

ORIGINAL ARTICLE



# Secretoneurin Is an Endogenous Calcium/Calmodulin-Dependent Protein Kinase II Inhibitor That Attenuates Ca<sup>2+</sup>-Dependent Arrhythmia

See Editorial by Morrow and Marx

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et al

**BACKGROUND:** Circulating SN (secretoneurin) concentrations are increased in patients with myocardial dysfunction and predict poor outcome. Because SN inhibits CaMKII $\delta$  (Ca<sup>2+</sup>/calmodulin-dependent protein kinase II $\delta$ ) activity, we hypothesized that upregulation of SN in patients protects against cardiomyocyte mechanisms of arrhythmia.

**METHODS:** Circulating levels of SN and other biomarkers were assessed in patients with catecholaminergic polymorphic ventricular tachycardia (CPVT; n=8) and in resuscitated patients after ventricular arrhythmia-induced cardiac arrest (n=155). In vivo effects of SN were investigated in CPVT mice (RyR2 [ryanodine receptor 2]-R2474S) using adeno-associated virus-9-induced overexpression. Interactions between SN and CaMKII $\delta$  were mapped using pull-down experiments, mutagenesis, ELISA, and structural homology modeling. Ex vivo actions were tested in Langendorff hearts and effects on Ca<sup>2+</sup> homeostasis examined by fluorescence (fluo-4) and patch-clamp recordings in isolated cardiomyocytes.

**RESULTS:** SN levels were elevated in patients with CPVT and following ventricular arrhythmia-induced cardiac arrest. In contrast to NT-proBNP (N-terminal pro-B-type natriuretic peptide) and hs-TnT (high-sensitivity troponin T), circulating SN levels declined after resuscitation, as the risk of a new arrhythmia waned. Myocardial pro-SN expression was also increased in CPVT mice, and further adeno-associated virus-9-induced overexpression of SN attenuated arrhythmic induction during stress testing with isoproterenol. Mechanistic studies mapped SN binding to the substrate binding site in the catalytic region of CaMKII $\delta$ . Accordingly, SN attenuated isoproterenol induced autophosphorylation of Thr287-CaMKII $\delta$  in Langendorff hearts and inhibited CaMKII $\delta$ -dependent RyR phosphorylation. In line with CaMKII $\delta$  and RyR inhibition, SN treatment decreased Ca<sup>2+</sup> spark frequency and dimensions in cardiomyocytes during isoproterenol challenge, and reduced the incidence of Ca<sup>2+</sup> waves, delayed afterdepolarizations, and spontaneous action potentials. SN treatment also lowered the incidence of early afterdepolarizations during isoproterenol; an effect paralleled by reduced magnitude of L-type Ca<sup>2+</sup> current.

**CONCLUSIONS:** SN production is upregulated in conditions with cardiomyocyte Ca<sup>2+</sup> dysregulation and offers compensatory protection against cardiomyocyte mechanisms of arrhythmia, which may underlie its putative use as a biomarker in at-risk patients.

**VISUAL OVERVIEW:** A [visual overview](#) is available for this article.

The full author list is available on page 12.

**Key Words:** calcium ■ calmodulin  
■ heart failure ■ secretoneurin  
■ tachycardia

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### WHAT IS KNOWN?

- Circulating SN (secretoneurin) levels are elevated in patients with myocardial dysfunction and predict poor outcome.
- SN inhibits the activity of CaMKII $\delta$  (Ca<sup>2+</sup>/calmodulin-dependent protein kinase II $\delta$ ), a known contributor to cardiomyocyte pathophysiology.

### WHAT THE STUDY ADDS?

- SN levels are increased in patients at risk for arrhythmia and may serve as a biomarker for identifying these individuals.
- Upregulation of SN is compensatory, as direct binding to CaMKII $\delta$  inhibits cardiomyocyte mechanisms of arrhythmia by attenuating over-activity of the ryanodine receptor.

CaMKII $\delta$  (calcium/calmodulin-dependent protein kinase II  $\delta$ ) is an important contributor to arrhythmogenesis in a broad range of cardiac pathologies, including heart failure (HF), atrial fibrillation, ischemia/reperfusion injury, and catecholaminergic polymorphic ventricular tachycardia (CPVT).<sup>1</sup> At the level of the single cardiac myocyte, CaMKII $\delta$  activation is linked to perturbation of Ca<sup>2+</sup> homeostasis and the generation of spontaneous action potentials. Such effects are particularly prominent during  $\beta$ -adrenergic stimulation, which leads to autophosphorylation of CaMKII $\delta$  at Thr287 and sustained kinase activity.<sup>2,3</sup> Although the proarrhythmic actions of CaMKII $\delta$  activation are complex, a central role of CaMKII $\delta$ -dependent phosphorylation of RyR2 (ryanodine receptor 2) is well established.<sup>4</sup> The resulting sensitization of the RyR2 promotes spontaneous Ca<sup>2+</sup> release and the generation of delayed afterdepolarizations (DADs), as released Ca<sup>2+</sup> is extruded by the Na<sup>+</sup>-Ca<sup>2+</sup> exchanger. Increased CaMKII $\delta$  activity has also been linked to increased risk of early afterdepolarizations (EADs) during the downstroke of the action potential and resulting extrasystoles. Various underlying mechanisms have been proposed, including CaMKII $\delta$ -dependent phosphorylation of the L-type Ca<sup>2+</sup> channel and a resulting increase in channel open probability.<sup>5,6</sup> Thus, enhanced CaMKII $\delta$  activity is linked to Ca<sup>2+</sup>-dependent arrhythmogenesis by multiple mechanisms.

The established role of CaMKII $\delta$  in arrhythmia has stimulated considerable interest in CaMKII $\delta$  inhibitors as potential therapeutics. Although such agents have been shown to protect against the generation of EADs, DADs, and in vivo arrhythmia,<sup>1,4</sup> these inhibitors typically exhibit significant off-target effects or limited uptake into cardiomyocytes, which precludes their applicability for use in patients.<sup>3</sup> We recently reported that the peptide SN (secretoneurin) likely serves as an endog-

enous CaMKII $\delta$  inhibitor by an unknown mechanism.<sup>7</sup> Produced by cleavage of the granin protein pro-SN (SgII [secretogranin II]),<sup>8</sup> we observed that SN is readily taken up into cardiomyocytes by endocytosis. We have also found increased circulating SN levels in various cohorts of patients with myocardial dysfunction, and that SN measurements add prognostic information to hs-TnT (high-sensitivity troponin T) and NT-proBNP (N-terminal pro-B-type natriuretic peptide) measurements.<sup>7,9–12</sup> In a manner analogous to BNP,<sup>13</sup> the biomarker capacity of SN may be indicative of its central role in cardiovascular disease, including possibly serving as a compensatory response to quell the proarrhythmic actions of CaMKII $\delta$  in at-risk patients. Accordingly, in this study, we hypothesized that patients prone to Ca<sup>2+</sup>-dependent arrhythmias would exhibit elevated SN levels and that SN would directly inhibit Ca<sup>2+</sup>-dependent arrhythmias both in vivo and in isolated cardiomyocytes.

### METHODS

A detailed Methods section is provided in the [Data Supplement](#). Requests by researchers to access the data, analytical methods, and study materials for the purposes of reproducing the results or replicating procedures can be made to the corresponding author.

All animal experiments were approved by the Norwegian Animal Research Committee and conformed to the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health (National Institutes of Health Publication No. 85-23, revised 1996). The clinical studies were approved by the Regional Ethics Committees and performed according to the Declaration of Helsinki. All subjects or next-in-kin provided written informed consent before study commencement.

### Patients With CPVT, Healthy Control Subjects and Patients With Ventricular Arrhythmias

Levels of SN and standard biomarkers were assessed in 2 clinical cohorts. First, we recruited 8 patients with genotype-verified CPVT from 3 families and 9 age- and sex-matched control subjects. Blood sampling of control subjects and patients with CPVT was conducted immediately before and after a maximal bicycle exercise stress test. Second, SN levels were measured in blood samples from 155 patients with ventricular arrhythmia—induced out-of-hospital cardiac arrest included in the FINNRESUSCI study, at a range of time points after hospitalization (<6, 24, 48, and 96 hours). Biomarker levels during the early phase of hospitalization were previously reported from this cohort.<sup>7</sup>

### SN Overexpression in CPVT Mice and In Vivo Testing of Arrhythmia Inducibility

Microarray analysis of left ventricular tissue was performed on female heterozygous RyR2-R2474S CPVT (RyR-RS) mice<sup>14,15</sup> and their wild-type (WT) littermates (age 8–12

weeks). Mice were treated with NaCl or 20 mg/kg isoproterenol sulfate for 3 days via an osmotic minipump, and myocardial RNA was prepared using TRIzol RNA isolation reagent (Invitrogen) following the manufacturer's instructions. Microarray analysis was performed using the Affymetrix GeneChip Mouse Gene 1.0 ST array (Affymetrix).

