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Lack of influence of sex hormones on Brugada syndrome-associated mutant Nav1.5 sodium channel

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ABSTRACT

Brugada syndrome (BS) is an autosomal dominant disease. The most common causes of BS are loss-of-function mutations occur in the SCN5A gene which encodes the sodium channel protein Nav1.5. BS has a higher incidence rate in males and the underlying mechanisms of the gender inequality are not yet fully understood. Considering sex hormones are among the most important factors behind gender differences and have previously been shown to regulate the activity of multiple cardiac ion channels, we hypothesized that sex hormones also affect Nav1.5 function which lead to BS predominantly affecting males. In this study, we investigate the protein expression level and current of Nav1.5 in the HEK293 cells cotransfected with SCN5A and sex hormone receptor plasmids using both wild-type SCN5A and BS-associated SCN5A channel mutants R878C and R104W. Our findings showed that sex hormones have no effects on the protein expression level and current of the wild-type Nav1.5, neither does it affect the protein expression level and current of BS-associated Nav1.5 mutants R878C and R104W, regardless of homozygous or heterozygous state. Our results suggest that the male preponderance of BS does not arise from the effects of the sex hormones on Nav1.5. Further studies are needed to explain the male preponderance of this disease.

Introduction

Brugada syndrome (BS) is an autosomal dominant disease with mutations in genes encoding cardiac ion channels that causes sudden death in young individuals. Loss-of-function mutations in the SCN5A gene, which encodes the α subunit of the cardiac sodium channel protein Nav1.5, are the most common genetic mutations found in this disease and have been identified in about 20%–25% of all cases [1,2]. The Nav1.5 protein contains four homologous transmembrane domains with six transmembrane segments (S1–S6) in each domain, which participate in the formation of the pore [3]. Despite SCN5A being inherited equally by males and females, the incidence rates of BS are higher in males [1,2]. Whether Nav1.5 is involved in the mechanisms of male preponderance of BS have not been studied.

Sex hormones are the most important factors behind gender differences. Testosterone and β-estradiol are the main sex hormones. Testosterone is considered to be the "male hormone" and is high in males, while β-estradiol is considered the "female hormone" and is high in females. Sex hormones, which can enter cells easily, exert their effects through the activation of cytosolic or membrane receptors and bind to various targets to exert multiple physiological effects [4].

The heart is one of the target organs of the sex hormones and accumulating evidence indicates that the sex hormones impact on human cardiac rhythm and arrhythmias [5–7]. Early reports described that diurnal ECG changes of patients with BS are associated with circadian rhythms of sex hormones and a higher level of testosterone may be related to the Brugada phenotype and its male preponderance [8–10]. Considering that BS is mainly caused by loss-of-function mutations in SCN5A, we hypothesized that β-estradiol may restore the reduced function of Nav1.5 thus lead to lower incidence of this disease, while testosterone may further inhibit the compromised Nav1.5, which lead to higher incidence rate.

According to the classical views, sex hormones only have genomic actions that they can only produce their effects by regulating transcriptional processes therefore influence gene expression after binding to cytosolic sex hormone receptors and translocating into the nucleus. However, accumulating evidence has shown that the sex hormones also have non-genomic actions that they acutely regulate cardiac ion

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channels and affect their current, which occurs too rapidly to be mediated by the synthesis of RNA and protein [4,6,11–14]. For example, the sex hormones can rapidly regulate L-type Ca^{2+} currents ($I_{Ca,L}$) and slowly activating delayed rectifier K^+ currents (I_{Ks}) via a non-genomic pathway after binding to membrane sex hormone receptors, which includes the sequential activation of c-Src, PI_3K, Akt, and eNOS and finally the release of NO, which activates the cAMP/PKA pathway to change the gating properties of these ion channels [5,15]. Sex hormones can also regulate the function of the HERG channel (I_{Kr}) by binding directly to a special site of the channel and influencing the gating [6,16].

In the present study, we examined the effects of β-estradiol and testosterone on wild-type and BS-associated mutant Nav1.5 via both genomic and non-genomic actions.

