978). The authors found that in primary SANCs, T-tubules are represented by short and narrow (about 60 nm in diameter versus 105 and 130 nm in atrial and ventricular myocytes, respectively) invaginations of the sarcolemma, which do not usually penetrate sufficiently far to contact the myofibrillae. Instead, SANCs are highly rich with caveolae structures, i.e., muscle-specific caveolin-3 (Cav-3) scaffolding protein containing a subpopulation of lipid rafts, representing small (50–100 nm in diameter) invaginations of the plasma membrane (**Figure 1**, '*caveolae*' microdomain). Caveolae density in rabbit SANCs is 2-times higher than in atrial and 4 to 5-times higher than in ventricular myocytes as estimated from electron microscopy photographs (Masson-Pevet et al., 1980). Through binding to caveolin-scaffolding domain, Cav-3 compartmentalizes and concentrates various proteins, including ion channels, transporters, G-protein subunits, kinases, endothelial nitric oxide synthase (eNOS), and others, many of which contribute to SAN pacemaking.

Through, it is quite difficult to localize the center SAN in electron microscopy studies without functional characterization of the leading pacemaker localization, Ayettey and Navaratnam highlighted the presence of some transitional cells within the

FIGURE 1 | Functional microdomains in SAN myocytes shown for primary *(left part of a model cell)* and subsidiary *(right part)* pacemaker cells, including caveolae [electron microscopy photograph used from (Masson-Pevet et al., 1980) with permission], surface sarcolemma with subsarcolemmal distribution of RyRs (immunofluorescent staining of primary SANCs for RyR2; from Le Scouarnec et al. (2008) with permission), and axial tubule junction both subsarcolemmal and striated distribution of RyRs (immunofluorescent staining of SANCs for RyR2; from Christel et al. (2012) with permission).

SAN region. These transitional myocytes resemble nodal cells in diminutiveness of size and lack of atrial granules and also possess a sparse and disorganized T-tubule system (Ayettey and Navaratnam, 1978). It appears that a sparse tubular system in SANCs is likely different from that in working ventricular myocytes, and may rather represent a 'super-hub' of Ca^{2+} signaling associated with axial tubule junctions that rapidly activate Ca^{2+} release through cell-specific molecular microdomain mechanisms and was recently proposed for atrial myocytes by the Lehnart's group (Brandenburg et al., 2016, 2018). The authors demonstrated that axial tubule junctions in atrial myocytes are highly enriched by cholesterol-rich nanodomains visualized by the fluorescent cholesterol analog dye Chol-PEG-KK114 in live cells (**Figure 1**, '*axial tubule junctions*'), in

contrast to ventricular myocytes where T-tubules rarely contain caveolae-shaped membrane structures. Axial tubule junctions form contact junctions with the sarcoplasmic reticulum (SR) and are associated with highly phosphorylated RyRs (Brandenburg et al., 2016) which may play an important role in SANC pacemaking as discussed below (see section "Ca Clock"). Axial tubule junctions and their role in atrial Ca^{2+} signaling have been shown in mouse, rat, rabbit, pig, and human myocytes (Brandenburg et al., 2018).

Such intrinsic structural heterogeneity of the SAN has been recently confirmed on the functional level as well. By performing consecutively measurement of I_f and $I_{Ca,L}$ from the rabbit pacemaker cells isolated from the intercaval region, including the SAN, Monfredi et al. (2018) found their significantly diverse

range. Importantly, I_f, but not $I_{Ca,L}$ current density was positively related to baseline beating rate. These data correlate well with the distribution of transversal-axial tubule system within the SAN: primary pacemakers with the fastest spontaneous beating rate do not have T-tubules, express the smallest $I_{Ca,L}$ which predominantly rely on extratubular LTCCs, and have the highest pacemaker current I_f, while subsidiary SAN pacemakers possess a rudimentary T-tubule network which results in a significant increase of $I_{Ca,L}$ and decrease in I_f. Subsequently, two recent reports from Lakatta's group demonstrated in guinea pig (Kim et al., 2018) and human SANCs (Tsutsui et al., 2018) several populations of cells which show rhythmic pacemaking activity, dysrhythmic firing, and no spontaneous activity (i.e., 'dormant' cells). Dysrhythmic and dormant SANCs have smaller and desynchronized LCR activity than rhythmic SANCs; however, in response to sympathetic stimulation, all dysrhythmic cells and a third of dormant SANCs increased their LCR activity and developed automaticity resulting in spontaneous electrical beating (Kim et al., 2018). Whether or not these cells are associated with different pacemaker clusters and responsible for certain ranges of heart rate, remains open to question. These, however, may provide mechanistic basis for dynamic pacemaker location shift within the SAN as it was observed experimentally in intact optically mapped mouse, canine, and human SAN preparations (Fedorov et al., 2010; Glukhov et al., 2010, 2013, 2015b; Li et al., 2017).

In the following sections, we describe microdomain-specific distribution, functioning, and remodeling of the main components of both membrane and Ca^{2+} clocks. Specifically, we focus on how the changes in scaffolding proteins affect functional pacemaker microdomains and results in SND. Though most of these changes are studied in transgenic mouse models, emerging evidence from SND patients harboring similar mutations, which are summarized in the review for available proteins, support the observed results and highlight microdomain-targeted remodeling as a new dimension to cardiovascular disease.

MEMBRANE CLOCK

Pacemaker Channels

The hyperpolarization-activated, cyclic nucleotide-gated (HCN) ion channels are responsible for generating the pacemaker current (funny current, I_f) which is the inward current that contributes to the early stage diastolic depolarization in the SAN (DiFrancesco, 1993). At diastolic potentials, I_f is predominantly carried by Na^+ (DiFrancesco, 1993). I_f is activated by hyperpolarizing voltage steps, with the threshold potential varies from -40 to -60 mV. The voltage dependence of the I_f activation is influenced by intracellular cyclic adenosine monophosphate (cAMP); a direct binding of cAMP to this channel increases the open probability via a depolarizing shift in the midpoint activation voltage ($V_{1/2}$) (DiFrancesco and Tortora, 1991).

