Modified sympathetic nerve regulation in AKAP5-null mice
(AKAP5-欠損マウスにおける交感神経の調節)

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Abstract

Genetic analyses have revealed an important association between A-kinase anchoring proteins (AKAPs) and the intracellular calcium modulating system. AKAP5, also known as AKAP79/150, is an anchoring protein between PKA and voltage-dependent calcium channels, ryanodine receptor-2, phospholamban and other molecules.

The aim of the present study was to elucidate the physiological importance of AKAP5 in the creation of cardiac rhythm using AKAP5-null mice.

ECG analysis showed a normal sinus rhythm and a decreased responsiveness to isoproterenol in AKAP5-null mice compared with wild-type mice. Analysis of heart rate variability revealed that the R-R interval was unstable in AKAP5-null mutants and that the low-frequency components had decreased, indicating that the tonus of the sympathetic nervous system was affected. Furthermore, the atrium of the AKAP5-null mice showed a decreased positive inotropic response to isoproterenol, indicating the involvement of AKAP5 in a PKA-dependent pathway. Thus, our present study revealed that AKAP5 plays a significant role in the regulation of sympathetic nerve activities.

Key words: PKA; Mouse; Sympathetic nerve; Parasympathetic nerve; Heart rate

Introduction

Sympathetic stimulation of the heart increases the force of contraction and the rate of ventricular relaxation by triggering phosphorylation of the target proteins by protein kinase (PK) A [1)]. Cardiovascular stress results in chronic overstimulation of the β-adrenergic

receptor pathway, cardiac hypertrophy, and ultimately cardiac failure [2, 3]. β-blockers are a widely used therapy for chronic heart failure. The efficiency of cyclic (cAMP)/PKA signaling is enhanced by various A-kinase anchoring proteins (AKAPs), which are scaffolding proteins that generate microsignaling domains [4, 5]. AKAPs are a family of structurally diverse proteins that share a common motif in their C terminus that binds to the regulatory subunits of PKA [6]. They form a family of functionally related proteins that contribute to intracellular signaling events by directing cAMP-dependent PKA to specific subcellular sites [7]. PKA is a Ser/Thr kinase that is activated by the binding of cAMP and phosphorylates a range of target proteins [8, 9]. Over 20 AKAPs have been identified in cardiomyocytes, although elucidating the specific role of each AKAP remains a long-term objective [1, 7]. In particular, AKAP5 (also referred to as AKAP150 in rodents and AKAP79 in humans) interacts with PKA holoenzymes containing RIIa or RIIb as well as calcineurin (PP2B), PKC, calmodulin, adenylyl cyclase type V/VI, voltage-dependent L-type calcium channels, and β-adrenergic receptors [10, 11]. Oliveria et al showed that CaV1.2 interacts directly with AKAP 79/150, which binds both PKA and the Ca^{2+}/calmodulin-activated phosphatase calcineurin. Co-targeting of PKA and calcineurin by AKAP79/150 confers bidirectional regulation of the L-type current amplitude in transfected HEK293 cells and hippocampal neurons [12]. AKAP5 knockout mice (AKAP5$^{-/-}$) lack persistent Ca^{2+} sparklets, have lower arterial wall intracellular calcium ($[Ca^{2+}]_i$) levels, and have decreased myogenic tone. AKAP5-null mice were hypotensive and did not develop angiotensin II–induced hypertension [7, 13]. Deletion of AKAP5 resulted in the loss of β-adrenergic-stimulated Ca^{2+} transients and the phosphorylation of substrates involved in Ca^{2+} handling [6].

The origin of cardiac pacemaker activity in the heart has been debated as a choice between two alternative mechanisms: the “membrane clock” and “Ca^{2+} clock”. The membrane clock is activated by hyperpolarization following I_f channel opening. The Ca^{2+}

clock is regulated by the timing of the uptake and release of Ca^{2+} by the sarcoplasmic reticulum (SR) [14]. Therefore, AKAP5-null mice should have a modulated "Ca^{2+} clock" because they have decreased levels of phosphorylated ryanodine receptor and phospholamban but have little influence on PKA-dependent calcium channel current augmentation [1]. Nevertheless, involvement of AKAP5 in sympathetic nerve regulation is still obscure. In the present study, we evaluated the cardiac phenotype of AKAP5-null mice to determine the physiological role of AKAP5 in the regulation of sympathetic nerve activities.

Materials and methods

Animals

"AKAP5-null" mice (NCrl.129-Akap5tm2Gsm/Mmucd; 034250-UCD), were purchased from the Mutant Mouse Regional Resource Center (MMRRC, Davis, CA, USA), a NCRR-NIH funded strain repository, and were donated to the MMRRC by Dr. G. Stanley McKnight of the University of Washington.

Throughout the study, the animals were housed under a constant 12-h light/dark cycle with free access to food and water. All experiments were conducted in accordance with the Guidelines for the Use of Laboratory Animals of Akita University School of Medicine.

