

Circulation: Arrhythmia and Electrophysiology

ORIGINAL ARTICLE

Thyrotropin Directly Affects Cardiac Electrophysiology and Is Associated With AF Prevalence

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BACKGROUND: Although hyperthyroidism is known to increase the risk of atrial fibrillation (AF), subclinical hypothyroidism (SH) is an often-underreported condition characterized by elevated thyroid-stimulating hormone (TSH) levels and normal fT₃/fT₄ levels. This study aimed to clarify the association between SH and AF and to identify potential direct electrophysiological effects of TSH.



METHODS: We retrospectively included 2311 patients diagnosed with SH between 2007 and 2020 who had an ECG within 7 days of diagnosis. Logistic regression analysis identified factors independently associated with AF in patients with SH. Effects of different TSH doses on ion channel mRNA and protein levels were analyzed in HL-1 and neonatal rat cardiomyocytes. Video analysis with MYOCYTER, patch-clamp, optical mapping, and computational modeling were used to study automaticity and action potential characteristics after TSH application.

RESULTS: AF was documented more often with higher TSH levels (4–10 mU/L TSH: 32.1% versus >10 mU/L TSH: 44.6%; $P<0.0001$). Multivariable regression identified elevated TSH levels as an independent risk factor for AF. TSHR (TSH receptors) were confirmed in cardiomyocytes, and exposure to TSH led to changes in ion channel expression levels that promoted action potential prolongation. TSH also increased the beating rate in neonatal rat cardiomyocytes. We identified a TSHR-mediated cascade involving cAMP, PKA (protein kinase A), and CREBH (cAMP-responsive element-binding protein H) as a potential regulator of cardiomyocyte electrical remodeling leading to the proarrhythmic effects that promote the development of AF.

CONCLUSIONS: Individuals with SH exhibit an increased prevalence of AF, which is likely in part due to a direct effect of TSH on ion channel expression in cardiomyocytes via the TSHR/cAMP/PKA pathway.

GRAPHIC ABSTRACT: A graphic abstract is available for this article.

Key Words: atrial fibrillation ■ hypothyroidism ■ ion channels ■ receptor, thyrotropin ■ thyrotropin

Atrial fibrillation (AF) is the most common persistent heart rhythm disorder encountered in clinical practice, with a prevalence of 1% to 3% in the general population.^{1,2} Demographic changes are expected to lead

to a further increase in its prevalence.^{1,3} Current guidelines for AF management highlight the potential role of thyroid dysfunction in AF development.⁴ Hyperthyroidism is strongly associated with AF,⁵ but AF is also more

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Supplemental Material is available at <https://www.ahajournals.org/doi/suppl/10.1161/CIRCEP.125.013775>.

For Sources of Funding and Disclosures, see page XXX.

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Circulation: Arrhythmia and Electrophysiology is available at www.ahajournals.org/journal/circep

WHAT IS KNOWN?

- Hyperthyroidism increases the risk of atrial fibrillation.
- Subclinical hypothyroidism is an often-underreported condition characterized by elevated thyroid-stimulating hormone levels and normal fT₃/fT₄ levels.

WHAT THE STUDY ADDS

- Thyroid-stimulating hormone exerts direct effects on cardiomyocytes via interaction with a thyroid-stimulating hormone receptor and activation of cAMP-dependent signaling, leading to ion channel remodeling.
- We show that elevated thyroid-stimulating hormone levels are an independent risk factor for atrial fibrillation.

Nonstandard Abbreviations and Acronyms

AF	atrial fibrillation
AP	action potential
K2P	2-pore domain K ⁺ -channels
CRE	cAMP response element
KCa	calcium-activated potassium channels
APD	action potential duration
CREBH	cAMP-responsive element-binding protein H
NRCM	neonatal rat cardiomyocytes
SH	subclinical hypothyroidism
TSH	thyroid-stimulating hormone
TSHR	thyroid-stimulating hormone receptor

common in cases of subclinical hypothyroidism (SH; Table S1).^{6,7} The prevalence of SH in the general population is reported to be 0.4% to 16.9%, with ≈2% to 5% of patients with SH progressing to clinically significant hypothyroidism.^{8,9} Patients with higher concentrations of thyroid antibodies and increased thyroid-stimulating hormone (TSH) levels are particularly susceptible to disease progression.¹⁰ However, whether elevated TSH concentrations present in SH correlate with AF and require therapeutic interventions remains a controversial issue and requires further in-depth investigation.^{11–16}

The influence of TSH on ion channels and key regulatory proteins in cardiomyocytes is largely unknown. In thyroïdal cells, TSH acts via a functional TSHR (TSH receptor), which activates intracellular signaling cascades, including stimulatory G-protein subunits (G_{α_s}), leading to an increase in cAMP concentration and PKA (protein kinase A).^{17–19} The CREB (cAMP response element-binding) pathway plays a crucial role in cellular signaling, and some studies have suggested a possible

link between the TSH signal transduction cascade and β-adrenergic stimulation.²⁰ Activation of this pathway, often initiated by TSH, triggers transcriptional regulation of numerous targets, including remodeling of regulatory kinases and adenylate cyclases, influencing key processes in cardiomyocytes.^{18,21} These regulatory cascades are essential for cellular responses in cardiac tissue and may also alter cardiomyocyte electrophysiology, potentially contributing to TSH-associated arrhythmogenesis.

We hypothesized that TSH directly regulates cardiac electrophysiology and AF prevalence. The objective of this study is to elucidate the direct effect of TSH on cardiac cellular electrophysiology, identify associations between TSH levels and AF prevalence, and determine if TSH is an independent risk factor for AF occurrence.

A deeper understanding of the pathophysiological mechanisms underlying direct TSH-induced proarrhythmic effects could lead to TSH concentration-dependent therapies and may offer new therapeutic options. This, in turn, might open the option for targeted individualized AF screening and therapy in this specific patient cohort.

METHODS



The data that support the findings of this study are not included in the study, and the [Supplemental Materials](#) are available from the corresponding author on reasonable request.

The study involving patient data was conducted in accordance with the Declaration of Helsinki, and the study protocol was approved by the Ethics Committee of the University of Heidelberg (Germany; institutional approval number S-276/2020). Animal experiments have been performed in accordance with the Guide for the Care and Use of Laboratory Animals from the US National Institutes of Health (publication No. 86-23, revised 1985) and with EU Directive 2010/63/EU, and the current version of the German Law on the Protection of Animals was followed. Experiments involving neonatal rat cardiomyocytes (NRCM; institutional approval numbers T-45/20; T-42/21; T-03/22; T-24/22) have been approved by the local animal welfare authority.

Patient Population

The retrospective patient collective was collected by the Research Data Warehouse of the Department of Cardiology at Heidelberg University. All adult patients who received thyroid hormone analysis between 2007 and 2020 and had elevated TSH (>4 mU/L) and normal fT₄ (8–18 ng/L) were included. Patients were divided into 2 groups (group 1: TSH levels of 4.0–10.0 mU/L and group 2: TSH levels >10.0 mU/L) based on guideline-based treatment recommendations for SH.¹⁵ Medical documents stored in the internal archive were analyzed. Additional inclusion and exclusion criteria and information on data collection can be found in the [Supplemental Methods](#) (Figure S1).

Collection of Human Heart Samples

Patients scheduled for cardiac surgery were included, and written informed consent for sample collection was obtained.

The atrial and ventricular samples were weighed, frozen using liquid nitrogen, and stored at -80°C . Frozen samples were subsequently cryosectioned. Details are presented in the [Supplemental Methods](#).

Cell Culture of HL-1, CHO Cells, and NRCM

HL-1 and CHO cells were cultured as described in detail in the [Supplemental Material](#). NRCMs were isolated from 1- to 3-day-old Wistar rats. Additional details of animal handling and cell isolation can be found in detail in the [Supplemental Material](#).

RNA Isolation and Quantitative Real-Time Polymerase Chain Reaction

HL-1 and NRCM were incubated with respective TSH concentrations for 24 hours. mRNA levels were studied using quantitative real-time polymerase chain reaction, as published.²²⁻²⁴ TaqMan Gene Expression Assays ([Table S2](#)), details of mRNA sequencing and data analysis are described in the [Supplemental Material](#).

Protein Isolation and Western Blotting

Proteins from cells and tissues were collected, and protein immunodetection was performed by Western blotting as described previously.^{23,24} Details on methods and antibodies ([Table S3](#)) are presented in the [Supplemental Methods](#).

Immunofluorescence Staining

HL-1 cells and neonatal NRCM were first plated out on 6-well plates equipped with 20 \times 20 mm cover glasses and incubated with different TSH concentrations for 24 hours. Human right atrial appendage, left ventricular tissue, and thyroid samples were cryosectioned and used for immunofluorescence staining. Technical details and antibodies ([Table S4](#)) can be found in the [Supplemental Methods](#).

RNA-Interference

Transfection of siRNA directed against TSHR (SR419940, OriGene) was performed with Lipofectamine RNAiMax (Thermo Fisher Scientific, Waltham, MA) in HL-1 cells according to the manufacturer's instructions. Details are presented in the [Supplemental Methods](#).

cAMP ELISA

cAMP levels were measured with a cAMP ELISA Kit (no. ADI-900-066; Enzo Life Sciences) in HL-1 cells 0, 4, 12, and 24 hours after application of different TSH concentrations. Details of the analysis can be found in the [Supplemental Methods](#).

Patch-Clamp Measurements in HL-1

Cardiac action potentials (APs) were recorded from HL-1 cells after 24-hour application of TSH concentrations using the whole-cell patch-clamp technique. Details on protocols and solutions are provided in the [Supplemental Methods](#).

