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Na/Ca exchange in the atrium: role in sinoatrial node pacemaking and excitation-contraction coupling

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Abstract

Na/Ca exchange is the dominant calcium (Ca) efflux mechanism in cardiac myocytes. Although our knowledge of exchanger function (NCX1 in the heart) was originally established using biochemical and electrophysiological tools such as cardiac sarcolemmal vesicles and the giant patch technique [1–4], many advances in our understanding of the physiological/ pathophysiological roles of NCX1 in the heart have been obtained using a suite of genetically modified mice. Early mouse studies focused on modification of expression levels of NCX1 in the ventricles, with transgenic overexpressors, global NCX1 knockout (KO) mice (which were embryonic lethal if homozygous), and finally ventricular-specific NCX1 KO [5–12]. We found, to our surprise, that ventricular cardiomyocytes lacking NCX1 can survive and function by engaging a clever set of adaptations to minimize Ca entry, while maintaining contractile function through an increase in excitation-contraction (EC) coupling gain [5, 6, 13]. Having studied ventricular NCX1 ablation in detail, we more recently focused on elucidating the role of NCX1 in the atria through altering NCX1 expression. Using a novel atrial-specific NCX1 KO mouse, we found unexpected

changes in atrial cell morphology and calcium handling, together with dramatic alterations in the function of sinoatrial node (SAN) pacemaker activity. In this review, we will discuss these findings and their implications for cardiac disease.

Keywords

sodium-calcium exchange; NCX1; excitation-contraction coupling; sinoatrial node; cardiac pacing; transverse axial tubules; small K channels; IP3 receptors; calcium dynamics

Introduction

Normal atrial function is essential for cardiac performance and accounts for about 20% of cardiac output (l/min). The contribution of atrial pumping can rise as high as 40% in patients with heart failure, including both heart failure with preserved or reduced ejection fraction (HFpEF or HFrEF) [14, 15]. Thus, the study of atrial cell contractility, and the role of NCX1 as a major regulator of Ca efflux (and thus sarcoplasmic reticulum [SR] load), is particularly important for these populations.

Moreover, because the right atrium harbors the SAN, the primary pacemaker of the healthy heart, abnormal Ca regulation in atrial cells may not only lead to contractile dysfunction, but also to the pathogenesis of common rhythm disturbances. These include atrial fibrillation, one of the leading causes of stroke (15–20% of all strokes) in the United States [16], and Sick Sinus Syndrome (SSS), a major cause of morbidity and mortality [17]. In most cases, the only available treatment is the placement of costly electronic pacemakers, which poses significant complication risks [16, 18]. Thus, a continued exploration of the basic mechanisms which drive cardiac pacemaking has the potential to have major therapeutic impacts in cardiac pathophysiology.

Over the last 15 years, several lines of evidence suggested that SAN pacemaker activity is generated by two cellular "clocks": a "membrane clock" generated by the funny current (I_f) through hyperpolarization-activated cyclic nucleotide-sensitive (HCN4) channels, and a "calcium clock" generated by intracellular Ca cycling [18–22]. I_f activates when the SAN cell repolarizes to its maximum diastolic membrane potential (~ -70 mV) [19, 20]. The inward sodium-mediated I_f depolarizes the cell in diastole until it reaches the threshold for activation of Ca current (I_{Ca}), which then triggers an action potential (AP) [23]. The "Ca-clock" is thought to depend upon spontaneous local Ca release (LCR) from the SR [19]. The released Ca is exchanged for Na by NCX1, producing an inward current which depolarizes the membrane [24]. The entrained membrane and Ca clocks, or "coupled-clocks," work together to maintain rhythmic cardiac activity [19, 25]. However, evidence in favor of a pivotal role of NCX1 in the Ca clock was originally based on pharmacologic approaches using non-specific agents against NCX1 [24] or computer simulation [26]. Here we review how we used the atrial-specific NCX1 KO mouse to more clearly define the role of NCX1 in the Ca clock without the limitations of pharmacology.

Generation of the atrial-specific NCX1 knockout mouse

A breakthrough methodology for generating an atrial-specific KO mouse was the development of the sarcolipin (SLN)-Cre mouse by the Nakano lab at UCLA [27]. These mice express Cre under the control of the endogenous SLN promoter, which is exclusively expressed in the atria of the murine heart. When mated with our previously described NCX1 exon 11 floxed mice (NCX1$^{fx/fx}$), the SLN-Cre mouse produces atrial-specific NCX1 KO mice [28]. These mice survive into adulthood with normal behavior and physical activity despite the complete absence of NCX1 in the atria, as verified by western blot and single cell electrophysiology [28]. Ventricles exhibit normal NCX1 expression and atria are dilated, while the alternative Ca efflux protein, plasma membrane Ca pump (PMCA) is increased [28].

NCX1 in SAN pacemaking

In-vivo electrocardiography

One of the most striking aspects of the atrial-specific NCX1 KO mouse is the lack of P-waves on surface electrocardiograms (ECG, Fig. 1B). This indicates an absence of atrial depolarization during normal cardiac function, which we confirmed by direct recordings of atrial electrograms. In addition, the heart rate is much slower in the NCX1 KO mouse than in wildtype (WT) (Fig. 1C), but with a narrow QRS, indicative of a "junctional" escape rhythm conducted normally through the His-Purkinje system [28]. We did not detect retrograde conduction to the atrium, suggesting either block in the atrioventricular (AV) node, or an inability of the atria to depolarize in the absence of NCX1. The latter seems unlikely since we were able to pace the atrial muscle tissue using external electrodes. These are topics of ongoing studies in our laboratories. Despite these alterations, we never detected atrial fibrillation in the atrial NCX1 KO mice. However, in an HCN4-cell-specific NCX1 KO mouse (which is not limited to the SAN) it has been reported that there are intermittent P waves on ECG, suggestive of the clinical bradycardia entity known in humans as "sick sinus syndrome" [29].