While HCN4 is the most predominant isoform expressed in rodent SANCs (Marionneau et al., 2005), recent reports showed that all three cardiac HCN isoforms (HCN1, HCN2, and HCN4)

are highly expressed in the human SAN (Li et al., 2015). Several studies demonstrated that HCN channels localize to caveolae based on the presence of HCN4 in low-density membrane fractions along with Cav-3 as well as the specific interaction of HCN4 with Cav-3 (Barbuti et al., 2007; Ye et al., 2008). Barbuti et al. (2012) reported that all HCN isoforms have a conserved caveolin-binding domain which impact both channel function and trafficking. Disruption of caveolae by reducing membrane cholesterol using methyl-β-cyclodextrin (MβCD) or by expression of dominant negative caveolin mutants alters the gating of HCN channels by shifting the voltage dependence of the activation by approximately 10 mV in the positive direction (Ye et al., 2008). In addition, β₂-adrenergic receptor (β₂AR) modulation of HCN4 channel is lost when caveolae are disrupted, which is supported by co-localization of β₂ARs and HCN4 channels in caveolae (Barbuti et al., 2007).

Recent studies indicate that caveolae-associated β₂AR-dependent stimulation of HCN4 channels relies on subcellular compartmentalization of cAMP signaling. First, it was found that HCN4 can be phosphorylated by cAMP-dependent protein kinase A (PKA) at the distal C-terminus, in addition to the well-studied cAMP binding to a conserved cyclic nucleotide binding domain in the proximal C-terminus (Liao et al., 2010). Moreover, PKA activity is necessary for cAMP-dependent signaling between β₂ARs and HCN channels in SANCs; inhibition of PKA with an inhibitory peptide, PKI, significantly reduced the shift in $V_{1/2}$ produced by β₂ARs stimulation while does not affect a direct stimulation of the channels by exogenous cAMP and Rp-cAMP (an analog than cannot activate PKA) (St Clair et al., 2013). St Clair and colleagues showed that PKA modulation of HCN4 channels depends on distinct cAMP signaling domains created by subcellular localization of cyclic nucleotide phosphodiesterases 3 and 4 (PDE3 and 4) which are responsible for cAMP degradation in the SANCs. The authors demonstrated that PDE4 inhibition in mouse SANCs produced a PKA-independent depolarizing shift in the $V_{1/2}$ of I_f at rest, likely via a direct binding of elevated cAMP to the channel, but did not remove the requirement for PKA in β₂AR-to-HCN signaling. In contrast, PDE3 inhibition produced PKA-dependent changes in I_f both at rest and in response to β₂AR stimulation (St Clair et al., 2017). Microdomain-specific localization and activity of PDEs in SANCs have been recently reviewed by Vinogradova et al. (2018) and highlight functional importance of local cAMP microdomains with high and low cAMP levels which are involved in local regulation of coupled-clock system components.

L-Type Ca²⁺ Channels

In the center of the SAN, where the AP upstroke is slow, and little or no Na^+ current is expressed, $I_{Ca,L}$ is principally responsible for the upstroke. Though $Ca_v1.2$ represents the major isoform of the L-type Ca^{2+} channel (LTCC) central pore subunit expressed in the heart, SANCs also express $Ca_v1.3$ isoform (Zhang et al., 2002). In comparison to $Ca_v1.2$ channels which are activated at -40 mV and mainly contribute to AP upstroke, channels formed by $Ca_v1.3$ are activated ∼20 mV more negatively than $Ca_v1.2$ and thus play an important role in the generation of diastolic depolarization. Knockout of $Ca_v1.3$ in mice decreased

$I_{Ca,L}$ density by 69% and resulted in severe bradycardia and highly erratic pacing rate in the SAN (Mangoni et al., 2003).

A number of important $Ca_v1.2$ subpopulations have been identified in cardiomyocytes that associate with unique macromolecular signaling complexes and scaffolding proteins (**Figures 1**, **2**) (Best and Kamp, 2012). These include channels localized to dyadic junctions (i.e., T-tubules) as well as extradyadic channels that reside in biochemically distinct regions of surface membrane, including caveolae, lipid rafts, and plasmalemma. Most of the studies performed on isolated mouse SANCs (Le Scouarnec et al., 2008; Christel et al., 2012) demonstrated a predominant plasma membrane localization of $Ca_v1.2$ channels; however, a ratio between caveolar and non-caveolar LTCCs in SANCs is unknown and requires additional studies. In contrast, immunofluorescence analysis of mouse SANCs showed $Ca_v1.3$ channels localized both on the plasma membrane (Le Scouarnec et al., 2008) and in sarcomeric structures (presumably, within a sparse T-tubule network observed in subsidiary pacemakers (Ayettey and Navaratnam, 1978) where they co-localize with RyRs (Christel et al., 2012). STimulated Emission Depletion (STED) co-immunofluorescence labeling of mouse atrial myocytes showed that $Ca_v1.3$ clusters are located close to Cav-3 clusters in axial tubule junctions where highly PKA phosphorylated clusters of RyR-pS2808 are identified (Brandenburg et al., 2018). Such highly phosphorylated, both at PKA- (P-Ser[2809]) and Ca^{2+}/calmodulin-dependent protein kinase II (CaMKII)-dependent (P-Ser[2815]), clusters of RyRs have been found in rabbit SAN and proposed to contribute to spontaneous LCRs. Along with a heterogeneous distribution of T-tubules in the SAN (compare primary vs. subsidiary pacemakers in **Figure 1**), this may indicate a variable contribution of $Ca_v1.3$ to $I_{Ca,L}$ in different SAN pacemaker clusters, their functional interaction with RyRs and thus their impact on heart rate. Indeed, Christel and colleagues demonstrated a significantly stronger inactivation of $Ca_v1.2$ versus $Ca_v1.3$ channels measured in mouse SANCs during strong (+80 mV) depolarization. The authors used a numerical model of mouse SAN automaticity and found that $Ca_v1.3$ voltage-dependent facilitation enhanced recovery of pacemaker activity after pauses and positively regulated pacemaking during slow heart rate (Christel et al., 2012). In a subsequent study from the same group, Torrente et al. (2016) showed that $Ca_v1.3$ deficiency strongly impaired $[Ca^{2+}]_i$ dynamics, reducing the frequency of local $[Ca^{2+}]_i$ release events and preventing their synchronization. These data highlight an importance of microdomain-specific localization of $Ca_v1.3$ channels in calcium-voltage clock coupling and in opposing abnormal slowing of heart rate.