In vivo antiarrhythmic effects were tested by overexpressing SN in newborn (3–5 days old) male and female RyR2-R2474S mice<sup>14,15</sup> using a single injection of adeno-associated virus 9, an approach that leads to robust expression in the heart into adulthood.<sup>16</sup> When the adeno-associated virus-9-infected mice reached adulthood, telemetric ECG transmitters were implanted as previously described.<sup>15</sup> A stress test was performed using an intraperitoneal injection of 20 mg/kg isoproterenol, whereas ECG recordings were collected. After the stress test, animals were euthanized and ventricular tissue was snap-frozen in liquid nitrogen for subsequent validation of adeno-associated virus-9-induced expression of SN or control peptide.

### Transfection of Human Embryonic Kidney 293 Cells, Pull-Down Experiments, Structure Modeling and ELISA

Human embryonic kidney 293 cells were cultured as described previously<sup>17</sup> and transiently transfected with various constructs of FLAG-CaMKII $\delta$ . For pull-down experiments, biotinylated synthetic SN peptide was coupled onto monoclonal anti-biotin-conjugated beads, incubated with the human embryonic kidney 293 lysate and analyzed by Western blotting. Atomic coordinates for human CaMKII $\delta$  were used to model putative sites of SN binding with the substrate-binding domain (S-site). Model validity was tested by creating SN mutants and analyzing effects on SN-CaMKII $\delta$  binding using an ELISA assay.

### Langendorff Preparations and Western Blotting Experiments

Mouse hearts were mounted on a Langendorff setup by cannulating the aorta and perfused with Hepes Tyrode solution supplemented with isoproterenol (100 nmol/L) or with isoproterenol and SN (2.8  $\mu$ mol/L). After perfusion, the left ventricle was rapidly excised, frozen in liquid nitrogen, and stored at  $-70^{\circ}\text{C}$ . Protein lysates and Western blotting were performed as previously reported.<sup>7</sup>

### Cardiomyocyte Isolation, Ca<sup>2+</sup>-Dependent Fluorescence, and Patch-Clamping Experiments

Cells were enzymatically isolated from C57BJ/6 mice by perfusing the heart with collagenase type II via a Langendorff setup as previously described.<sup>7</sup> Ca<sup>2+</sup> sparks were recorded in resting cardiomyocytes using a confocal microscope in line-scan mode, with testing of 2 doses of SN (2.8  $\mu$ mol/L, 2.8 nmol/L). Whole-cell Ca<sup>2+</sup> transients and Ca<sup>2+</sup> waves were examined by wide-field fluorescence, whereas patch-clamp experiments were performed to record action potentials and L-type Ca<sup>2+</sup> currents (Methods in the [Data Supplement](#)).

## Statistical Analyses

Statistical tests used are noted in the figure legends accompanying each data set. Statistical differences between patients with CPVT and control subjects were examined by a Mann-Whitney *U* test (Figure 1A, Table I in the [Data Supplement](#)). Correlations between SN levels and other biomarkers were examined by Spearman correlation (Table II in the [Data Supplement](#)). In patients resuscitated from ventricular arrhythmias, differences between baseline biomarker concentrations and later time points were assessed by the Friedman test for repeated measures of nonparametric data (Figure 1B). Comparison of patient characteristics according to 1-year mortality was examined by a Mann-Whitney *U* test (Table III in the [Data Supplement](#)), and correlation coefficients were examined by Spearman correlation (Table IV in the [Data Supplement](#)). Multivariable linear regression analysis was used to identify variables associated with changes in biomarker concentration, including age, sex, body mass index, creatinine clearance, established coronary artery disease, hypertension, previous HF, diabetes mellitus, witnessed cardiac arrest, performed bystander CPR, therapeutic hypothermia, Simplified Acute Physiology Score II, and Sequential Organ Failure Assessment score (Table V in the [Data Supplement](#)). In experiments examining WT and CPVT mice, statistical significance for microarray data was determined by a false discovery rate  $<0.001$  (Figure 2A). Polymerase chain reaction analysis of mCherry fusion protein, the frequency of arrhythmia events, and arrhythmia score were tested by 1-way ANOVA on Ranks with Dunn test for multiple comparisons (Figure 2C, 2E, and 2F). The incidence of ventricular tachycardia was tested with a Fisher exact test (Figure 2G). Results from mutagenesis studies (Figure 3E) were examined by 1-way ANOVA with Bonferroni test for multiple comparisons. In cardiomyocyte experiments (Figures 4 and 5, Figures I and II in the [Data Supplement](#)), statistical differences between groups were calculated by Mann-Whitney tests, except for differences in the L-type Ca<sup>2+</sup> current-voltage relationship, which were tested by 2-way ANOVA with Bonferroni test for multiple comparisons. Western blot data were examined with Student *t* test (Figures 3F and 4A, Figure IIB in the [Data Supplement](#)). A *P* value of  $<0.05$  was considered statistically significant.

## RESULTS

### SN Levels Are Increased in Subjects With CPVT and Patients With Out-of-Hospital Cardiac Arrest

Based on our previous observations that SN regulates cardiomyocyte Ca<sup>2+</sup> homeostasis,<sup>7</sup> we hypothesized that SN may be specifically linked to Ca<sup>2+</sup>-dependent arrhythmias. To this end, we examined circulating SN levels in patients and mice with CPVT, who exhibit dysfunctional cardiomyocyte Ca<sup>2+</sup>-handling during  $\beta$ -adrenergic activation. Baseline circulating levels of SN were elevated in patients with CPVT compared with age- and sex-matched control subjects (Figure 1A and

Table I in the [Data Supplement](#)). In contrast, levels of catecholamines, hs-TnT, NT-proBNP, and chromogranins were not higher in patients with CPVT compared with controls (Figure 1A, Table I in the [Data Supplement](#)). All of the patients with CPVT, but none of the control subjects, were treated with  $\beta$ -blockers (mean dose  $50 \pm 32\%$  of recommended target dose). No statistically significant linear correlations were observed between circulating SN levels and levels of catecholamines, established cardiac biomarkers, chromogranins, or  $\beta$ -blocker dose (Table II in the [Data Supplement](#)), and SN levels were not influenced by a short-term bicycle exercise stress test (Figure 1A). We also found increased myocardial pro-SN mRNA concentrations in CPVT mice compared with controls (Figure 2A), which were not treated with  $\beta$ -blockers.

We next compared the temporal profile of SN levels with the profiles of hs-TnT and NT-proBNP in patients that were hospitalized after resuscitation for ventricular arrhythmia-induced out-of-hospital cardiac arrest. Circulating SN levels were elevated upon hospital admission (<6 hours after hospitalization) compared with later time points (Figure 1B, Table III in the [Data Supplement](#)), when the risk of subsequent ventricular arrhythmias and mortality are high.<sup>18</sup> SN levels then rapidly declined and remained low during the subsequent days of follow-up. In contrast, NT-proBNP levels increased during the first days after ventricular arrhythmia, whereas hs-TnT levels did not markedly change (Figure 1B, Table III in the [Data Supplement](#)). We found no statistically significant linear correlations between SN levels and other biomarkers, or between changes in SN levels and other biomarkers after ventricular arrhythmia (Table IV in the [Data Supplement](#)). Moreover, variables associated with change in SN levels, as assessed by multivariable linear regression analysis, differed from the variables associated with change in hs-TnT and NT-proBNP levels after ventricular arrhythmia (Table V in the [Data Supplement](#)). These observations link SN expression more closely to cardiomyocyte  $\text{Ca}^{2+}$  handling and support that SN elevation may better distinguish patients at risk for arrhythmia than established cardiac biomarkers, identifying additional myocardial pathophysiology.