Isolated SAN cell electrophysiology in the atrial-specific NCX1 KO

In order to resolve the Ca versus membrane clock debate, we used the current clamp mode of the whole cell patch clamp technique to record spontaneous activity from single SAN cells enzymatically isolated from WT and NCX1 KO mice. We found that spontaneous APs occurred regularly in WT SAN cells but were completely absent in the slightly depolarized NCX1 KO SAN cells (Fig. 1D). Although the cells were indeed capable of generating APs in response to current injection, lowering the external potassium concentration to hyperpolarize the cells to WT diastolic membrane potential did not restore normal rhythm. We also confirmed in patch clamped cells that there was no reduction in HCN4 current and only subtle changes in voltage dependence and activation kinetics, indicating that I_f by itself is insufficient to produce spontaneous action potentials in SAN cells. Interestingly, we observed almost a 50% reduction in I_{Ca} (Fig. 1E), caused by Ca-dependent inactivation [28], a phenomenon which also occurs in our ventricular NCX1 KO [12]. These data indicate that

NCX1 is essential for spontaneous pacemaking activity in isolated SAN cells and is consistent with the Ca clock hypothesis.

Finally, we observed that Ca cycling of the clock initiation mechanism was not disrupted in the NCX1 KO. Indeed, confocal line scans of isolated SAN cells revealed LCRs (Fig. 5) or Ca waves [28] (Fig. 2B) occurring in a periodic pattern and at a similar rate to the spontaneous APs observed in WT mice (Fig. 2A). Our findings therefore support the essential role of NCX1 as a bridge between internal Ca cycling, LCRs and membrane depolarization to generate pacemaker APs in synchrony with the membrane clock. In other work, we were able to use the atrial NCX1 KO to demonstrate that IP$_3$R agonists and antagonists, such as 2-aminoethoxydiphenyl borate (2-APB), can alter pacing rate by influencing the Ca clock, in a manner completely independent to the membrane clock [30] (Fig. 2). The role of IP$_3$Rs in pacing is well described in embryonic stem cell-derived cardiomyocytes [31, 32].

SAN and atrial depolarization in the multicellular ex vivo tissue preparation

Although we found no evidence of spontaneous APs in SAN cells isolated from NCX1 KO mice, and no P waves or atrial electrical activity recorded in live mice and explanted Langendorff preparations, we felt it was important to examine the SAN in intact tissue. For this purpose we used an *ex vivo* SAN/atrial tissue preparation which keeps the SAN and atrial muscle structures intact, but removes potentially electrically-interfering tissue, such as the AV node [33]. Using optical voltage mapping, we demonstrated that there is indeed spontaneous depolarization of the intact SAN in the NCX1 KO preparation. However, the depolarizations were irregular and intermittent, following a burst/pause pattern that was not conducted to the surrounding atrial tissue [33] (Fig. 3A). To explain these findings, we explored the extent of tissue fibrosis through Masson's Trichrome staining. Consistent with our previously reported findings in the ventricular-specific NCX1 KO, there was increased fibrosis in the NCX1 KO atria [9] (Fig. 5). This atrial fibrosis could be a consequence of Ca accumulation caused by reduced Ca efflux in the absence of NCX1. Indeed, Ca accumulation is associated with the activation of apoptotic pathways, which could lead to fibrosis [34, 35]. However, we were unable to explain why isolated SAN cells are silent even though the intact SAN tissue demonstrates spontaneous electrical activity.

To better understand the pacemaker activity generated by the intact NCX1 KO SAN, we examined the SAN tissue preparation using high speed 2D confocal Ca imaging. Because the SAN in the mouse is only one or two cell layers thick, live confocal imaging of this structure is possible by loading the whole preparation with the Ca indicator Cal520 AM. Using this approach, we observed regular Ca transients in WT preparations, but in the NCX1 KO we saw intermittent Ca transients, interrupted by periods of quiescence during which we observed an abundance of intracellular Ca waves [33] (Fig. 3B). Ca waves are often a sign of intracellular Ca overload, which would not be surprising in the absence of NCX1. Elevated cellular and subsarcolemmal Ca could alter the behavior of several families of ion channels in a way that might negatively affect the ability of the cell to spontaneously depolarize. To address this possibility, we buffered intracellular Ca using the cell permeable form of BAPTA (BAPTA AM). Under this buffered condition, we demonstrated improved/longer

trains of Ca transients. Conversely, when we used the Ca channel agonist BayK to increase I_{Ca} and Ca entry, we further depressed SAN function. This experiment points to the importance of Ca efflux to maintain the right conditions for pacemaker generation. But it also suggests that the reduced I_{Ca} amplitude in the NCX1 KO does not independently inhibit spontaneous APs (Fig. 3).