Along with distinct functional roles that LTCCs play in cellular microdomains, emerging evidence indicate that subpopulations of LTCCs may possess different biophysical properties resulted from the difference in channel's structure and/or subcellular microenvironment influence, and therefore experience diverse pathological remodeling. For example, extratubular LTCCs measured in rat and human atrial myocytes, which express both $Ca_v1.2$ and $Ca_v1.3$, demonstrate low single channel activity (Glukhov et al., 2015a) indicating either a different
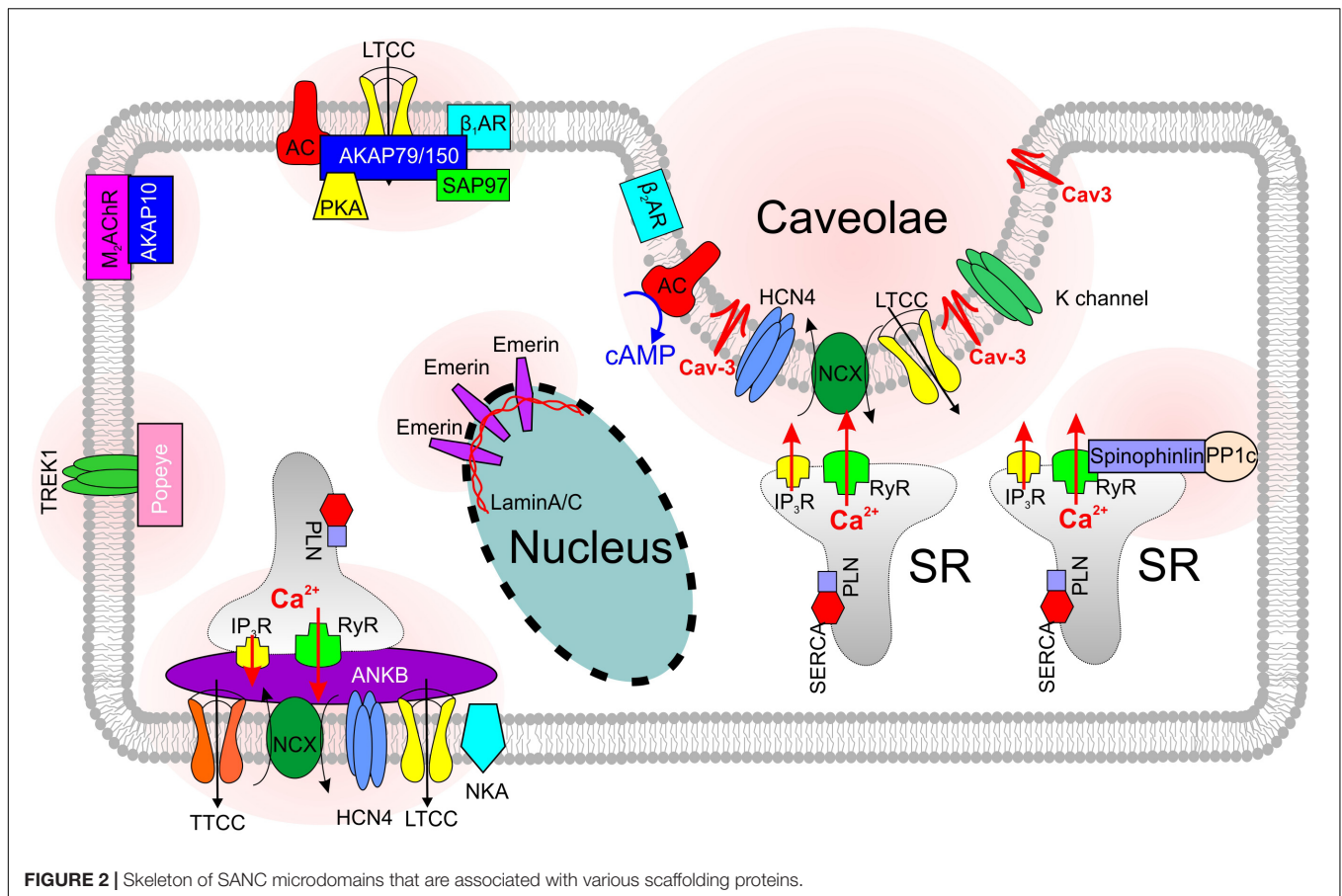
LTCC structure (both on pore-forming $Ca_v1.2$ and $Ca_v1.3$ or regulatory $Ca_v\beta_{1-4}$ and $\alpha_2\delta$ (Foell et al., 2004) subunits) or microdomain-specific regulation, including the association with various scaffolding proteins. β-subunits enhance channel trafficking to the distinct plasma membrane microdomains, increase the channel's open probability, cause a hyperpolarization shift in the voltage-dependence of activation, and affect voltage-dependent inactivation (Foell et al., 2004). Though $Ca_v\beta_2$ is the most abundant β subunit expressed in the heart, both $Ca_v\beta_1$ and $Ca_v\beta_3$ mRNA expression have been found in mouse (Marionneau et al., 2005) and rat (Yanni et al., 2011) SAN, and their mRNA levels were higher than those measured in atrial and ventricular myocardium. Similar to $Ca_v\beta$, $\alpha_2\delta$ subunits are also involved in channel's trafficking via interactions with other proteins, such as extracellular matrix and other membrane proteins involved in cell adhesion (Whittaker and Hynes, 2002).

Along with spatial compartmentalization, LTCCs show a microdomain-specific cAMP- mediated and PKA-dependent regulation, both at baseline and during autonomic stimulation [reviewed in details elsewhere (Best and Kamp, 2012; Vinogradova et al., 2018) and discussed below in 'Ca^{2+} clock' section]. The specificity of cAMP signaling is modulated by the binding of PKA to A kinase-anchoring proteins (AKAPs) (**Figure 2**), which target the kinase to specific intracellular locations and LTCC pools, and provide spatiotemporal control over cAMP signaling events. Various AKAPs serve as a scaffold to assemble proteins involved in Ca^{2+} signaling regulation via microdomain-specific phosphorylation of different subpopulations of LTCCs thus adding another level of complexity to $I_{Ca,L}$ and Ca^{2+} signaling regulation (see section "SND and Scaffolding Proteins").

T-Type Ca^{2+} Channels

Hagiwara et al. (1988) reported the existence of both long-lasing ($I_{Ca,L}$) and transient ($I_{Ca,T}$) Ca^{2+} currents in isolated rabbit SANCs. The authors described their distinct biophysical properties, including voltage dependencies for activation/inactivation and pharmacology. Threshold for activation of $I_{Ca,T}$ was around −60 mV, and $I_{Ca,T}$ was fully activated at about −10 mV. Because activation range of $I_{Ca,T}$ overlapped the pacemaker potential of SAN, the potential involvement of $I_{Ca,T}$ in the generation of the diastolic depolarization in SANCs was proposed. Indeed, selective inhibition of $I_{Ca,T}$ by Ni^{2+} (Hagiwara et al., 1988) or mibefradil (Protas and Robinson, 2000) resulted in significant slowing of spontaneous activity of isolated rabbit SANCs by selective suppression of the later phase of pacemaker depolarization in a concentration-dependent manner, with no effects on AP amplitude and duration.