Video Analysis With Myocytex

To determine the contraction frequency of NRCM, Makro Myocytex (v. 1.3) was used as a plug-in for the image processing software ImageJ (v. 1.52b, Wayne Rasband; National Institutes of Health). Details can be found in the [Supplemental Methods](#).

Computational Modeling of AP Morphology

The experimentally observed changes in mRNA levels in response to different concentrations of TSH were used to scale individual ion currents in a state-of-the-art model of the adult human atrial cardiomyocyte electrophysiology. AP properties were assessed during steady-state pacing at 1, 2, and 3 Hz.²⁵ The changes in ion currents are summarized in [Table S5](#). A 1-factor-at-a-time sensitivity analysis was performed by introducing changes in each ion current individually.

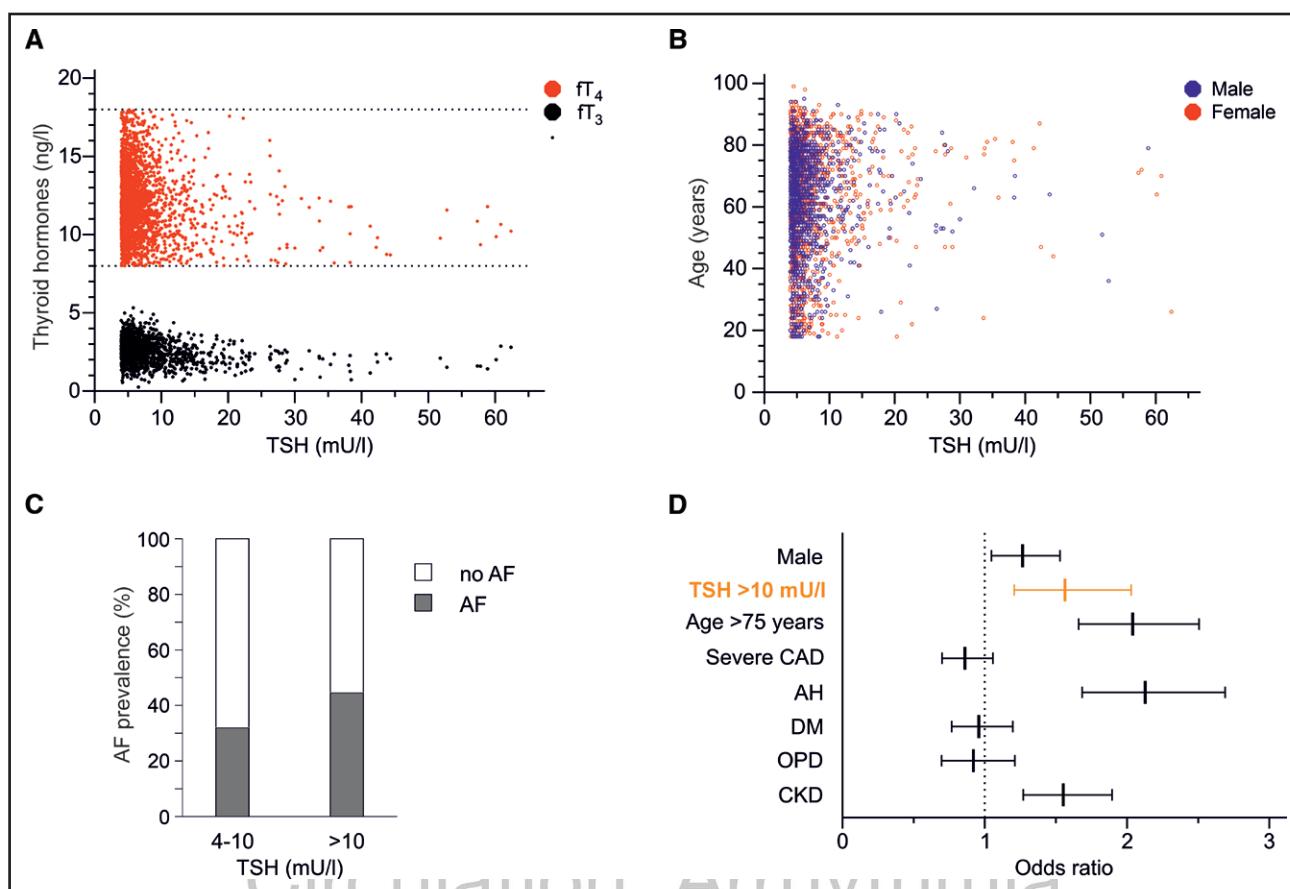
Statistics

Statistical analyses were performed using Prism 10 (GraphPad Software, San Diego, CA) and Origin2023 (OriginLab Corporation, Northampton, MA). Normality was assessed using the Kolmogorov-Smirnov test. Depending on the variable type (categorical variables or continuous variables), distribution, and the underlying question, data were expressed as count (n) with percentage (%), median, and interquartile ranges. We applied χ^2 test, Fisher exact test, Student *t* test, Mann-Whitney *U* test, ANOVA, or Kruskal-Wallis test, as appropriate. A *P* value of <0.05 was considered statistically significant. Details on the logistic regression model can be found in the [Supplemental Methods](#).

RESULTS

Incidence of AF in Patients With Elevated TSH

In the examined group of patients, the fT_4 values spanned from 8.00 to 17.92 ng/L, ([Figure S1](#)). The $\text{fT}3$ values ranged from 0.26 to 5.32 ng/L ([Figure 1A](#)), whereas the TSH levels varied between 4.01 and 62.41 mU/L ([Figure 1B](#)). The patient cohort was stratified into 2 distinct groups based on TSH levels corresponding to different SH treatment recommendations.¹⁵ Group 1 comprised 2006 patients with mildly elevated TSH levels (4.0–10.0 mU/L), whereas group 2 consisted of 305 patients with TSH levels >10.0 mU/L. Patients in this cohort ranged from 18 to 99 years, illustrating a typical age distribution for an industrialized nation ([Figure 1B](#)). Individuals in group 2 were slightly older, with mean ages of 62.2 ± 17.6 years in group 1 compared with 65.6 ± 16.3 years in group 2 ($P < 0.01$), whereas body mass index was not statistically significantly different between the 2 groups (27.4 ± 5.7 versus 27.5 ± 7.2 kg/m², $P = 0.89$; [Table](#)). Of note, a higher proportion of patients in group 2 received antiarrhythmic medications (78.0% compared with 69.9% in group 1, $P < 0.01$), including β -blockers and amiodarone. The indication for amiodarone was AF in 53.7% versus 47.7% in both groups ([Figure S2](#)). TSH

**Figure 1. xxx.**

A, Visualization of the fT_3 and fT_4 levels of patients included in the study in relation to their respective thyroid-stimulating hormone (TSH) levels. The normal range of fT_4 is marked with black lines ($n=2311$). **B**, Visualization of the age distribution of patients, separated by both sexes (blue male, red female), in relation to their respective TSH levels, age ranges from 18 to 99 years ($n=2311$). **C**, Proportion of patients with reported atrial fibrillation (AF) in their medical history. The patient collective was divided into 2 groups according to their TSH level. There was a significant difference between the 2 groups regarding AF prevalence ($P<0.0001$). Statistical significance was calculated using the χ^2 test ($n=2311$). **D**, Odds ratios of the independent variables of the multiple logistic regression. AF was the dependent variable. CKD (chronic kidney disease): the renal function was considered impaired if the eGFR was below 60 mL/min per 1.73m². AH indicates arterial hypertension; CAD, coronary artery disease; DM, diabetes; and OPD, obstructive pulmonary disease.

values before and under amiodarone are shown in Figure S3. AF was documented in 643 patients (32.1%) in group 1 and in 136 patients (44.6%) in group 2 ($P<0.0001$; Figure 1). The significant difference in AF prevalence between TSH groups remained robust when excluding patients receiving amiodarone or patients with a known thyroid disease or L-thyroxine intake (Tables S6 and S7; Figures S4 and S5).

Logistic regression (Figure 1F; Table S8) identified TSH >10 mU/L, as well as advanced age, arterial hypertension, renal dysfunction, and male sex as independent risk factors associated with AF. A TSH level exceeding 10.0 mU/L was associated with an odds ratio for AF of 1.6 (95% CI, 1.2–2.0), comparable to other recognized risk factors for AF. The independent association between elevated TSH and AF remained after excluding patients receiving amiodarone or L-thyroxin, or with preexisting thyroid disease (Tables S9 and S10). On the other hand, diabetes and coronary artery disease did not achieve

statistical significance within our selected patient cohort. Similarly, no significant differences were noted in terms of heart rate, PQ interval, or QRS duration. However, a slight QTc interval prolongation was observed in group 2 patients with TSH values exceeding 10 mU/L (group 1: 418 ± 31.9 ms, $n=1881$ versus group 2: 426 ± 38.8 ms, $n=275$, $P<0.001$; Table S11). Similarly, we did not observe significant differences in left atrial diameter, left ventricular end-diastolic diameter, or the presence of diastolic dysfunction in the subset of patients for whom echocardiography data were available. However, patients with higher TSH levels exhibited a slightly lower left ventricular ejection fraction compared with those in group 1 (40.8% versus 36.9%, $P=0.04$; Table S12).