Genotyping

The AKAP5 gene was amplified using the primers "AKAP5-for" (5'-ATGGAGACCAGCGTTTCTGA-3') and "AKAP5-rev" (5'-TTAGGCAAGGAAACTTAAGTCT-3') to detect the wild-type allele. The mutated AKAP5 gene was amplified using the primers "neo-for" (5'-TGAATGAACTGCAGGACGAG-3') and

“neo-rev” (5’-AACGCTATGTCCTGATAGCG-3’) to detect the neomycin-resistant gene, which was inserted into the mutated allele. The PCR product amplified by the “AKAP5-for” and “AKAP5-rev” primers resulted in a 373-bp band, while that by the “neo-for” and “neo-rev” primers resulted in a 499-bp band (Supplementary Fig. 1).

Reverse transcription–polymerase chain reaction (RT-PCR)

Poly(A)+ RNA was isolated from cells using TRIzol reagent (Invitrogen, Carlsbad, CA, USA) and Oligotex-dT30 (TaKaRa, Shiga, Japan). The RT reaction was performed using a first-strand cDNA synthesis kit (SuperScript II Reverse Transcriptase; Invitrogen). PCR amplification was performed using the GoTaq Green Master Mix (Promega, Madison, WI, USA).

Specific sequences of adrenergic β1, β2, and muscarinic 2 receptors were amplified by PCR (37 cycles). Specific sequences of CREB1 were amplified by PCR (36 cycles). As an internal control, β-actin sequences were amplified by PCR (35 cycles). More detailed information is included in the Supplementary Information.

Histological examination

Tissues of wild-type and AKAP-null mice were excised under ether anesthesia, fixed immediately in 4% (v/v) paraformaldehyde in 0.1 M sodium phosphate buffer (pH 7.2) overnight at 4°C, embedded in paraffin wax, and stained with hematoxylin-eosin periodic acid Schiff. Tissues were then examined under a light microscope.

Cross-sectional area (CSA) measurements were obtained from a minimum of 50 cardiac myocytes from wild-type and AKAP5-null mice. Four images from non-overlapping regions of each tissue CSA stained with HE were used for CSA measurements. Mean fiber CSA of the respective fiber types was determined by planimetry.

General anesthesia

The mice (12–16 weeks of age) were anesthetized in an induction chamber (25 × 25 × 14 cm) containing 4% isoflurane (Forane; Abbott Japan Co., Ltd., Tokyo, Japan) and room air. Anesthesia was maintained for 45 min (anesthetic maintenance state) using 2% isoflurane inhalation anesthesia at an airflow rate of 0.5 L/min. Ten minutes after the induction of anesthesia, the baseline electrocardiogram (ECG) and echocardiography were recorded for 5 min, followed by the pharmacological tests. All experiments were conducted between 10:00 and 16:00 h.

ECG evaluation

ECG, heart rate, and the R-R interval were measured simultaneously (ML846 Power Lab system; AD Instruments, Dunedin, New Zealand) [15]. An M-button connector was connected to the electrode [16]. We used heart rate variability (HRV) as a measure of cardiac autonomic nerve control [15-17] (detailed information is provided in the Supplementary Information).

Echocardiography

Mice were anesthetized with 2% inhaled isoflurane. They were allowed to breathe spontaneously during the echocardiographic studies. A commercially available echocardiography machine equipped with a 10-MHz transducer (Aspen, Acuson, Stockton, CA, USA) was used to record the M-mode echocardiogram in the mid-portion of the left ventricle. Echocardiographic recordings were analyzed to determine the left-ventricular inner diameter at end-diastole and -systole and the left-ventricular ejection fraction using the software supplied with the system [18].

Inotropic atrium contraction

Left atria were dissected free of ventricular tissue and placed in an oxygenated 37°C tissue bath containing Tyrode's solution (123.8 mM NaCl, 5.0 mM KCl, 2.0 mM $CaCl_2$, 1.2 mM $MgCl_2$, 25.0 mM $NaHCO_3$, and 11.2 mM glucose).

Isolated left atria were stimulated at 2 Hz at a voltage just above the threshold for basal contraction (1-ms duration) for 30 min to stabilize the basal contraction. The isometric contractile force was measured using a force transducer (CD200; Nihon Koden).

Statistical analysis

All data are presented as means ± SEMs. The statistical significance of an observed difference was determined by analysis of variance (ANOVA) followed by the application of Dunnett's test, and *p*-values less than 0.05 were considered significant.

Results

Genotyping

The AKAP5-null and wild-type alleles were confirmed using PCR (Supplementary Fig. 1A).

