

RESEARCH ARTICLE

# Molecular Cloning and Functional Expression of the Equine K<sup>+</sup> Channel K<sub>v</sub>11.1 (Ether à Go-Go-Related/KCNH2 Gene) and the Regulatory Subunit KCNE2 from Equine Myocardium

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## Abstract

The *KCNH2* and *KCNE2* genes encode the cardiac voltage-gated K<sup>+</sup> channel K<sub>v</sub>11.1 and its auxiliary β subunit KCNE2. K<sub>v</sub>11.1 is critical for repolarization of the cardiac action potential. In humans, mutations or drug therapy affecting the K<sub>v</sub>11.1 channel are associated with prolongation of the QT intervals on the ECG and increased risk of ventricular tachyarrhythmia and sudden cardiac death—conditions known as congenital or acquired Long QT syndrome (LQTS), respectively. In horses, sudden, unexplained deaths are a well-known problem. We sequenced the cDNA of the *KCNH2* and *KCNE2* genes using RACE and conventional PCR on mRNA purified from equine myocardial tissue. Equine K<sub>v</sub>11.1 and KCNE2 cDNA had a high homology to human genes (93 and 88%, respectively). Equine and human K<sub>v</sub>11.1 and K<sub>v</sub>11.1/KCNE2 were expressed in *Xenopus laevis* oocytes and investigated by two-electrode voltage-clamp. Equine K<sub>v</sub>11.1 currents were larger compared to human K<sub>v</sub>11.1, and the voltage dependence of activation was shifted to more negative values with V<sub>1/2</sub> = -14.2±1.1 mV and -17.3±0.7, respectively. The onset of inactivation was slower for equine K<sub>v</sub>11.1 compared to the human homolog. These differences in kinetics may account for the larger amplitude of the equine current. Furthermore, the equine K<sub>v</sub>11.1 channel was susceptible to pharmacological block with terfenadine. The physiological importance of K<sub>v</sub>11.1 was investigated in equine right ventricular wedge preparations. Terfenadine prolonged action potential duration and the effect was most pronounced at slow pacing. In conclusion, these findings indicate that horses could be disposed to both congenital and acquired LQTS.

**Competing Interests:** The authors have declared that no competing interests exist.

## Introduction

In equine medicine, sudden deaths are a well-known problem and often the cause of death cannot be determined by necropsy [1]. Spontaneous arrhythmias have been shown to occur in horses [2], but studies coupling specific arrhythmias to sudden cardiac death (SCD) are sparse [3]. In humans, SCD in athletes has been linked to the Long QT Syndrome (LQTS) [4]. LQTS is characterized by a delayed repolarization of cardiac action potentials, a prolongation of the QT interval on the surface ECG and development of ventricular tachyarrhythmia of the *tor-sades de pointes*-type, which can progress to SCD. The presence of LQTS in veterinary patients has been suggested [5], however, reference values of the QT interval are not available for many companion animals and often the causes of unexpected deaths are not investigated in details [6]. We have recently established the normal QT interval in standard and warmblood horses [7,8]

Repolarization of the cardiac action potential in larger mammals, including humans and horses, has been shown to be dependent on the rapid and slow activating delayed rectifier K<sup>+</sup> currents,  $I_{Kr}$  and  $I_{Ks}$  [6,9,10]. Loss of function mutations in the genes encoding proteins mediating  $I_{Kr}$  or  $I_{Ks}$  are common causes of congenital LQTS in humans and pharmaceutical blockage of  $I_{Kr}$  is a well described cause of acquired LQTS.  $I_{Kr}$  is mediated by the pore-forming protein K<sub>v</sub>11.1 [11], which has been proposed to interact with the axillary  $\beta$ -subunit KCNE2 in cardiac cells [12]. K<sub>v</sub>11.1 is encoded by the *KCNH2* gene, also known as the *ether à go-go related gene* (ERG) due to similarities to the *Drosophila ether à go-go* (EAG) gene product [13]. The protein KCNE2 is encoded by the *KCNE2* gene [12]. Mutations in *KCNH2* have been linked to the long QT syndrome type 2 (LQT2) and mutations in *KCNE2* can give rise to LQT6 [12,14,15]. The K<sub>v</sub>11.1 channel is susceptible to pharmacological block by many compounds, including antiarrhythmic drugs as well as a variety of non-cardioactive drugs, which has been linked to acquired LQTS [16]. Acquired LQTS might also be of relevance to equine patients as horses are often treated with drugs known to prolong cardiac repolarization in other species, including quinidine, cisapride, erythromycin, and trimethoprim-sulfamethoxazole.

The aim of this study was to obtain a full length cDNA sequence of the equine *KCNH2* and *KCNE2* genes and to characterize the electrophysiological properties of equine K<sub>v</sub>11.1 and K<sub>v</sub>11.1/KCNE2 using the human isoform as reference. We found that the overall electrophysiological properties of the cloned equine channels are similar to those of the human isoform. However, equine K<sub>v</sub>11.1 currents were larger compared to the human homologue. We also found that  $I_{Kr}$  is physiologically important for cardiac repolarization in multicellular preparations of equine right ventricle. Our results elucidate the function of equine  $I_{Kr}$  in cardiac repolarization and indicate that congenital or acquired LQTS could potentially underlie unexpected deaths in horses.

## Materials and Methods

### Ethics Statement

Horses were donated to the Department of Veterinary Clinical and Animal Science, University of Copenhagen. The horses were euthanized by captive bolt gun and bleeding. No permit for animal testing was necessary under Danish law (The Animal Experimentation Act 1253 of 8<sup>th</sup> of March 2013) to collect the equine tissue used for the experiments. In total, 7 horses were included, 3 geldings, 1 stallion 3 mares, age 9–10 years and of mixed breed. The prevalent reason for euthanasia was lameness and the horses had no history of any cardiac illnesses.

*Xenopus laevis* oocytes for heterologous expression were surgically removed from anesthetized frogs (anesthetic compound—0.2% 3-aminobenzoate methanesulfonate). The procedure

was approved by the Danish animal experimentation board (approval number: 2012-15-2934-00625).

## PCR Primer Design

Predicted equine *KCNH2* and *KCNE2* gene sequences were obtained by using the Nucleotide Basic Local Alignment Search Tool (BLAST) [17] to compare the human *KCNH2* and *KCNE2* sequences (GenBank [18] accession numbers: NM\_000218 and NM\_172201.1, respectively) with the genomic sequence data from the EquCab2.0 *Equus caballus* genome project [19]. The predicted equine *KCNH2* and *KCNE2* sequences were used as the query sequence in BLAST covering all species with an annotated *KCNH2* and *KCNE2* gene. Following, portions with a high conservation were used as a basis for manual primer design. Primers were synthesized by Eurofins MWG Operon (Ebersberg, Germany).

## Bioinformatics

For further analysis of conservation, all species with an annotated *KCNH2* and *KCNE2* gene were found using the GenBank database or UniProt database [20] and these sequences were included in multiple sequence alignments generated using MAFFT [21] (Figs 1 and 2).

## Cloning of Full-length K<sub>v</sub>11.1 and KCNE2 cDNA

Equine myocardial tissue was sampled from the left ventricular free wall less than two minutes after euthanasia. Total RNA was isolated using Trizol reagent (Invitrogen). cDNA was synthesized using the SuperScript III First-Strand Synthesis SuperMix (Invitrogen) or, for Rapid Amplification of cDNA Ends (RACE), the FirstChoice RLM-RACE kit (Ambion) was used. Size of PCR products were confirmed by gel electrophoresis and subsequently cloned into the pCR4-TOPO vector (Invitrogen) using the TOPO TA cloning kit (Invitrogen). Following, the ligated vectors were transformed into One Shot Top10 and DH5 $\alpha$ -T1<sup>R</sup> competent cells (Invitrogen). Plasmid DNA was purified using the GenElute Plasmid Miniprep and kit (Sigma-Aldrich) and sequenced (Eurofins MWG Operon). Ligation of overlapping coding PCR products was done using T4 DNA ligase after cutting with relevant restriction enzymes (New England Biolabs). 5' RACE PCR was unsuccessful for *KCNE2*. Based on our sequencing results from conventional PCR, the predicted equine *KCNE2* sequenced obtained from EquCab2.0 [19], and sequence alignment with *KCNH2* from other species, it was rationalized that the three amino acids (MPT) initiate the equine *KCNE2* protein sequence at the amino terminus. Based on this assumption the predicted full equine *KCNE2* was synthesized by GenScript (Piscataway). To facilitate expression in *Xenopus laevis* oocytes, the equine K<sub>v</sub>11.1 and *KCNE2* cDNA was subcloned into the pXOOM expression vector [22] and sequenced (Eurofins MWG Operon). Human K<sub>v</sub>11.1 (NM\_000218) and *KCNE2* (NM\_172201.1) in pXOOM were kind gifts from Dr. Thomas Jespersen.

## In Vitro Transcription of mRNA

Following linearization of the expression constructs with XbaI (New England Biolabs), mRNA was produced using the mMessage mMachine kit and purified with MEGAclean (Ambion). mRNA was stored at -80°C until use.