Two out of three T-type Ca^{2+} channel α-subunits, namely $Ca_v3.1$ (α_{1H}) and $Ca_v3.2$ (α_{1I}), were detected in the heart. In contrast to Ca_v1, Ca_v3 channels produce large currents in the absence of co-expressed accessory β or $\alpha_2\delta$ subunits, and therefore these proteins are not obligate auxiliary subunits for Ca_v3 channels. Genetic inactivation of $Ca_v3.1$ channels significantly slowed the intrinsic *in vivo* heart rate, prolonged the SAN recovery time, and slowed pacemaker activity of

FIGURE 2 | Skeleton of SANC microdomains that are associated with various scaffolding proteins.

mouse SANCs through a reduction of the slope of the diastolic depolarization (Mangoni et al., 2006). In contrast, $Ca_v3.2$-deficient mice displayed normal sinus rhythm without arrhythmias (Chen et al., 2003).

To date, experimental data on T-type Ca^{2+} channels subcellular distribution, including SAN, is limited. In mice with cardiac-specific, conditional expression of the $Ca_v3.1$ channels, immunocytochemical labeling revealed their presence primarily on the surface sarcolemma of ventricular myocytes, with less staining within the T-tubules (Jaleel et al., 2008). Osmotic shock, which selectively eliminates T-tubules, induced a greater reduction in L- versus T-type Ca^{2+} currents. Ca^{2+} influx through T-type Ca^{2+} channels also did not induce normal SR Ca^{2+} release which suggests that T- and L-type Ca^{2+} channels are located in different portions of the sarcolemma (Jaleel et al., 2008). Similarly, in wild type mouse atrial myocytes, Curran et al. (2015) showed both $Ca_v3.1$ and $Ca_v3.2$ expressed on the plasma membrane and, surprisingly, over the atrial Z-line while their association with T-tubules is questionable. Co-immunoprecipitation analysis suggested an association of $Ca_v3.1$ and $Ca_v3.2$ channel isoforms with caveolae scaffolding protein Cav-3 in neonatal mouse ventricular myocytes (Markandeya et al., 2011). Interestingly, co-expression of Cav-3 significantly decreased the peak $Ca_v3.2$ current density in HEK293 cells, whereas co-expression of Cav-3 did not alter peak $Ca_v3.1$ current density. It is possible that $Ca_v3.1$

and $Ca_v3.2$ channels are located on the surface sarcolemma, in association with different structural microdomains (**Figure 1**).

Na$^+$/Ca^{2+} Exchanger

NCX plays a critical role in SAN pacemaking by producing a depolarizing current and boosting depolarization rate in late diastole when local Ca^{2+} released by RyRs beneath the cell surface membrane (**Figure 1**, *primary pacemaker*) (Lakatta et al., 2010). NCX1 is the predominant isoform expressed in the heart. Atrial-specific NCX knock-out in mice resulted in severe pacemaker abnormalities manifested by "tachy-brady" arrhythmias and associated with quiescent isolated SANCs where, however, copious intracellular Ca^{2+} waves were present but failed to trigger APs (Torrente et al., 2015). These highlight a key role of NCX in synchronizing Ca^{2+} and membrane clocks.

Though data on NCX distribution in SANCs is limited, studies on atrial and ventricular myocytes indicate that NCX membrane localization might be associated with different targeting components. Immunohistological evidence from rabbit SANCs indicate significant submembrane co-localization of NCX1 and RyRs which exceeds that measured in atrial and ventricular myocytes (Lyashkov et al., 2007) where NCX1 labeling is present throughout the cell (attribute to an extensive T-tubular network) (compare 'caveolae' vs. 'axial tubule junction' compartments in **Figure 1**). Such localization allows

crosstalk between RyR LCRs and NCX (**Figure 1**, '*surface sarcolemma*' compartment). Several groups have identified NCX associated with Cav-3 by both co-immunoprecipitation and immunolabeling (Scriven et al., 2005; Camors et al., 2006) with the latter finding those complexes enriched on the cell surface and little in the T-tubules (Scriven et al., 2005). It was also reported that NCX1 has several caveolin binding motifs and that the NCX1 protein associates with Cav-3 (Bossuyt et al., 2002). Others, using the same techniques, have found little, if any, association (Cavalli et al., 2007).

Other Channels

Besides the ion channels discussed above, there are other ion channels that participate in the SAN pacemaking. These include (1) ion channels involved in store-operated Ca^{2+} entry (SOCE); (2) SR-Ca^{2+} activated non-selective Na^+ transient receptor potential melastatin 4 (TRPM4) ion channel; (3) sodium channels $Na_v1.5$ which are likely expressed the periphery but not in the center of the SAN; and (4) chloride channels. All these channels provide an additional inward current during spontaneous diastolic depolarization and thus may contribute to SAN pacemaking. Though these channels may not play a prominent role in SAN activity, the mutations of the genes that encode them are reported to associate with SND observed clinically, suggesting their contributions to the SAN pacemaking. Importantly, most of those channels are associated with different structural proteins and have been linked to distinct microdomains, including caveolae/lipid rafts, intercalated disks, luminal SR, etc., and thus may require a precise spatial arrangement to support their functional coupling and integrity. Disruption in one of the protein localization could affect other proteins involved in a complex protein–protein interaction and thus disturb pacemaker automaticity. Finally, potassium ion channels, including a rapidly recovering transient outward current I_{to} ($K_v4.2$), a rapidly, I_{Kr} (ERG), and slowly activating, I_{Ks} (K_vLQT1), delayed rectifier currents, acetylcholine-activated K^+ current $I_{K,ACh}$ ($K_{ir}3.1$ and $K_{ir}3.4$), and ATP-sensitive K^+ current $I_{K,ATP}$ ($K_{ir}6.2$), are expressed in the SAN and also demonstrate microdomain-specific distribution and regulation, as reviewed in details elsewhere (Balycheva et al., 2015).