Role of small conductance Ca-activated potassium channels

The family of Ca-activated K channels plays an important role in the regulation of membrane potential in several dynamic systems, particularly in neuroendocrine tissues such as chromaffin cells [33, 36–38]. Some of these Ca-activated channels, e.g. the large and medium conductance K channels, have been implicated in the generation of SAN pacemaker activity [39, 40]. Moreover, alternations of bursts and pauses with associated spike adaptation are signatures of Ca-dependent activation of small conductance K (SK) channels in neuroendocrine tissues [36]. Although SK channels have been characterized previously in ventricular and atrial tissue, they had not been identified in SAN cells. Thus, we proceeded to look for evidence of SK channels in the SAN using pharmacologic, immunologic, and electrophysiological approaches. We found that SK channels are indeed present in the SAN of the mouse [41]. Furthermore, we were able to demonstrate that selectively blocking these channels by apamin restored a more regular rhythm with reduced adaptation and shorter pauses [41]. Thus, we conclude that 1) SK channels are present in the SAN and 2) activation of SK channels by elevated intracellular Ca in the NCX1 KO mouse is the most likely cause of spike adaptation and subsequent pauses interrupting the bursts of action potentials perpetuated by I_f.

Excitation-contraction coupling and the transverse-axial tubule system in the atrial NCX1 KO

Transverse-axial tubules (TATs) are invaginations of surface sarcolemma in cardiomyocytes that allow LCCs on the surface membrane to reside in close proximity to the intracellular RyRs decorating the junctional SR, thereby forming Ca release units, or "couplons." The presence of couplons along TATs allows for synchronous triggering of RyRs and release of peripheral and central Ca to facilitate coordinated contraction of the cell [42]. TATs are universally present in ventricular cardiomyocytes. When the Ca release process is studied in live cells using the line-scan mode of the laser scanning confocal microscope, a normal synchronized Ca release pattern in ventricular myocytes is typically observed to be "I"-shaped. Until recently, TATs were assumed to be sparse or absent in atrial myocytes (especially from small animals such as rats and cats), producing a non-uniform "V"-shaped Ca release pattern when studied with confocal line-scan imaging. The "V" shape in the absence of TATs is a consequence of rapid Ca release triggered by APs at the cell surface, followed by a slower spread of Ca into the cell center by diffusion-mediated triggering of ryanodine receptors [43, 44]. As we began to characterize Ca release patterns in NCX1 KO atrial myocytes, we were surprised to discover that WT mouse atrial myocytes had extensive TATs expression (male>female) observed with Di-4 ANEPPS. We also found corresponding "I"-shaped Ca transients on confocal line scans (Fig. 4A), resembling those in ventricular

cells [45]. In contrast, we found a dramatic loss of TATs in NCX1 KO atrial myocytes before and after isolation [46] (Fig. 5). As a consequence of TAT loss, Ca transients began at the periphery of the cell and then propagated to the center, producing the classical "V"-shaped line scan confocal activation pattern (Fig. 4B). To assess the influence of NCX1 and its loss on Ca transients in atrial myocytes, we used a formamide-induced detubulation method to produce atrial cells with normal NCX1 but no TATs, which we compared with the NCX1 KO atrial myocytes that also lacked TATs [47]. In WT cells, loss of TATs corresponded to a slowing and lower amplitude of central Ca release (Fig. 4C) [46]. These relative changes were similar in NCX1 KO myocytes. Indeed, the kinetics of Ca propagation from the periphery to cell center was not altered in KO cells. However, Ca transient decay rates close to the sarcolemma were much slower in the NCX1 KO cells compared to WT detubulated cells, consistent with lack of NCX1 and reduced extrusion rate of Ca from the subsarcolemmal space (Fig. 4D) [46].

Since humans are known to have extensive atrial TATs, our identification and characterization of heterogeneous TATs expression in WT mouse atrial myocytes supports the relevance of using murine models of human disease to study atrial excitation-contraction coupling and arrhythmogenesis [48]. TAT depletion in the atrial-specific NCX1 KO mouse suggests that insufficient Ca efflux and consequent Ca overload promotes TAT remodeling through a Ca-sensitive process, a phenomenon which requires further study.

Conclusion

Knockout mouse models of NCX1 in the ventricle have provided a treasure trove of knowledge about the role of NCX1 in Ca regulation, as well as the clever adaptations that cells use to maintain contractility while keeping Ca overload at bay in the absence of the dominant Ca efflux protein [6, 12, 13]. Using the atrial-specific NCX1 KO mouse, we find that NCX1 is essential for normal SAN function, that pacemaker rate can be influenced by manipulation of IP_3Rs, and that lack of NCX1 is associated with loss of TATs in atrial myocytes along with changes in the kinetics of Ca transients [28, 30, 33, 46].

There are two aspects of SAN dysfunction that are produced by the lack of NCX1 (Fig. 5). First, the absence of NCX1 disables the ability of the Ca clock to depolarize the membrane. It is important to note that in the absence of a functioning Ca clock, I_f is unable to produce spontaneous depolarization in single SAN cells. Second, in the intact SAN/atrial tissue preparation, the cooperative mechanism created by the network of cells and I_f along with I_{Ca} appears to be sufficient to generate intermittent beating. However, the absence of NCX1 produces Ca overload, which interrupts pacing by 1) activating Ca-sensitive SK channels and 2) by reducing the amplitude of depolarizing Ca current. These findings may be important for understanding SAN dysfunction in humans. Human SAN disease is frequently attributed to fibrosis [49], which we also observe in our KO model. This fibrosis could be prompted in part by Ca overload similar to what we observe in our ventricle-specific NCX1 KO model. In addition, our studies highlight how Ca overload itself can interrupt normal pacemaker function simply by hyperactivating pathological K^+ currents. As of yet, NCX1 mutations have not been identified in patients with SAN dysfunction. Whether NCX1 is

essential for normal AV node function is unknown at this time, and is a subject of ongoing study in our laboratory.