Materials and methods

Materials

β-Estradiol and testosterone (Sigma, USA) were dissolved in ethanol as stock solutions. The final ethanol concentration was no more than 0.2%. Given that the concentrations of β-estradiol and testosterone in patients with BS are within the normal ranges, the concentrations of both of these hormones used in the study were also chosen to mimic their physiological concentration ranges in humans. Cells were treated with β-estradiol or testosterone for 24 h since previous studies indicated that 24 h is sufficient for sex hormones to impact on the expression of proteins [13,14].

Cell culture and transfection

The human SCN5a-pEGFP-N2 plasmid was provided by Dr. Yanmin Zhang (University of Manchester, UK), the BS-associated SCN5A mutations, R878C and R104W, were generated by site-directed mutagenesis (QuickChange Lightning Site-Directed Mutagenesis Kit; Agilent Technologies, CA, USA). Human embryo kidney (HEK293) cells, which have no sex hormone receptors, were cultured in DMEM cell culture medium (GIBCO, USA) supplemented with 10% fetal bovine serum (GIBCO, USA) at 37 °C in a 5% CO_2 atmosphere. Wild-type and BS-associated mutant SCN5A, androgen receptor (AR; pWPI-AR, NM_000044, a kind gift from Dr. Lei Li, First Affiliated Hospital of Xi'an Jiaotong University, China), and estrogen receptor alpha (ERα; pCMV3-Flag-ESR1, NM_001122742.1, Sino Biological Inc., China) were transiently transfected into HEK293 cells using Lipofectamine3000 (Invitrogen, USA), following the manufacturer's protocol.

Whole-cell patch clamp recording

Sodium currents (I_{Na}) was recorded using a MultiClamp 700B patch amplifier (Axon Instrument Inc., USA) at room temperature (20–22 °C). pClamp 9.2 software (Axon Instrument Inc., USA) was used to analyze data. Glass pipettes were pulled using an electrode puller (P97; Sutter, USA) and maintained at a resistance of 3–5 MΩ. The extracellular solution (in mM) was as follows: NaCl 140, KCl 4, $MgCl_2$ 1, $CaCl_2$ 1.8, HEPES 10 and glucose 10, with the pH titrated to 7.3 using NaOH. The pipette solution (in mM) was as follows: NaCl 10, CsCl 130, HEPES 10, and EGTA 10, with the pH titrated to 7.3 using CsOH. Currents were digitized at a sampling rate of 10 kHz and filtered at 2 or 5 kHz (Digidata 1440A, Axon Instruments Inc.). I_{Na} was recorded by step depolarization for 20 ms to different potentials between −80 and +60 mV from a holding potential of 140 mV.

Western blotting analysis

After treatment, cells were washed twice with ice-cold PBS and then solubilized in ice-cold RIPA plus protease inhibitor cocktail (Roche, Germany). Protein samples were separated on an 8% SDS-PAGE gel, and then transferred to polyvinylidene difluoride membranes (Millipore, USA). These membranes were blocked in blocking solution (5% nonfat dry milk and 0.1% Tween 20 in TBS) for 1 h at room temperature. Then, the membranes were incubated with rabbit polyclonal anti-Nav1.5 (1:1000 dilution; Alomone, Israel), anti-AR (1:1000 dilution; Santa Cruz, USA), and anti-ERα (1:1000 dilution; Santa Cruz, USA) overnight at 4 °C. GAPDH or β-actin (1:1000 dilution; Santa Cruz, USA) was used as a control. The membranes were then washed with TBST (0.1% Tween 20 in TBS) and incubated with the horseradish peroxidase-conjugated goat anti-rabbit secondary antibody (1:10,000 dilution; Santa Cruz, USA) for 2 h at room temperature. Protein bands were detected using a chemiluminescence imaging system (Chemidoc XRS; BioRad, USA).

Statistical analysis

All values are presented as means ± SD. One-way ANOVA and Student's *t*-test were used for statistical analysis of the data and *p*-value less than 0.05 was considered to be statistically significant.

Results

β-Estradiol and testosterone do not affect the wild-type SCN5A channel

First, we investigated the effects of β-estradiol and testosterone on the wild-type Nav1.5 channel using HEK293 cells cotransfected with wild-type SCN5A and sex hormone receptor plasmids. These hormones were not found to affect the protein level of the wild-type Nav1.5 channel (Fig. 1). This indicates that β-estradiol and testosterone have no genomic effects on the wild-type Nav1.5 channel.