RT-PCR analysis

Expression of PKA-related molecules was analyzed in the heart by RT-PCR analysis. The reduced responsiveness to sympathetic nervous stimulation in AKAP5-null mutants might be due to modified expression levels of genes involved in the PKA-dependent pathway. Therefore, we explored the expression levels of various PKA-related molecules in the heart (Fig. 1A). The expression of two adrenergic receptors, β1 and β2, was unchanged by AKAP ablation. The expression of the cholinergic M2 receptor was also unchanged. We also examined CREB1, which is a post-receptor molecule in the adrenergic pathway, and no significant changes were observed. β-actin expression was examined as a control. Taken together, our analysis revealed no significant changes in the expression of PKA-related molecules by AKAP ablation.

Histology

Persistent ablation of AKAP5 might decrease responsiveness to sympathetic nerve stimulation. As catecholamines are major factors in cardiac hypertrophy in chronic heart failure, elimination of the AKAP5 gene might cause hypotrophic effects on cardiac structure and function. Therefore, we performed a histological analysis (hematoxylin and eosin staining) of the heart. Gross examination revealed that the hearts of 12-week-old AKAP5-null mice showed no apparent changes, although the AKAP5-null heart tended to be hypotrophic. To better understand the cardiomyopathic changes in the heart, a CSA was determined by planimetry (Fig. 1Bii). There were no significant differences between the wild-type and AKAP5-null mice.

Modified pharmacological response in AKAP5-null mice

As AKAP5 is essential for PKA-dependent phosphorylation of ryanodine receptors and phospholamban, significant changes in heart rate and contraction were expected. Therefore,

we analyzed ECG and echocardiography recordings. During the basal state, we observed no difference between the wild-type and the AKAP5-null mice (Fig. 2A). There were also no significant differences related to heart rate (Fig. 2Bi). Interestingly isoproterenol (4 μg/kg), a β-adrenergic agonist, resulted in a decreased response in AKAP5-null mice (Fig. 2Bii), while no significant changes were observed in the ejection fraction between the two groups (data not shown). However, propranolol, a β-adrenergic antagonist, showed no significant effect on heart rate between the two groups (Fig. 2Biii). We also observed no significant difference in the ejection fraction between the two groups in response to propranolol (data not shown). As US recordings showed no significant difference between the two groups, which might be due to the difficulty in accurately measuring the ejection fraction, we focused on the ECG recordings. We examined dose-dependent responses to propranolol (0.1-0.8 mg/kg), albeit without significance (Supplementary Fig. 1B). These data suggest no significant difference in the responsiveness to a β-adrenergic antagonist between the wild-type and AKAP5-null mice, while the responsiveness to a β-adrenergic agonist was significant in the AKAP5-null mice.

Modified heart rate variability

As AKAP5-null mice showed a decreased responsiveness to isoproterenol, we performed ECG analysis to explore HR regulation in these using the Power Lab HRV software. Fig. 3A shows representative Poincaré plots for wild-type (left panel) and AKAP5-null mice (right panel). Wild-type mice exhibited stable changes in R-R intervals, whereas AKAP5-null mice had relatively unstable R-R intervals (shown as dots), suggesting unstable pacemaking.

Power spectral analysis (Fig. 3B) showed that the low frequency component (LF) was decreased in the AKAP mutants (15.7 ± 2.4%, n = 8) compared with wild-type mice (25.1 ± 4.2%, n = 10). Because the AKAP5-null mutant exhibited low LF, the LF/high frequency

component (HF) ratio was also significantly lower (0.20 ± 0.03, n = 8) than that of wild-type mice (0.38 ± 0.07, n = 10). The significant decreases in the LF and LF/HF of AKAP-mutant mice suggest that sympathetic nerve control is affected in these animals. Together, the data show that the tonus of sympathetic nerve control was lower in the AKAP mutant. As AKAP5 is essential for PKA-dependent phosphorylation of the ryanodine receptor and phospholamban, our present results are consistent with previous studies [1)].

Inotropic Atrium contraction

Because modified sympathetic signal transduction in AKAP5-null cardiac myocytes might decrease responsiveness, as observed by heart rate changes in the ECG recordings, we assessed sympathetic responsiveness by measuring inotropic responses to electrical field stimulation in isolated left atria. Fig. 4 shows dose-dependent changes in response to isoproterenol (1-100 nM) in the wild-type and AKAP5-null mouse atria. Isoproterenol caused dose-dependent changes in force in the atrium from wild-type and AKAP5-null mice, while AKAP5-null atria showed a decreased responsiveness at high doses.

Discussion

In the present study, we explored the phenotypes of AKAP5-null mice. Upon pharmacological ECG analysis, AKAP5-null mice showed a decreased response to isoproterenol. However, they showed normal responsiveness to propranolol (a β-blocker). HRV analysis showed that the AKAP5-null mice had unstable R-R intervals and decreased LF, resulting in a low LH/HF ratio, indicating that the tonus of the sympathetic nerves had

been modified. In addition, the AKAP5-null atrium showed a decreased contractile response to isoproterenol. Our present results suggest that AKAP5-null mice exhibit a modulated sympathetic nerve response.