Our results also show that IP$_3$Rs have a measurable effect on spontaneous pacing rate, a consequence of modulating the Ca clock, which supports development of IP$_3$ signaling modulators for regulation of heart rate [30]. This is particularly relevant to heart failure where IP$_3$Rs are upregulated.

We find that NCX1 ablation produces profound morphological changes in atrial cardiomyocytes, i.e. extensive loss of TATs, a phenomena we do not observe in ventricular myocytes isolated from the ventricular-specific NCX1 KO [46]. Presumably, loss of TATs in the atrial model is a consequence of abnormal Ca regulation and activation of a Ca-dependent remodeling program. Why this does not occur in the ventricular NCX1 KO, even though there is hypertrophy and fibrosis, is not clear. Neonatal cardiomyocytes in rat, and presumably other mammalian cardiomyocytes, are devoid of TATs, which develop postnatally over 2–3 weeks [50]. We do not know if TATs are present at any stage during the life of the atrial-specific NCX1 KO mouse. Further insights into these questions may be obtained using an inducible KO system in adult mice, an ongoing project in our laboratory.

Glossary

AP	action potential
2-APB	2-aminoethoxydiphenyl borate
Ca	calcium
HCN4	hyperpolarization activated cyclic nucleotide-gated cation channel 4
I$_{Ca}$	calcium current
I$_f$	funny current
IP$_3$	inositol-1,4,5-trisphosphate
IP$_3$R	IP$_3$ receptor
K	potassium
KO	knockout
LCR	local Ca^{2+} release
Na	sodium
NCX1	cardiac sodium–calcium exchanger
RyR	ryanodine receptor
SAN	sinoatrial node
SERCA	sarcoplasmic/endoplasmic reticulum Ca^{2+} ATPase

|	
SR	sarcoplasmic reticulum
TATs	transverse-axial tubules
WT	wildtype

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Highlights

- We made an atrial-specific NCX1 KO mouse to study SAN automaticity and EC coupling.

- Isolated SAN cells from the NCX1 KO lack spontaneous action potentials.

- In NCX1 KO SAN tissue, Ca-activated small K channels cause abnormal sinus pauses.

- NCX1 is thus essential for normal operation of the so-called "calcium clock."

- Similar to humans, mouse atrial myocytes exhibit transverse axial tubules (TATs).

- TATs are lost in the atrial myocytes of the atrial-specific NCX1 KO mouse.