## Isolation of Oocytes and Injection of RNA for Heterologous Expression

Preparation of oocytes from *Xenopus laevis* was performed as previously described [23]. In brief, ovarian lobes were excised from the abdominal cavity of anaesthetized frogs. The ovarian

	1				50
eKv11.1	MPVRRGHVAP	QNTFLDTIIR	KFEGQSRKFI	IANARVENCA	VIYCNDGFCE
hKv11.1	MPVRRGHVAP	<u>QNTFLDTIIR</u>	<u>KFEGQSRKFI</u>	<u>IANARVENCA</u>	<u>VIYCNDGFCE</u>
	51	$\alpha$		PAS	100
eKv11.1	LCGYSRAEVM	QRPCDCFLH	GPRTQRRAAA	QIAQALLGAE	ERKVEISFYR
hKv11.1	<u>LCGYSRAEVM</u>	<u>QRPCDCFLH</u>	<u>GPRTQRRAAA</u>	<u>QIAQALLGAE</u>	<u>ERKVEIAFYR</u>
	101				150
eKv11.1	KDGSCFLCLV	DVVPVKNEGD	AVIMFILNFE	VMEKDMVGS	PARDTNHRGP
hKv11.1	<u>KDGSCFLCLV</u>	<u>DVVPVKNEGD</u>	<u>AVIMFILNFE</u>	<u>VMEKDMVGS</u>	<u>PAHDTNHRGP</u>
	151				200
eKv11.1	PTSWLATGRA	KTFRLKLPAL	LALTARESTV	RPGGAGSTGA	PGAVVVDVDL
hKv11.1	PTSWLATGRA	KTFRLKLPAL	LALTARESSV	RSGGAGGAGA	PGAVVVDVDL
	201				250
eKv11.1	TPAAPSSSEL	ALDEVTAMDN	HVAGLGPAAE	RRALVGPSP	PACAPIPHPS
hKv11.1	TPAAPSSSEL	ALDEVTAMDN	HVAGLGPAAE	RRALVGPSP	PRSAPOQLPS
	251				300
eKv11.1	PRAHSLNPD	SGSSCSLART	RSRESCASVR	RASSADDIEA	MRTG LPPPP
hKv11.1	PRAHSLNPD	SGSSCSLART	RSRESCASVR	RASSADDIEA	MRAGVLPPPP
	301				350
eKv11.1	RHASTGAMHP	LRSGLLNSTS	DSDLVRYRTI	SKIPQITLNF	VDLKGDFFLA
hKv11.1	RHASTGAMHP	LRSGLLNSTS	DSDLVRYRTI	SKIPQITLNF	VDLKGDFFLA
	351				400
eKv11.1	SPTSREIIRA	PKIKERTHN	TEKVTQVLSL	GADVLPEYKL	QAPRIHRWTI
hKv11.1	SPTSREIIRA	PKIKERTHN	TEKVTQVLSL	GADVLPEYKL	QAPRIHRWTI
	401				450
eKv11.1	LHYSPPKAVW	DWLILLLVII	TAVFTPYSA	FLLKETEEL	PATDCGYACQ
hKv11.1	<u>LHYSPPKAVW</u>	<u>DWLILLLVII</u>	<u>TAVFTPYSA</u>	<u>FLLKETEEL</u>	<u>PATECGYACQ</u>
	451	S1			500
eKv11.1	PLAVVDLIVD	IMFIVDILIN	FRTTYVNANE	EVVSHPGRIA	VHYFKGWFLI
hKv11.1	<u>PLAVVDLIVD</u>	<u>IMFIVDILIN</u>	<u>FRTTYVNANE</u>	<u>EVVSHPGRIA</u>	<u>VHYFKGWFLI</u>
	501	S2			550
eKv11.1	DMVAIIPFDL	LIFGSGSEEL	IGLLKTARLL	RLVRVARKLD	RYSEYGAAVL
hKv11.1	<u>DMVAIIPFDL</u>	<u>LIFGSGSEEL</u>	<u>IGLLKTARLL</u>	<u>RLVRVARKLD</u>	<u>RYSEYGAAVL</u>
	551	S3	S4		600
eKv11.1	FLLMCTFALI	AHWLACIWYA	IGNMEQPHMD	SRIGWLHNLG	DQIGKPYNSS
hKv11.1	<u>FLLMCTFALI</u>	<u>AHWLACIWYA</u>	<u>IGNMEQPHMD</u>	<u>SRIGWLHNLG</u>	<u>DQIGKPYNSS</u>
	601	S5			650
eKv11.1	GLGGPSIKDK	YVTALYFTFS	SLTSVGFNGV	SPNTNSEKIF	SICVMLIGSL
hKv11.1	GLGGPSIKDK	YVTALYFTFS	SLTSVGFNGV	SPNTNSEKIF	SICVMLIGSL
	651		Pore		700
eKv11.1	MYASIEGNVS	AIQRLYSGT	ARYHTQMLRV	REFIRFHQIP	NPLRQLEEY
hKv11.1	<u>MYASIEGNVS</u>	<u>AIQRLYSGT</u>	<u>ARYHTQMLRV</u>	<u>REFIRFHQIP</u>	<u>NPLRQLEEY</u>
	701	S6			750
eKv11.1	FQHAWSYTNG	IDMNAVLKGF	PECLQADICL	HLNRSLLQHC	KPFRGATKGC
hKv11.1	FQHAWSYTNG	IDMNAVLKGF	PECLQADICL	HLNRSLLQHC	KPFRGATKGC
	751				800
eKv11.1	LRALAMKFKT	THAPPGDTLV	HAGDLLTALY	FISRGSIEIL	RGDVVVAILG
hKv11.1	<u>LRALAMKFKT</u>	<u>THAPPGDTLV</u>	<u>HAGDLLTALY</u>	<u>FISRGSIEIL</u>	<u>RGDVVVAILG</u>
	801		CNBD		850
eKv11.1	KNDIFGEPLN	LYARPGKSN	DVRALTYCDL	HKIHRDDLLE	VLDMPYEFSD
hKv11.1	<u>KNDIFGEPLN</u>	<u>LYARPGKSN</u>	<u>DVRALTYCDL</u>	<u>HKIHRDDLLE</u>	<u>VLDMPYEFSD</u>
	851				900
eKv11.1	HFWSLEITF	NLRDTNMIPG	SPGSTELEGG	FNRQRKRKLS	FRRRTDKDPE
hKv11.1	<u>HFWSLEITF</u>	<u>NLRDTNMIPG</u>	<u>SPGSTELEGG</u>	<u>FNRQRKRKLS</u>	<u>FRRRTDKDPE</u>
	901			PIP2	950
eKv11.1	QPGEVSALGP	GRAGAGPSSR	GRPGGPWGES	PSSGPSSPES	SEDEGPGRSS
hKv11.1	QPGEVSALGP	GRAGAGPSSR	GRPGGPWGES	PSSGPSSPES	SEDEGPGRSS
	951				1000
eKv11.1	SPLRLVPFSS	PRPPGEPPGG	EPLIEDCEKS	SDTCNPLSGA	FSGVSNIFSF
hKv11.1	SPLRLVPFSS	PRPPGEPPGG	EPLMEDCEKS	SDTCNPLSGA	FSGVSNIFSF
	1001				1050
eKv11.1	WGDSRGRQYQ	ELPRCPAPAP	SLLNIPSSP	GRRPRGDVES	RLDALQRQLN
hKv11.1	WGDSRGRQYQ	ELPRCPAPT	SLLNIPSSP	GRRPRGDVES	RLDALQRQLN
	1051				1100
eKv11.1	RLETRLSADM	ATVLQLLQRO	MTLVPPAYSA	VTPPGPGPTS	TSPLLPVSP
hKv11.1	RLETRLSADM	ATVLQLLQRO	MTLVPPAYSA	VTPPGPGPTS	TSPLLPVSP
	1101				1150
eKv11.1	PTLTLDLSLQ	VSQFMACEEL	PPGAPQLPQD	GPTRRLSLPG	QLGALTSQPL
hKv11.1	PTLTLDLSLQ	VSQFMACEEL	PPGAPQLPQE	GPTRRLSLPG	QLGALTSQPL
	1151				
eKv11.1	HRHGSDFGS				
hKv11.1	HRHGSDFGS				

**Fig 1. Alignment of human and equine K<sub>v</sub>11.1 protein sequences.** Genbank accession number: Human NP\_000229, horse ADK92992/ NP\_001180587.1. The transmembrane domains S1–S6 are underlined in red. The α helix at residues 13–23, the PAS domain, the signature sequence at residues 620–629, the Y-652 and the IFG residues in S6, the cyclic nucleotide binding domain (CNBD) at residues 749–872 and the PIP2 binding domain are underlined in blue. Green boxes mark the equine amino acid in position A97S as this substitution in the PAS domain could be important for channel gating and position 444 as the E444D mutation has been published as a cause of long QT syndrome in humans.