Ca-CLOCK

Besides ion channels that depolarize SAN V_m during diastole, spontaneous Ca^{2+} oscillations also play a critical role in pacemaking (Lakatta et al., 2010). Attributed to the 'Ca-clock' component of the pacemaking system, this $[Ca^{2+}]_i$ cycling activity is comprised of spontaneous local Ca^{2+} releases from the RyRs (i.e., AP-independent Ca^{2+} releases, or LCRs, in contrast to AP-triggered SR Ca^{2+} releases, or Ca^{2+} transients, CaT) and Ca^{2+} reuptake back to the SR via SERCA. Emerging evidence shows that disruption of LCR activity leads to dramatic changes in SAN pacemaking (Vinogradova and Lakatta, 2009; Gao et al., 2010). LCRs occur during the late phase of the diastolic depolarization. They generate small increments in the

local $[Ca^{2+}]_i$ which activates NCX to pump Ca^{2+} out of the cell in exchange for Na^+ ions at the ratio of 1 Ca^{2+}: 3 Na^+, therefore leading to a net positive charge influx and a subsequent depolarization of the V_m to the threshold of the next beat. Unlike AP-triggered CaT, LCRs are V_m-independent, rhythmic activities that are localized near the sarcolemmal membrane (Vinogradova et al., 2004). Such highly localized LCR patterns are associated with a unique distribution of RyR found in SANCs. In contrast to atrial and ventricular myocytes, immunofluorescent staining studies performed on SANCs demonstrated that RyRs are exclusively expressed beneath the sarcolemma in rabbit (Musa et al., 2002), guinea-pig (Rigg et al., 2000) and mouse (Le Scouarnec et al., 2008) SANC from a central SAN area. Internal labeling of RyRs that followed a striation pattern was also observed by other groups in mouse and guinea-pig SANs (Rigg et al., 2000; Christel et al., 2012) as well as in rabbit myocytes isolated from a peripheral SAN area (Musa et al., 2002) (compare immunofluorescent staining images for RyR2 distribution in primary and subsidiary pacemakers in **Figure 1**).

Molecular mechanisms that underlie the generation and regulation of LCR activity, are still incompletely understood. Multiple factors have been proposed to contribute to these spontaneous SR Ca^{2+} releases including an elevated SR Ca^{2+} load (i.e., $[Ca^{2+}]_{SR}$) as well as hyperactivity of RyRs due to their hyperphosphorylation or hypersensitization via IP_3R-mediated Ca^{2+} release.

SR Ca^{2+} release depends on the level of $[Ca^{2+}]_{SR}$ which affects the sensitivity of RyR2 (Bassani et al., 1995). Though no accurate $[Ca^{2+}]_{SR}$ was reported in SANCs, atrial myocytes possess a threefold higher SR Ca^{2+} load compared to ventricular myocytes (Walden et al., 2009). Though amplitude of caffeine-evoked CaT in rabbit central SANCs is ∼50% lower than that in atrial myocytes (Jones et al., 2008), SANCs have a dramatically smaller size than atrial myocytes and thus a higher single SR Ca^{2+} load might be expected in SANCs. Such "Ca^{2+} overload" may lead to spontaneous Ca^{2+} releases such as LCRs. "Ca^{2+} overload" then gets restored after Ca^{2+} is replenished by SERCA from AP-induced CaT, thus the next leak follows and rhythmic LCRs are generated.

SR Ca^{2+} load is tightly associated with a SR protein calsequestrin (Casq2). Casq2 is a low-affinity, high-capacity Ca^{2+} binding protein expressed in cardiomyocytes (Knollmann et al., 2006). Overexpression of Casq2 in transgenic mice (Jones et al., 1998) causes a significant increase in the SR Ca^{2+} load and SR Ca^{2+} release. With such increase, a higher heart rate was indeed observed in mice overexpressing *Casq2* (Jones et al., 1998). Accordingly, bradycardia was reported in *Casq2* knockout mice (Glukhov et al., 2015b).

IP_3Rs may also contribute to the LCR generation via hypersensitization of RyRs. IP_3Rs are another type of SR Ca^{2+} releasing channels which are activated by IP_3 through the hydrolysis of phosphatidylinositol-(4,5)-bisphosphate by phospholipase C. Recent studies demonstrated that this process might be confined within different microdomains including lipid rafts (Delos Santos et al., 2015) and dorsal ruffles

(Gu and Neel, 2003). Stimulation of IP$_3$Rs was found to accelerate a spontaneous beating rate of mouse SANCs likely through regulation of Ca^{2+} spark parameters and RyR open probability (Ju et al., 2011). In heart failure rabbit atrial myocytes, upregulation of IP$_3$R-induced Ca^{2+} releases was detected and linked to the enhanced spontaneous SR Ca^{2+} releases (Hohendanner et al., 2015). Unlike RyRs, IP$_3$Rs seem to express both beneath the sarcolemma membrane and inside SANCs (**Figure 1**), with the ones near membrane found partially co-localized with HCN4, RyR, and SERCA (Ju et al., 2011). In canine ventricular myocytes, a direct interplay between IP$_3$-mediated G$_q$ protein coupled receptor signaling pathway and Cav-3 has been demonstrated (Guo et al., 2011), linking IP$_3$-dependent non-junctional Ca^{2+} activities to subsarcolemmal caveolae. Significant decrease in the occurrence of spontaneous Ca^{2+} sparks was reported in rat and human atrial myocytes treated with MβCD, which depletes cholesterol and destroys caveolae structures (Glukhov et al., 2015a). Stimulation of IP$_3$Rs through sphingosine kinase 1 is shown to control the Ca^{2+} release in caveolar microdomains (Pulli et al., 2015). IP$_3$Rs were also associated with structural protein AnkB: their expression and membrane targeting were found to be significantly reduced in AnkB$^{+/-}$ mice (Le Scouarnec et al., 2008).

Phosphorylation of RyRs, SERCA and phospholamban (PLN), are also suggested to regulate LCRs. cAMP-mediated, PKA- (Vinogradova et al., 2006) and CaMKII-dependent phosphorylation (Vinogradova et al., 2000; Li et al., 2016) of RyRs have been reported to affect the size and rhythm of LCRs in rabbit SANCs. At the same time, knock-in alanine replacement of RyR phosphorylation sites PKA (S2808) or CaMKII (S2814) did not affect heart rate responses to isoproterenol *in vivo* or in isolated SANCs in mice (Wu et al., 2016). Furthermore, Wu et al. (2016) demonstrated that selective mutations of PLN phosphorylation sites PKA (S16) or CaMKII (T17) in mice also did not affect heart rate. Therefore, although phosphorylation of SR Ca^{2+} proteins may contribute in SAN LCRs, they appear to not affect heart rate by one single target site governing SR Ca^{2+} uptake or release. Recent studies found that RyR phosphorylation follows a highly localized pattern and was linked to specific subcellular microdomains including lipid rafts (Younes et al., 2008; Lukyanenko et al., 2016).

cAMP

Although cardiac pacemaking, at rest and during the sympathetic fight-or-flight response, was shown to depend on cAMP signaling in SAN myocytes, cAMP does not directly regulate Ca-clock and SR Ca^{2+} cycling (Lakatta et al., 2010). Instead, it regulates the membrane clock functioning via a direct binding to HCN4 channels. In addition, cAMP modulates the Ca-clock via the regulation of PKA-dependent phosphorylation of SR proteins including RyR, SERCA and PLN. SANCs have a high basal level of cAMP due to a high constitutive activation of adenylyl cyclase (AC), the enzyme that converts adenosine triphosphate to cAMP (Vinogradova et al., 2006). Such high basal level of cAMP may facilitate the periodical LCR activities in the SAN.