### SN Overexpression Reduces Arrhythmia in CPVT Mice

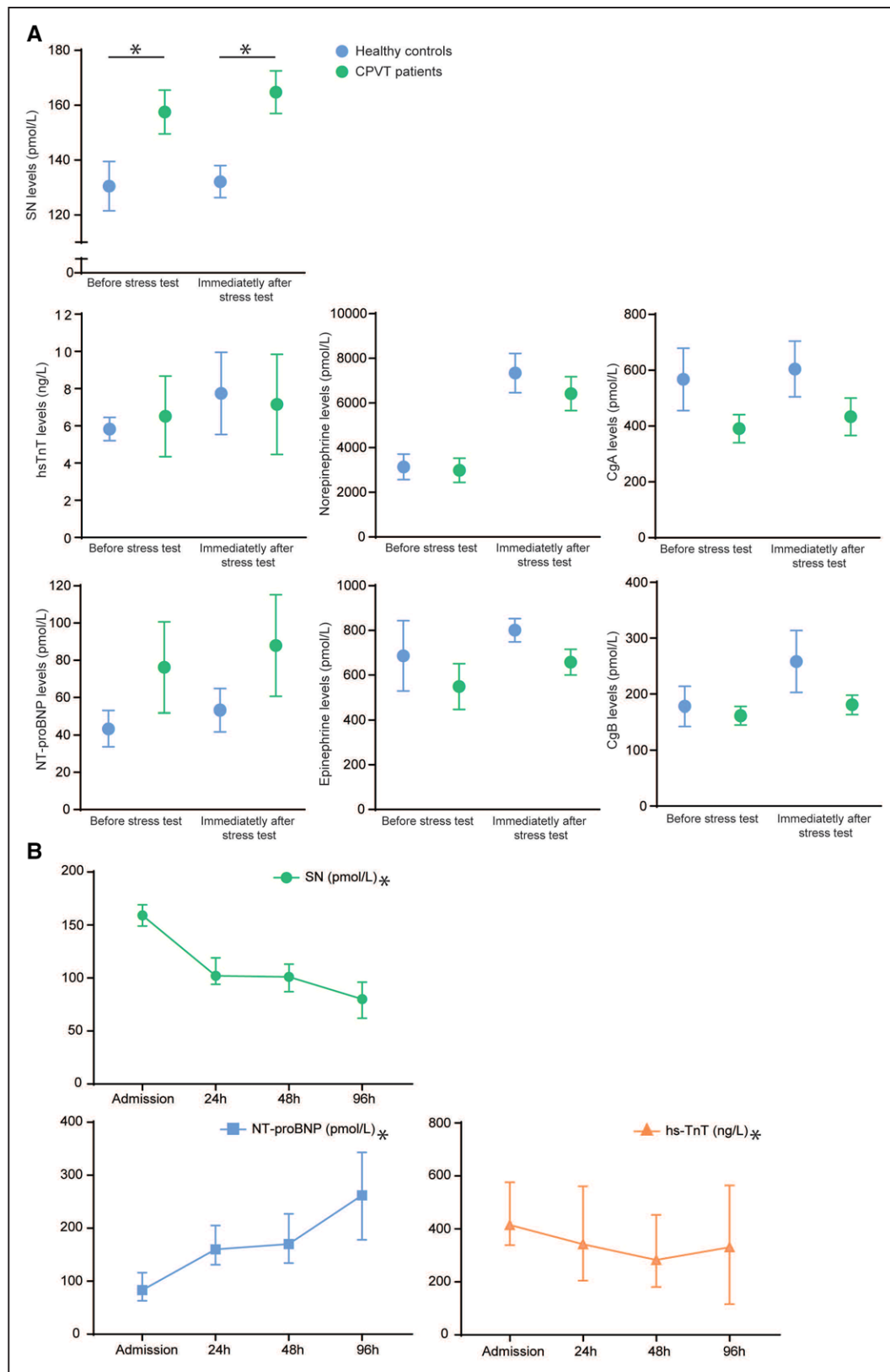
Based on the above findings in subjects with increased prevalence of arrhythmia, and our prior data indicating that SN can inhibit CaMKII $\delta$ ,<sup>7</sup> we hypothesized that SN elevation has compensatory, antiarrhythmic actions. To investigate this, we used CPVT mice (RyR2-R2474S mutation). CPVT mice exhibited an upregulation of SgII at baseline (Figure 2A). SN was further overexpressed in 3- to 5-day-old animals using a single, intrathorax injection of adeno-associated virus 9, which has strong tro-

pism for cardiac tissues.<sup>16</sup> A scrambled construct served as control. Imaging and polymerase chain reaction analysis of the mCherry fusion protein documented robust expression of both SN and scrambled construct peptides in cardiomyocytes into adulthood (Figure 2B and 2C). During stress testing with isoproterenol, telemetric recordings revealed that few arrhythmias were induced in WT mice (Figure 2D and 2E). A statistically significant higher number of arrhythmic events was observed in CPVT animals transfected with scrambled peptide, but not SN (Figure 2E). An arrhythmia score assigned based on the severity of the event observed (ventricular extrasystoles, bigeminy, couplets, or ventricular tachycardia, described in the legend of Figure 2F) similarly showed a statistically significant higher score in CPVT mice transfected with scrambled peptide but not SN, in comparison with WT controls. Of particular prevalence was a lower incidence of ventricular tachycardia in CPVT animals treated with SN, both in terms of number of events (Figure 2E) and proportion of affected animals (Figure 2G). This finding supports compensatory antiarrhythmic actions by SN upregulation in subjects with CPVT.

### SN Interacts With the S-Site in the Catalytic Region of CaMKII $\delta$ and Reduces Autophosphorylation of CaMKII $\delta$ During $\beta$ -Adrenergic Challenge

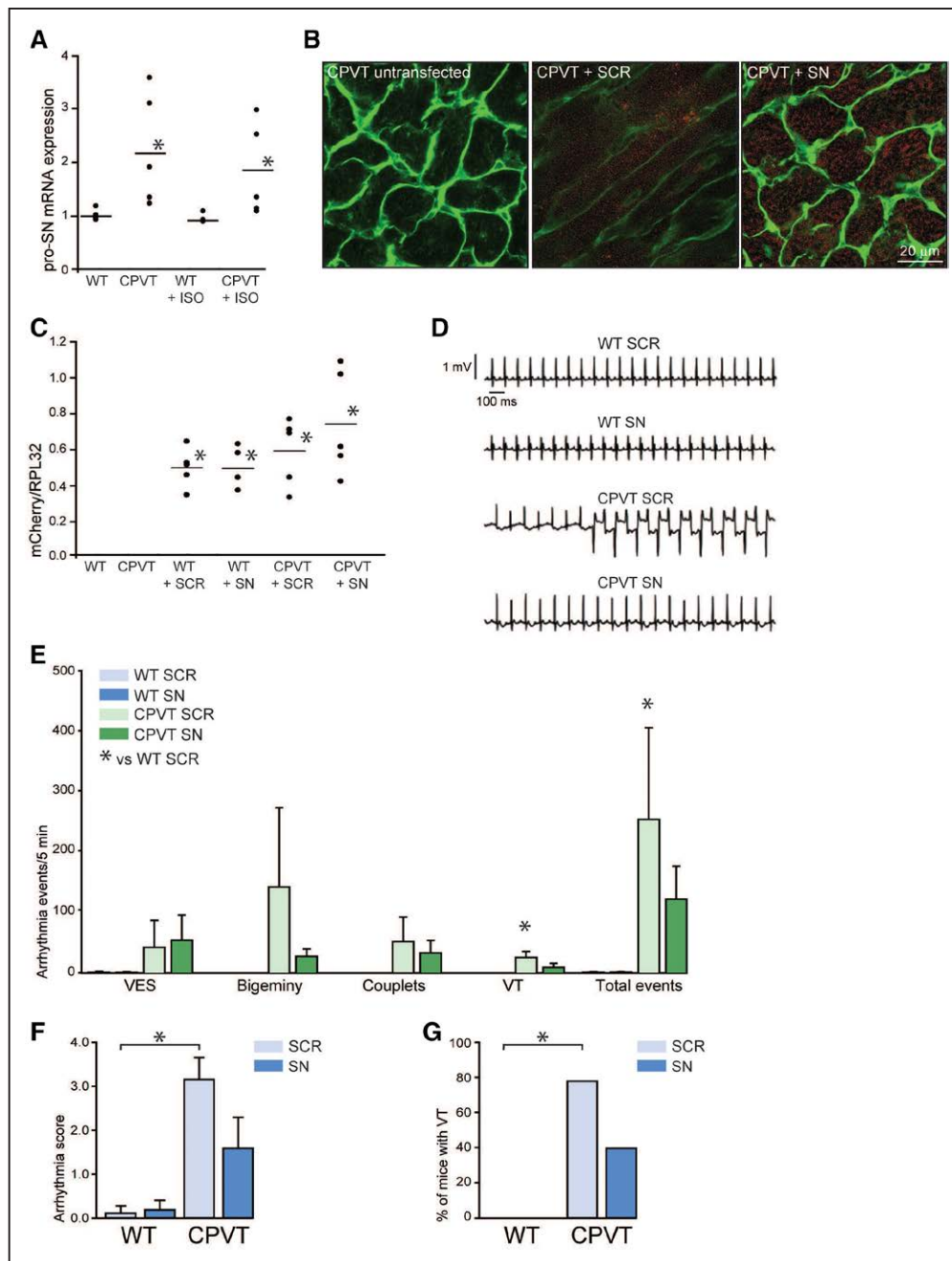
Based on the above findings in patients and mice at risk for arrhythmia, and our prior data indicating that SN can inhibit CaMKII $\delta$ ,<sup>7</sup> we examined the mechanism by which SN elevation protects against  $\text{Ca}^{2+}$ -dependent arrhythmogenesis. To this end, we first aimed to identify SN binding sites on CaMKII $\delta$  based on established knowledge of its domain organization (Figure 3A). 3xFLAG-CaMKII $\delta$  full length (T287D mutated, constitutive active) and deletion variants were designed and expressed in human embryonic kidney 293 cells. Pull-down experiments with a biotinylated SN peptide revealed that all 3xFLAG-CaMKII $\delta$  variants precipitated with SN, except 3xFLAG-CaMKII $\delta$  (1–50), which lacks the S-site (also known as docking site A<sup>3</sup>) in the catalytic domain (Figure 3B, upper). This finding suggests that SN binds to the S-site. Indeed, bioinformatics revealed 2 sequence homologies between SN and the S-site binding region of the regulatory domain, which contains the autophosphorylation site Thr287 (Figure 3C). In line with this finding, modeling of SN binding to CaMKII $\delta$  revealed 2 possible SN-S-site interactions, centered either at Thr163 (model 1) or Thr169 (model 2) in SN/SgII (Figure 3C and D, upper and lower, respectively). Both models contained favorable hydrophobic interactions (blue dashed lines) and hydrogen bonds (yellow dashed lines) between SN and the S-site (Figure 3D). However, model 2 was favored