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lobes were digested with collagenase and incubated with hypertonic phosphate buffer to clear the follicle cell layer from the oocytes. Stage V or VI oocytes were selected and injected with approximately 50 nl mRNA solution (10 ng for K<sub>v</sub>11.1 alone and 10/20 ng for K<sub>v</sub>11.1/KCNE2) using a micro-injector (Nanoject, Drummond Broomall). Following, the oocytes were incubated approximately 48 hours at 19°C in kulori medium (in mM): 90 NaCl, 1 KCl, 1 MgCl<sub>2</sub>, 1 CaCl<sub>2</sub>, 5 HEPES, pH 7.4

### Two-Microelectrode Voltage Clamp of Oocytes

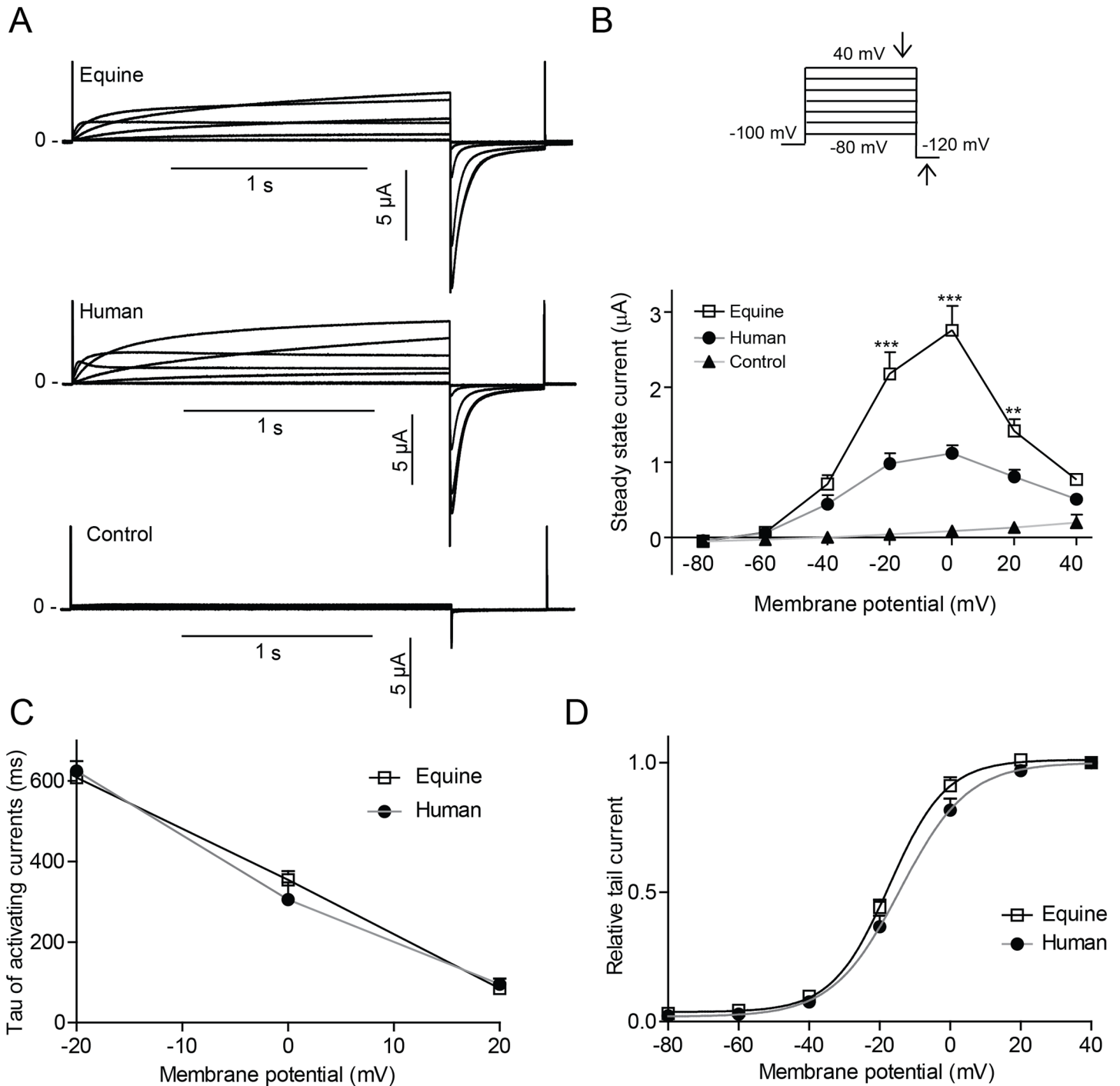
Currents were recorded by two-electrode voltage clamp using an Oocyte Clamp amplifier (OC-725 B, Warner Instruments) and a PC-interface (Axon Digidata 1440A, Molecular Devices). Data were sampled at 2 kHz using pClamp (Axon V. 10.2.14, Molecular Devices). Electrodes were pulled from capillary glass (TW 120–3, WPI) on a programmable micropipette puller (P-97, Sutter Instrument, Novato, CA, USA). Electrodes were filled with 1 M KCl and electrode resistance ranged from 0.5 to 1.5 MΩ. Experiments were performed at room temperature (19–21°C) in an air-conditioned room with the oocytes in a bath under a continuous flow of kulori medium. The experiments were repeated in at least three different batches of oocytes and qualitatively similar results were obtained.

Data were analyzed using Clampfit (Axon 10.4, Molecular Devices) and Prism 5 (GraphPad Software). The rate of activation of equine vs. human K<sub>v</sub>11.1 channel and K<sub>v</sub>11.1/KCNE2 channel complex was described by fitting a single exponential function  $I(t) = A_e e^{-(t/\tau)} + C$  to the initial 500 ms of the activating currents in Fig 3A. Only currents at -20 to 20 mV were included due to a prominent onset of inactivation at higher voltages. To address the voltage dependence of activation (Fig 3D), normalized peak tail current amplitude were plotted as a function of test potentials and a Boltzmann function,  $I = 1/(1 + e^{[(V_{1/2} - V_t)/k]})$  was fitted to the data.  $V_{1/2}$  is the voltage required for half maximal activation of current,  $V_t$  is the test potential, and  $k$  is the slope

	1					50
eKCNE2	<u>M</u> <u>P</u> <u>T</u> LSNLTQT	LEDVFKKIFI	TYMNNWRNT	TAEQEALQAK	VDAENFYIVI	
hKCNE2	<u>M</u> <u>S</u> <u>T</u> LSN <u>F</u> TQT	LEDVFRRIFI	TYMDNWRQNT	TAEQEALQAK	VDAENFYIVI	
	51					100
eKCNE2	LYLMVMIGMF	SFIIIVAILVS	TVKSKRREHS	NDPYHQYIVE	DWQEKYRSQI	
hKCNE2	<u>LYLMVMIGMF</u>	<u>SFIIIVAILVS</u>	<u>TVKSKRREHS</u>	NDPYHQYIVE	DWQEKYKSQI	
	101	S1	123			
eKCNE2	LNLEEPKATI	HKNISATEFQ	MSP			
hKCNE2	LNLEESKATI	HENIGAAGFK	MSP			

**Fig 2. Alignment of equine and human KCNE2 protein sequences.** Genbank accession number: Human NP\_751951, horse AHH41329. The predicted three amino acids (MPT) initiating the equine KCNE2 protein sequence and the N6 and N29 glycosylation sites and the T71 and S74 phosphorylation sites are underlined in blue. The transmembrane region is underlined in red.