Previous work has shown that an increase in the rate and amplitude of Ca^{2+} sparks during β-adrenergic receptor activation is linked to increased SR Ca^{2+} load [19, 20]. Therefore, the intracellular calcium clock system might modulate cardiac rhythm and contraction. Nichols et al reported decreased PKA-dependent phosphorylation of RyR_2 (Ser2808 on RyR_2) and phospholamban (Ser16 on phospholamban) but not CaV1.2 under basal and β-adrenergic receptor-stimulated conditions in AKAP5-null cardiac myocytes [1].

Phosphorylation of phospholamban by PKA should induce dissociation of phospholamban from sarcoplasmic reticulum Ca^{2+}-ATPase (SERCA), increasing the rate of Ca^{2+} reuptake into the SR [21]. Therefore, AKAP5-null mice should have decreased calcium-induced calcium release and decreased calcium sequestration via SERCA, resulting in a significant effect on the intracellular calcium clock system. If voltage-dependent calcium channel phosphorylation is not changed by AKAP5-gene manipulation, our present finding indicated that decreased sensitivity to isoproterenol in AKAP5-null mice is due to decreased phosphorylation of ryanodine receptors and phospholamban. This suggests the importance of the intracellular calcium clock (calcium modulation system), which is based on calcium-influx through voltage-dependent calcium channels (unchanged by AKAP5-gene manipulation), the ryanodine receptor, and other calcium regulators, such as the cardiac SERCA2, which is a target for phosphorylated phospholamban. Nichols et al also reported that isoproterenol (100 nM) treatment increased

the Ca^{2+} spark rate only in wild-type, but not in AKAP5-null, cardiac myocytes [1]. Their results indicate that AKAP5 is important in the sympathetic regulation of the heart from the point of the intracellular calcium clock system. Our data showed modified sympathetic nerve regulation and a decreased response to isoproterenol, which is consistent with their results.

In mice carrying the catecholaminergic polymorphic ventricular tachycardia–linked mutation in the ryanodine receptor ($RyR2^{R4496C}$) had slowed pacemaker activity and a reduced response to catecholamines compared with myocytes from wild-type mice [22]. They also reported frequent pauses in the generation of spontaneous action potentials in $RyR2^{R4496C}$ mice, indicating an important role of the calcium clock in cardiac rhythm. There is a parallel correlation between the timing of the Ca^{2+} clock and the degree of phosphorylation and dephosphorylation of functionally important proteins such as L-type Ca^{2+} channels and phospholamban [23]. In the present study, AKAP5-null mice, which have decreased levels of phosphorylated ryanodine receptor and phospholamban [1], showed a decreased heart rate with increasing isoproterenol dose, giving similar results, although the AKAP5-null mice showed a regular heart rhythm. These modulated cardiac responses might suggest a modulatory role of the intracellular calcium clock system.

In the present study, we analyzed sympathetic nerve tonus mainly by ECG HRV analysis, while HR variability is not dependent on the autonomic nervous system alone. Both the sympathetic and parasympathetic nervous systems modulate LF and HF [24]. R-R intervals are also influenced by other factors, such as the respiratory cycle. Accordingly, complex LF, HF and LF/HF data are produced by calculated HRV parameters. Calculated LF/HF data may not be appropriate to quantify the cardiac “sympatho-vagal balance” accurately [24],

while one advantage of HRV analysis is that it assesses the effect of various manipulations using simple ECG recordings [25].

AKAP5 (also known as 79/150) is involved in the trafficking and signaling of the myocardial β1-adrenergic receptor [6]. Li et al reported that shRNA-mediated down-regulation of AKAP5 in HEK-293 cells inhibited recycling of the β1-adrenergic receptor. They also reported an increased contraction rate in response to isoproterenol in primary neonatal cardiac myocyte cultures in AKAP5-null mice as well as in those in wild-type mice, inconsistent with our results. The reason for this discrepancy is not clear. Li et al prepared primary neonatal cardiac myocyte cultures, while we conducted our study on whole mice or isolated atrium tissue. Nevertheless, due to differences in experimental conditions (neonatal vs. adult, isolated primary cultures vs. *in vivo* experiments), further analysis is required.

In the present study, we found no significant difference in the expression levels of adrenergic β receptors in the heart. The AKAP5-null mice showed normal basal heart pacemaking activity. However, pharmacological manipulation by isoproterenol revealed lower responses in terms of heart rhythm and contraction force in AKAP5-null mice. These results indicate that β-adrenergic signal transduction was affected in the AKAP5-null mutant heart. As both heart rhythm and contraction in response to isoproterenol were affected, the signaling domain created by AKAP5 must play an important role in sympathetic nerve regulation.

AKAPs are of particular interest as potential drug targets due to their specific and modulating roles in systems governed by effectors that also have many other functions.