With a progress in the development of biosensor techniques, different pools of cAMP activities between the plasma membrane and the bulk cytoplasmic compartment have been observed. Recent studies unveiled that cAMP activity may be more compartmentalized within discrete subcellular microdomains, including lipid rafts and caveolae (Younes et al., 2008). It was shown that activities of some ACs (Younes et al., 2008) and PDEs (Senzaki et al., 2001) that are responsible for cAMP synthesis and degradation, respectively, are restricted to lipid rafts in SANCs. In the SAN, high basal PDE activity (Vinogradova et al., 2008; Hua et al., 2012) is one of the control checkpoints. Inhibition of basal PDEs in SANCs markedly elevates cAMP-mediated phosphorylation of membrane and Ca-clocks' proteins, resulting in an acceleration of spontaneous AP firing rate in the rabbit SAN (Vinogradova et al., 2008). PDE2-4 are the most abundant in the mouse SAN (Hua et al., 2012) where they regulate the beating rate of atrial preparations (Galindo-Tovar and Kaumann, 2008), AP firing rate, and I_{Ca} in SANCs (Hua et al., 2012). In rat ventricular myocytes, inhibition of PDE4, similar to MβCD treatment, results in loss of confined βAR-mediated cAMP signaling, resulting in a cell-wide cAMP signal propagation patterns (Nikolaev et al., 2010). In SANCs, caveolae may localize cAMP signaling to confined subsarcolemmal microdomains, similar to that observed in ventricular myocytes, and thus participate in compartmentalized regulation of both clocks. Downregulation of caveolae scaffolding protein Cav-3 in failing ventricular myocytes (Nikolaev et al., 2010) as well as expression of the dominant negative Cav-3 mutant (Wright et al., 2014), or disruption of caveolae by MβCD (Calaghan et al., 2008), converts the sarcolemmal-confined cAMP signal to a global signal that targets proteins of the SR and myofilaments, increasing cell contractility and CaT amplitude. This was not linked to the modulation of $I_{Ca,L}$, but instead to a discrete PKA-mediated phosphorylation of PLN (Macdougall et al., 2012). Recently, by using a PLN-linked cAMP biosensor, Sprenger et al. (2015) uncovered the existence of compartmentalized SERCA phosphorylation mediated by cAMP in adult mouse ventricular myocytes. Because SERCA activity is critical in the regulation of [Ca^{2+}]$_i$ balance and SR Ca^{2+} cycling in SANCs (Logantha et al., 2016), one could expect that this compartmentalized phosphorylation may be important for the determination of distribution and size of LCRs in the regulation of the Ca-clock.

CaMKII

Ca^{2+}/calmodulin-dependent protein kinase II (CaMKII) is another important component regulating the coupled-clock system. CaMKII is involved in the regulation of both clocks and participates in both physiological (Vinogradova et al., 2000; Li et al., 2016) and pathological (Luo et al., 2013) activities of the SAN. CaMKII is a Ser/Thr protein kinase and is known as one of the major downstream targets of Ca^{2+} signaling. In SANCs, CaMKII senses subcellular Ca^{2+} changes and is activated via binding to Ca^{2+}-calmodulin (CaM) complex at the CaM regulatory domain (Wu and Anderson, 2014). Activated CaMKII

catalyzes phosphorylation of both L- and T-type Ca^{2+} channels, PLN (Grimm and Brown, 2010) and RyRs (Wehrens et al., 2004).

Recent studies suggested that CaMKII activity may be confined to some specific membrane microdomains. Caveolae-specific activation of CaMKII was also detected in cardiomyocytes and linked to caveolae-localized phosphorylation of LTCCs (Tonegawa et al., 2017). In ventricular myocytes, a local Ca^{2+}/reactive oxygen species (ROS) function microdomain was reported, where a cluster of RyRs directly couples to CaMKII and ROS and thus gets a direct modulation from local CaMKII activity (Dries et al., 2013). In such localized microdomains, CaMKII is found to translocate to specific target compartments, and spatial barriers to CaMKII phosphorylation could be overcome by its translocation and anchoring to the substrate itself or to nearby target protein within the localized compartments (Tsui et al., 2005).

These findings highlight a critical role of localized CaMKII-mediated regulation of Ca^{2+} handling proteins and suggest a potential role of microdomain-specific activity of CaMKII in the regulation of SAN pacemaking. Furthermore, CaMKII- and cAMP-mediated regulation are reported to share microdomain location in SAN myocytes where a cross talk between CaMKII and PDE1 is found (Lukyanenko et al., 2016). Deciphering the disruptions of such localized regulation in SAN may create a new direction for SND treatment in the future.

COMPARTMENTALIZED AUTONOMIC REGULATION IN SANCs

Compartmentalized β-Adrenergic Receptors

$β_1$- and $β_2$ARs are the primary sympathetic receptors in the heart and play different roles in the regulation of cardiac rhythm. In early 2000s, researchers already found that both ARs show a compartmentalized distribution in cardiomyocytes: $β_2$ARs are predominantly concentrated in T-tubules and caveolar structures, whereas $β_1$ARs are mainly localized in the non-caveolar membrane and non-lipid raft heavy fractions of plasma membrane (Rybin et al., 2000) (**Figure 1**). Distinct functional domains then were found in cardiomyocytes to conduct $β_1$- and $β_2$-adrenergic signaling (Shcherbakova et al., 2007). The cardiomyocyte membrane was reported to develop into specialized zones associated with scaffold proteins SAP97 and AKAP79/150, where $β_1$ARs are found enriched, whereas, $β_2$ARs are excluded from such microdomains (Shcherbakova et al., 2007) (**Figure 2**). Disrupted compartmentalized pattern of βARs was shown to be involved in ventricular myocyte remodeling associated with heart failure (Nikolaev et al., 2010).