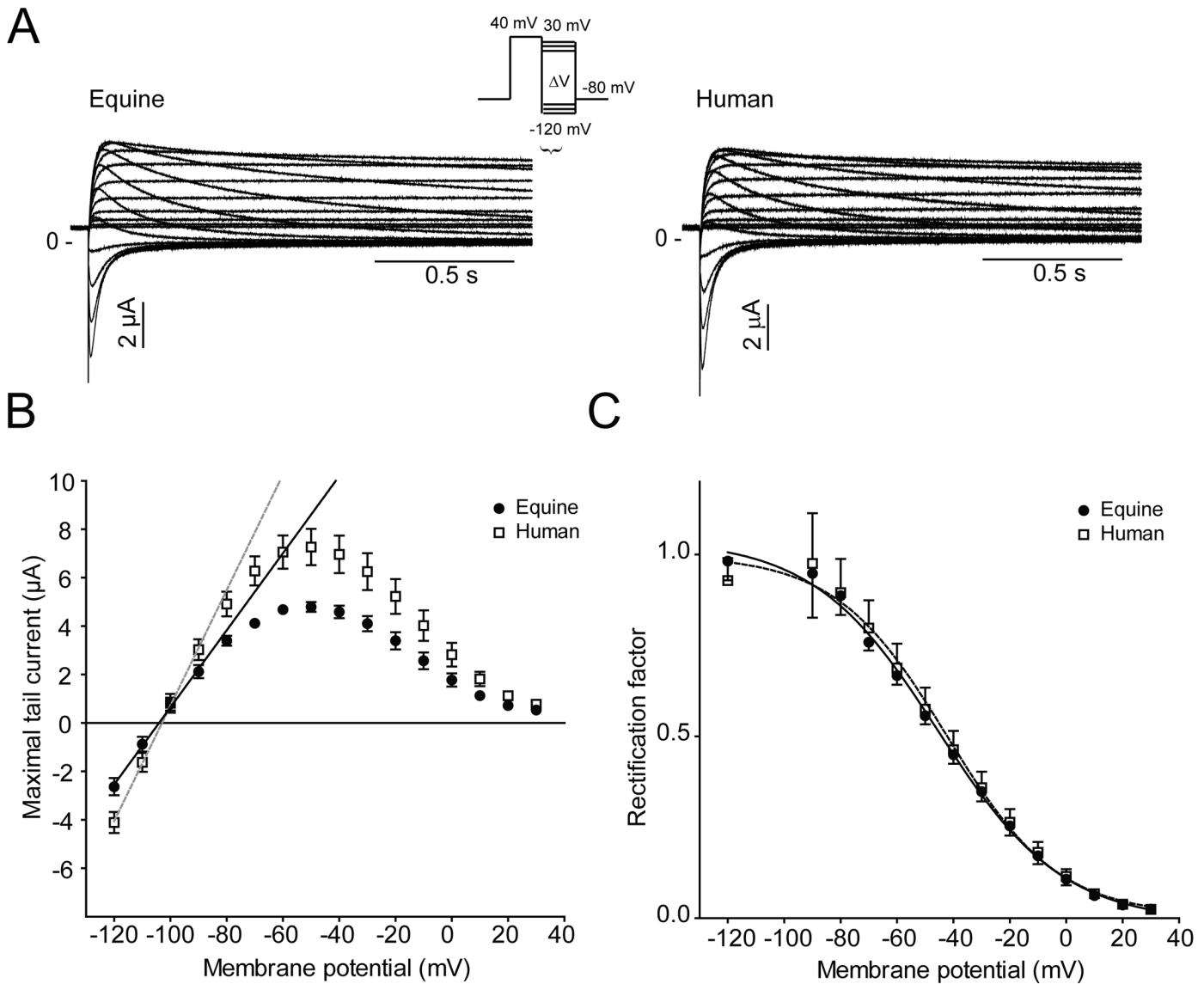
doi:10.1371/journal.pone.0138320.g002



**Fig 3. Equine and human  $K_v11.1$  expressed in *Xenopus laevis* oocytes (A) Representative recordings of equine (n>14) and human (n>13)  $K_v11.1$  expressed in *Xenopus laevis* oocytes as well as uninjected controls (n = 15). (B) Steady-state currents (indicated by downward pointing arrow on the protocol) as a function of voltage. (C) Time-constants (Tau) of the activating currents. (D) Peak tail currents (indicated by upward pointing arrow) normalized to maximal amplitude as a function of the voltage at the preceding step.**

doi:10.1371/journal.pone.0138320.g003

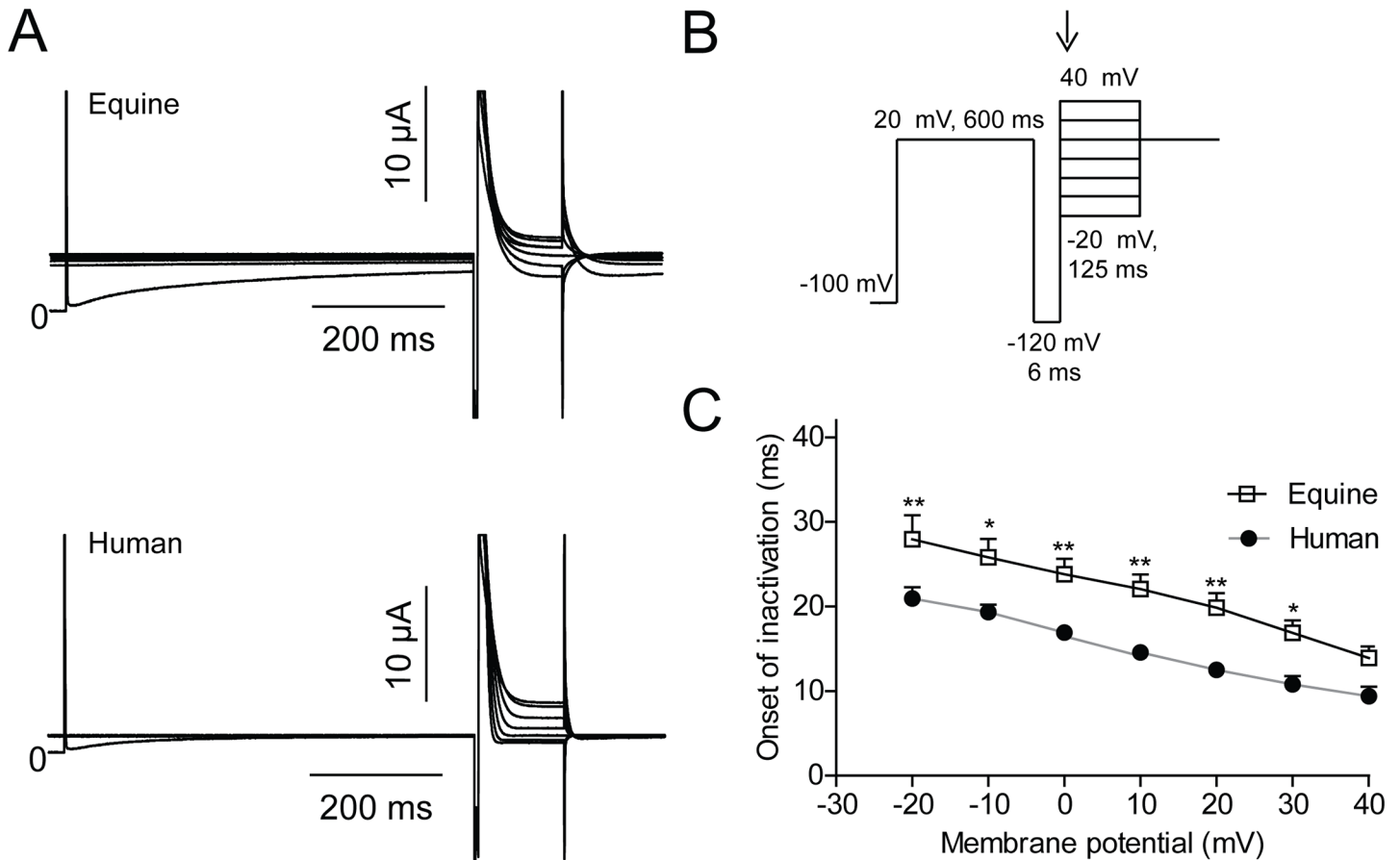
factor. The rate of deactivation of equine vs. human  $K_v11.1$  channel and  $K_v11.1/KCNE2$  channel complex was obtained by fitting a double exponential function  $I(t) = \sum Ae^{(-t/\tau)} + C$  to the



**Fig 4.  $K_v11.1$  channel rectification and voltage dependence of inactivation.** (A) Representative recordings of equine ( $n = 10$ ) and human  $K_v11.1$  ( $n = 10$ ) expressed in *Xenopus laevis* oocytes. (B) Fully activated current-voltage ( $I$ - $V$ ) relationship of the equine and human  $K_v11.1$  channels. The maximal conductance ( $G$ ) of the tail currents was determined as the slope of a linear fit to maximal tail current amplitudes at potential between  $-120$  to  $-90$  mV. (C) Voltage dependence of rapid inactivation of equine and human  $K_v11.1$ . The rectification factor ( $R$ ) at each potential was calculated using the current amplitudes plotted in Panel (B) (see [Methods](#) for calculation). Data were fitted with a Boltzmann equation.

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deactivating currents in [Fig 4A](#). Voltage dependence of equine and human  $K_v11.1$  channel rectification from fast inactivation was determined by comparison of the fully activated  $I$ - $V$  relationship for  $K_v11.1$  current with the  $I$ - $V$  relationship expected for an Ohmic conductor ([Fig 5A](#)). A linear fit of current amplitudes between  $-110$  mV and  $-90$  mV describes the  $I$ - $V$  relationship that would be found in the absence of rectification (Ohmic conduction). The slope determines the maximum conductance of  $K_v11.1$  tail currents, which can be used to calculate voltage dependence of channel rectification, as the rectification factor is given by  $R = I_{K_v11.1} / (G * n * (V_t - E_{rev}))$  where  $G$  is the maximal conductance of  $K_v11.1$  tail currents,  $n$  is the activation variable at  $+40$  mV (1.0),  $V_t$  is the test potential, and  $E_{rev}$  is the reversal potential. The rate of onset of



**Fig 5. Time constants of onset of  $K_v11.1$  inactivation.** Equine ( $n = 16$ ) and human ( $n = 20$ )  $K_v11.1$  expressed in *Xenopus laevis* oocytes. (A) Representative recordings. (B) Voltage-clamp protocol. (C) Mono-exponential functions were fit to the inactivating currents as indicated by the arrow on the protocol and the obtained time constants were plotted as a function of voltage.