- Absent atrial TATs, triggered Ca release occurs only near the sarcolemma.
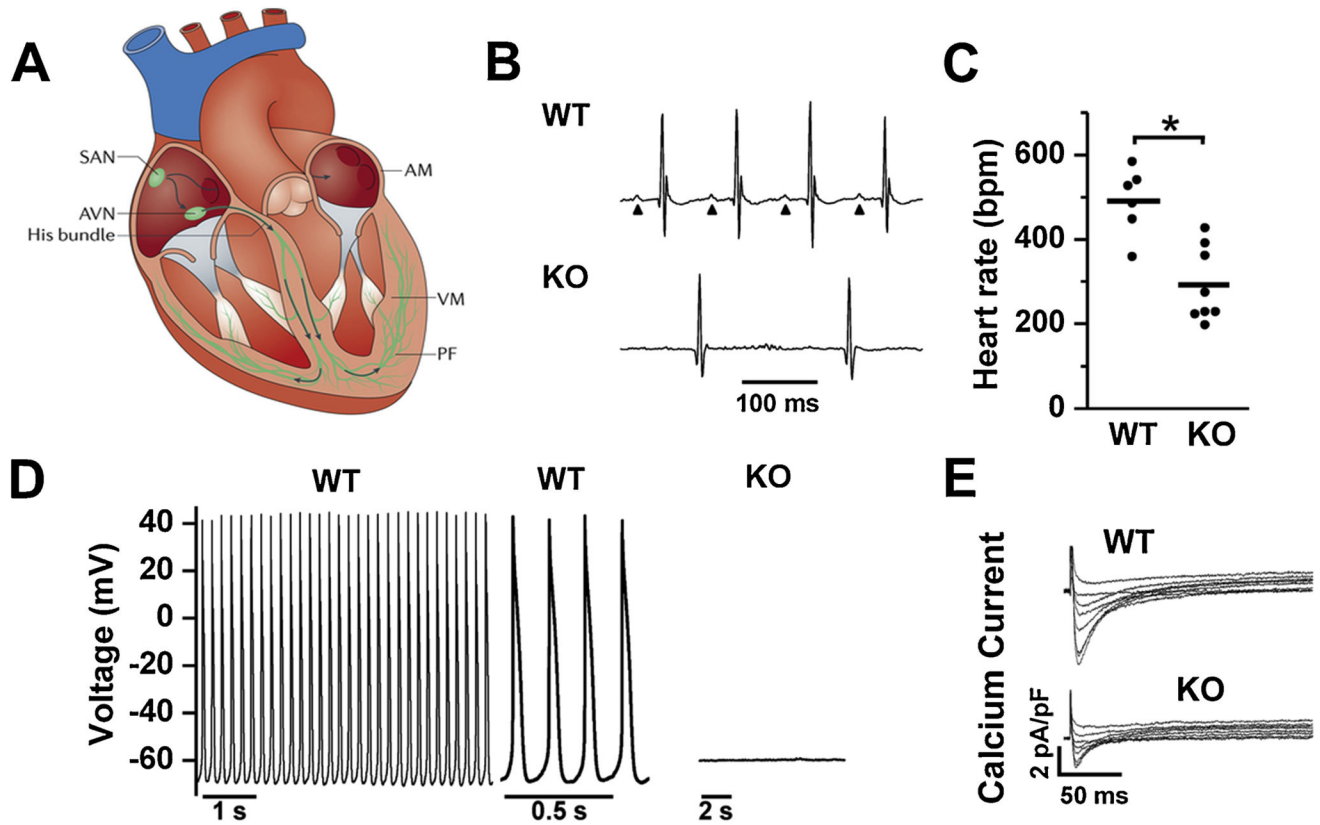
**Figure 1. Sinus arrest and junctional rhythm in atrial-specific NCX1 KO mice.**
A. Schematic representation of the anatomy and cellular electrophysiology of the cardiac conduction system. The cardiac impulse (arrows) originates in the sinoatrial node (SAN), travels across the atrial myocardium (AM), and moves through the atrioventricular node (AVN), the His bundle, and left and right bundle branches. The simultaneous activation of both bundle branches and their terminal Purkinje fibers (PF) provides antegrade activation of the ventricular myocardium (VM) in a synchronized fashion. **B**. Representative telemetry ECGs from WT and atrial-specific NCX1 KO mice. WT mice were in normal sinus rhythm, with each P wave (arrows) followed by a typical murine QRS complex. In KO mice, P waves were conspicuously absent and a slow junctional escape rhythm (narrow QRS) was present. **C**. Mean ventricular (heart) rate in KO mice was lower than in WT mice (thick line, *<P,0.001). **D**. Spontaneous action potentials occurred at regular intervals in patch clamped WT SAN cells, but were absent in NCX1 KO SAN cells. **E**. Raw traces showing L-type Ca current (normalized to cell capacitance) recordings from representative whole cell patch clamped WT and KO SAN cells depolarized from a holding potential of −40 mV to test potentials ranging from −30 to +40 mV at 10 mV intervals. Modified from Groenke et al, 2013 [28].
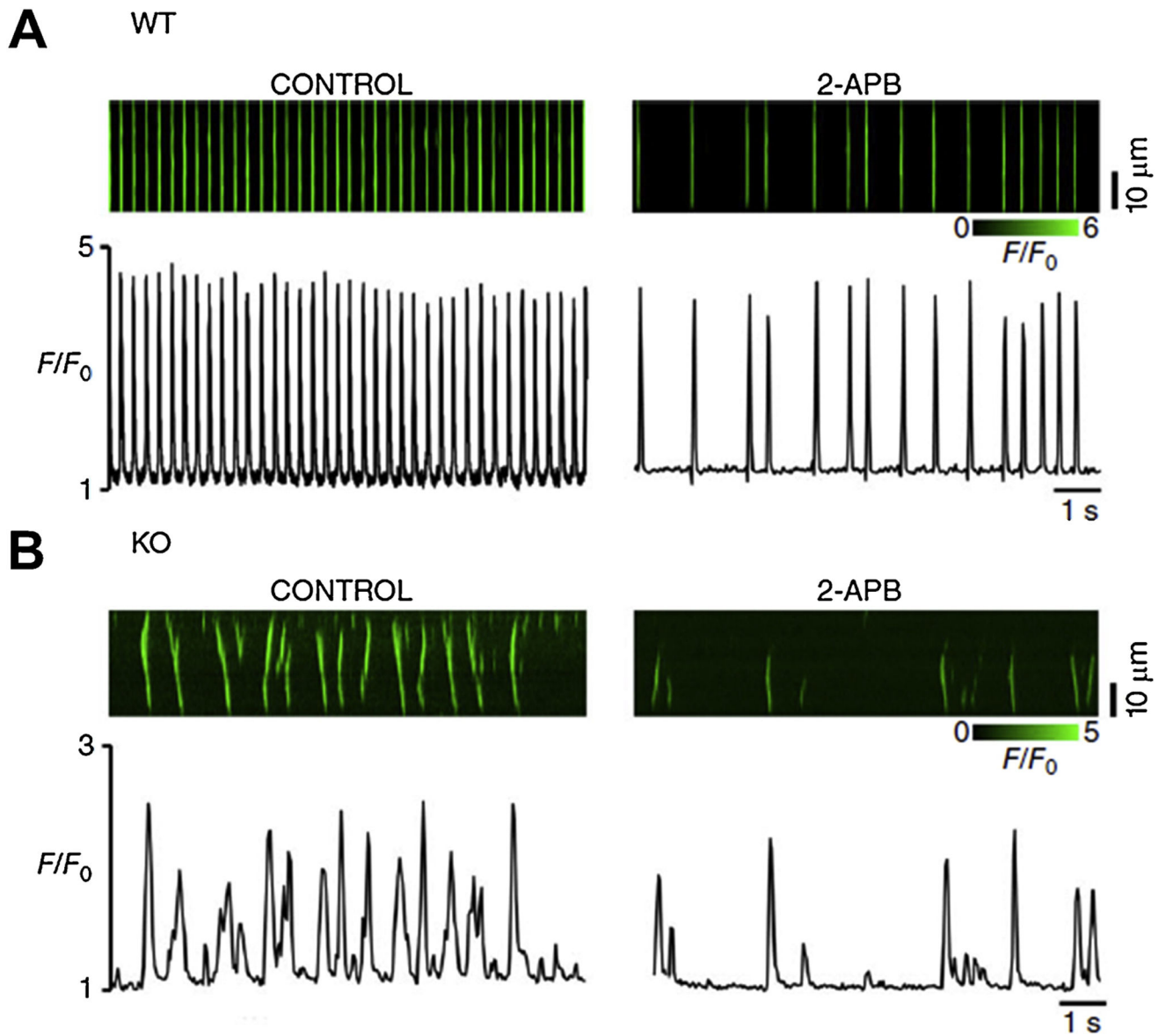
Figure 2. Ca oscillations in WT and NCX KO SAN cells loaded with fluo-4: involvement of IP$_3$ Receptors.