Coordinated handling of Ca^{2+} in cardiac myocytes is essential for efficient contraction and relaxation of the heart. Sympathetic control of the heart through β-adrenergic stimulation increases both the rate and force of contraction and relaxation of cardiac muscle by regulating Ca^{2+} handling at the level of the L-type Ca^{2+} channel, the ryanodine receptor, a Ca^{2+} activated Ca^{2+} release channel, and cardiac SERCA2.

AKAP5 organizes a signaling complex around the L-type voltage-dependent calcium channel in the cardiac myocyte that is critical to the β-adrenergic response. Nichols et al found in the AKAP5 knockout heart that the organization of the signaling complex is disrupted, adenylyl cyclase 5/6 no longer associates with caveolin 3 in the T-tubules, and noncaveolin 3–associated calcium channels become phosphorylated following β-adrenergic stimulation, although this does not lead to enhanced levels of transient calcium. It was surprising to find that AKAP5 knockout cardiomyocytes lost the ability to modulate calcium transients in response to isoproterenol but still retained isoproterenol-dependent increases in whole cell L-type voltage-dependent calcium channel currents. Nevertheless, further studies will be needed to clarify the role of AKAP5 in sympathetic nerve regulation. Cheng et al reported that ablation of AKAP150 restores normal gating in CaV1.2-Timothy syndrome mutant channels, which is a dominant gain-of-function G406R mutation in the cytoplasmic loop of CaV1.2 channels, and causes long QT syndrome 8 (LQT8), resulting in protection of the heart from arrhythmias [26]. Therefore, AKAP might be a therapeutic target.

In conclusion, we analyzed the phenotypes of AKAP5-null mice. ECG analysis showed that the AKAP5-null mutant showed a decreased response to isoproterenol on ECG. In the contraction analysis, the AKAP5-null atrium showed a decreased responsiveness to isoproterenol. HRV analysis revealed an unstable R-R interval in the AKAP5-null mutant.

AKAP5-null mice showed decreased LF components, resulting in a decreased LF/HF ratio, indicating modified sympathetic nerve regulation. Our results strongly suggest that AKAP5 plays a significant role in the sympathetic nerve regulation.

Acknowledgments

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Figure legends

Fig. 1

A) Typical RT-PCR data from the wild-type (left) and AKAP5-null (right) mice. The amplified sequences are indicated (WT: wild type, AKAP5-null: AKAP5-null mutant). Identification of sympathetic β-adrenergic receptor (β1 and β2)-specific transcripts in the heart (WT, wild-type mice; AKAP5-null, AKAP5-null mutants). Cholinergic M2 receptor and CREB1

transcripts in the heart were also evaluated. Expression of β-actin was evaluated as a control. The primer sets used for PCR amplification are shown.

B) Histological analysis of hearts from 12-week-old WT and AKAP5-null mice. Hearts were sectioned transversely and stained with hematoxylin and eosin. The black arrow indicates hypotrophic muscle wall of the AKAP5-null heart ventricle.

C) Cross-sectional area (CSA) of 12-week-old WT (open bar) and AKAP5-null (closed bar) mice. CSA measurements were obtained from a minimum of 200 cardiac myocytes from wild-type and AKAP5-null mice. *$P < 0.05$ vs. wild-type control.

Fig. 2

A) Representative echocardiography and ECG traces of WT (left panel) and AKAP5-null mice (right panel). Scale bars: 1.0 mV and 100 ms. LVES (left ventricular end-systolic diameter) and LVED (left ventricular end-diastolic diameter) were indicated.

B) Statistical analysis of the heart rates of WT (open bars) and AKAP5-null (closed bars) mice.

There was no significant difference in the heartrate between the wild-type and AKAP5-null mice at baseline (i). The pharmacological responses to isoproterenol (ii) and propranolol (iii) are shown. The AKAP mutant mice had a reduced responsiveness to isoproterenol (4.0 μg/kg, i.p.). There was no significant difference in the response to propranolol (0.8 mg/kg, i.p.) between the wild-type and AKAP5-null mice. *$p < 0.05$, $n = 8–10$.