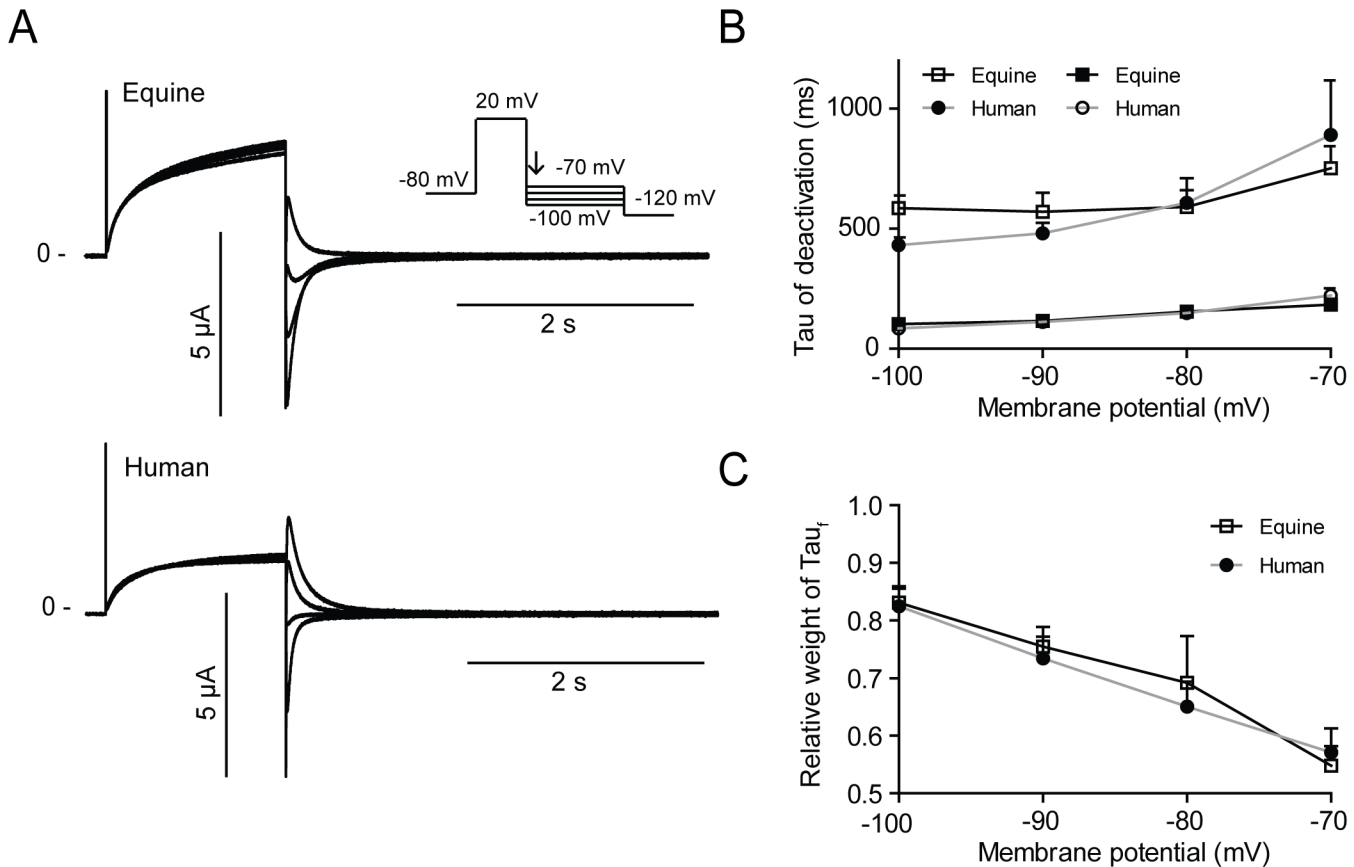
doi:10.1371/journal.pone.0138320.g005

inactivation was described by fitting a single exponential function to the data (Fig 6). To describe pharmacological block of the  $K_v11.1$  channel, terfenadine concentrations (0.01, 0.03, 0.1, 0.3, 1.0, 3.0, 10.0  $\mu M$ ) were transformed to  $\log(\text{concentrations})$  and plotted against current. A non-linear regression was fitted to the data, to obtain the  $IC_{50}$ .

### Multicellular Right Ventricular (Wedge) Preparations

The right ventricle was excised and perfused with 4 x 50 mL heparinized (5 IE/L) cardioplegic solution (in mM: 129 NaCl, 12 KCl, 0.9  $NaH_2PO_4$ , 20  $NaHCO_3$ , 1.8  $CaCl_2$ , 0.5  $MgSO_4$ , 5.5 glucose, pH 7.4, 4°C) through the coronary artery immediately after isolation and transported in 5 L of heparinized cardioplegic solution. Transmural wedges (4 x 3 x 2 cm) from the right ventricular wall were dissected, cannulated and perfused arterially with cardioplegic solution. Leaks were ligated and the perfusion cannula was sutured to the wedge using 5-0 Mersilene suture (Ethicon GmbH). Only right ventricles were used as thickness of the left ventricle made it difficult to obtain viable preparations. The wedge was transferred to a tissue bath and perfused with oxygenated (95%  $O_2$  and 5%  $CO_2$ ) Tyrode's solution (in mM: 129 NaCl, 4 KCl, 0.9  $NaH_2PO_4$ , 20  $NaHCO_3$ , 1.8  $CaCl_2$ , 0.5  $MgSO_4$ , 5.5 glucose, pH 7.4) at  $36 \pm 1^\circ C$  and at a constant flow rate using Reglo Digital MS-4/8 tubing pump (Ismatic). The flow was set to 15–20 mL/min depending on wedge size. The wedge was paced from the endocardial surface using DS3 Constant





**Fig 6. Time constants of  $K_v11.1$  deactivation.** Equine ( $n = 25$ ) and human ( $n = 25$ )  $K_v11.1$  expressed in *Xenopus laevis* oocytes. (A) Representative recordings. (B) Bi-exponential functions were fitted to the decaying currents (indicated on the protocol by an arrow) and the time constants  $\tau_{fast}$  and  $\tau_{slow}$  were plotted as a function of voltage. (C) The relative weight of the fast time constant ( $\tau_{fast}$ ).

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Current Isolated Stimulator (Digitimer Ltd). Basic cycle lengths (BCLs) of 4000, 2000, 1000, 500, 333 and 250 ms were used. Recordings from the midmyocardium were made 30 min after cannulation (control) and 30 min after application of terfenadine ( $10 \mu\text{M}$ ) using a floating microelectrode made from 1B100F-4 glass capillaries with filament (WPI). Microelectrodes were pulled on a Model P-97 Micropipette Puller (Sutter Instruments) to a resistance of  $\approx 55 \text{ M}\Omega$ , when filled with 3 M KCl and connected to a Model 3100 Intracellular Electrometer (A-M Systems). A transmural ECG was recorded using Ag/AgCl half cells (Warner Instruments) mounted approximately 1 cm from the endo- and epicardial surfaces of the wedge and connected to an ISO-80 Isolated Bio-Amplifier (WPI). Data was recorded and digitized using PowerLab 4/20 (ADInstruments) and analyzed using LabChart v8.0.2 (ADInstruments). Chemicals other than enzymes and kits were obtained from Sigma-Aldrich.

### Statistics

All data are expressed as mean  $\pm$  SEM. Normal distribution was tested prior statistical analysis with KS test (Kolmogorov- Smirnov test with Dallal-Wilkinson Lillie for p-value) and all data was normal distributed except for Tau values describing  $K_v11.1$  deactivation, where the non-parametric Kruskal Wallis and Man Whitney tests were used. Single outliers being more than three times the SD from the mean were removed from the dataset. Unpaired t- test was used to compare the equine and human half maximum activation  $V_{1/2}$  and the slope factors k. All other

data were analyzed using two way ANOVA followed by a Bonferroni test. Statistical level of significance on figures are shown with \*:  $p \leq 0.05$ , \*\*:  $p \leq 0.01$ , \*\*\*:  $p \leq 0.001$ . All data was analyzed in Prism5 (GraphPad).

## Results

The full sequence of the equine K<sub>v</sub>11.1 and the partial sequence of the equine KCNE2 cDNA sequence were submitted to GenBank with the accession numbers HM641824 and KF937396, respectively. As the K<sub>v</sub>11.1 channel sequence is 3477 base pairs and highly GC rich it was difficult to amplify. Therefore, the full sequences was based on a full sequence from only one horse, which was verified by >50 partial sequences (50–500 base pairs, all with 100% homology) from five other horses covering different regions, but not the full sequence. Furthermore, our equine K<sub>v</sub>11.1 sequence have undergone validation (NP\_001180587.1) by Genbank based on comparison to genomic sequence data (AAWR02041039.1) and the transcript is supported by transcript alignments and orthologous data. The submitted equine KCNE2 sequence corresponds to a predicted transcript (XP\_001494244) based on computational analysis of a genomic sequence, using Gnomon (NW\_001867397.1). The equine K<sub>v</sub>11.1 cDNA sequence had 93% similarity to the human cDNA and the encoded protein sequence had 99% similarity to the human (Fig 1). The equine K<sub>v</sub>11.1 cDNA sequence had 88% similarity to the human gene. The pore, the transmembrane domains S1-S6, and the cyclic nucleotide homology binding domain (CNBD) of equine K<sub>v</sub>11.1 were identical to human K<sub>v</sub>11.1. Most deviations were found in the N-terminus proximal to the Per-Arnt-Sim (PAS) domain. A single substitution was found within the PAS domain. The PAS domain is important for channel assembly and for the slow deactivation gating kinetics of the channel [24] and, interestingly, the p.E444D mutation has been published as a cause of long QT syndrome in humans [25]. The equine KCNE2 sequence had 90% similarity to the human KCNE2 (Fig 2). The transmembrane domains were identical in equine and human KCNE2 and the differences in sequence were found in the distal N- and C-termini.