β-Estradiol does not affect the homozygous mutant SCN5A channel

Considering that BS is caused by mutant genes encoding cardiac ion channels, we hypothesized that the sex hormones may influence mutant but not wild-type Nav1.5. Thus, we investigated the effects of the sex hormones on homozygous mutant Nav1.5 using HEK293 cells cotransfected with mutant SCN5A and sex hormone receptor plasmids. The effect of β-estradiol on the mutant Nav1.5 current was examined first. As shown in Fig. 2a and b, β-estradiol had no influence on the current of both types of mutant Nav1.5, which indicates that this hormone has no non-genomic effect on this mutant protein. Next, we tested whether β-estradiol had a genomic effect on mutant Nav1.5. Again, the findings showed that β-estradiol did not change the protein level of both types of mutant Nav1.5 (Fig. 2c, d).

Testosterone has no effect on the homozygous mutant SCN5A channel

The results showed that testosterone does not influence the mutant Nav1.5 current and has no non-genomic effect on mutant Nav1.5 (Fig. 3a, b). In the next part of the study, the genomic effect of testosterone on mutant Nav1.5 was also determined. Again, testosterone was shown to have no influence on the protein level of both types of mutant Nav1.5 (Fig. 3c, d).

Sex hormones do not affect heterozygous SCN5A mutants

The mutation of SCN5A in BS patients is always in heterozygous form. Considering that the sex hormones may cause a male preponderance of BS by influencing heterozygous SCN5A channels, we investigated their effects on heterozygous Nav1.5 mutants. The whole-cell patch clamp technique showed that the sex hormones have no influence on the current of heterozygous mutant Nav1.5 (Fig. 4a, b and Fig. 5a, b), indicating that these hormones have no non-genomic effect on this mutant in heterozygous form. Next, we determined the genomic effect of sex hormones on heterozygous mutants. The results showed

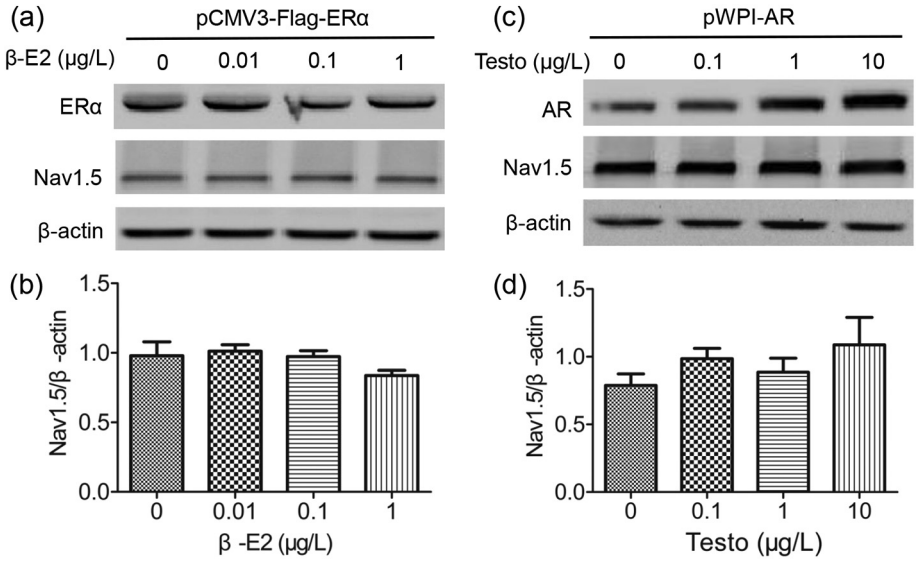

Fig. 1. Effects of β-estradiol and testosterone on wild-type Nav1.5 channel. (a) Representative Western blot image of the Nav1.5 protein expression upon treatment with different concentrations of β-estradiol (0, 0.01, 0.1, 1 μg/L) for 24 h. β-Actin served as an internal control. (b) The relative expression rate of Nav1.5 protein (Nav1.5/β-actin). ERα, estrogen receptor alpha; β-E2, β-estradiol. (c) Representative Western blot image of the Nav1.5 protein expression upon treatment with different concentrations of testosterone (0, 0.1, 1, 10 μg/L) for 24 h. β-Actin served as an internal control. (d) The relative expression rate of Nav1.5 protein (Nav1.5/β-actin). AR, androgen receptor; Testo, testosterone.

that the sex hormones do not change the protein expression level and thus also have no genomic effect on heterozygous mutant Nav1.5 (Fig. 4c, d and Fig. 5c, d).