A-B. Confocal linescan images and corresponding fluorescence intensity plots of representative WT (**A**) and NCX1 KO SAN cells (**B**) before (CONTROL) and after superfusion with the IP$_3$ antagonist 2-APB (2 μM). Note that both spontaneous action potential-mediated Ca transients (in WT) and Ca waves (in KO) slow in response to 2-APB, consistent with modulation of the Ca clock by IP$_3$ receptors. Modified from Kapoor et al, 2015 [30].
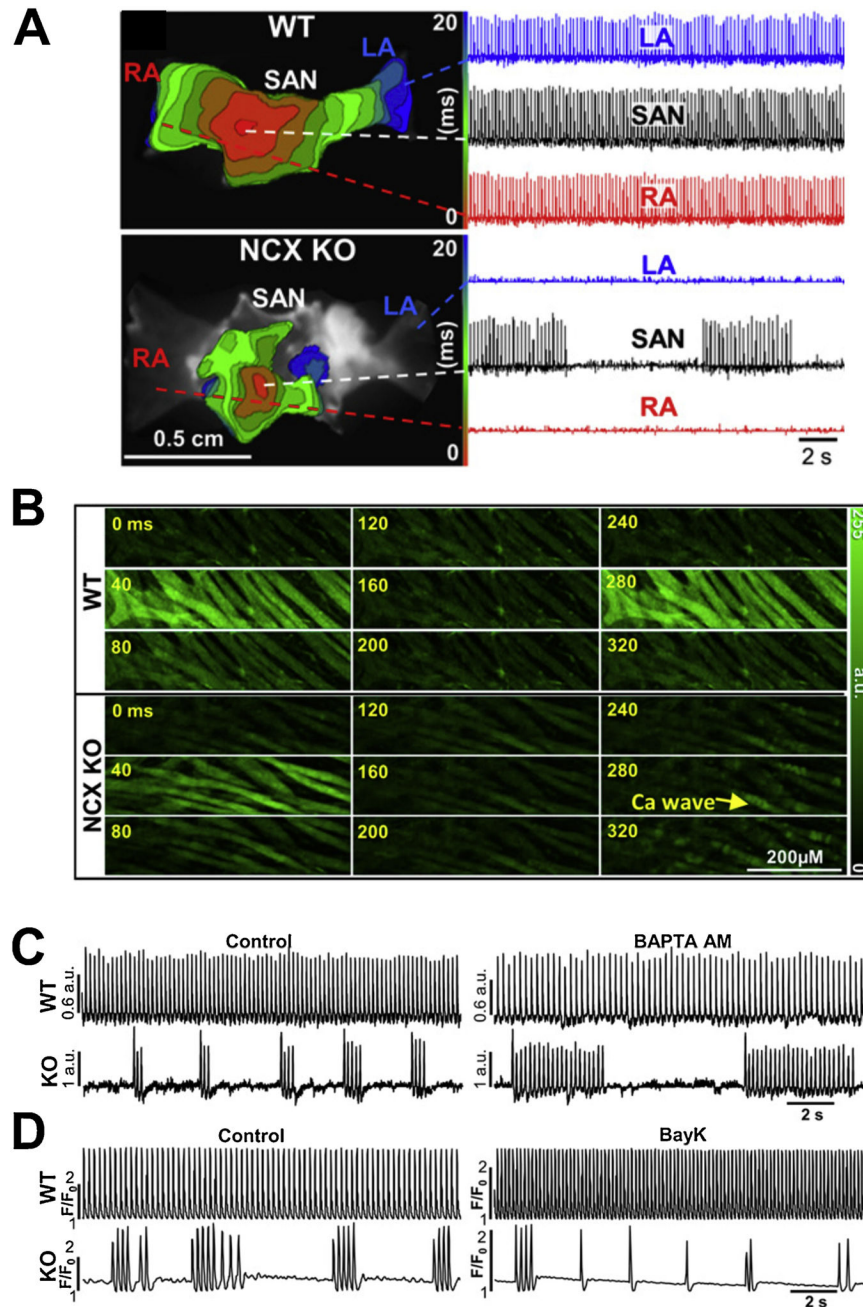
Figure 3. Optical voltage mapping of explanted SAN/atrial tissue and Ca dynamics in the intact SAN tissue.
A. Isochronal voltage maps from WT and NCX KO tissue (Left) and corresponding optical recordings of APs from discreet locations (Right). **B**. Time series of 2D confocal images of Ca [Cal-520 fluorescence in arbitrary units (a.u.); 50-Hz acquisition speed] in WT and NCX KO SANs. **C**. Optical recordings of spontaneous APs in WT (Upper) and NCX KO (Lower) SAN tissue, before (Left) and after (Right) incubation with BAPTA AM (2 μM). **D**. Confocal recordings of Ca transients in WT (Upper) and NCX KO (Lower) SAN tissue, before (Left) and after (Right) perfusion with BayK (1 μM). Modified from Torrente et al, 2015 [33].