TSHR Expression in Cardiomyocytes

To exert direct effects on cardiomyocytes, TSH must interact with a specific TSHR on the plasma membrane

Table. Baseline Characteristics of Patients, Their Medication, and Their Medical History (n=2311)

Variables	All (n=2311)	TSH 4–10 mU/L (n=2006)	TSH >10 mU/L (n=305)	P value
Demographic				
Male sex, n (%)	1248 (54.0)	1128 (56.2)	120 (39.3)	<0.0001*
Age, y (mean±SD)	62.6 (±17.5)	62.2 (±17.6)	65.6 (±16.3)	<0.01
Body mass index, kg/m ² (mean±SD)	27.4 (±5.8)	27.4 (±5.7)	27.5 (±7.2)	0.89
Medication				
Antiarrhythmic drugs, n (%)	1634 (70.7)	1396 (69.6)	238 (78.0)	<0.01*
Class I, n (%)	17 (0.7)	14 (0.7)	3 (1.0)	0.59
Class II, n (%)	1573 (68.1)	1345 (67.0)	228 (74.8)	<0.01*
Class III, n (%)	191 (8.3)	147 (7.3)	44 (14.4)	<0.0001*
Class IV, n (%)	85 (3.7)	71 (3.5)	14 (4.6)	0.40
Cardiac glycosides, n (%)	156 (6.8)	132 (6.6)	24 (7.9)	0.56
Ivabradine, n (%)	51 (2.2)	48 (2.4)	3 (1.0)	0.12
L-thyroxine, n (%)	743 (32.2)	545 (27.2)	198 (64.9)	<0.0001*
Medical history				
Thyroid disease, n (%)	1104 (47.8)	868 (43.3)	236 (77.4)	<0.0001*
Atrial fibrillation, n (%)	779 (33.7)	643 (32.1)	136 (44.6)	<0.0001*
Severe CHD, n (%)	897 (38.8)	786 (39.2)	111 (36.4)	0.35
Hypertension, n (%)	1571 (68.0)	1351 (67.3)	220 (72.1)	0.60
Lipid metabolism disorder, n (%)	955 (41.3)	830 (41.4)	125 (41.0)	0.90
diabetes, n (%)	507 (21.9)	430 (21.4)	77 (25.2)	0.13
insulin-dependent, n (%)	202 (8.7)	170 (8.5)	32 (10.5)	0.25
Asthma or COPD, n (%)	282 (12.2)	234 (11.7)	48 (15.7)	0.04*
Impaired renal function, n (%)	741 (32.4)	589 (29.7)	152 (50.3)	<0.0001*
eGFR, mL/min per 1.73m ² (mean±SD)	74.8 (±29.6)	76.4 (±29.2)	64.3 (±29.8)	<0.0001*

The collective was divided into 2 groups: the first group comprising patients with a TSH level of 4 to 10 mU/L (n=2006) and the second group comprising patients with a TSH level >10 mU/L (n=305). Metric data are expressed as mean±SD. Dichotomous data are expressed as absolute and relative frequencies. Statistical significance was calculated using the 2-tailed *t* test for metric data and the χ^2 test for dichotomous data. CHD was classified as severe if stenoses >50% were present or revascularization was required. Renal function was classified as impaired if the eGFR was <60 mL/min per 1.73m². CHD indicates coronary heart disease; eGFR, estimated glomerular filtration rate; and TSH, thyroid-stimulating hormone.

*  FIRST PROOF ONLY

of these cardiomyocytes. We confirmed the presence of TSHR expression in HL-1, NRCM, and human cardiac tissue through multiple methods including mRNA sequencing, quantitative real-time polymerase chain reaction, Western blots, and immunofluorescence analysis (Figure 2). Immunofluorescent staining provided visual evidence of TSHR expression at the protein level in both HL-1 and NRCM cells.

Impact of TSH on Expression Levels of Cardiac Ion Channels

To analyze the direct influence of TSH on mRNA expression profiles of ion channels in cardiomyocytes, HL-1 cell cultures were incubated with 15, 30, or 60 mU/L TSH for 24 hours. TSH induced dose-dependent expression changes (Figure S6), including changes in cardiac ion channel subunits after 24 hours (Figure 3A and 3B). These findings were subsequently validated using

quantitative real-time polymerase chain reaction: After TSH incubation with 15 mU/L, there was a significant upregulation of *mCacna1c* (+28±12%; $P=0.034$; n=9), as well as a downregulation of *mCacna1s* (-47±6%; $P=0.0008$; n=6) and *mKcnq1* (-23±4%; $P=0.017$; n=6) expression (Figure 3C). Incubation with 30 mU/L TSH similarly increased mRNA of *mCacna1c* +27±9% ($P=0.012$; n=6) while decreasing *mScn5a* -25±5% ($P=0.049$; n=6) and *mSlc8a1* -17±6% ($P=0.043$; n=6) expression (Figure 3D). Incubation with 60 mU/L TSH led to increased expression of *mScn5a* (+25±9%; $P=0.045$; n=6), *mCacna1c* (+52±7%; $P=0.0004$; n=6), *mKcnq1* (+38±11%; $P=0.021$; n=6), *mKcn1* (+93±10%; $P=0.009$; n=6), and *mKcnk3* (+77±15%; $P=0.005$; n=6) expression (Figure 3E). Moreover, a selective siRNA-induced knockdown of the TSH receptor (-54±5%; $P=0.0001$; n=18) in HL-1 cells led to an upregulation of *mKcnk3* (+86±42%; $P=0.0001$; n=12) and *mCacna1s* (+173±64%; $P=0.0001$; n=15) mRNA

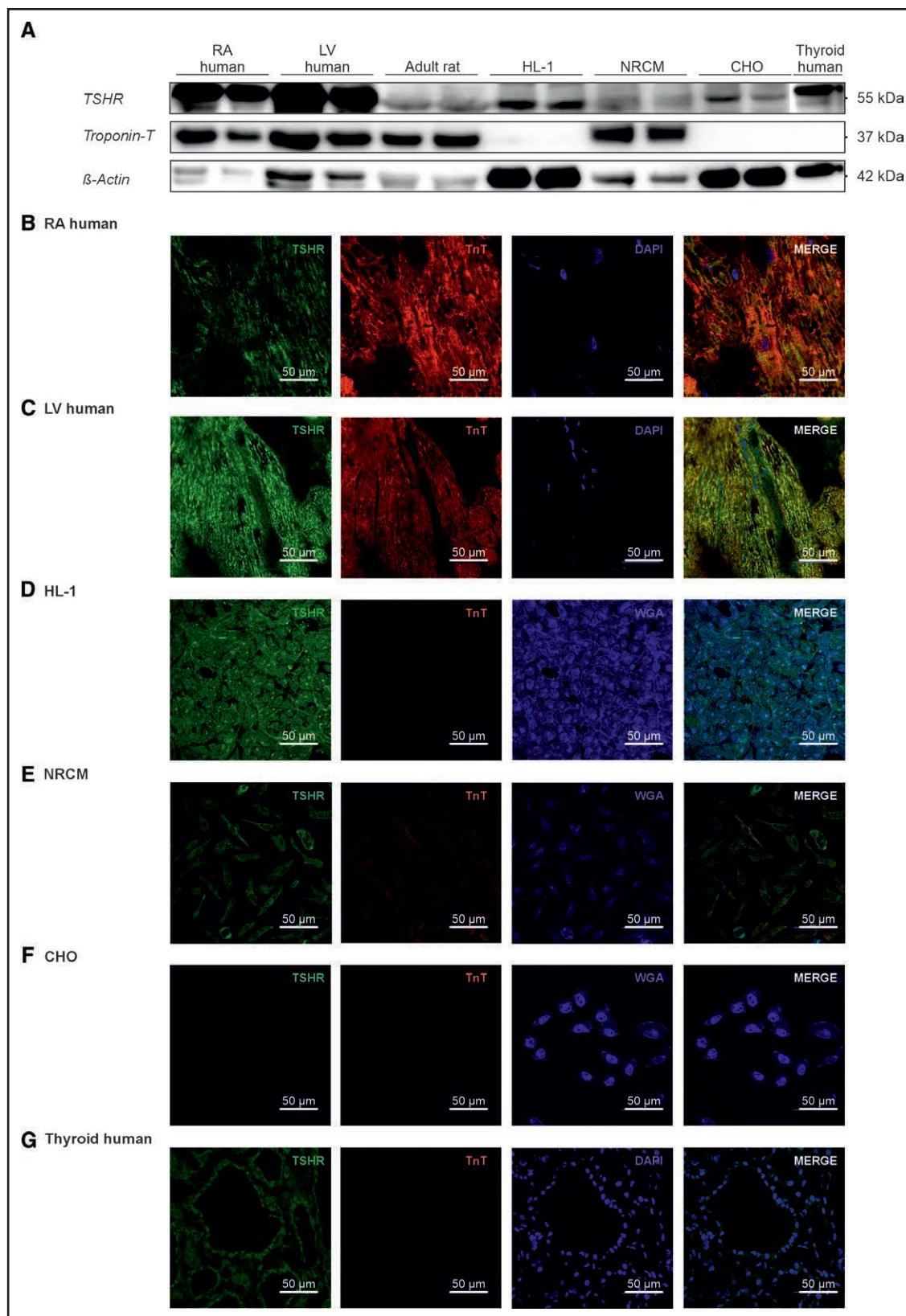


Figure 2. TSHR (thyroid-stimulating hormone [TSH] receptor) expression in cardiomyocytes.

A, Western blot of TSHR expression in human right atrium (RA) and left ventricle (LV), adult rat cardiomyocytes, CHO, HL-1 and neonatal rat cardiomyocytes (NRCM). Human protein lysate from thyroid glands served as positive control. **B** through **F**, Confocal images of TSHR staining (AlexaFluor 488, green) together with membrane (WGA [wheat germ agglutinin], blue) or DNA staining (DAPI [4,6-diamidino-2-phenylindole], blue) show that the TSHR is expressed at the protein level in human atrial (**B**) and ventricular tissue (**C**), HL-1 cells (**D**) and in NRCM (**E**), and human thyroid (**G**). TnT (troponin T) marked in red. CHO cells (**F**) serve as a negative control and show no expression of the TSHR or troponin (n=3).