Discussion

The purpose of this study is to determine whether the male preponderance of BS results from the effects of sex hormones on Nav1.5. Although sex hormones affect the functions of many cardiac ion channels, such as HERG, $I_{Ca,L}$ and I_{Ks}, our data indicate that they have no effects on both wild-type and BS-associated mutant Nav1.5.

Specifically, we explored the effects of β-estradiol and testosterone on SCN5A channels by transiently cotransfecting SCN5A and sex hormone receptor plasmids into HEK293 cells. Previous studies demonstrated that these overexpressed ERα and AR are actually functional. For example, Zhang et al. transfected ERα and c-Fos plasmids into

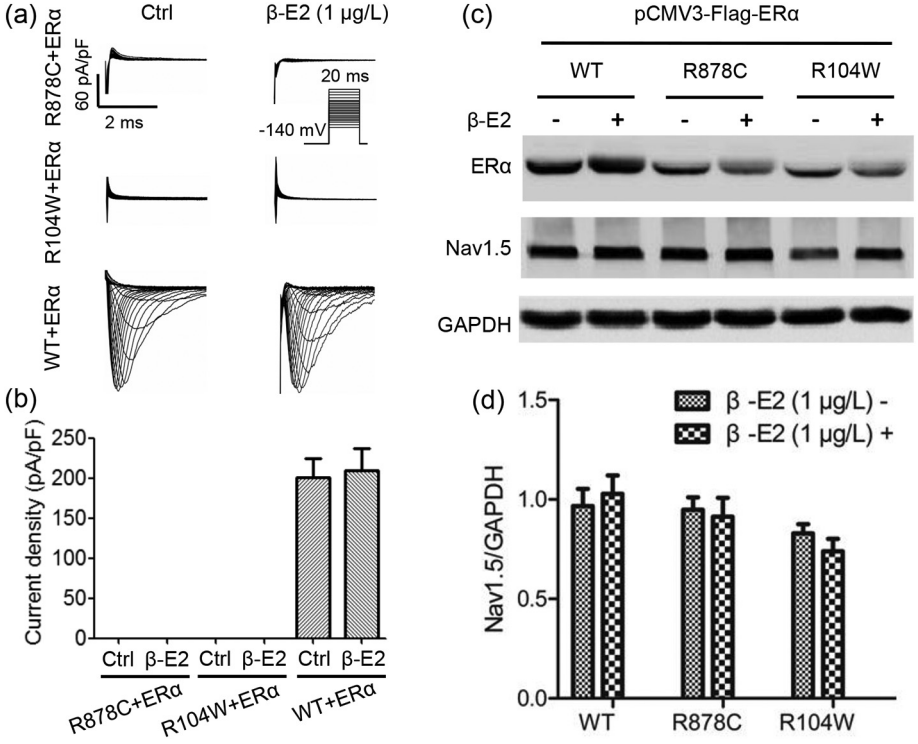

Fig. 2. Effect of β-estradiol on homozygous mutant Nav1.5 channel. (a) Representative current traces in HEK293 cells which were transiently cotransfected with mutant SCN5A and ERα plasmids upon treatment with β-estradiol (1 μg/L). (b) Peak I_{Na} (pA/pF) of each experimental situations. (c) Representative Western blot image of the mutant Nav1.5 protein upon treatment with β-estradiol (1 μg/L) for 24 h. GAPDH served as an internal control. (d) The relative expression rate of mutant Nav1.5 protein (Nav1.5/GAPDH). ERα, estrogen receptor alpha; β-E2, β-estradiol.