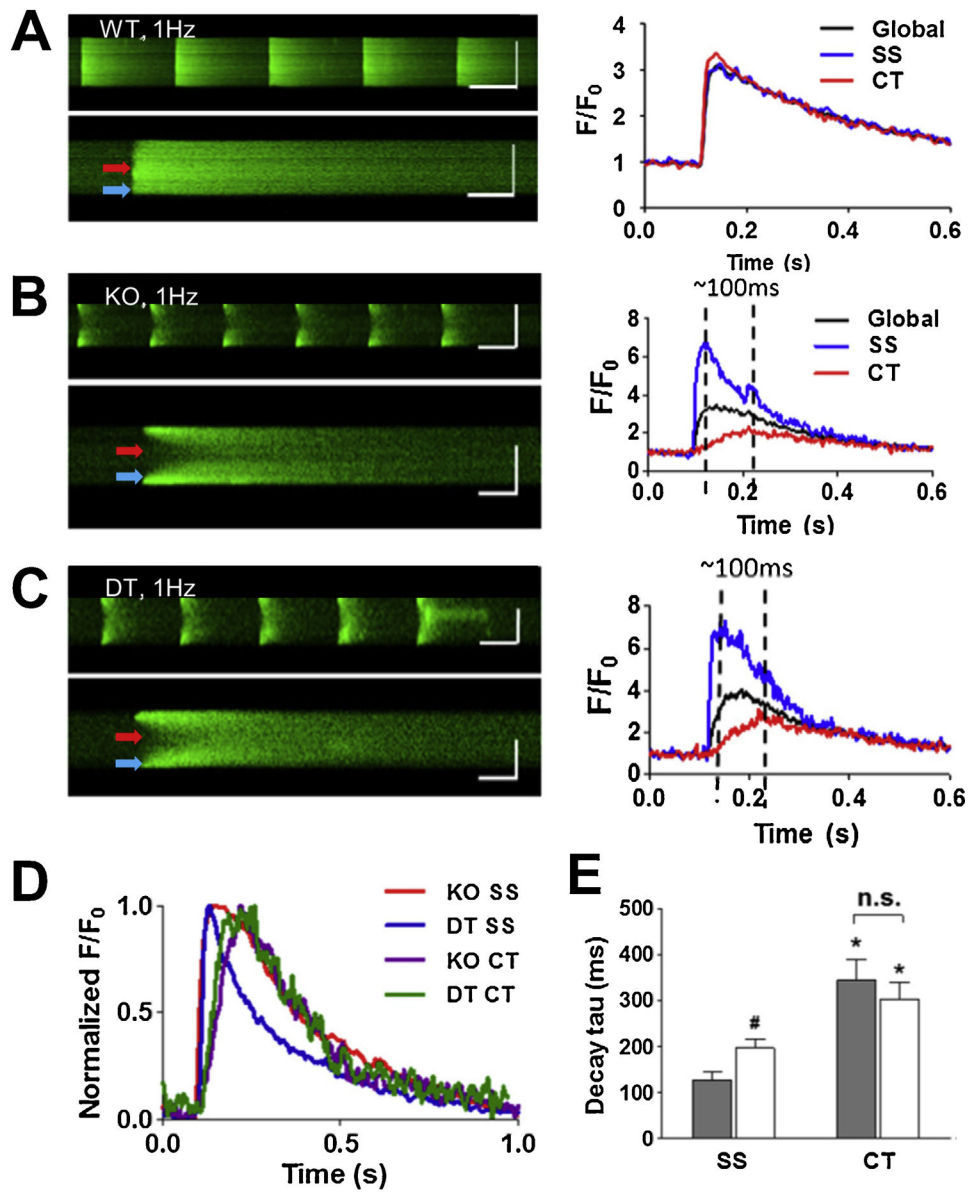
Figure 4. Ca release in control and NCX1 KO atrial myocytes.
A. Left, Confocal line-scan images of five Ca transients (CaT) from a representative atrial myocyte loaded with Fluo-4-AM and field stimulated at 1 Hz (upper image), and a single CaT taken from the same cell but shown on an expanded time scale (lower image). Horizontal scale bars represent 500 ms (upper panel) and 100 ms (lower panel), while vertical scale bars represent 10 μm for both images. Right, fluo-4 fluorescence intensity (F/F_0) plots over time from the cell in A, at 1.5 μm from the subsarcolemmal space (SS, blue) or in the cell center (CT, red). Color coded arrows on the image correspond to the locations where the fluorescence was sampled for intensity plots. The global CaT is shown in black. Note the typical synchronous Ca release and uptake at both locations (with the global trace overlapping the SS and CT traces). **B**. Left, representative line-scan images collected as in A, but for an NCX1 KO atrial myocyte. Note the dramatically different