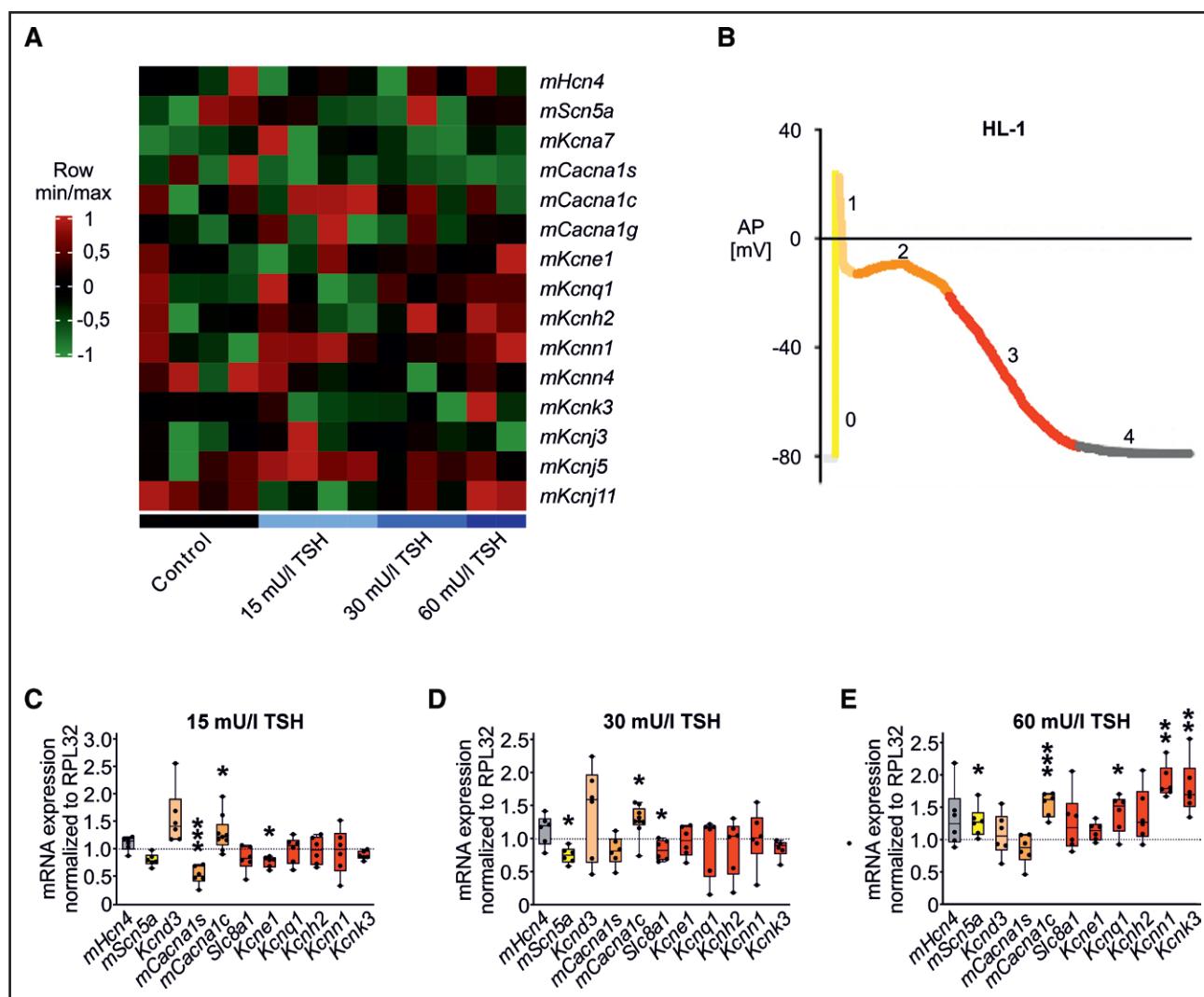


Figure 3. Remodeling of ion channels and subunits after 24-hour incubation with 15, 30, 60 mU/L thyroid-stimulating hormone (TSH) on mRNA expression obtained in HL-1 cells.

A, mRNA sequencing analysis of HL-1 (**A**) reveals dose-dependent expression changes. **B**, Schematic representation of a human atrial action potential (AP) showing phase 0 (yellow), phase 1 (beige), phase 2 (orange), phase 3 (red), and phase 4 (gray). **C** through **E**, mRNA expression of ion channels and subunits after incubation with respective TSH concentrations analyzed by quantitative real-time polymerase chain reaction: 15 mU/L (**C**), 30 mU/L (**D**), and 60 mU/L (**E**) TSH in HL-1 cells. The ion channels and subunits of the phases of the action potential are color-coded accordingly. Data are visualized as box plots and dots representing raw data* $P<0.05$, ** $P<0.01$, *** $P<0.001$ vs control (*mRPL32*; Student *t* tests).

transcripts compared with control with scrambled siRNA (Figure S7).

To analyze changes in protein expression HL-1 cell cultures were incubated for 24 hours with 15, 30, or 60 mU/L TSH, and expression levels were assessed using Western blot (Figure 4). After incubation with 15 mU/L TSH, *mCa_v1.2* (+18±5%; $P=0.011$; $n=4$) was upregulated, whereas *mCa_v1.1* –81±9% ($P=0.022$; $n=4$) and *mKcne1* (–65±21%; $P=0.033$; $n=4$) were downregulated (Figure 4A and 4B). Incubation with 30 mU/L TSH similarly resulted in increased expression of *mCa_v1.2* (+53±10%; $P=0.008$; $n=4$), as well as decreased expression of *mNa_v1.5* (–31±10%; $P=0.045$; $n=8$) and *mNCX1* –32±7% ($P=0.017$; $n=6$; Figure 4C and 4D). Application of 60 mU/L TSH increased expression

of *mNa_v1.5* (+102±23%; $P=0.006$; $n=4$), *mCa_v1.2* (+145±7%; $P=0.031$; $n=4$), *mK_v7.1* (+223±82%; $P=0.043$; $n=4$), *mK_{Ca}2.1* (+207±74%; $P=0.044$; $n=4$) and *mK_{2P}3.1* (+56±19%; $P=0.02$; $n=12$; Figure 4E and 4F). The changes in protein expression could be confirmed in immunofluorescence staining in HL-1 cells (Figure 4G). These data suggest a consistent TSH-dependent upregulation of L-type Ca²⁺-channel subunit expression that may affect cellular electrophysiology.

APs in HL-1 Are Prolonged After TSH Incubation

Patch-clamp experiments were performed to identify possible TSH-induced changes of atrial AP morphology