**Figure 1. SN (secretoneurin) levels are increased in patients with catecholaminergic polymorphic ventricular tachycardia (CPVT) and patients after out-of-hospital cardiac arrest.**

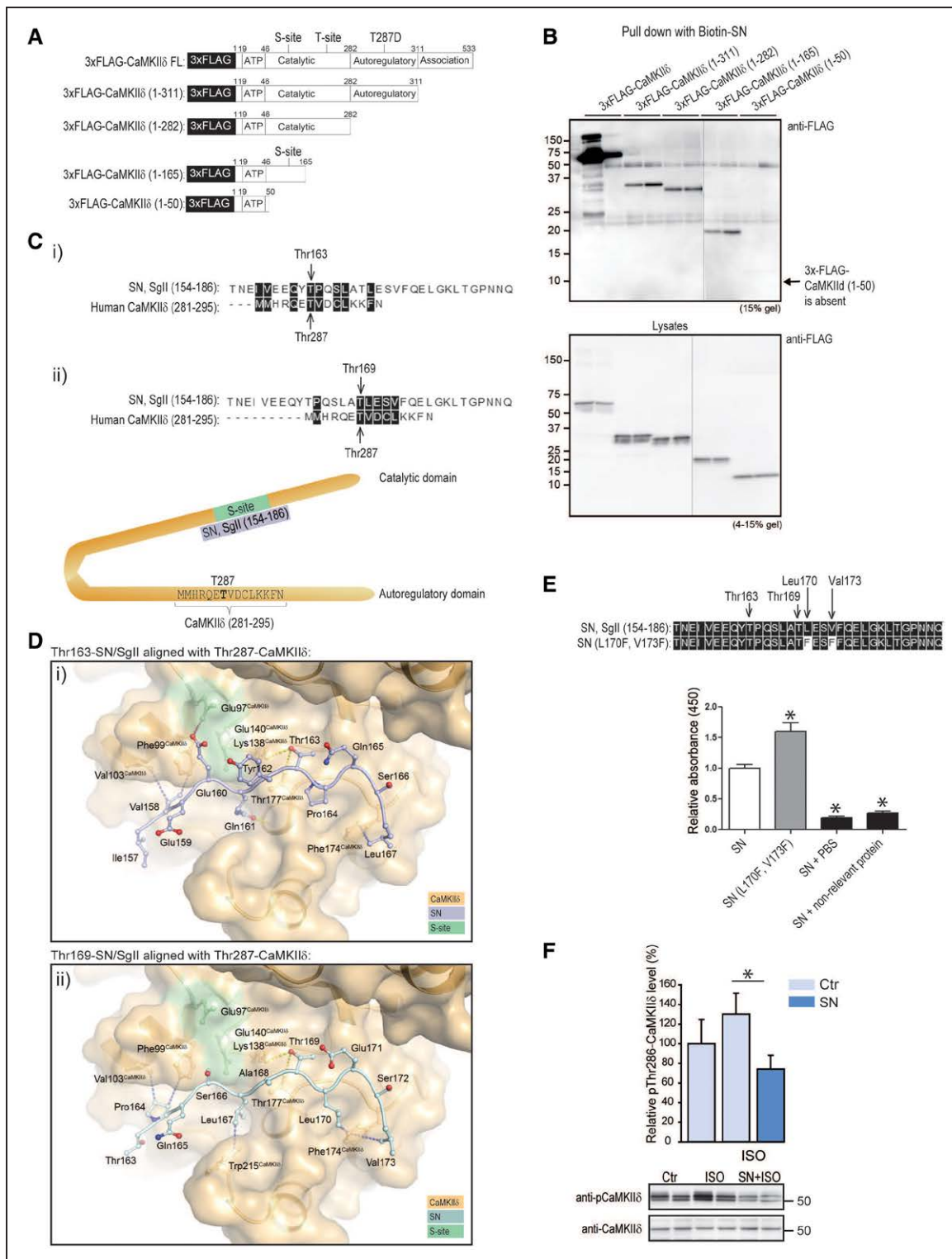
**A**, Circulating SN levels were higher in patients with CPVT (n=8) compared with control subjects (n=9), both at rest and after a short-term exercise test. Levels of catecholamines, hs-TnT (high-sensitivity troponin T), NT-proBNP (N-terminal pro-B-type natriuretic peptide), and CgA, CgB (chromogranins A and B) were unchanged. \* $P < 0.05$ , by Mann-Whitney  $U$  test. **B**, Circulating SN levels were elevated after resuscitation for ventricular arrhythmia-induced cardiac arrest (n=155), when the risk of subsequent ventricular arrhythmias and mortality are highest, followed by a decline of circulating SN levels after 24 h when the risk is low. In contrast, hs-TnT levels did not markedly change, and NT-proBNP levels increased during the first days after cardiac arrest. Presented biomarker levels are median (interquartile range). \* $P < 0.05$ , by Friedman test.