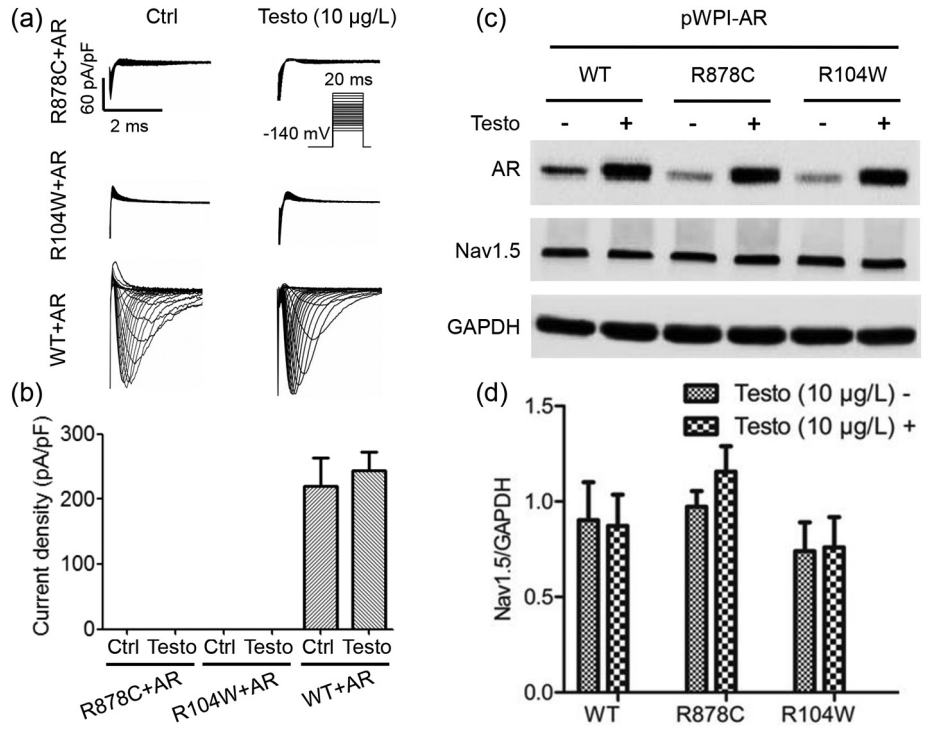

Fig. 3. Effect of testosterone on homozygous mutant Nav1.5 channel. (a) Representative current traces in HEK293 cell which was transiently cotransfected with mutant SCN5A and AR plasmids and upon treatment with testosterone (10 μg/L). (b) Peak I_{Na} (pA/pF) of each experimental situations. (c) Representative Western blot image of the mutant Nav1.5 protein upon treatment with testosterone (10 μg/L) for 24 h. GAPDH served as an internal control. (d) The relative expression rate of mutant Nav1.5 protein (Nav1.5/GAPDH). AR, androgen receptor; Testo, testosterone.

HEK293 cells. Upon treatment with β-estradiol, the expression level of c-Fos protein in the group cotransfected with ERα and c-Fos was higher than in the other groups [17]. In addition, Tang et al. transiently cotransfected CYP7B1 promoter-luciferase reporter gene constructs and AR plasmid into HEK293 cells and found that CYP7B1 promoter activity and CYP7B1-mediated catalysis were suppressed upon treatment with DHT [18]. In our study, the western blotting data showed increased expression of ERα and AR upon treatment with β-estradiol and testosterone respectively, which were consistent with the findings in previous studies [11,19,20].