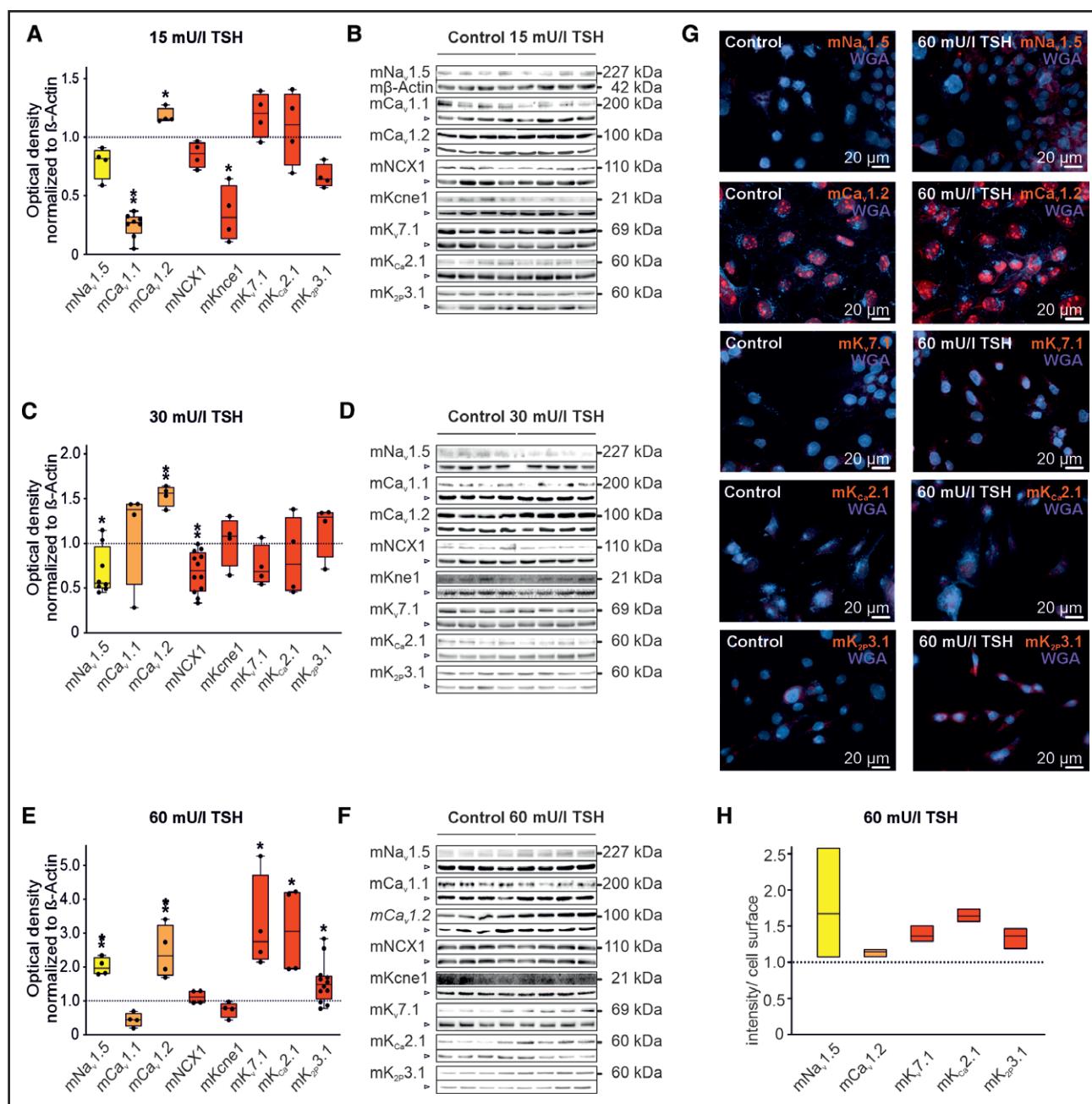


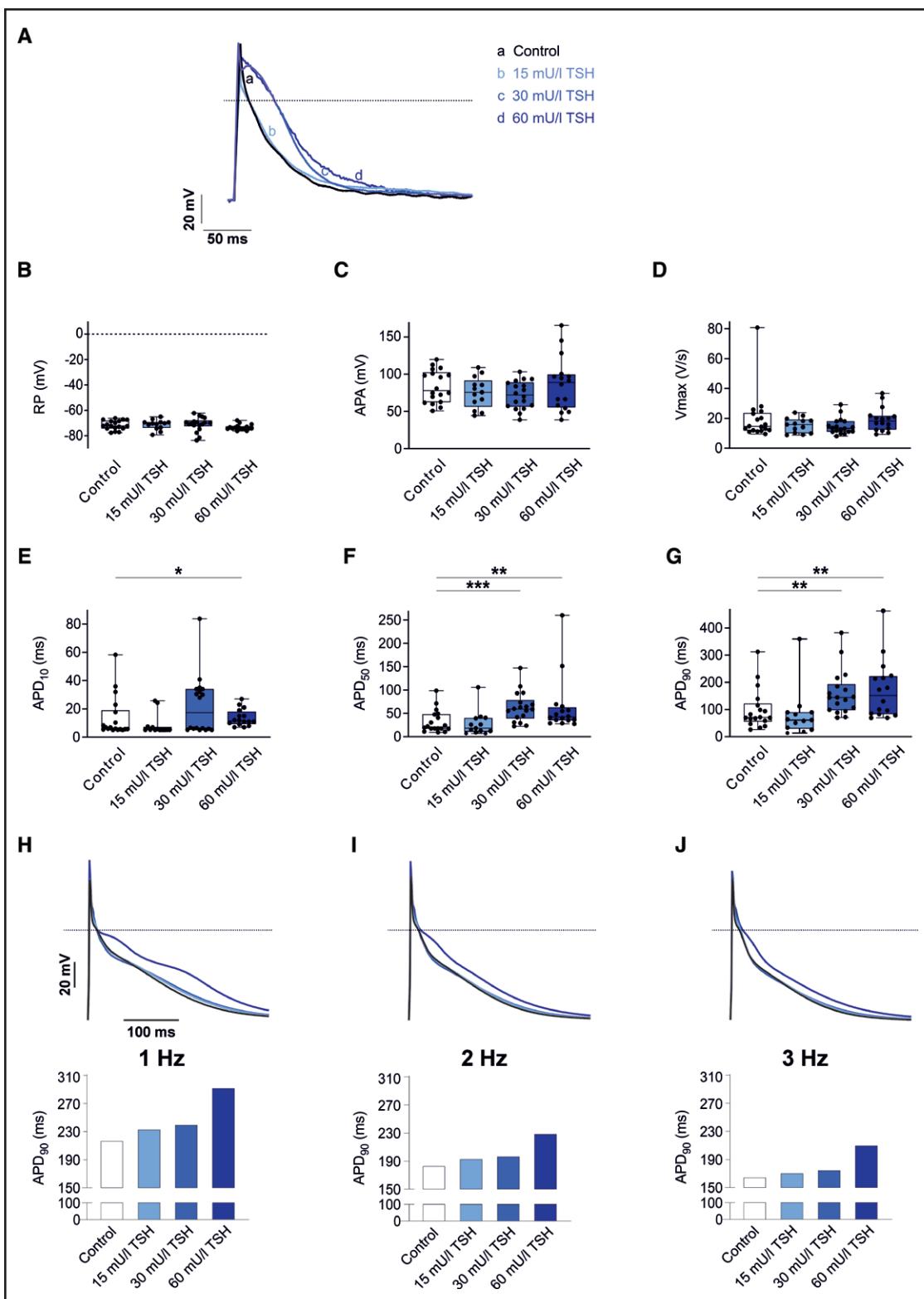
Figure 4. Remodeling of selected ion channels and subunits on protein level after 24-hour incubation with thyroid-stimulating hormone (TSH) in HL-1 cells.

Expression changes after incubation with 15 mU/L (A and B), 30 mU/L (C and D), and 60 mU/L TSH (E and F). Shown are cutout bands from exemplary stripped Western blots. Data are presented as boxplots with data points ($n=4-12$) representing raw data. G, Exemplary immunofluorescence stainings (G) of HL-1 cells after incubation with 60 mU/L TSH and (H) quantification of protein levels ($n=3$). * $P<0.05$, ** $P<0.01$ vs control ($m\beta$ -Actin, indicated by □; Student *t* tests). Complete Western blots can be found in the [Supplemental Material](#). WGA indicates wheat germ agglutinin

in HL-1 cells (Figure 5A; Figure S8). The resting membrane potential, AP amplitude, and maximum upstroke velocity of the AP were not significantly affected by TSH (Figure 5B through 5D). By contrast, AP duration (APD)₅₀ and APD₉₀ were significantly prolonged after incubation with 30 mU/L TSH (Figure 5E and 5F) by 96±23% ($P=0.0008$; $n=18$) and 60±20% ($P=0.009$; $n=16$), respectively. Similar results were obtained with

60 mU/L TSH (Figure 5E and 5F), with APD₅₀ increased by 98±15% ($P=0.003$; $n=18$) and APD₉₀ by 73±27% ($P=0.007$; $n=16$).

We subsequently used computational modeling to evaluate whether the TSH-induced changes could also influence AP alterations in adult human cardiomyocytes and to examine their frequency dependence. Incorporation of the combined experimentally observed changes in

**Figure 5. xxx.**

A, Action potential (AP) measurements in HL-1 cells (a) without thyroid-stimulating hormone (TSH; n=18), (b) with 15 mU/L (n=13), (c) 30 mU/L (n=18), and (d) 60 mU/L TSH (n=16). The resting membrane potential (RP; **B**), the action potential amplitude (APA; **C**), the maximum upstroke velocity of the AP (V_{max} ; **D**), and the time interval at 10% (**E**), at 50% (**F**) and at 90% (**G**) of maximum repolarization (action potential duration [APD]) were determined. Data are presented as boxplots with raw data points; *P<0.05, **P<0.01, ***P<0.001 compared with control (Student *t* tests). **H** through **J**, Computational modeling for adult human cardiomyocytes produced a prolongation of APD that was particularly pronounced at 60 mU/L TSH. Dotted lines denote 0 mV.

ion currents (Table S5) produced a prolongation of APD that was particularly pronounced at 60 mU/L (Figure 5H through 5J). A sensitivity analysis incorporating individual changes in ion currents one at a time, identified I_{CaL} as the driving force underlying APD prolongation (Figure S9). In a population-of-models approach simulating cell-to-cell variability in ionic properties, the TSH-associated electrical remodeling induced a concentration-dependent increase in early afterdepolarization formation as a potential proarrhythmic mechanism (Figure S10). Modeled antiarrhythmic drug supplementation of 10 μ mol/L flecainide or 2 μ M amiodarone to the TSH-induced APD changes did not produce major additional changes in APD₉₀ (Figure S11).

TSH Increases Beating Rate in NRCM

The influence of TSH on spontaneous beating rate of NRCM was investigated. First, to detect the influence of TSH on mRNA expression profiles of ion channels, NRCM were incubated with 15, 30, or 60 mU/L TSH for 24 hours. TSH induced dose-dependent expression changes in cardiac ion channels and subunits after 24 hours of incubation (Figure 6A). The *Hcn2* and *Hcn4* mRNA levels underlying hyperpolarization-activated cyclic nucleotide-gated pacemaker channels were significantly increased in TSH-treated cells (Figure 6A). Number and cell density of fibroblasts did not increase significantly during the 24-hour interval (Figure 6C and 6D), and ion channels in isolated cardiac fibroblasts did not change after 24 hours (Figure S12). However, after 24 hours of incubation with 60 mU/L TSH, the cycle length significantly shortened by $-24 \pm 0.05\%$ ($P=0.004$; $n=6$) compared with control conditions (Figure 6E). Expression levels of connexins *Gja1* ($+31 \pm 23\%$; $P=0.038$; $n=6$) and *Gja5* ($+87 \pm 10\%$; $P=0.0026$; $n=6$) showed a significant increase after incubation with 15 mU/mL TSH (Figure S13).