Fig. 3

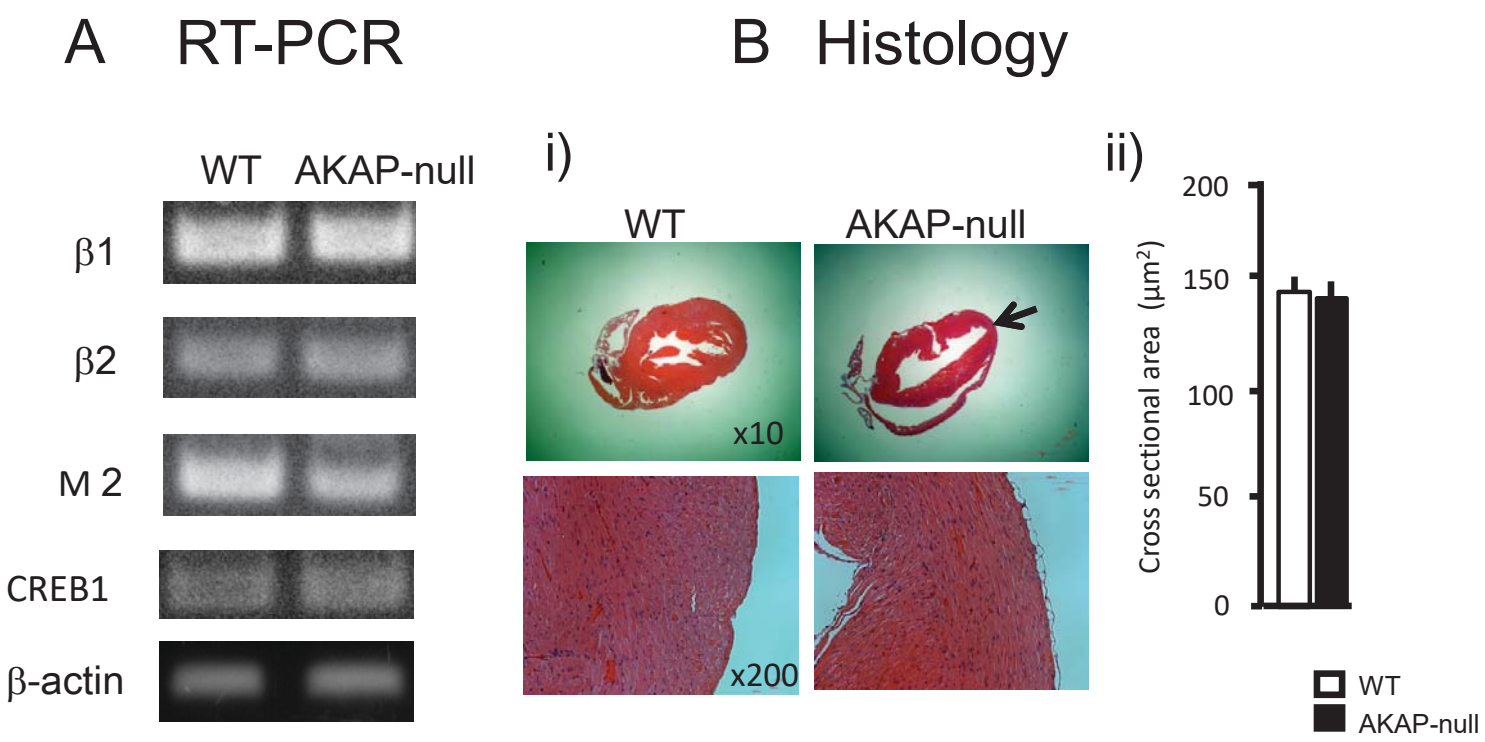
A) Representative ECG Poincaré plots of WT (left panel) and AKAP5-null mice (right upper panel).

B) Representative power spectrum analysis of WT (left panel) and AKAP5-null mice (right panel).

C) Statistical analysis of the power spectra (i-iii). The LF (i) and HF (ii) components and the LF/HF ratios (iii) of WT (open bars) and AKAP5-null mice (closed bars). *p < 0.05 vs. wild-type, n = 8–10. Error bars indicate SEMs.

Fig. 4

Dose-dependent contractile changes in the response to isoproterenol (1.0-100 nM) of wild-type (open circles) and AKAP5-null (closed circles) atria. Error bars indicate S.E.M. *P < 0.05, difference between wild-type and AKAP5-null atria.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B Histology
WT
AKAP-null
β1
β2
M 2
CREB1
β-actin
i)
ii)
WT
AKAP-null
x10
x200
Cross sectional area (μm²)
200
150
100
50
0
WT
AKAP-null