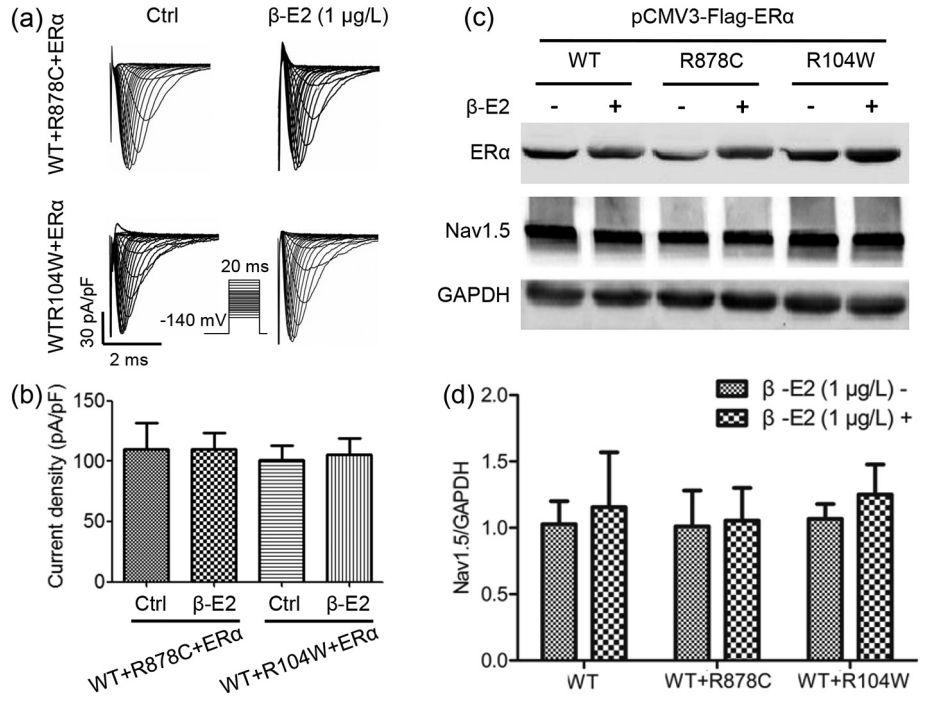

Fig. 4. Effect of β-estradiol on heterozygous mutation of SCN5A. (a) Representative current traces in HEK293 cells which was transiently cotransfected with mutant SCN5A and ERα plasmids upon treatment with a physiological concentration of β-estradiol (1 μg/L). (b) Peak I_{Na} (pA/pF) of each experimental situations. (c) Representative Western blot image of the heterozygous mutation of SCN5A protein upon treatment with β-estradiol (1 μg/L) for 24 h. GAPDH served as an internal control. (d) The relative expression rate of heterozygous mutation of SCN5A protein (Nav1.5/GAPDH). ERα, estrogen receptor alpha; β-E2, β-estradiol.