kinetics of the SS (directly triggered) versus CT (propagated) locations. **C**. Similar to A and B but in a detubulated (DT) WT myocyte. **D**. Normalized, field stimulation-induced intracellular CaTs from representative DT and NCX1 KO myocytes at the SS regions (DT, blue; NCX1 KO, red), as well as the CT regions (DT, green; NCX1 KO, purple). **E**. Summary plot of the local CaT decay tau from SS and CT regions in DT (grey) and KO (white) atrial myocytes. *P < 0.05, CT vs. SS, #P < 0.05, KO vs. DT, unpaired Student's t-test, n = 9 cells from 3 animals for DT, n = 27 cells from 6 animals for KO. Note the slowed decay in the SS region of NCX1 KO compared to DT. Modified from Yue et al, 2017 [46].
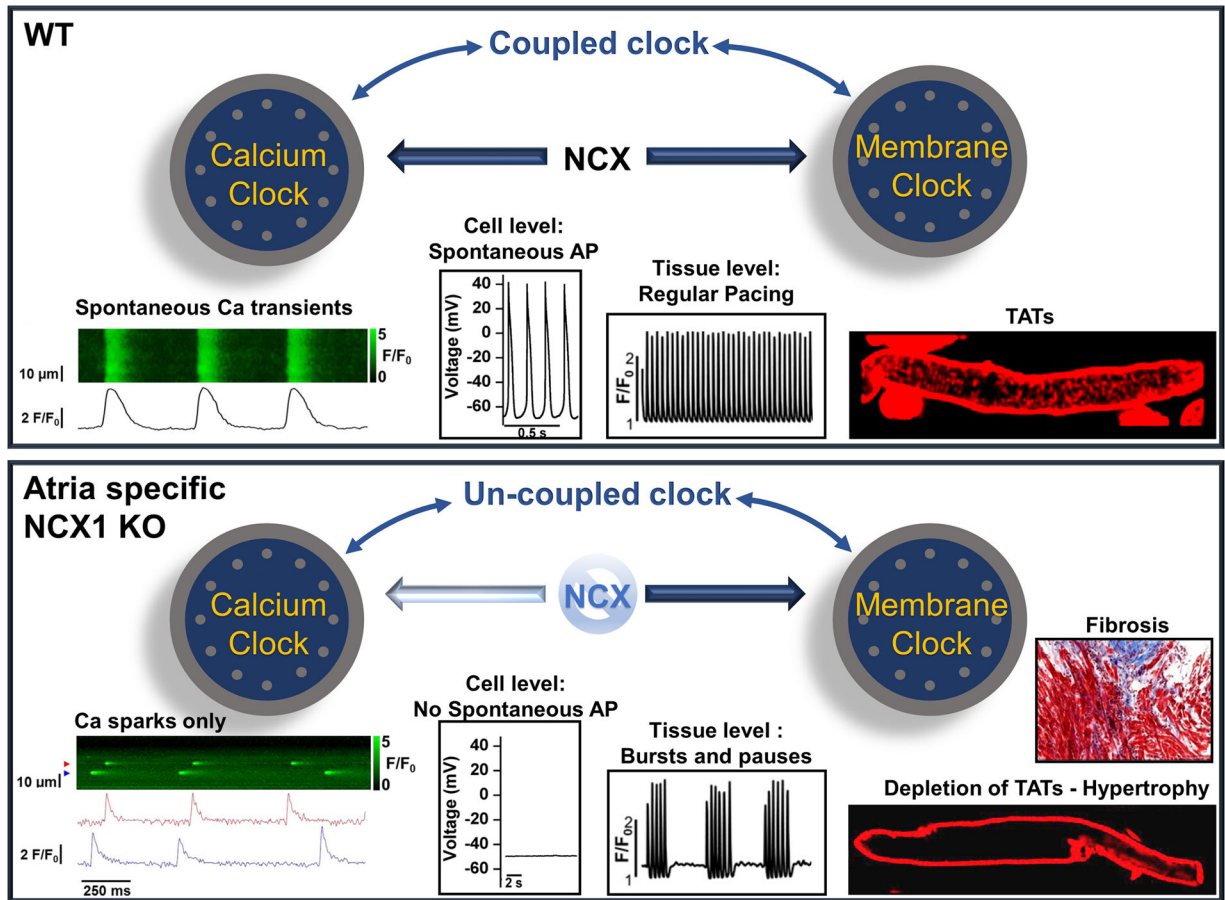
Figure 5. Summary comparison of SAN function in WT versus NCX1 KO.
In WT mice (upper panel), a calcium clock based on NCX1 activation by LCRs depolarizes the membrane at rhythmic intervals, which leads to a large Ca transient. This in turn generates regular action potentials both at the cell and tissue level. In addition, extensive TATs contribute to the microdomain co-localization between LCRs and the membrane. In the KO, the absence of NCX1 disengages the link between the Ca clock and membrane depolarization, illustrated by normal LCRs but no calcium transient or spontaneous action potentials. Ablation of NCX1 also promotes remodeling characterized by loss of TATs, hypertrophy and fibrosis.