**Figure 2. SN (secretoneurin) overexpression attenuates in vivo arrhythmia in catecholaminergic polymorphic ventricular tachycardia (CPVT) mice.** **A**, Microarray analysis revealed increased myocardial pro-SN mRNA concentrations in CPVT mice, both in animals treated with NaCl or 20 mg/kg isoproterenol (ISO) sulfate for 3 d via an osmotic minipump. Wild-type (WT) control vs CPVT control: false discovery rate (FDR) <0.001, fold change 1.95. WT ISO vs CPVT ISO: FDR <0.001, fold change 1.67. **B**, SN levels were further increased in newborn mouse hearts via intrathorax injection of adeno-associated virus 9. Comparison was made with scrambled construct (SCR). Robust ventricular expression of the constructs was observed into adulthood, as demonstrated by imaging of the mCherry fusion protein (co-staining of cell membranes with wheat-germ agglutinin) and **(C)** polymerase chain reaction analysis. \**P*<0.05 by 1-way ANOVA on Ranks with Dunn test for multiple comparisons. **D**, Telemetric recordings during ISO stress testing revealed frequent arrhythmias in CPVT mice expressing SCR but not SN peptide. **E**, Arrhythmia events were categorized and tallied by a blinded observer during the first 5 min of stress testing. \**P*<0.05 by 1-way ANOVA on Ranks with Dunn test for multiple comparisons. **F**, An arrhythmia score was assigned based on the severity of the event; ventricular extrasystole (VES)=1 point, bigeminy=2 points, couplets=3 points, or ventricular tachycardia (VT)=4 points. \**P*<0.05 by 1-way ANOVA on Ranks with Dunn test for multiple comparisons. **G**, In comparison with WT mice, VT was observed in a higher proportion of CPVT mice transfected with SCR but not SN. \**P*<0.05 by Fisher exact test. (CPVT mice: SCR: n=9, SN: n=10, WT mice: SCR: n=7, SN: n=5)

over model 1, as the latter also contained an unfavorable repulsive interaction between Glu160-SN/Sgll and Glu97-CaMKII $\delta$  (red dashed line). Model 2 was further supported by SN mutational analysis showing

that biotin-SN (L170F, V173F) bound more strongly than SN to His-CaMKII $\delta$  (69–282; Figure 3E). The 2 aromatic residues (phenyl-alanines) in L170F/V173 may enhance peptide binding to CaMKII $\delta$  by aromatic



**Figure 3. SN (secretoneurin) interacts with the S-site in the catalytic region of CaMKIIδ (Ca<sup>2+</sup>/calmodulin-dependent protein kinase IIδ) and inhibits autophosphorylation of CaMKIIδ during β-adrenergic challenge.**

**A**, Schematic diagram of the various Flag-CaMKIIδ constructs expressed in human embryonic kidney 293 cells used to examine SN binding. **B**, Western blot analyses demonstrated co-precipitation of SN with CaMKIIδ only when the S-site was present (upper panel). **Lower**: Shows equal expression of all Flag-CaMKIIδ variants (control blot; n=2). **C**, Sequence alignments of SN, centered either at Thr163 or Thr169 (numbering according to SgII [secretogranin II]) to the substrate binding Thr287-segment in CaMKIIδ (upper schematic in lower). The S-site spans residues E97 and E140 in CaMKIIδ (E96 and E139 in CaMKIIα).<sup>18</sup> **D**, Structural models of the 2 possible SN-Site interactions, centered either at Thr163-SN/SgII (model 1, upper) or Thr169-SN/SgII (model 2, lower). SN is shown as a ball-and-stick model, with S-site residues Glu97 and Glu140 in light green; CaMKIIδ is shown in yellow. **Upper**: Putative favorable hydrophobic interactions between Val158-SN/SgII and Phe99/Val103-CaMKIIδ, as well as Val167-SN/SgII and Phe174-CaMKIIδ are shown as blue dashed lines. Hydrogen bonds to SN side chains are shown in yellow. An unfavorable repulsive interaction between Glu160-SN/SgII and Glu97-CaMKIIδ is indicated in red. **Lower**: Putative favorable hydrophobic interactions between Pro164-SN/SgII and Phe99/Val103-CaMKIIδ, Leu167-SN/SgII and Trp215-CaMKIIδ, and Leu170/Val173-SN/SgII with Phe174-CaMKIIδ are (Continued)

stacking with Phe174. SN binding was greatly reduced when CaMKII $\delta$  (69–282) was omitted or replaced with a nonrelevant protein without any S-site (negative controls). Thus, our data support that a region of SN centered around Thr169 binds within a pocket of the S-site of CaMKII $\delta$ . As the CaMKII $\delta$ -S-site is responsible for autophosphorylation of Thr287 in the neighboring CaMKII $\delta$  molecule in the dodecamer,<sup>3</sup> we postulated that SN binding to the S-site blocks autophosphorylation and thereby limits kinase activity. Indeed, SN was observed to inhibit isoproterenol-induced Thr287 phosphorylation in Langendorff hearts perfused with 100 nmol/L isoproterenol (Figure 3F).

### SN Inhibits CaMKII $\delta$ -Dependent Phosphorylation of RyR2 and Ca<sup>2+</sup> Sparks

In agreement with an observed inhibition of CaMKII $\delta$  activity by SN, SN treatment also reduced isoproterenol-induced CaMKII $\delta$ -dependent phosphorylation of RyR2. Although isoproterenol perfusion increased phosphorylation at the known CaMKII $\delta$  and PKA (protein kinase A) regulatory sites (pSer2814-RyR2 and pSer2808-RyR2, respectively), this effect was statistically only significantly reversed by SN at the CaMKII $\delta$  site (Figure 4A). Consistent with inhibition of CaMKII $\delta$ -dependent phosphorylation of RyR2, SN treatment (2.8  $\mu$ M) tended to reduce both the frequency and dimensions of Ca<sup>2+</sup> sparks in isolated cardiomyocytes (Figure 4B). These effects were exaggerated in the presence of isoproterenol (Figure 4B). More modest effects were observed for a 1000X lower dose of SN (2.8 nmol/L, Figure I in the [Data Supplement](#)), which is in the range of the highest circulating levels observed in patients with out-of-hospital cardiac arrest (>2 nmol/L).

### SN Inhibits Ca<sup>2+</sup> Waves and DADs

In line with a reduction in RyR2 activity, SN treatment increased sarcoplasmic reticulum (SR) Ca<sup>2+</sup> content at baseline (Figure II in the [Data Supplement](#)). SN also decreased the incidence of spontaneous Ca<sup>2+</sup> waves, both at baseline and during  $\beta$ -adrenergic challenge (Figure 5A). In patch-clamp experiments, these effects were manifested as an observed reduction in DAD frequency in cells paced at 1 Hz (Figure 5B). Generated DADs were frequently observed to trigger spontaneous action potentials, and SN treatment tended to reduce the occurrence of these events (Figure 5B).

### SN Inhibits EADs and L-type Ca<sup>2+</sup> Current (I<sub>CaL</sub>)

Given the role of CaMKII $\delta$  in promoting EADs, we examined whether SN treatment attenuated arrhythmogenesis by this mechanism. In patch-clamp experiments, isoproterenol markedly increased EAD generation in paced cardiomyocytes; an effect that was strongly inhibited by SN (Figure 5C). SN also reduced L-type Ca<sup>2+</sup> current (Figure 5D), an important CaMKII $\delta$ -sensitive EAD trigger<sup>5,6</sup> but did not alter kinetics of current decay ( $\tau_{\text{fast}}$  = 18.4 $\pm$ 1.4 ms, 24.1 $\pm$ 3.2 ms in control, SN;  $P$ =not significant by  $t$  test). Thus, our results support that elevation of SN levels reduces arrhythmogenesis during isoproterenol stimulation by inhibiting cellular mechanisms underlying both EADs and DADs, suggesting a compensatory role of SN in patients at high risk for arrhythmias.