In SAN myocytes, $β_2/β_1$ expression ratio is higher comparing to atrial and ventricular myocytes (Brodde et al., 1982), and a higher efficiency of $β_2$ARs was observed (Brodde et al., 2001). Co-immunoprecipitation and immunocytochemistry studies found in rabbit SANCs that $β_2$ARs co-localized with HCN4 channels in the caveolar microdomain (**Figure 1**), and their disruption via cholesterol depletion by MβCD completely abolished the effect

of $β_2$-adrenergic stimulation, while the effect of $β_1$-adrenergic stimulation still maintained (Barbuti et al., 2007). Altogether, these suggest that the β-adrenergic stimulation in the SAN is presented in a compartmentalized pattern, in which, $β_1$- and $β_2$ARs localize and function in distinct microdomains.

Compartmentalized Muscarinic Receptors

M_2 muscarinic receptors are the primary type of muscarinic receptors expressed in cardiac myocytes. It was shown that M_2 receptors are located outside of caveolar fractions of plasma membrane (Feron et al., 1997). Upon parasympathetic stimulation, the translocation of M_2 receptors to caveolar fractions was detected using a PKA FRET-based biosensor (Warrier et al., 2005). It is reported that the M_2 receptor-mediated cAMP response is associated with distinct AC isoforms expressed in different membrane microdomains (Iancu et al., 2007).

SND AND SCAFFOLDING PROTEINS

Etiology of SND may include both intrinsic and extrinsic reasons. Prevailing intrinsic factors leading to SND are associated with mutations or dysfunctions of key components in the coupled clock systems including HCN4 channel, potassium channel (*KCNQ1*), sodium channel (*SCN5A*), RyR and others. Emerging evidence has shown that dysfunction of structural or scaffolding proteins could also result in SND (**Table 1**). Below, we summarize several scaffolding proteins which dysfunctions could lead to SND.

Ankyrin-B

Ankyrin-B (AnkB) is an important multifunctional scaffolding protein that is essential for membrane structure organization as well as trafficking and localization of various pacemaker proteins. AnkB syndrome, i.e., a type 4 long QT syndrome, is a rare cardiac arrhythmia syndrome, which is associated with a loss-of-function mutation of AnkB in the heart (Mohler et al., 2004). Dysfunctions in AnkB result in SND in human (Le Scouarnec et al., 2008) and mice (Le Scouarnec et al., 2008; Glukhov et al., 2010) and are associated with atrial fibrillation (Le Scouarnec et al., 2008; Cunha et al., 2011).

Heterozygous knocking out AnkB in mice results in significantly reduced expression of NCX, NKA and IP$_3$Rs in the SAN (**Figure 2**); furthermore, AnkB$^{+/-}$ SANCs show abnormal localization of Ca$_v$1.3 and NCX proteins. In contrast to the homogenous membrane distribution of Ca$_v$1.3 channels in wild type SANCs, Ca$_v$1.3 expression in AnkB$^{+/-}$ cells was limited to an internal perinuclear distribution (Le Scouarnec et al., 2008). This was associated with a concomitant decrease in a whole-cell $I_{Ca,L}$ density. Cunha et al. (2011) demonstrated that AnkB directly associates with Ca$_v$1.3, and this interaction is regulated by a short, highly-conserved motif on the C4 region of the C-terminus of Ca$_v$1.3. Importantly, no changes in Ca$_v$1.2 expression and localization were found in AnkB$^{+/-}$ SAN and atrial myocytes. Similar, AnkB$^{+/-}$ mice showed significant

TABLE 1 | SAN pacemaking abnormalities linked to mutations in scaffolding proteins and associated remodeling of the coupled-clock pacemaking system.

Protein	Gene	Species	Condition	Dysfunction	Associated pacemaking component remodeling		Reference
					Membrane clock	Calcium clock	
AKAP10	*AKAP10*	Human	646V	Fast HR; low HRV	AChR-mediated targets	AChR-mediated targets	Tingley et al., 2007
		Mouse	Global I646V	Bradycardia; sinus pauses	AChR-mediated targets	AChR-mediated targets	Tingley et al., 2007
Ankyrin-B	*ANK2*	Human	E1425G	Bradycardia; AF	NCX; NKA	IP$_3$R	Mohler et al., 2003; Le Scouarnec et al., 2008
		Mouse	Global AnkB$^{+/-}$	Bradycardia; high HRV	NCX($\downarrow I_{NCX}$); NKA; Cav1.3 ($\downarrow I_{CaL}$)	IP$_3$R	Le Scouarnec et al., 2008
Caveolin-3	*CAV3*	Human	T78M	Bradycardia; tarchycardia; AF	Kv1.5; HCN4	cAMP signaling	Campostrini et al., 2017
Emerin	*EMD*	Human	Lys37del	Bradycardia; AF	?	?	Karst et al., 2008
Lamin A/C	*LMNA*	Human	A331G	Bradycardia; AF	?	?	Hoorntje et al., 2017
MHC-α	*MYH6*	Human	A721T	Sick sinus syndrome	?	?	Holm et al., 2011
Popeye	*POPDC1.2*	Mouse	Global Popdd, 2$^{-/-}$	Bradycardia; sinus pauses	I_K, I_{Na}		Froese et al., 2012
Spinophilin /neurabin	*PPP1R9B*	Mouse	Global Sp$^{-/-}$	Enhanced bradycardiac response to a-adrenergic stimulation	cAMP-mediated targets	RyR; cAMP signaling	Lu et al., 2010; Chiang et al., 2014

HR: Heart Rate; HRV: Heart Rate Variability

reduction of I_{NCX} current density in SANCs (Le Scouarnec et al., 2008). Altogether, these resulted in SAN pacemaking abnormalities observed both in single SANCs (Le Scouarnec et al., 2008) and isolated SAN preparations (Glukhov et al., 2010).

Caveolin-3

Cav-3, the integral membrane protein that is essential in formation of caveolae (**Figures 1**, **2**), is also linked with SND (Lang et al., 2016; Campostrini et al., 2017). As discussed in the previous sections, Cav-3 is involved in the regulation of multiple ion channels and transporters, including those involved in pacemaking. Cav-3 F97C and S141R mutations have been linked to the long QT type 9 inherited arrhythmia syndrome (LQT9) causing AP duration prolongation which had been attributed to an increase in late Na$^+$ current (Vatta et al., 2006). In spontaneously beating neonatal cardiomyocytes, the expression of the T78M mutation in *CAV3* gene significantly increased AP peak-to-peak variability without altering neither the mean rate nor the maximum diastolic potential (Campostrini et al., 2017) and was associated with a positive shift of activation of HCN4 channels, in a dominant way. The authors also identified a small cohort of patients with supraventricular arrhythmias including sinus tachycardia, bradycardia, and atrial fibrillation where the T78M Cav-3 variant is more frequent than in the general population. Recent preliminary findings from out laboratory indicate that cardiac-specific conditional knock-out of Cav-3 in mice results in significant beat-to-beat heart rate lability linked with suppressed SAN function, enhanced atrial ectopy and paroxysms of alternating periods of tachycardia-bradycardia rhythm (Lang et al., 2016). These results highlight the importance of Cav-3 in supporting functional integrity of the SAN pacemaker complex.