Figure 1

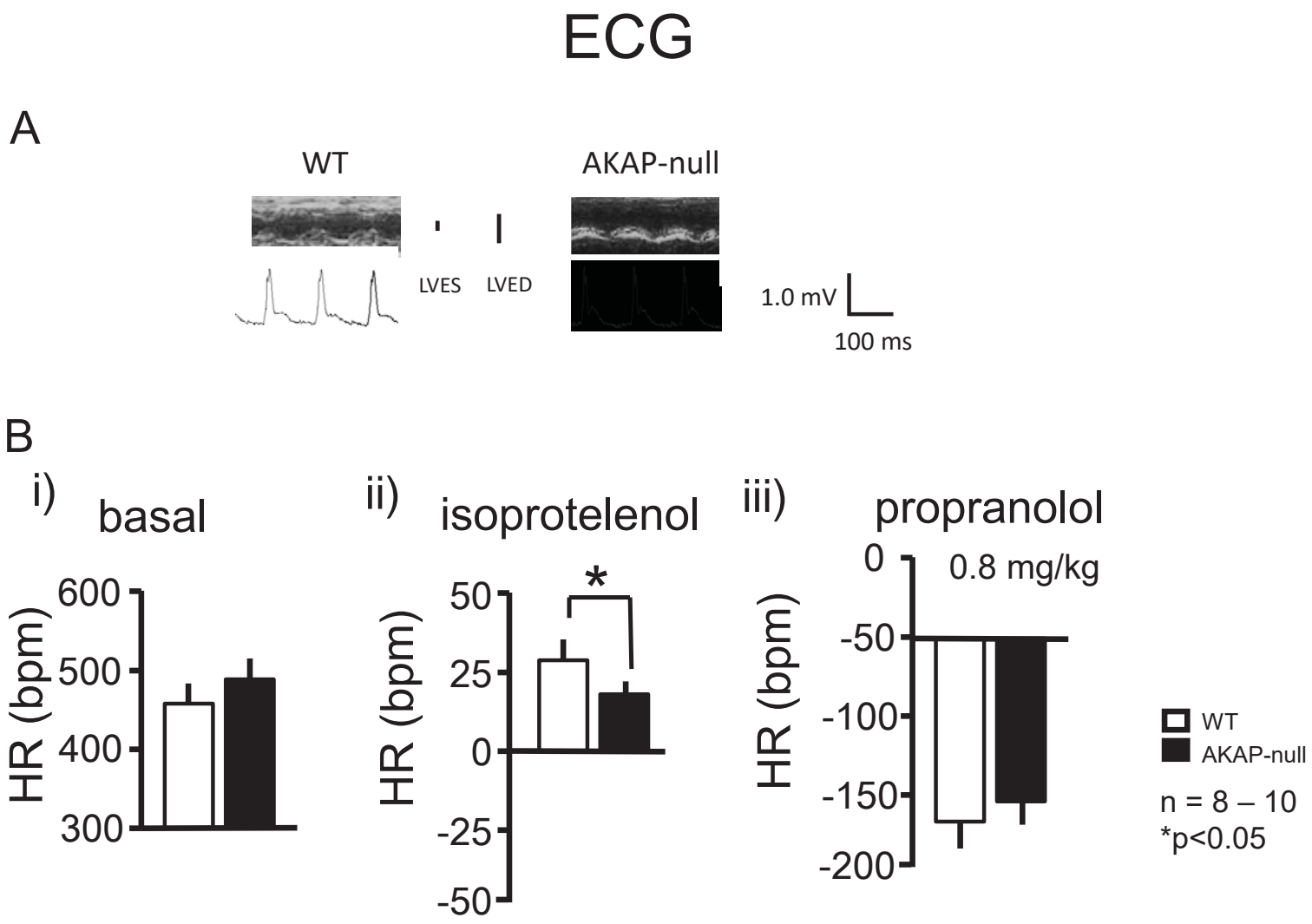

ECG
A
WT
AKAP-null
LVES
LVED
1.0 mV
100 ms
B
i)
basal
HR (bpm)
600
500
400
300
ii)
isoprotelenol
*
HR (bpm)
50
25
0
-25
-50
iii)
propranolol
0.8 mg/kg
HR (bpm)
0
-50
-100
-150
-200
WT
AKAP-null
n = 8 – 10
*p<0.05