TSH Signaling Pathway Is Mediated via cAMP and PKA in HL-1 Cardiomyocytes

Alterations in the mRNA expression profiles of proteins implicated in the cAMP signal transduction pathway were examined by mRNA sequencing after TSH stimulation of HL-1 cells. Exposure to TSH resulted in significant modifications in the regulatory kinases and adenylate cyclases within cardiomyocytes (Figure 7A). To determine whether these TSH-induced changes affected cAMP signaling, we measured cAMP levels in cardiomyocytes with an ELISA assay after 1-, 4-, or 24-hour incubation with different TSH concentrations. We could show that TSH leads to a time-dependent increase in cAMP levels (Figure 7B). Incubation with TSH also led to increased phosphorylation of the enzymatic PKA alpha subunit after 60 mU/L TSH for 24 h ($+105 \pm 33\%$;

$P=0.027$; $n=4$) and of CREB ($+13 \pm 3\%$; $P=0.034$; $n=4$) after 30 mU/L TSH, highlighting dose-dependent effects on downstream proteins. These results strongly suggest the existence of a TSH-TSHR-cAMP cascade in cardiomyocytes.

DISCUSSION

In this study, we explored the association between thyroid dysfunction, particularly SH, and cardiac arrhythmias, with a focus on AF. SH, characterized by elevated TSH levels despite normal fT₃/fT₄ levels, was analyzed in a retrospective monocentric study involving 2311 patients from 2007 to 2020. Our findings demonstrated a significant increase in AF prevalence among patients with SH with higher TSH levels compared with those with moderately elevated TSH levels. Logistic regression analysis identified TSH levels as an independent risk factor for AF in patients with SH.

Further experimental analyses revealed direct electrophysiological effects of TSH on cardiomyocytes, with TSHR expression confirmed in HL-1 cells, NRCM, and human cardiac tissue. TSH exposure led to alterations in ion channel expression, which in turn affected APD and beating rate in NRCM. We identified the TSHR-mediated cAMP/PKA signaling cascade as a likely mechanism underlying TSH-induced electrical remodeling. These findings suggest that TSH-induced electrical remodeling is a novel mechanism linking thyroid dysfunction to cardiac arrhythmias.

Clinical Association Between Thyroid Dysfunction and AF

The relationship between TSH and AF risk is controversial. Previous studies have shown no significant change in AF incidence in patients with SH compared with patients with euthyroidism.^{6,26} In our analysis, the prevalence of AF was significantly higher in patients with TSH levels above 10.0 mU/L compared with those with TSH levels of 4.0 to 10.0 mU/L, highlighting the heterogeneity of the SH population and suggesting the existence of subgroups with elevated AF risk. Consistent with our findings, studies investigating the association between TSH levels and AF frequency have produced conflicting results.^{7,27} Although some studies report an increased risk of AF in patients with overt hyperthyroidism or SH, others suggest a U-shaped relationship between TSH levels and cardiovascular risk.²⁸

However, it is challenging to determine the isolated effect of TSH on AF in the clinical setting due to multiple potentially influencing factors.²⁹ Our logistic regression analysis revealed that TSH levels above 10 mU/L were independently associated with the occurrence of AF. This stayed a robust risk factor even when excluding patients under treatment with amiodarone and patients with

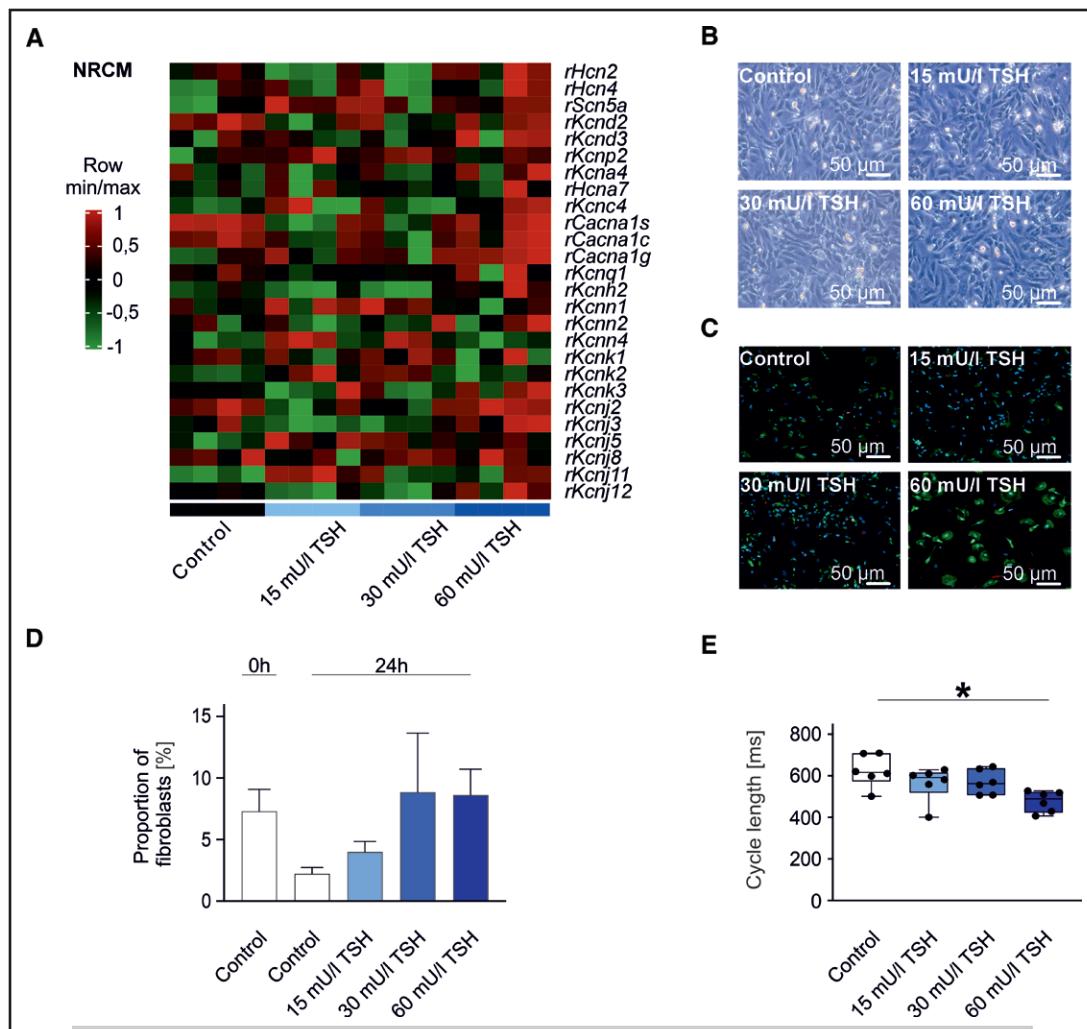


Figure 6. Thyroid-stimulating hormone (TSH) effects on neonatal rat cardiomyocytes (NRCM).

Remodeling of ion channels and subunits after 24 hours of incubation with 15, 30, and 60 mU/L TSH on mRNA expression obtained in NRCM. **A**, mRNA sequencing analysis of NRCM reveals dose-dependent expression changes. **B**, Transmission light images of NRCM with and without TSH, scale bar 50 µm. **C**, Representative immunofluorescence staining of NRCM before and after incubation for 24 hours with different TSH concentrations, respectively. TnT (troponin T; green), vimentin (red), and DAPI (4',6-diamidino-2-phenylindole; blue). **D**, Cell counting revealed significant increases in cell numbers during 24 hours. **E**, TSH induces an increased beating rate in neonatal cardiomyocytes after 24 hours of Incubation. Cycle length shortened after 15, 30, and 60 mU/L TSH. Data are presented as boxplots with each point ($n=6$) representing the mean of 1 video; 12 cells were analyzed per video, ** $P<0.01$ vs control (Student *t* tests).

prediagnosed thyroid disease or L-thyroxin treatment. We also identified age >75 years, male sex, arterial hypertension, and impaired renal function to be associated with increased odds of AF in patients with SH, which is consistent with epidemiological studies in diverse populations.^{30–32} By contrast, diabetes and insulin resistance, previously associated with AF, did not show a significant increase in the odds ratio for AF in this study, possibly due to inconsistent diagnosis and documentation of diabetes in the electronic health records.³³ Similarly, coronary artery disease did not significantly increase the risk of AF in this study, which contrasts with previous findings.³⁴ Possible explanations include differences in the severity of the disease, the protective effect of drug treatment for coronary artery disease against AF, or selection bias due

to undocumented AF in patients presenting with angina symptoms as a primary event.

Elevated TSH Levels Cause Complex Cardiac Electrical Remodeling as a Molecular Basis for Arrhythmogenesis

In our study, patients with higher TSH levels showed prolonged QTc times. These findings are consistent with previous research reporting prolonged QTc time and increased QTc dispersion in patients with SH.^{35,36} Moreover, studies have shown that QTc time is related to TSH levels across the entire range.³⁷ Importantly, both QTc time prolongation and increased QTc dispersion have been found to be reversible with effective treatment of