Spinophilin

Another scaffolding protein involved in the SAN pacemaking and dysfunction is spinophilin. Spinophilin is ubiquitously expressed (**Figure 2**) and interacts with a variety of target proteins essential for Ca^{2+} homeostasis and cellular contraction in adult ventricular myocytes (Petzhold et al., 2011). Spinophilin mediates the targeting of protein phosphatase 1 to RyR (Ragusa et al., 2010). Single RyR channel's open probability was observed to increase in spinophilin knockout mice (Chiang et al., 2014). A recent study reported a bradycardic response to α$_2$-adrenergic stimulation found in spinophilin null mice; however, no significant heart rate changes comparing to wild type mice were observed at baseline condition (Lu et al., 2010).

Popdc Protein

The Popeye domain containing (Popdc, *POPDC1-3*) gene family displays preferential expression in skeletal muscle and the heart, and encode membrane proteins harboring an evolutionarily conserved Popeye domain, which functions as a binding domain for cAMP. Popdc proteins are abundantly present in intercalated disks, lateral membranes and T-tubules (**Figure 2**). In the heart, atrial expression of Popdc1 is higher than in the ventricle, and the entire cardiac conduction system, including the SAN, displays the most intense expression levels (Froese et al., 2012). Null mutations of members of the Popdc gene family in mice are associated with a stress-induced sinus bradycardia and prominent sinus pauses. Moreover, the phenotype develops in an age-dependent manner, being absent in the young animal and becoming increasingly severe, as the animals grow older. In addition to cAMP binding site, Popeye domain of POPDC1 has the binding sites of KCNK2 (TREK-1, a member of two-pore K$^+$ channels family) (Froese et al., 2012) and Cav-3 (Alcalay et al., 2013) proteins. It was found that TREK-1 current was

increased twofold in the presence of Popdc1-3 proteins. Along with a prevalent SAN phenotype characterized by bradycardia with frequent episodes of sinus pause following stress in cardiac-specific TREK-1-deficient mice (Unudurthi et al., 2016), this may contribute to SND observed in Popdc-null mice. Finally, Popdc1-null cardiomyocytes showed a statistically significant 70% reduction in caveolae number (Alcalay et al., 2013) which can additionally contribute to SND phenotype via modulation of various pacemaker ion channels in transporters discussed in previous sections.

A-Kinase Anchor Proteins

Another scaffolding protein family linked to SND, is AKAPs. AKAPs localize PKA to a different subcellular compartment, permitting a higher degree of selectivity and specificity of phosphorylation for different downstream PKA substrates. More than 14 different AKAPs have been shown to be expressed in both rodent and human heart tissue including: AKAP5 (AKAP150/79), AKAP7 (AKAP 15/18), gravin (AKAP12), AKAP9 (yotiao) and mAKAP. Though AKAPs' function in the SAN is not clear, those proteins are critically important for $[Ca^{2+}]_i$ regulation and thus may contribute to Ca-clock regulation and SAN pacemaking.

Throughout all AKAPs, AKAP150/79 (AKAP5) is probably the most studied in the heart. AKAP150/79 targets PKA and phosphatases to regions near $Ca_v1.2$ (**Figure 2**) increasing the probability of long openings and coupled gating events between channels. Enzymes known to associate with AKAP150/79 include PKA, protein kinase C, CaM, AC5/6, and PP2B. Sympathetic stimulation of adult cardiomyocytes requires association of AKAP150/79 with a subpopulation of LTCCs coupled with Cav-3 (Nichols et al., 2010). Ablation of AKAP150 in mice with long QT syndrome 8 (LQT8), a disease also known as Timothy syndrome characterized by sinus bradycardia, prolonged QT interval and lethal arrhythmias, restores normal gating in $Ca_v1.2$-LQT8 channels and protects the heart from arrhythmias (Cheng et al., 2011). Dysfunction in AKAP10 has been associated with SAN pacemaking and SND in both mice and humans by effecting vagal regulation of the heart (Tingley et al., 2007).

Other Structural Proteins

A rare variant in MYH6, which encodes protein myosin heavy chain α isoform (MHC-α) is also reported associated with high risk of sick sinus syndrome (Holm et al., 2011). There are also some nuclear structural proteins that have been reported to be involved in the SND. A type II integral membrane protein that anchored to the inner nuclear membrane, emerin (**Figure 2**), whose mutation is known to develop Emery-Dreifuss Muscular Dystrophy (Emery and Dreifuss, 1966), is reported to associate

with SND with underlying mechanism yet to illustrate (Karst et al., 2008). Mutations in the lamin A/C gene LMNA are reported to cause a variety of heart diseases including SND (**Figure 2**) (Hoorntje et al., 2017). Though the direct mechanisms of nucleus-associated scaffolding protein-induced modulation of SAN activity are unknown, it might be linked to pacemaker protein expression and/or trafficking.

SUMMARY

Contemporary evidence clearly demonstrates an emerging role of compartmentalized, i.e., associated with distinct, spatially-confined microdomains, organization of pacemaker signaling complexes in the SANCs. Disruption in subcellular targeting of pacemaker proteins and associated signaling molecules upon structural remodeling of the SAN, may affect their biophysical properties and neurohormonal regulation as well as protein–protein interactions within the pacemaker signaling complex disturbing rhythmic generation of APs and thus contributing to the pathophysiology of the SND. These are clear from patients and animal models with genetic defects of scaffolding proteins which are closely associated with SND via the indirect changes of key components in the coupled-clock systems in terms of protein expression, functioning and membrane localization. This extends beyond the classical concept of electrical remodeling, according to which dysfunction can be explained by straightforward increases or decreases in protein expression alone, and adds a new dimension to cardiovascular disease. It thus introduces a novel framework for therapeutic approaches for pacemaker dysfunction treatment targeted at preventing the degradation of cardiac cytoarchitecture.

AUTHOR CONTRIBUTIONS

AG and DL substantially contributed to the conception and design of the work; the acquisition, analysis or interpretation of the data and literature; drafting the work critically for important intellectual content; provide approval for publication of the content; agree to be accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved.

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