Figure 2

HRV analysis

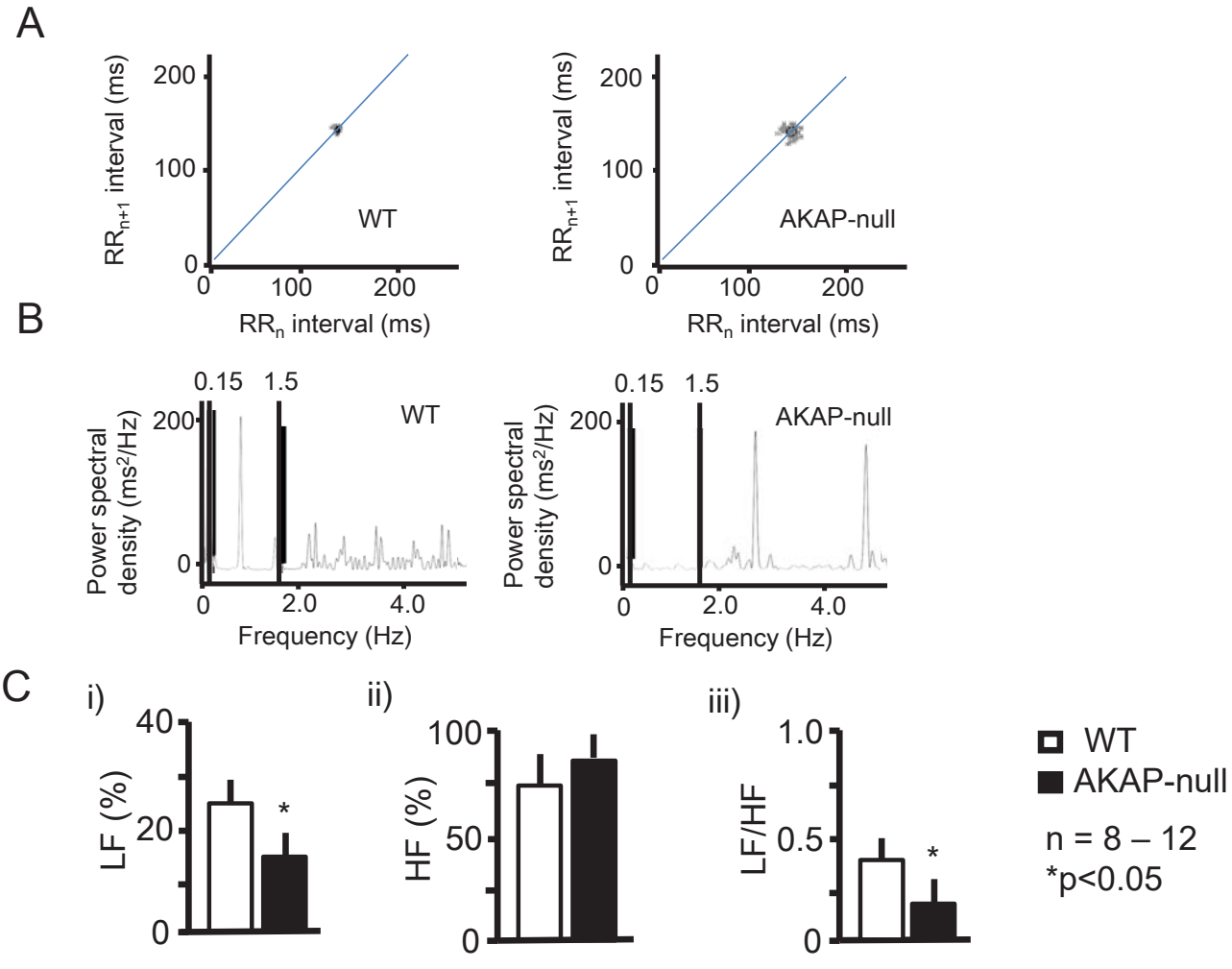


Figure 3

Atrium contraction (pacing)

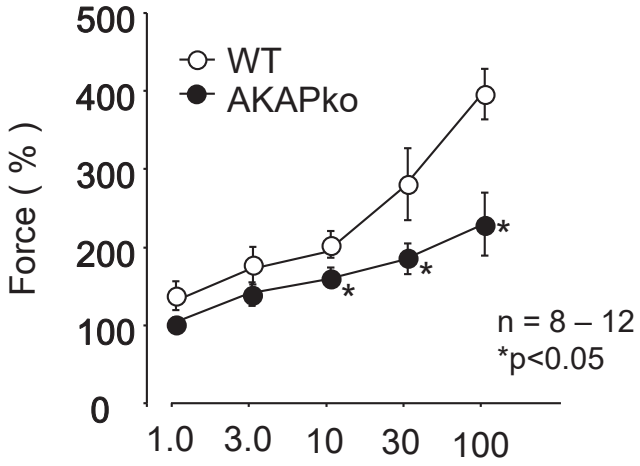
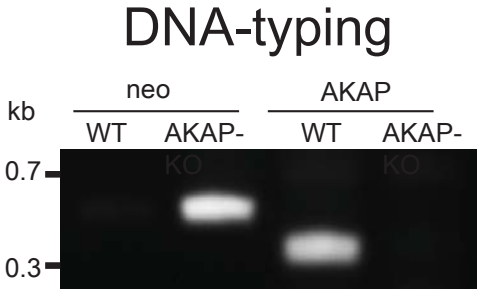


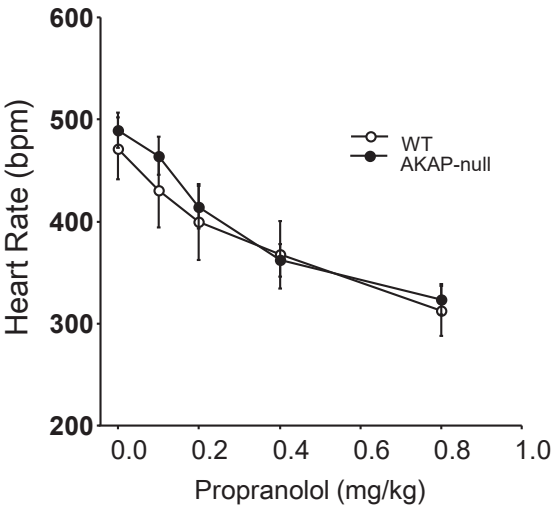
Figure 4

AKAP5-null mouse

A



B



Supplemental Figure 1