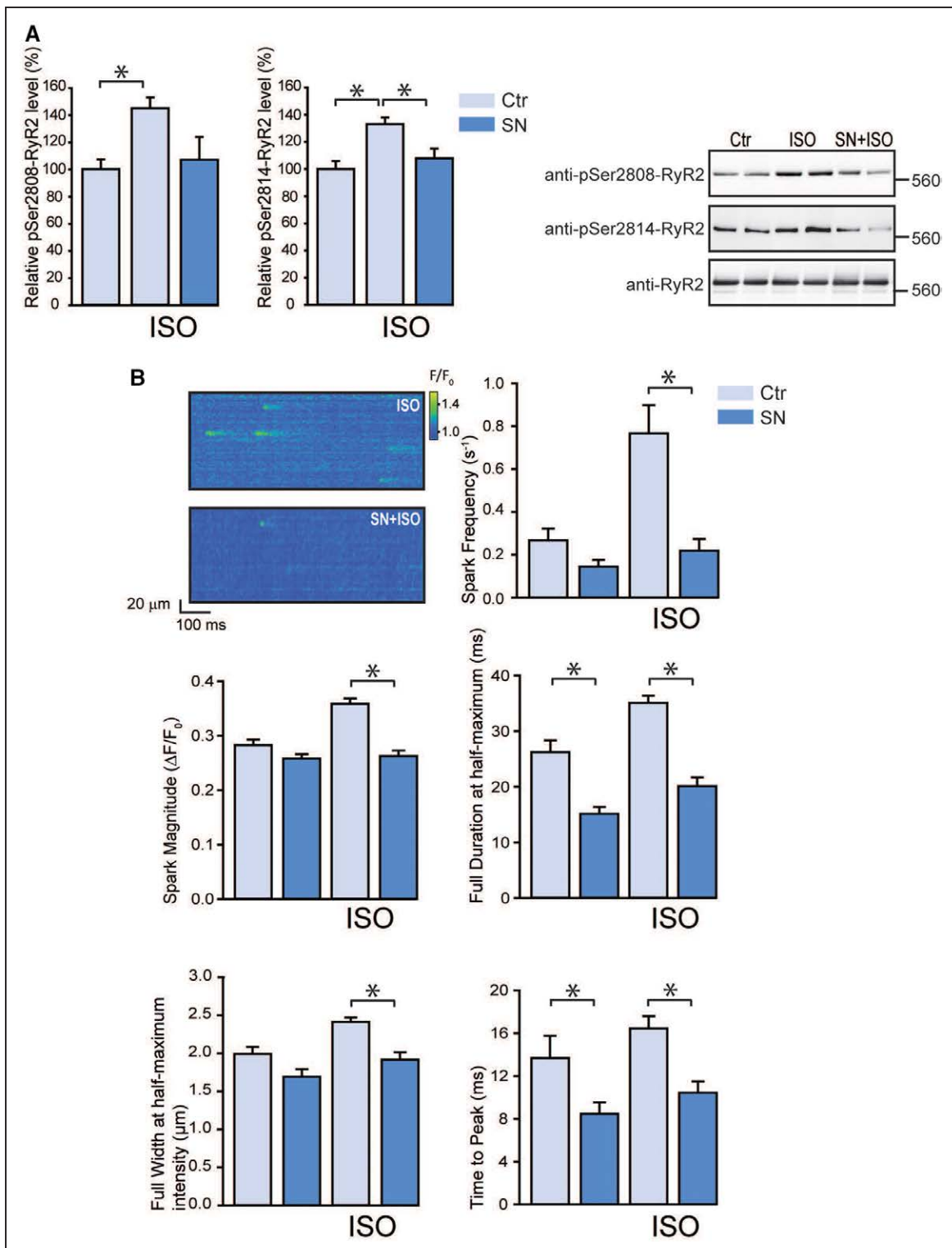
## DISCUSSION

The principal findings of this study are summarized in the graphic abstract. Circulating SN levels were increased in patients with CPVT, who exhibit pathological cardiomyocyte Ca<sup>2+</sup> handling. High SN levels were also observed in cardiac arrest patients during the early phase following arrhythmia when risk of a new arrhythmia is high and then normalized within 24 hours. This temporal profile of SN levels after ventricular arrhythmia differed from the profile of hs-TnT and NT-proBNP, suggesting that SN levels may better distinguish patients at risk for arrhythmia and reflect additional myocardial pathophysiology compared with established cardiac biomarkers. A compensatory role of SN upregulation was indicated in CPVT mice, as myocardial pro-SN synthesis was increased in animals with CPVT, and adeno-associated virus-9–induced SN overexpression reduced arrhythmic incidence and severity during stress testing. In experimental studies aimed at identifying the arrhythmic implications of SN upregulation, we observed that SN binds to the S-site of CaMKII $\delta$ , a well-established contributor to arrhythmogenesis in cardiomyocytes.<sup>1</sup> SN binding was found to reduce CaMKII $\delta$  autophosphorylation and phosphorylation of its target proteins and to lower the prevalence of DADs and EADs during  $\beta$ -adrenergic stimulation. These findings indicate that SN upregulation offers compensatory protection against ventricular arrhythmia, which underlies its putative use as a prognostic biomarker in at-risk patients.

To be clinically relevant, novel biomarkers should reflect pathophysiology of relevance for morbidity and

**Figure 3 Continued.** shown as blue dashed lines. Hydrogen bonds to Thr169-SN/SgII are shown in yellow. **E**, Using the ELISA technique, mutagenesis studies supported the validity of model 2, as SN binding was greatly reduced when CaMKII $\delta$  69–282 was omitted (SN+PBS) or replaced by a nonrelevant amino acid sequence. \* $P$ <0.05, examined by 1-way ANOVA with Bonferroni Multiple Comparison test. (SN+CaMKII 69–282:  $n$ =12; SN (L170F, V173F) + CaMKII 69–282:  $n$ =6; SN+PBS:  $n$ =6; SN+nonrelevant protein:  $n$ =6). **F**, SN reduced ISO-induced autophosphorylation of Thr287–CaMKII $\delta$  in Langendorff-perfused hearts. \* $P$ <0.05, examined by Student  $t$  test. (Control [Ctr]:  $n$ =5; ISO:  $n$ =4; SN+ISO:  $n$ =3).



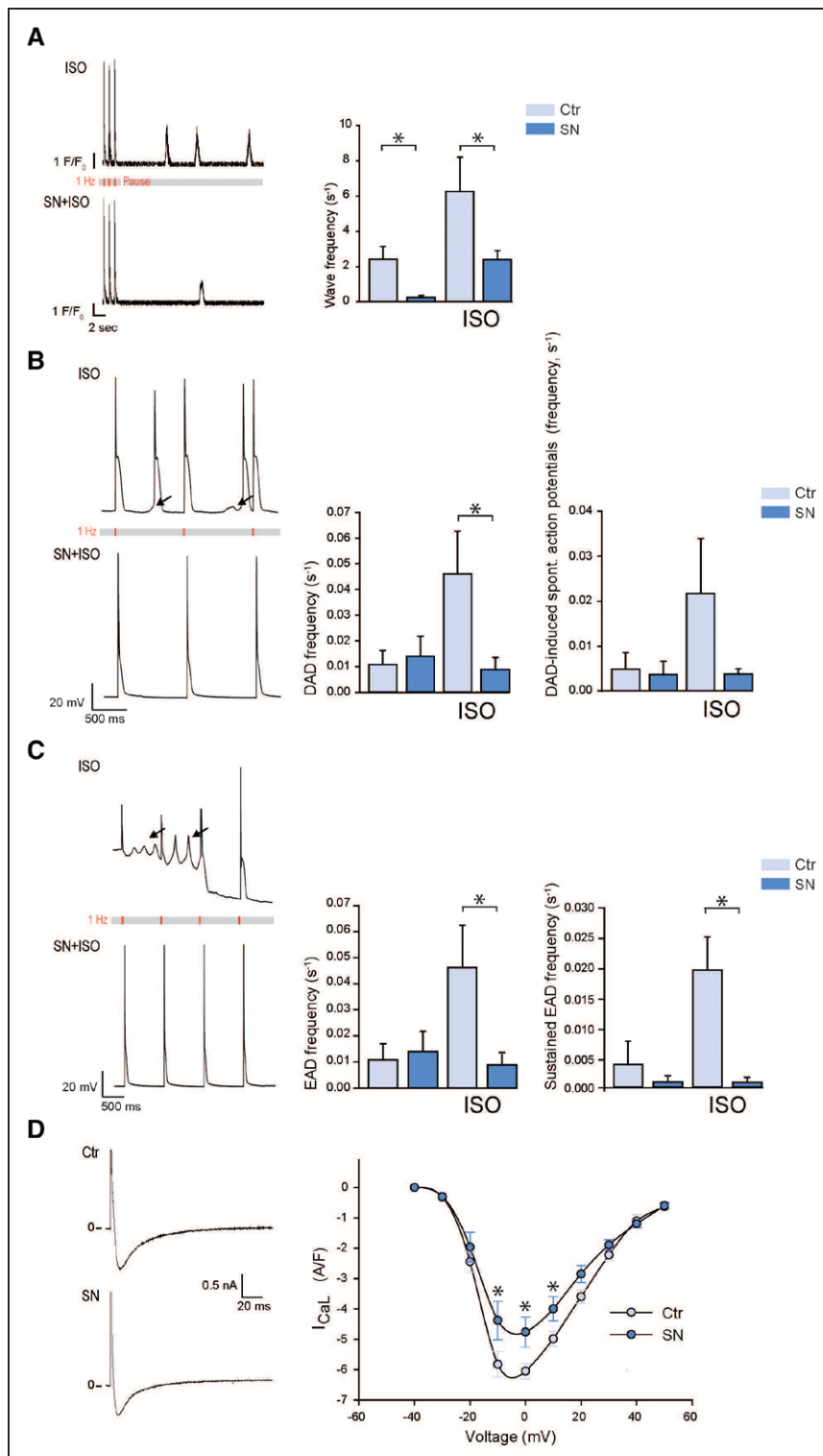


**Figure 4.** SN (secretoneurin) reduces CaMKII $\delta$  (Ca<sup>2+</sup>/calmodulin-dependent protein kinase II $\delta$ )-dependent phosphorylation of RyR2 (ryanodine receptor 2) and Ca<sup>2+</sup> sparks.