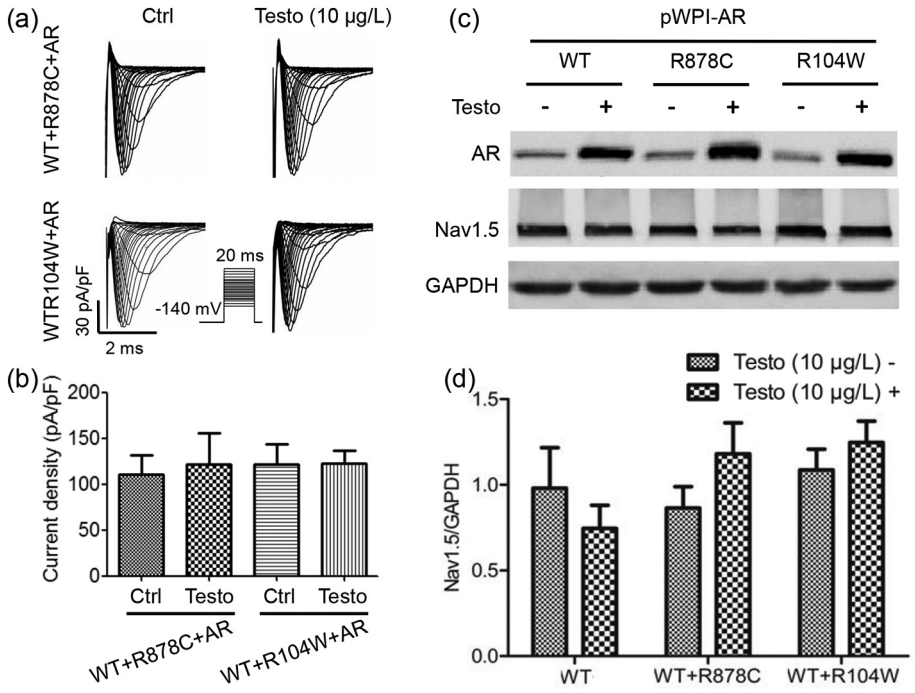

Fig. 5. Effect of testosterone on heterozygous mutation of SCN5A. (a) Representative current traces in HEK293 cells cotransfected with mutant SCN5A and AR plasmids upon treatment with a physiological concentration of testosterone (10 μg/L). (b) Peak I_{Na} (pA/pF) of each experimental situations. (c) Representative Western blot image of the heterozygous mutation of SCN5A protein upon treatment with testosterone (10 μg/L) for 24 h. GAPDH served as an internal control. (d) The relative expression rate of heterozygous mutation of SCN5A protein (Nav1.5/GAPDH). AR, androgen receptor; Testo, testosterone.

Findings from previous studies can help to interpret the effect of sex hormones on wild-type Nav1.5 as identified in the present work. For example, Gaborit et al. found that the protein expression levels of Nav1.5 do not differ between normal male and female human heart, indicating that wild-type Nav1.5 is not affected by sex hormones [21]. In addition, Clemens et al., who investigated concentration-related block of wild-type Nav1.5 by estradiol, found that estradiol cannot reduce the current of Nav1.5 [7].

The effects of sex hormones on BS-associated Nav1.5 channel mutations R878C and R104W have not previously been reported. R878C was first identified in a Chinese family [22]. The proband was an 8-year-old boy and there was a tendency for a male preponderance among the affected individuals within the pedigree. R878C is located within a highly conserved region of domain II S5–S6 encoding the pore of the channel and this mutation from arginine to cysteine leads to a missense mutation. The residues between S5 and S6 form the pore of the channel, which controls the selectivity and permeation of sodium ions, and thus R878C is a gating-defective mutant [3,22]. In contrast, the proband of R104W was a male (33 years old) with a spontaneous ECG pattern of BS type 1 and the affected individuals in this family were all male [23]. R104W is a missense mutation in the N-terminal region of domain I, involving arginine being changed to tryptophan, which leads to retention and degradation of most of the channels. In our study, sex hormones were shown to have no non-genomic effects on Nav1.5 and did not influence the Nav1.5 current, which was probably due to free of influence on the properties of Nav1.5 after the activation of the downstream signaling pathways of membrane ERα and AR. We also found that the sex hormones cannot regulate the protein expression level of the SCN5A channel and have no genomic actions on Nav1.5. It can thus be speculated that the activation of cytosolic ERα and AR does not interfere with the signaling pathways that are involved in regulating of the synthesis, trafficking, and degradation of Nav1.5.

Sex differences are based not only on differences in sex hormones but also on differences in sex chromosomes and sex-specific gene expression in the autosomes. The male preponderance of BS may thus be the result of the effects of sex hormones on other genes expressed on sex chromosomes or autosomes. In addition, sex hormone-independent gene expression from the sex chromosomes may also participate in the male preponderance of this disease. Previous studies have suggested that the male predominance of Brugada phenotype is related to the presence of a more prominent I(to) in males versus females [24]. The presence of a more prominent Ito-mediated notch in the Epi of males may predispose males to the development of the Brugada phenotype and that a smaller Epi notch in females relegates them to development of progressive conduction problems [25]. However, I(to)-mediated action potential notch appears just to be a prerequisite and are not involved in most cases of the BS [24]. Though our results indicate that sex hormones have no effects on wild-type SCN5A and BS-associated SCN5A mutants R878C and R104W, our research is based on in-vitro testing and in-vivo testing was not performed, which is a potential limitation. As a consequence of these limitations, further studies are still needed to clarify the reason for the male preponderance of this disease.

In conclusion, our study showed that sex hormones have no effects on wild-type SCN5A and BS-associated SCN5A mutants R878C and R104W, indicating that the male preponderance of BS may not result from the effect of sex hormones on the wild-type SCN5A and SCN5A channel mutants R878C and R104W.

Author contributions

GDY, AQM, TZW conceived and designed the experiments. GDY, JL conducted the experiments. GDY, YD, YW analyzed data. GDY, TZW, AQM wrote the manuscript. All the authors read and approved the manuscript.

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Disclosures

None.

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