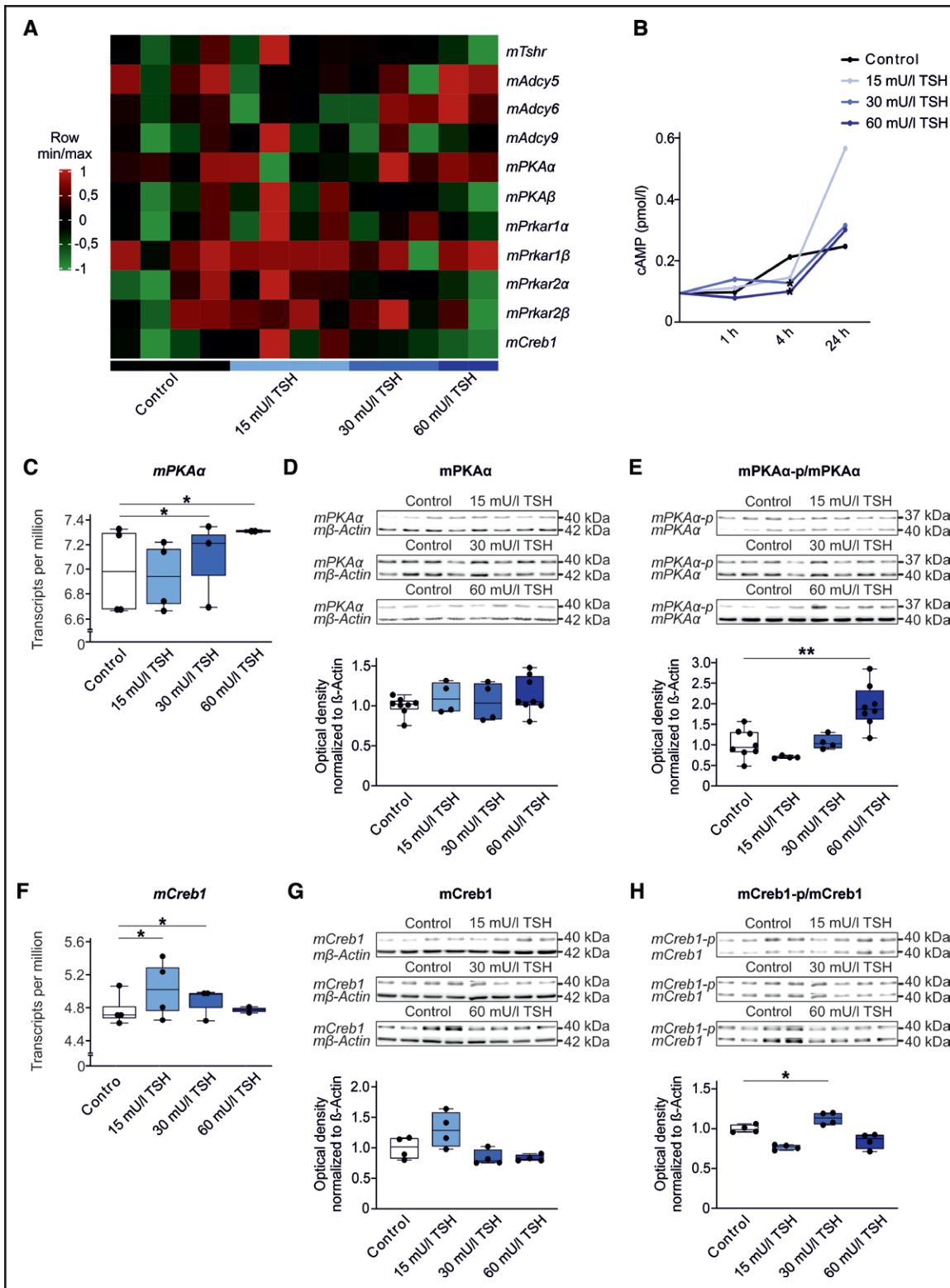


Figure 7. Altered mRNA and protein expression of protein kinases and adenylate cyclases after 24-hour incubation with thyroid-stimulating hormone (TSH) in HL-1 cells.

A, mRNA sequencing results of protein kinase A, adenylate cyclases, and CREB. **B**, Four-hour incubation with 30 mU/L ($n=6$, $P=0.002$) and 60 mU/L ($n=6$, $P=0.0001$) TSH led to significant changes in cAMP concentrations. **C**, *mPKA α* mRNA expression increased on TSH stimulation. **D** and **E**, TSH (60 mU/L) caused increased phosphorylation of *mPKA α* ($n=4$; $P=0.027$). **F** through **H**, *mCrb1* expression is changed on mRNA, but not on the protein level, but phosphorylation increased at 30 mU/mL incubation for 24 hours ($n=4$, $P=0.034$). Data are shown as boxplots with dots representing raw data. * $P<0.05$ ** $P<0.01$ vs control (β -Actin; Student *t* tests).

SH.^{35,36} However, it should be noted that while the difference between the groups was statistically significant, the absolute magnitude of the difference was not large, at ≈ 8 ms. Other factors, such as variations in the use of class III antiarrhythmic drugs, might also contribute to the observed discrepancy.

We, therefore, analyzed TSH-related electrical remodeling in cardiomyocyte cell lines, specifically HL-1, representing mouse atrial cardiomyocyte-like cells, and NRCM, primarily reflecting rat ventricular cardiomyocytes. Because ion channels are differentially expressed in the atrium and ventricle, the APs of different regions also differ.³⁸ Therefore, TSH-dependent remodeling in HL-1 cells also varies from NRCM. However, both cell types showed altered ion channel expression pattern after 24-hour incubation with TSH, indicating that TSH leads to modified gene expression in cardiomyocytes across species and cardiac chambers. The choice of a 24-hour incubation period was based on a previous study by Alonso et al³⁹ in which this duration for TSH experiments in rat cardiomyocytes was used, allowing for TSH-dependent transcriptional regulation of ion channel subunits.

Changes in the expression of ion channels play a crucial role in the physiology and pathophysiology of AF.⁴⁰ Both AP shortening and prolongation can be proarrhythmic and contribute to AF.⁴⁰ Excessive prolongation of APD may promote early afterdepolarizations and initiate arrhythmogenic mechanisms in AF development, whereas shortened APD may lead to increased reentry, promoting AF.^{40,41} In addition, APD prolongation, particularly when induced by increased $I_{Ca,L}$, may promote calcium overload and proarrhythmic calcium-handling abnormalities.

Thyroid hormones have a significant impact on the protein expression of ion channels in cardiomyocytes, and early studies showed that thyroid dysfunction alters APD. Hyperthyroid animals exhibited shortened APD in both, atrial and ventricular myocytes, whereas hypothyroid animals showed prolonged APD.^{42–45}

In contrast to previous studies, our study examined the effects of different concentrations of TSH on ion channel remodeling in cardiomyocytes. Elevated TSH levels, as present in patients with SH, had concentration-dependent effects on the expression of various ion channels, with the consequence of disrupting cellular homeostasis, altering APD, and potentially contributing to the development of AF.

A TSH concentration of 30 mU/L resulted in reduced expression of *mScn5a* and *mNa_v1.5*, underlying the fast sodium current responsible for AP initiation and contributing significantly to cardiac conduction.^{46,47} Conversely, a TSH concentration of 60 mU/L led to increased expression of both, *mScn5a* and *mNa_v1.5*.

The repolarization phase of the human cardiac AP is facilitated by outward currents through various

potassium channels, which include delayed rectifiers (I_{Kr} and I_{Ks}), inward rectifying I_{K1} , and K_{2P} (2-pore domain K⁺-channels). Studies have indicated that downregulation of *Kcne1* can reduce potassium current, resulting in APD prolongation.^{48,49} Incubation with 15 mU/L TSH-induced downregulation of *Kcne1* in both HL-1 cells and NRCM. K_{2P} channels are responsible for conducting a background potassium current that plays a significant role in modulating cellular excitability and repolarization of the cardiac AP.⁵⁰ Increased expression of the $K_{2P}3.1$ channel, as observed in patients with long-standing persistent AF, has been found to shorten APD and promote the development of AF.^{51–53} Administration of 60 mU/L TSH increased the expression of *mK_{2P}3.1*, which indeed shortened APD in our computational model (Figure S9) and may be proarrhythmic.

The small conductance calcium-activated potassium channels (K_{Ca} , SK) contribute to atrial repolarization and may be involved in the pathophysiology of AF. These channels generate the cardiac I_{KCa} current. Reduced expression of $K_{Ca}2$ channels has been observed in patients with persistent and permanent AF, and *Kcnnk3* SNPs have also shown an association with AF.^{54,55} Additionally, pharmacological inhibition of $K_{Ca}2$ channels has been found to prolong atrial refractoriness and APD, suppressing atrial arrhythmias.⁵⁶ In our investigations, incubation with 60 mU/L TSH resulted in increased expression of *mKcnn1* and *mK_{Ca}2.1* in atrial myocytes (HL-1), which was associated with APD shortening in our simulations.

During the plateau phase of the AP (phase 2), L-type voltage-gated calcium channels, encoded by the *CACNA1C* gene, are active and contribute to an inward calcium current ($I_{Ca,L}$). In patients with AF, the mRNA and protein expression of these L-type calcium channels was reduced.^{57,58} However, TSH administration resulted in increased expression of *mCACNA1C* and *mCa_v1.2*, potentially leading to a prolonged APD and refractory period. Intriguingly, *mCa_v1.1*, another calcium channel expressed mainly in skeletal muscle, as well as in murine atrial and ventricular cardiomyocytes, was found to be downregulated by a concentration of 15 mU/L TSH in HL-1 cells. However, the specific impact on APD is yet to be determined.^{59–61} This is likely one of the key mechanisms, as supported by consistent findings showing that all concentrations of TSH—both at the mRNA and protein levels, as well as through computational modeling—primarily contribute to the observed prolongation of APD.

These findings suggest that TSH levels can influence ion channel expression in cardiomyocytes and exert effects on APD, which may have implications for cardiac function and arrhythmogenesis, including AF susceptibility in patients with varying thyroid hormone levels.^{62,63} Prolongation of the APD can promote early afterdepolarizations, as shown in our computational modeling analysis (Figure S10), which can trigger ectopic impulses from ectopic foci and cause AF. Delayed afterdepolarizations

could also be caused by abnormalities in calcium balance. In particular, when APD prolongation is induced by increased $I_{Ca,L}$, this may promote calcium overload and proarrhythmic abnormalities in calcium handling.^{40,41,64,65} However, delayed afterdepolarizations have not been assessed in the present work and remain speculative.