**A**, Isoproterenol (ISO) stimulation of Langendorff-perfused hearts increased RyR2 phosphorylation at both CaMKII- and PKA (protein kinase A)-dependent sites (Serine2814 [Ser2814] and Ser2808, respectively). SN treatment inhibited phosphorylation at Ser2814 only. \* $P < 0.05$ , examined by Student  $t$  test. (Control [Ctr]:  $n = 5$ ; ISO:  $n = 4$ ; SN+ISO:  $n = 3$ ). **B**, SN treatment of cardiomyocytes reduced the frequency and geometry of spontaneous Ca<sup>2+</sup> sparks, particularly in the presence of ISO. \* $P < 0.05$ , examined by Mann-Whitney test. (Ctr:  $n_{\text{hearts}} = 5$ ,  $n_{\text{cells}} = 35$ ; SN:  $n_{\text{hearts}} = 5$ ,  $n_{\text{cells}} = 43$ ; ISO:  $n_{\text{hearts}} = 6$ ,  $n_{\text{cells}} = 80$ ; SN+ISO:  $n_{\text{hearts}} = 5$ ,  $n_{\text{cells}} = 37$ ).

mortality in cardiovascular disease. Our present data link SN directly to cardiomyocyte Ca<sup>2+</sup> mishandling, which mechanistically supports the previously reported asso-

ciation to poor outcome in patients with myocardial dysfunction.<sup>7-10,12,19</sup> Specifically, our earlier work identified SN elevation in blood samples from patients with acute



**Figure 5. SN (secretoneurin) inhibits the generation of delayed afterdepolarizations (DADs) and early afterdepolarization (EADs) during  $\beta$ -adrenergic challenge.**

**A**,  $Ca^{2+}$  waves were recorded by whole-cell fluorescence in cardiomyocytes after termination of 1 Hz field stimulation. Including 2.8  $\mu$ mol/L SN in the superfusate reduced  $Ca^{2+}$  wave frequency, both at baseline and during isoproterenol (ISO)-treatment.  $*P < 0.05$ , by Mann-Whitney test. (Control [Ctr]:  $n_{hearts} = 7$ ,  $n_{cells} = 19$ ; SN:  $n_{hearts} = 6$ ,  $n_{cells} = 24$ ; ISO:  $n_{hearts} = 7$ ,  $n_{cells} = 27$ ; SN+ISO:  $n_{hearts} = 4$ ,  $n_{cells} = 22$ ). **B**, In concordance with attenuated wave generation, SN treatment reduced the frequency of DADs (arrows) observed during ISO challenge in patch-clamped cells paced at 1 Hz. The frequency of DAD-induced spontaneous action potentials (APs) also tended to be reduced in the ISO + SN group.  $*P < 0.05$ , by Mann-Whitney test. (Ctr:  $n_{hearts} = 6$ ,  $n_{cells} = 9$ ; SN:  $n_{hearts} = 3$ ,  $n_{cells} = 11$ ; ISO:  $n_{hearts} = 6$ ,  $n_{cells} = 10$ ; SN + ISO:  $n_{hearts} = 3$ ,  $n_{cells} = 11$ ). **C**, SN treatment decreased the appearance of EADs (arrows) induced by ISO stimulation. The occurrence of sustained EADs lasting an entire cycle length was also attenuated.  $*P < 0.05$ , by Mann-Whitney test. (Ctr:  $n_{hearts} = 6$ ,  $n_{cells} = 9$ ; SN:  $n_{hearts} = 3$ ,  $n_{cells} = 11$ ; ISO:  $n_{hearts} = 6$ ,  $n_{cells} = 10$ ; SN + ISO:  $n_{hearts} = 3$ ,  $n_{cells} = 11$ ). **D**, SN reduced L-type  $Ca^{2+}$  current ( $I_{CaL}$ ), a known CaMKII ( $Ca^{2+}$ /calmodulin-dependent protein kinase II)-sensitive trigger of EAD generation.  $*P < 0.05$  by 2-way ANOVA with Bonferroni test for multiple comparisons. (Ctr:  $n_{hearts} = 3$ ,  $n_{cells} = 9$ ; SN group:  $n_{hearts} = 3$ ,  $n_{cells} = 9$ ).

HF,<sup>7,11</sup> critically ill patients with infections,<sup>9</sup> and in patients with cardiovascular related-acute respiratory failure.<sup>10</sup> Importantly, SN levels provided additional prognostic information to established risk scores and biomarkers in these patients, whereas SN levels did not improve risk assessment in patients with pulmonary disease.<sup>7,10,12</sup> The cardiac syndromes examined in these previous studies are associated with excessive  $\beta$ -adrenergic stimulation

and CaMKII $\delta$  activation. We presently show that myocardial and circulating SN levels are increased also in subjects with CPVT, a condition in which the mutated, leaky RyR2 activates CaMKII $\delta$ , and the resulting phosphorylation of RyR2 further sensitizes the channel in a positive-feedback loop.<sup>20,21</sup> These effects are exaggerated during  $\beta$ -adrenergic stimulation,<sup>2</sup> and therefore, a system that could counteract the detrimental effects of excessive

CaMKII $\delta$  activity is expected to be protective in patients with CPVT. The early increment in circulating SN levels after ventricular arrhythmia-induced cardiac arrest also fits a model of SN as an endogenous CaMKII $\delta$  inhibitor as the risk of new ventricular arrhythmic events is highest early after hospital admission.<sup>7</sup> In contrast to SN levels, which rapidly decreased after patients had been resuscitated for ventricular arrhythmias, NT-proBNP levels increased over time, and hs-TnT levels did not markedly change. Still, as dysregulated cardiomyocyte Ca<sup>2+</sup> homeostasis may impact both the risk of ventricular arrhythmias and systolic and diastolic function, it is currently not clear whether SN assessment in HF patients could distinguish patients at risk of arrhythmias from those at risk of worsening HF with pulmonary congestion. Accordingly, there is a need for additional clinical and experimental studies to explore this question more closely. We also cannot completely rule out that the difference in  $\beta$ -blocker use between patients with CPVT and control subjects may have altered SN concentrations as  $\beta$ -blockers also influence cardiomyocyte Ca<sup>2+</sup> homeostasis. However, we have previously found that  $\beta$ -blocker treatment was not associated with increased SN concentrations in patients with acute HF,<sup>7</sup> and we did not presently find a correlation between  $\beta$ -blocker dose and SN concentration in our patients with CPVT (Table II in the [Data Supplement](#)). The increase in myocardial pro-SN synthesis in CPVT mice, which were not treated with  $\beta$ -blockers, also supports that the difference in medication alone cannot explain our results. Hence, we believe that differences in SN concentrations between patients with CPVT and control subjects are most likely rooted in the disease pathophysiology.

As SN is formed by the cleavage of SgII, elevation of circulating SN levels in CPVT and cardiac arrest patients could result from increased SgII production. Indeed, we observed augmented SgII production in cardiomyocytes from CPVT mice at baseline (Figure 2A), and we have previously observed increased SgII transcription in postinfarction HF.<sup>8</sup> Upregulation of SgII has also been shown to be induced in mice during hypoxia.<sup>22</sup> Thus, SgII upregulation may be a common feature of various cardiac pathologies. However, it is difficult to verify this claim in our present patient cohorts because we did not have access to myocardial biopsies and because it is mainly the SN cleavage product that is measured in blood (>90%).<sup>23,24</sup> Alternatively, processing of SgII to SN may be increased in the patient populations examined, paralleling our previous observations in HF, where enhanced SgII cleavage was attributed to upregulation of proprotein convertase 1/3 and proprotein convertase 2.<sup>8</sup> Finally, SN may be released from existing stores in cardiac cells in CPVT and cardiac arrest patients, or there may be leakage of SN into the circulation from other organs, such as the lung or brain, as previously suggested.<sup>19</sup> Although the precise source of SN in our patient cohorts is unclear, so too is

the initiating trigger. One possibility is that elevation of SN may be signaled by rising Ca<sup>2+</sup> levels. The cytosolic Ca<sup>2+</sup> concentration is high during arrhythmias and myocardial injury, and high cytosolic Ca<sup>2+</sup> concentration is a well-known activator of CaMKII $\delta$ . Thus, increased SN levels could provide a feedback mechanism to prevent excessive CaMKII $\delta$  activation.