The cloned equine K<sub>v</sub>11.1 was functionally expressed in *Xenopus laevis* oocytes and representative equine and human K<sub>v</sub>11.1 currents recorded by TEVC are shown in Fig 3A. Similarly to the human isoform, the steady-state current-voltage relationship was bell-shaped (Fig 3B), however, we repeatedly observed larger currents for equine K<sub>v</sub>11.1 compared to human (>6 different batches of *Xenopus laevis* oocytes, 3 different preparations of mRNA). Endogenous currents from control oocytes were insignificant (~0.2 μA) and did not affect measurements. As the proximal domain between the PAS and transmembrane segment 1 has been reported to be crucial for setting human K<sub>v</sub>11.1 activation kinetics, time constants (Tau) of the activating currents were determined by fitting a mono-exponential equation to initial 500 ms of the current. No differences in Tau values were found (Fig 3C). It should be noted that since there is an overlap of the activation and inactivation processes both with regard to time- and voltage-dependence, the resulting Tau values reflect both these processes. The voltage dependence of activation for K<sub>v</sub>11.1 was addressed by plotting normalized currents measured at the -120 mV step as a function of the preceding test potential and a Boltzmann equation was fitted to the data (Fig 3D). Test pulses below -60 mV did not result in time-dependent currents. The half maximal activation ( $V_{1/2}$ ) was significant different ( $p = 0.0178$ ) with  $V_{1/2} = -17.3 \pm 0.7$ ,  $k = 8.1 \pm 0.7$ ,  $n = 24$  for equine and  $V_{1/2} = -14.2 \pm 1.1$ ,  $k = 9.6 \pm 1.0$ ,  $n = 16$  for human. The slope factors  $K$  were not significantly different ( $p = 0.2143$ ).

The degree of channel inactivation was determined from a fully activated K<sub>v</sub>11.1 current-voltage relationship (Fig 4). Currents were activated by a +40 mV step and peak tail currents were plotted as a function of potential (Fig 4B). The maximal conductance of the tail currents ( $G$ )

was determined as the slope of a linear fit to maximal tail current amplitudes at potential between -120 and -90 mV ( $G_{equine} = 159 \pm 13 \mu S$  and  $G_{human} = 234 \pm 18 \mu S$ ,  $n = 10$ ). Voltage dependence of inactivation was estimated by the rectification factor  $R$  (Fig 4C). Reversal potentials were found to be  $E_{rev, equine} = -103$  mV and  $E_{rev, human} = -104$  mV,  $n = 10$ .

The speed of onset of inactivation was determined by activating  $K_{V11.1}$  currents by a +20 mV step followed by a brief step to -120 mV to release inactivation. A series of steps ranging from -20 mV to 40 mV resulted in a rapid inactivation of  $K_{V11.1}$  (Fig 5A). To obtain time constants, mono-exponential functions were fitted to the inactivating currents. The onset of inactivation was significantly slower for equine  $K_{V11.1}$  compared to human (Fig 5C).

Deactivation was addressed by activating channels by a +20 mV step and releasing inactivation by a series of hyperpolarizing steps from -100 to -70 mV (Fig 6A). Fast and slow time constants ( $\tau_{fast}$  and  $\tau_{slow}$ ) were found by fitting bi-exponential equations to the decaying currents and were similar for equine and human  $K_{V11.1}$  (Fig 6B). For both equine and human  $K_{V11.1}$ ,  $\tau_{fast}$  predominated over  $\tau_{slow}$  at more hyperpolarized voltages (Fig 6C).

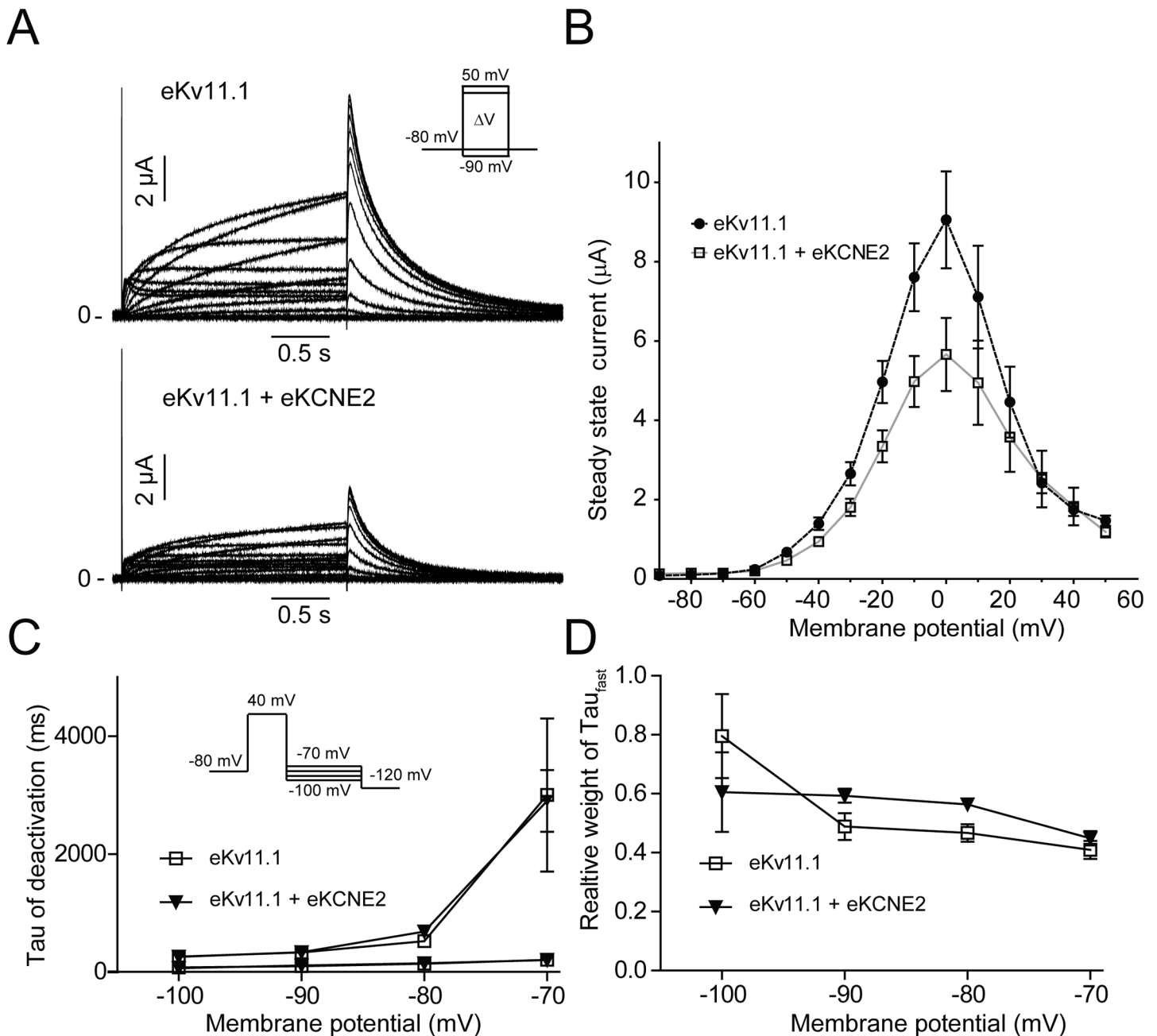
$K_{V11.1}$  and KCNE2 have been proposed to interact in cardiac cells [12]. Co-expression of equine  $K_{V11.1}$  and KCNE2 resulted in a reduction of the average steady-state current by  $42.3 \pm 6.4\%$  (Fig 7A and 7B). The gating properties were similar to those of homomeric  $K_{V11.1}$  channels (Fig 7A) and there were no effects on current deactivation (Fig 7C and 7D).

Pharmaceutical block of  $K_{V11.1}$  can cause arrhythmias and sudden cardiac death [26]. Aromatic residues in the S6 transmembrane segment, Y-652 and F-656 are critical for the interaction with pharmaceutical compounds [27,28]. These residues are also present in equine  $K_{V11.1}$ . Pharmacological block of the equine  $K_{V11.1}$  channel was tested with the histamine H1 receptor antagonist terfenadine. A concentration-dependent block of  $K_{V11.1}$  by terfenadine (0.01, 0.03, 0.1, 0.3, 1, 3 and 10  $\mu M$ ) was found (Fig 8). The  $IC_{50}$  was  $0.42 \pm 0.14 \mu M$  for steady state currents and  $0.37 \pm 0.06 \mu M$  for tail currents,  $n = 4$ .