The NRCM analysis revealed a significant shortening of the cycle length after TSH stimulation.⁶⁶ Increased spontaneous activity may be partly explained by changes in ion channel expression, especially HCN and $Na_v 1.5$, which can be proarrhythmic and potentially lead to arrhythmias.⁶⁴ Studies have shown an increased incidence of ventricular arrhythmias in both rat models and humans with primary hypothyroidism, even with only mildly elevated TSH levels.^{67–70}

TSH Affects Expression of Ion Channels via the PKA/CREB Pathway

The TSH receptor, known for its role in the thyroid gland, is also expressed outside the thyroid and appears to have physiological significance (Table S13). The reasons for the expression of extrathyroidal TSH receptors remain uncertain, but could be related to their involvement in promoting proliferation and hypertrophy during fetal development.^{71,72} TSH acts on the TSH receptor, initiating intracellular signaling pathways, including an increase in cAMP concentration. Here, we confirmed the expression of the TSH receptor in human atrial and ventricular tissue and NRCM. Moreover, we show for the first time the presence of the TSH receptor in HL-1 cells, indicating their potential as a model system to analyze the direct effects of TSH on cardiomyocytes.

In thyrocytes, the TSH receptor can activate either the cAMP or the phosphatidylinositol 4,5-bisphosphate/Ca²⁺ signaling cascade.¹⁹ In cardiomyocytes of a rat hypothyroidism model (30 mU/L TSH), expression changes of ion channels induced by TSH can be rescued by PKA inhibition.⁶⁷

PKAα is expressed dominantly in atrial and ventricular myocytes compared with PKAβ and PKAγ and has been implicated in the development of cardiovascular diseases and cardiomyopathies.^{73,74} An increased TSH concentration of 60 mU/L promotes phosphorylation of PKAα in our study. Interestingly, patients with SH and a TSH level >10 mU/L have shown increased cardiovascular mortality in various studies.^{75–78}

In addition to intracellular and membrane-bound proteins, PKA can phosphorylate various transcription factors, including CREB, which controls DNA synthesis, cell activity, and pleiotropic functions.^{79–84} CREB is a widely known nuclear transcription factor that is activated by an increased intracellular cAMP concentration via phosphorylation of Ser133 and binds to a nuclear CRE (cAMP response element) modulator and modifies gene expression.^{84,85} CREB can be activated by multiple kinases, including PKA.^{85–87}

CREB has been implicated in the pathogenesis of AF. A lack of CREB regulation, facilitated by the CREB repressor, has been associated with atrial dilation and the occurrence of spontaneous AF.^{88,89} Additionally, rapid electrical stimulation of atrial myocytes activated the cAMP/PKA/CREB signaling cascade.⁹⁰ Furthermore, transcription alterations in patients with AF revealed an increased susceptibility to downregulation of target genes belonging to the CREB/CRE family.⁹¹

Clinical Implications

Key research priorities in the field of cardiac dysfunction related to thyroid disease include developing optimized thyroid function tests to better identify subgroups of patients with hypothyroidism, improving arrhythmia management in the context of thyroid dysfunction, and exploring novel biomarkers for electrophysiological changes induced by thyroid hormones.⁹² It is also notable that a significant underreporting of SH occurs in clinical practice, shown by patients with SH without a diagnosis of thyroid disease or L-thyroxin intake. A deeper understanding of the role of extrathyroidal TSH receptors could offer valuable insights into both physiological and pathophysiological mechanisms. The TSH concentration groups, studied in this article, are frequently measured in clinical settings and should be considered as separate patient groups due to their association with certain pathologies, including AF.^{93–97} Consequently, a potential rise in SH prevalence may be accompanied by an increase in AF prevalence in the future.⁷ Our data, supported by a review of the current literature, suggest that SH leads to electrical remodeling due to elevated TSH levels, resulting in changes of APD and promoting proarrhythmic effects. Currently, there are no specific guidelines for managing AF in the context of SH. However, understanding the endocrine control of AF could lead to the development of new therapeutic options for both cardiac and endocrine diseases.⁹⁸ The initiation of L-thyroxine therapy for SH is a subject of debate.^{16,75–78,99–101} Recent guidelines do not recommend L-thyroxine therapy, citing insufficient clinically relevant benefits for quality of life or thyroid-related symptoms.²⁹ However, morbidity and mortality due to cardiovascular disease are not adequately addressed in these guidelines. Further research is needed to establish clear guidelines for managing endocrine AF related to SH and to evaluate AF screening in patients with SH to detect asymptomatic AF and consider L-thyroxin or future specific cardiac TSHR-targeted therapies to avoid SH-related changes in cardiac electrophysiology.^{102–106}

Potential Limitations

This study is limited by its retrospective design, which allows for identifying associations but does not establish causation. Therefore, our clinical findings should be

interpreted as hypothesis-generating, requiring validation in future prospective trials.

A key limitation of this study is the potential under-detection of AF episodes, as continuous monitoring with an implantable loop recorder was not performed in all patients.

The recording of previous diseases and risk factors from physicians' letters carries the risk of being incomplete. As evidenced by the number of thyroid diseases reported (Table), seemingly minor diagnoses are often overlooked. Furthermore, detailed information on the severity, time course of TSH levels, and prior medical conditions was unavailable in most cases. Consequently, the chronology of AF and its relationship to TSH levels and other conditions remain uncertain. Echocardiographic data were obtained by several different physicians as part of routine clinical care. In numerous cases, only selective individual parameters were measured, and ejection fraction was often visually assessed.

This study focused on ion channels and downstream remodeling. Although RNA sequencing variability in our cell culture models is acknowledged, the confirmation by independent experimental approaches (quantitative real-time polymerase chain reaction, Western blot) supports our conclusions. Nevertheless, only APs were measured. Detailed characterization of single ionic currents, for example, $I_{Ca,L}$, remains a subject for future studies. Increased PKA phosphorylation on TSH stimulation observed in our study could also explain discrepancies in ion channel expression patterns and currents due to acute phosphorylation or changes in subcellular localization of cardiac ion channels.^{62–66} We acknowledge that epigenetic mechanisms and analysis of other signal transduction cascades might be of interest but were beyond the scope of the present work and therefore require investigation in separate approaches.

Finally, the data specifically refer to a clinically relevant AF subtype in the context of SH and should be regarded as hypothesis-generating to consider TSH-level-dependent treatment in patients with AF and SH in the future.

Conclusions

This study provides new insights into the physiological effects of TSH on cardiac electrophysiology. It reveals that SH is associated with an increased risk of AF, likely in part due to cAMP/PKA/CREB-dependent pathway promoting atrial electrical remodeling. Notably, the increased expression of L-type calcium channels ($Ca_v1.2$) at both the mRNA and protein levels appears to be a key mechanism, as APD prolongation was observed in both computer-simulated ion current modeling and patch-clamp measurements of HL-1 cells.

Currently, there is a recommendation to treat SH with levothyroxine if TSH levels exceed 10 mU/L to prevent progression to overt hypothyroidism. With the findings

from this research and further investigations, this recommendation may evolve into a clinical guideline, given the increased risk of cardiac arrhythmias associated with SH. SH therapy could be tailored based on the level of TSH concentration and the presence of other medical conditions. Moreover, considering the potential higher risk of AF in patients with SH, more frequent AF screening in these individuals might be worth considering.

ARTICLE INFORMATION

Received January 16, 2025; accepted October 28, 2025.

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Acknowledgments

The authors gratefully acknowledge the excellent technical assistance of Patrizia Lo Vetere, Nicole Westerhorstmann, Miriam Baier, and Chiara Heß. The work was performed at Medical University Hospital Heidelberg, Heidelberg University, and Mannheim University Hospital.

Sources of Funding

This study was supported in part by research grants from the University of Heidelberg, Faculty of Medicine (Postdoctoral Fellowships to Drs Lugenbiel and Rahm, Olympia Morata Fellowship to Dr Rahm), from the German Cardiac Society (Fellowships to Dr Rahm and Lugenbiel), from the Ernst und Berta Grimmke-Stiftung (to Drs Lugenbiel and Syren), from the German Heart Foundation/German Foundation of Heart Research (F/08/14 to Dr Thomas, Fellowship to Dr Rahm, Kaltenbach-Promotionsstipendium to Drs Wunsch and Gampf), from the German Internal Medicine Society (Clinician-Scientist-Program to Dr Rahm), from the Joachim Siebenreicher Foundation (to Dr Thomas), from the Deutsche Forschungsgemeinschaft (German Research Foundation; TH 1120/7-1 and TH 1120/8-1 to Dr Thomas), and from the Ministry of Science, Research and the Arts Baden-Württemberg (Sonderlinie Medizin to Dr Thomas). Drs Wunsch, Pfeiffer, and Gampf were supported by the Cardiology Career Program of the Department of Cardiology, University of Heidelberg. Dr Heijman is supported by the Netherlands Organization for Scientific Research (NWO/ZonMW Vidi 09150171910029), the Dutch Heart Foundation (grant number 01-002-2022-0118, EmbRACE consortium).

Disclosures

Dr Rahm reports educational support from Boston Scientific, Johnson & Johnson, Abbott, and Medtronic. Dr Thomas reports receiving lecture fees/honoraria from Bayer Vital, Boehringer Ingelheim Pharma, Bristol-Myers Squibb, Daiichi Sankyo, Medtronic, Pfizer Pharma, Sanofi-Aventis, St. Jude Medical, and ZOLL CMS. Dr Lugenbiel reports receiving lecture fees from Bayer Vital and Pfizer Pharma and educational support from Boston Scientific and Johnson & Johnson. The other authors report no conflicts.

Supplemental Material

Supplemental Methods

Tables S1–S13

Figures S1–S13

Western Blot Raw Data

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