A growing consensus linking CaMKII $\delta$  activation to cardiac arrhythmia during  $\beta$ -adrenergic stimulation has indicated an involvement of both cAMP/exchange factor directly activated by cAMP (Epac)-dependent and cAMP-independent signaling pathways.<sup>2</sup> Numerous previous reports have linked CaMKII $\delta$ -dependent activation of RyRs and L-type Ca<sup>2+</sup> channels to generation of DADs and EADs and shown that CaMKII $\delta$  inhibition protects against such Ca<sup>2+</sup>-dependent arrhythmogenesis.<sup>1</sup> The present study is, to the best of our knowledge, the first to show such antiarrhythmic effects of an endogenous CaMKII $\delta$  inhibitor. We have recently reported that SN is readily taken up into cardiomyocytes by endocytosis,<sup>7</sup> whereas limited cardiomyocyte uptake of other CaMKII $\delta$  inhibitors precludes their possible use in patients.<sup>1</sup> We now present data showing that SN binds within a pocket of the S-site of CaMKII $\delta$ , with modeling predicting 2 possible interaction sites. Follow-up mutagenesis experiments specifically supported that the binding region of SN centers around Thr169. Such binding may directly interfere with the ability of the kinase domain to phosphorylate its targets, including Ser2814 on RyR2. SN blockade of the S-site also seems to limit autophosphorylation at Thr287 and thereby kinase activity. It is interesting to note that although we previously reported that SN provides rather modest reduction in CaMKII $\delta$  activity,<sup>7</sup> our present results show more striking effects on CaMKII $\delta$  phosphorylation targets and the incidence of Ca<sup>2+</sup> sparks, waves, DADs, and EADs during isoproterenol stimulation. The reason for this discrepancy may be because of feed-forward mechanisms present in cells not accounted for in ex vivo assays. In cells, CaMKII $\delta$  is stimulated by local Ca<sup>2+</sup> levels. Therefore, by inhibiting CaMKII $\delta$  and thus RyR2 leak, SN is expected to reduce the pool of dyadic Ca<sup>2+</sup> which regulates local CaMKII $\delta$ . In electrically stimulated cells, inhibition of L-type Ca<sup>2+</sup> current by SN may additionally reduce this local dyadic Ca<sup>2+</sup> pool. Of note, previous work has linked CaMKII $\delta$  activity to facilitation of Ca<sup>2+</sup> current, manifested as a proarrhythmic slowing of current kinetics.<sup>25</sup> As this phenomenon is predominantly present at physiological resting potentials, our present measurements of L-type currents using depolarized holding potentials may, in fact, underestimate the extent of SN-dependent CaMKII $\delta$  inhibition.

Although CaMKII $\delta$ -dependent activation of RyRs and L-type Ca<sup>2+</sup> channels promotes the generation of DADs and EADs, it is important to point out that these effects can be codependent and that the presently observed actions of SN may reflect these overlap-

ping roles. For example, although CaMKII $\delta$ -dependent phosphorylation of the L-type Ca<sup>2+</sup> channel increases the likelihood of EADs,<sup>5,6</sup> increased L-type current also loads the SR and promotes spontaneous RyR2 Ca<sup>2+</sup> release (DADs).<sup>1</sup> Similarly, although increased RyR2 open probability promotes Ca<sup>2+</sup> waves/DADs during diastole, Ca<sup>2+</sup> release during action potential repolarization can promote EADs.<sup>26</sup> Protection against EADs and DADs by SN may also involve additional CaMKII $\delta$ -regulated proteins and include inhibition of late Na<sup>+</sup> current and various K<sup>+</sup> channels.

Although we have presently observed encouraging effects of SN treatment in CPVT mice, additional studies are required to assess the potential of exogenous SN as a therapeutic drug. It is interesting to note that overall Ca<sup>2+</sup> homeostasis was not negatively affected by SN treatment. A baseline reduction in Ca<sup>2+</sup> leak was associated with increased SR content and Ca<sup>2+</sup> transient magnitude in the SN group (Figure IIA in the [Data Supplement](#)). Such differences were not observed during isoproterenol, where SR content and release were markedly elevated but similar in SN-treated and untreated cells.  $\beta$ -adrenergic stimulation augments SR function via phosphorylation of phospholamban at Ser16 and Thr17, which disinhibits sarcoendoplasmic reticulum Ca<sup>2+</sup>-ATPase. SN treatment reversed isoproterenol-dependent phosphorylation at the CaMKII $\delta$ -dependent Thr17 site (Figure IIB in the [Data Supplement](#)). Thus, effects of CaMKII $\delta$  inhibition by SN seem to balance reductions in SR Ca<sup>2+</sup> leak and reuptake during  $\beta$ -adrenergic challenge, resulting in maintained SR content and Ca<sup>2+</sup> transients while inhibiting Ca<sup>2+</sup> wave/DAD generation. Patient groups such as those with HF might, therefore, benefit from the antiarrhythmic effects of SN without risk of reduced contractility.

In conclusion, our results support antiarrhythmic actions of the endogenous peptide SN via inhibition of CaMKII $\delta$  activity, L-type Ca<sup>2+</sup> channel opening, and the generation of EADs and DADs. We suggest that these actions underlie SN's applicability as a biomarker and, with further work, that patients at risk for arrhythmia may potentially benefit from exogenous SN.

## ARTICLE INFORMATION

Received November 26, 2017; accepted February 12, 2019.

The Data Supplement is available at <https://www.ahajournals.org/doi/suppl/10.1161/CIRCEP.118.007045>.

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## Acknowledgments

We thank the Section of Comparative Medicine at Oslo University Hospital Ullevål for expert animal care, and the FINNICU (Finnish Intensive Care Unit) network and Clinical Trial Unit, Division of Medicine, Akershus University Hospital for blood sampling. We also thank Inger Olsson, Uppsala University for granin protein measurements and Professor Tor-Arne Hagve, Division of Diagnostics and Technology, Akershus University Hospital and the University of Oslo for hs-TnT and NT-proBNP measurements.

## Sources of Funding

Generous funding was received from the Norwegian National Association for Public Health (Dr Røsjø), the Centre for Heart Failure Research, the K. G. Jebsen Foundation (Dr Ottesen), and the European Union's Horizon 2020 research and innovation programme (Consolidator Grant, No 647714, Dr Louch). Additional support was received from The South-Eastern Norway Regional Health Authority (Dr Louch), Anders Jahre's Fund for the Promotion of Science, The Norwegian Institute of Public Health, Oslo University Hospital Ullevål (Dr Louch), the University of Oslo (Drs Louch and Carlson), the Research Council of Norway (Drs Carlson, Omland, Røsjø, Lunde), and Akershus University Hospital (Drs Ottesen, Omland, Røsjø). We gratefully acknowledge funding from the South-Eastern Norway Regional Health Authority (Regional Core Facility for Structural Biology; Grant 2015095, BD), and Grant to the Laboratory of Clinical Molecular Biology, Akershus University Hospital (South-Eastern Regional Infrastructure for Clinical Translational Research [SERIT]).

## Disclosures

Drs Stridsberg, Omland, Christensen, and Røsjø are partners in a patent application filed by the University of Oslo regarding the use of SN (secretoneurin) as a biomarker in cardiovascular disease and in patients with critical illness. Dr Røsjø, Christensen, and Omland have financial interests in CardiNor AS, which holds the license to commercialize secretoneurin. Dr Ottesen, Stridsberg, Omland, and Røsjø have also received personal payments from CardiNor AS. Dr Omland has served on advisory boards and received speaker's honoraria and travel funding from Roche Diagnostics, and Roche Diagnostics provided hs-TnT (high-sensitivity troponin T) and NT-proBNP (N-terminal pro-B-type natriuretic peptide) kits at a reduced price via Akershus University Hospital. Drs Myhre, Omland, and Røsjø have also received personal fees from Novartis and Drs Omland and Røsjø from Thermo Fisher BRAHMS. The other authors report no conflicts.

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