To determine the physiological importance of  $K_{V11.1}$  currents in equine hearts, the effect of terfenadine (10  $\mu M$ ) was tested in arterially perfused sections of the right ventricle (RV), the wedge model. Action potentials were recorded from the midmyocardial region at different pacing rates (4000, 2000, 1000, 500, 333 and 250 ms BCL) using floating microelectrodes (Fig 9). In the presence of terfenadine, the action potential duration at 90% repolarization ( $APD_{90}$ ) was significantly increased at BCLs of 1000 and 2000 ms (Fig 9B). At 250 ms BCL we could not get capture in (5/6) wedges in controls, in the presence of terfenadine the prolonged action potential and concomitant increased refractory period prevented initiation of action potential at 250 ms BCL in 6/6 wedges.

## Discussion

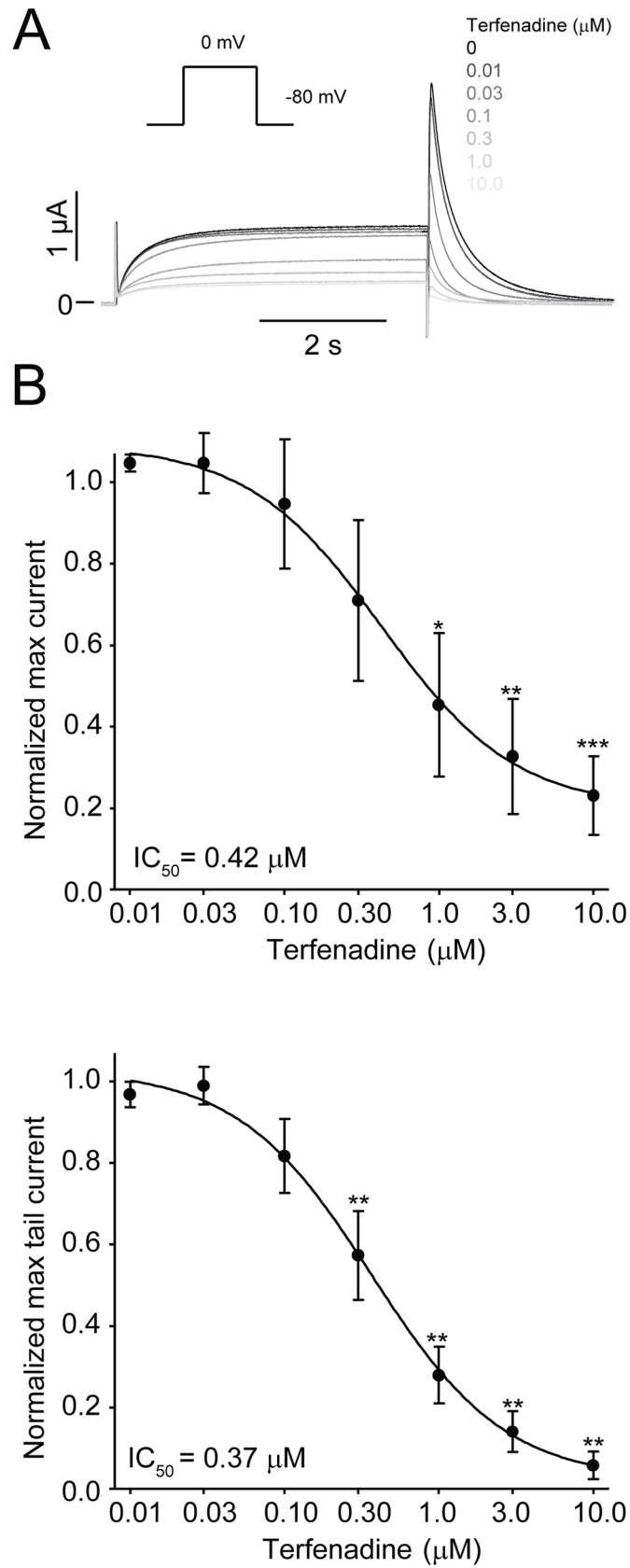
In this study we successfully cloned full equine  $K_{V11.1}$  and partial KCNE2 cDNA. Currents were characterized by TEVC. The electrophysiological gating properties of the equine  $K_{V11.1}$  channel and  $K_{V11.1}/KCNE2$  channel complex were found to resemble those of the human  $K_{V11.1}$  and  $K_{V11.1}/KCNE2$  channels. The currents showed a bell-shaped steady-state current-voltage relationship that peaked at 0 mV due to a prominent inactivation. Furthermore, hyperpolarization resulted in a rapid recovery followed by slow channel deactivation. These electrophysiological properties are hallmarks of the  $K_{V11.1}$  channel and  $K_{V11.1}/KCNE2$  channel complex [12,29]. However, equine currents were larger compared to human  $K_{V11.1}$  and the voltage dependence of activation was shifted to more negative values. The onset of inactivation was slower in equine  $K_{V11.1}$  compared to human  $K_{V11.1}$ , and we are speculating whether these differences in kinetics can contribute to the increase in  $K_{V11.1}$  current. There were no differences in the rectification of the  $K^+$  current, the voltage-dependence of inactivation,



**Fig 7. The effect of equine KCNE2 on equine  $K_v11.1$ .** Equine  $K_v11.1$  and  $K_v11.1/KCNE2$  expressed in *Xenopus laevis* oocytes. (A) Representative recordings. (B) Steady-state currents as a function of voltage,  $n = 10$ . (C) Time constants ( $\tau_{fast}$  and  $\tau_{slow}$ ) of deactivation of equine  $K_v11.1$  ( $n = 8$ ) and  $K_v11.1/KCNE2$  ( $n = 10$ ) plotted as a function of voltage. (D) The relative weight of the fast time constant ( $\tau_{fast}$ ).

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reversal potential or deactivation kinetics between equine and human  $K_v11.1$ . Co-expression of KCNE2 resulted in a reduction of currents for both equine and human  $K_v11.1$ . In accordance with Y-652 and F-656 being present in equine  $K_v11.1$ , currents were blocked by terfenadine in the same concentration range as the human  $K_v11.1$  currents [28]. Finally we demonstrated that  $K_v11.1$  plays a functional role in cardiac repolarization in equine right ventricle, as application of terfenadine resulted in a prolongation of  $APD_{90}$  which was most prominent at slower pacing rates.

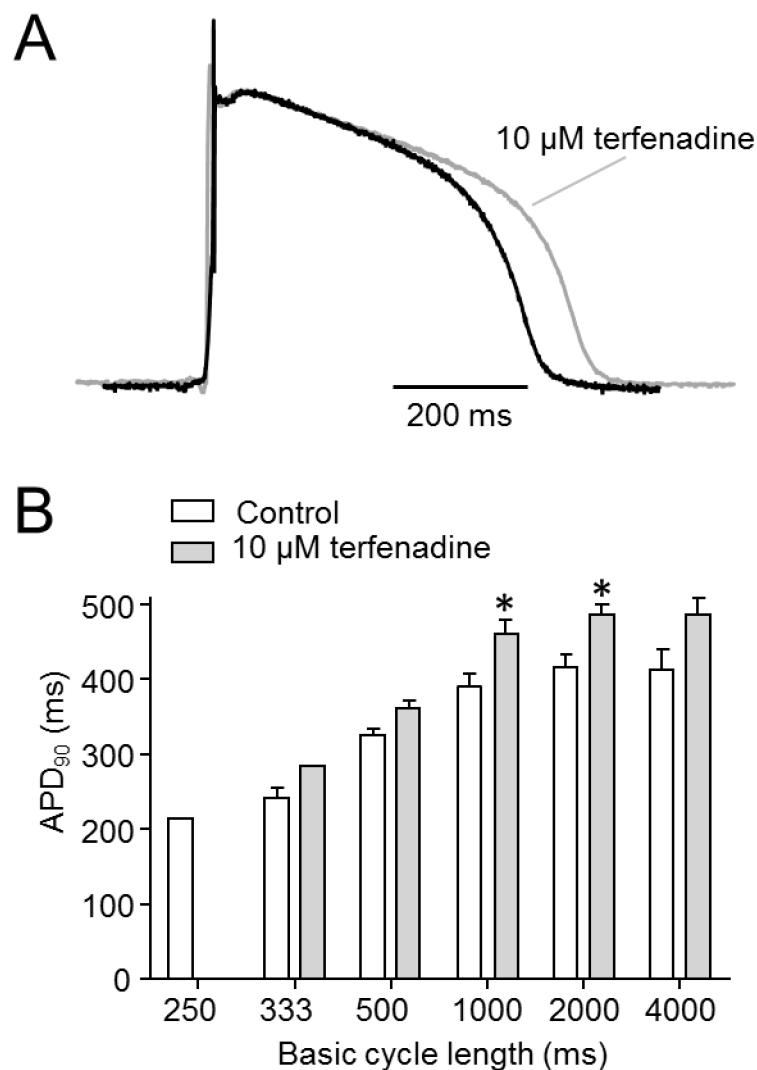


**Fig 8. Equine  $K_v11.1$  channels are blocked by terfenadine.** Equine ( $n = 4$ )  $K_v11.1$  expressed in *Xenopus laevis* oocytes. Currents were activated by a repeated depolarization to 0 mV from a holding of -80 mV. (A) Representative recordings in control and in the presence of 0.01, 0.03, 0.1, 0.3, 1, 3 and 10  $\mu$ M terfenadine. Currents got successively smaller as concentrations were increased. (B) Dose-response for the effect of terfenadine on the equine  $K_v11.1$  steady-state currents at the end of a depolarizing step to 0 mV. (C) Dose-response for the effect of terfenadine on the equine  $K_v11.1$  peak tail current after repolarization from 0 mV to -80 mV.  $K_v11.1$  currents are expressed as a fractional value ( $I_{drug}/I_{control}$ ). On the X-axis values non-transformed values are shown. A non-linear regression was fitted to the data.

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### Sequence and electrophysiological properties

The equine  $K_v11.1$  polypeptide sequence was highly similar to the human equivalent (99% similarity). This homology is greater than for any other previously sequenced animal  $K_v11.1$  polypeptide. Importantly, the signature sequence which shapes the selectivity filter of the channel was conserved. This part is essential to maintain the selectivity towards  $K^+$  and thereby the



**Fig 9. Physiological importance of KV11.1 in equine right ventricle.** Action potentials were recorded from the midmyocardium in right ventricular wedges in absence or presence of terfenadine (10  $\mu$ M). (A) Representative recordings at 2000 ms BCL. (B) Action potential duration at 90% repolarization (APD<sub>90</sub>) in absence or presence of terfenadine as a function of basic cycle length,  $n = 6$ .

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appropriate function of the channel [30] and in line with this, we found similar reversal potentials for equine and human  $K_v11.1$ . Likewise, 100% conservation was seen for the transmembrane segments S1-S3, the S4 segment, which is essential for voltage sensing, and the pore forming S5 and S6 segments, which are hallmarks of voltage dependent  $K^+$  channels [31]. In the PAS domain, a p.A97S substitution was found in the equine  $K_v11.1$  polypeptide encoded by both the genomic sequence from the EquCab2.0 project [19] and the cDNA sequence obtained here. This substitution has not been reported in other species. Functionally the PAS domain is of importance for the deactivation kinetics [32], likely by interaction between residues 26–135 in the PAS-domain and residues 749–872 in the CNBD [33]. However, no differences in deactivation kinetics were found between equine and human  $K_v11.1$ . A serine to alanine substitution is regarded as conserved suggesting that this substitution may be of little significance. To the authors' knowledge, this mutation has not been published as a cause for altered properties of the  $K_v11.1$  channel. The CNBD domain was found to be 100% identical. Another important region of the  $K_v11.1$  channel is the amphipathic alpha helix at residues 13–23, which is involved in protein-protein and protein-membrane interactions [33]. This region was also found to be 100% identical in equine and human  $K_v11.1$  isoforms. The majority of the 17 amino acid changes in the equine versus the human sequence map to the proximal domain between the PAS and the first transmembrane segment (S1). This region has been reported to be important for activation kinetics [34,35]. We found a left shift in the voltage dependence of current activation, however, the time constants of activation were similar for equine and human  $K_v11.1$ . One interesting finding was the presence of a p.E444D substitution positioned in the extracellular loop between S1 and S2 in the equine  $K_v11.1$ . This substitution is present in the peptide sequence of most species, except in humans and rabbits. p.E444D has previously been associated with long QT syndrome in a Chinese family [25], however, the electrophysiological properties of the substitution has not been tested *in vitro* [25] and the importance of the substitution remains speculative.

## Regulation of $K_v11.1$ by KCNE2

The equine KCNE2 protein sequence was quite similar to the human equivalent (90% homology). The transmembrane regions were identical and the N6 and N29 glycosylation sites, as well as the T71 and S74 phosphorylation sites were conserved [36]. Co-expression with KCNE2 caused an approximately 40% reduction in steady-state equine  $K_v11.1$  currents, in agreement the effects of KCNE2 on human  $K_v11.1$  amplitude [12,37]. The reports describing the effects of KCNE2 on  $K_v11.1$  kinetics are not consistent (Reviewed in [36]). The role of KCNE2 in  $I_{Kr}$  has been questioned as co-expression of  $K_v11.1$  and KCNE2 does not recapitulate native  $I_{Kr}$  [38] and recently it has been proposed that KCNE2 is modulating  $K_v11.1$  by accelerating degradation of the  $K_v11.1$  protein [37]. It is, however, possible that interactions with other regulatory subunits affect the conductance and gating of the equine  $K_v11.1$  channel [32,39,40]. In humans, an association of the  $K_v11.1$  channel with the auxiliary  $\beta$  subunit KCNE1 has been proposed to regulate the  $K^+$  conduction of the channel [41] and in horses one study has described a possible interaction between  $K_v11.1$  and KCNE1 but no electrophysiological measurements have been performed on this equine channel complex [42]. Another reason for the discrepancy between native  $I_{Kr}$  and expressed  $K_v11.1$  could be the presence of different splice variants in native tissue. In humans, splice variants encoding a  $K_v11.1$  channel with a truncated N terminal (hERG1b) [43] and a c-terminal splice variant ERG<sub>USO</sub> [40][44] has been reported. The ERG1b or the ERG<sub>USO</sub> variants were not detected in horse hearts [42] but other splice variants could be speculated to be present.

## Pharmacology

The aromatic residues Y-652 and F-656 located in the central cavity of the  $K_V11.1$  channel have been demonstrated to be critical sites of interaction with structurally diverse drugs. Mutations of these residues have been shown to drastically decrease susceptibility of the  $K_V11.1$  channel to pharmacological block by various compounds [26,27]. These residues were conserved in the equine  $K_V11.1$  sequence. To test if equine  $K_V11.1$  is equally sensitive to pharmacological blockade as the human isoform, we tested the effect of the histamine H1 receptor antagonist terfenadine. Terfenadine has been shown to block the human  $K_V11.1$  channel at concentrations relevant to its therapeutic levels [45], and it was removed from the market after frequent reports of syncope, QT prolongation and *torsade de pointes* in humans. The equine  $K_V11.1$  channel exhibited a dose-dependent block by terfenadine with an  $IC_{50}$  value of 0.42  $\mu$ M, which is comparable to 0.36  $\mu$ M described for the human  $K_V11.1$  channel [28].

## Physiological Importance of Equine $K_V11.1$

The physiological importance of  $K_V11.1$  in equine hearts was determined by addition of terfenadine (10  $\mu$ M) to arterially perfused wedges from the right ventricle. Terfenadine application resulted in a prolongation of the  $APD_{90}$  most prominently at slower pacing rates. At 2000 ms BCL, the  $APD_{90}$  was increased by 17% indicating that  $I_{Kr}$  plays an important role in action potential repolarization in equine right ventricle. These results are in agreement with Finley et al. that found a similar prolongation of  $APD_{90}$  after application of the  $I_{Kr}$  blocker cisapride to epicardial slice preparations of equine hearts [42]. Taken together, this suggests that LQTS, both in the congenital and acquired form could be of relevance in equine patients as in humans. In humans it has been proposed that a “repolarization reserve” exists [46]. Many different currents contribute to repolarization and as a consequence loss of one component (such as  $I_{Kr}$ ) normally does not result in repolarization failure, but if several components of the repolarization reserve are reduced, this may result in failure of repolarization [46]. It will thus be of great importance to determine other currents that are contributing to repolarization in equine cardiomyocytes.

## Conclusions

This study has shown that the equine  $K_V11.1$  and KCNE2 cDNA sequences are highly similar to the human and that they encode a functional  $K_V11.1$  and  $K_V11.1/KCNE2$  channel complex in *Xenopus laevis* oocytes. Equine currents were larger compared to human  $K_V11.1$  and the voltage dependence of activation was shifted to more negative values ( $V_{1/2} = -14.2 \pm 1.1$  mV and  $-17.3 \pm 0.7$ , respectively). The onset of inactivation was slower in equine  $K_V11.1$  compared to human  $K_V11.1$ . Furthermore, the equine channel is susceptible to the H1 receptor antagonist terfenadine in the same dose range as the human  $K_V11.1$  channel. Finally, application of terfenadine to a right ventricular wedge resulted in a prolongation of  $APD_{90}$ , indicating that the  $K_V11.1$  channel plays a pivotal role in repolarization of the equine heart, as it does in the human heart.

The findings in this study suggest that horses could be disposed to both congenital LQT2, LQT6 and acquired LQTS. This is of importance to veterinary pathologists examining cases of sudden death in horses and to veterinary practitioners treating horses with drugs known to block the  $K_V11.1$  channel.

## Limitations

The experiments were performed at room temperature using *Xenopus laevis* oocytes. In *Xenopus laevis* oocytes, as in all heterologous expression systems, endogenous factors may affect the currents.



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## Author Contributions

Conceived and designed the experiments: PJP KC DAK MAT FH. Performed the experiments: PJP KBT ERO KLP SG KC. Analyzed the data: PJP KBT ERO KLP SG KC MAT DAK. Contributed reagents/materials/analysis tools: PJP KC MAT DAK. Wrote the paper: PJP KBT KLP SG KC MAT DAK RB.

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