Dissection of a Quantitative Trait Locus for PR Interval Duration Identifies Tnni3k as a Novel Modulator of Cardiac Conduction

Elisabeth M. Lodder1, Brendon P. Scicluna1,5, Annalisa Milano1, Albert Y. Sun2, Hao Tang3, Carol Ann Remme1, Perry D. Moerland4, Michael W. T. Tanck6, Geoffrey S. Pitt2, Douglas A. Marchuk3, Connie R. Bezzina1*

1 Heart Failure Research Center, Department of Experimental Cardiology, Academic Medical Center, University of Amsterdam, Amsterdam, The Netherlands, 2 The Ion Channel Research Unit, Department of Medicine, Duke University, Durham, North Carolina, United States of America, 3 Department of Molecular Genetics and Microbiology, Duke University, Durham, North Carolina, United States of America, 4 Department of Clinical Epidemiology, Biostatistics, and Bioinformatics, Academic Medical Center, University of Amsterdam, Amsterdam, The Netherlands

Abstract

Atrio-ventricular conduction disease is a common feature in Mendelian rhythm disorders associated with sudden cardiac death and is characterized by prolongation of the PR interval on the surface electrocardiogram (ECG). Prolongation of the PR interval is also a strong predictor of atrial fibrillation, the most prevalent sustained cardiac arrhythmia. Despite the significant genetic component in PR duration variability, the genes regulating PR interval duration remain largely elusive. We here aimed to dissect the quantitative trait locus (QTL) for PR interval duration that we previously mapped in murine F2 progeny of a sensitized 129P2 and FVBN/J cross. To determine the underlying gene responsible for this QTL, genome-wide transcriptional profiling was carried out on myocardial tissue from 109 F2 mice. Expression QTLs (eQTLs) were mapped and the PR interval QTL was inspected for the co-incidence of eQTLs. We further determined the correlation of each of these transcripts to the PR interval. Tnni3k was the only eQTL, mapping to the PR-QTL, with an established cardiac-specific expression pattern and a significant correlation to PR interval duration. Genotype inspection in various inbred mouse strains revealed the presence of at least three independent haplotypes at the Tnni3k locus. Measurement of PR interval duration and Tnni3k mRNA expression levels in six inbred lines identified a positive correlation between the level of Tnni3k mRNA and PR interval duration. Furthermore, in DBA/2J mice overexpressing hTNNI3K, and in DBA.AKR.hrfm2 congenic mice, which harbor the AKR/J "high-Tnni3k expression" haplotype in the DBA/2J genetic background, PR interval duration was prolonged as compared to DBA/2J wild-type mice ("low-Tnni3k expression" haplotype). Our data provide the first evidence for a role of Tnni3k in controlling the electrocardiographic PR interval indicating a function of Tnni3k in atrio-ventricular conduction.

Introduction

Atrio-ventricular (AV) conduction delay describes the impairment of the electrical continuity between the atria and the ventricles and is characterized by prolongation of the PR interval on the surface electrocardiogram (ECG). AV delay of varying severity is a common feature in Mendelian rhythm disorders and is associated with sudden cardiac death [1]. PR interval prolongation is also a strong predictor of atrial fibrillation (AF) [2] and is therefore considered an intermediate phenotype for this condition [3]. AF is the most commonly observed sustained cardiac arrhythmia, with an age dependent prevalence of up to 9% [4]. Identification of genetic determinants of AV conduction delay is essential for understanding the underlying molecular mechanisms and for the possibility of development of targeted treatments and prevention strategies.

There is a strong heritable component in the variability of the PR interval [5–7] and although genome-wide approaches have highlighted several causal loci [3,6,7] a major proportion of the heritability and the underlying genes remains elusive. The identification of these genetic factors in the human population has been difficult owing to wide genetic heterogeneity and an uncontainable environment. We here exploit the homogeneous genetic background and controlled environment of inbred laboratory mouse strains to identify a novel genetic modifier of the PR interval.

We have previously detected a quantitative trait locus (QTL) for the PR interval (PR-QTL) on chromosome 3 in a conduction...
Author Summary

Atrio-ventricular (AV) conduction disease (delay), characterized by prolongation of the PR interval on the surface electrocardiogram (ECG), is a common feature in Mendelian rhythm disorders and is associated with sudden cardiac death. Prolongation of the PR interval is also a strong predictor of atrial fibrillation (AF), the most common sustained cardiac arrhythmia. Although there is a substantial heritable component to the variability of the PR interval, the causative genes remain largely elusive. The identification of these genetic factors in the human population has been difficult owing to wide genetic heterogeneity and an uncontrollable environment. We here exploited the homogeneous genetic background and controlled environment of inbred laboratory mouse strains to detect a genetic modifier of the PR interval. We identify Tnni3k as prime candidate for the modulation of the PR interval duration and suggest a new role for this gene, in the modulation of atrio-ventricular conduction.

Results

F2 screen for identifying genetic modifiers of cardiac conduction

The identification of a main-effect PR interval QTL in the distal portion of chromosome 3 has been reported previously [8]. In brief, we combined ECG and genome-wide genotypic data in 502 F2 progeny generated from an FVB/N/J-Scn5a\(^{1798\text{ins}D+/+}\) × 129P2-Scn5a\(^{1798\text{ins}D-/-}\) intercross to map QTLs for ECG parameters. The most significant SNP association with PR interval was rs13477506; explaining 3.9% (\(p=7.71 \times 10^{-5}\)) of the observed variance in PR interval [8].

Identification of eQTLs for the PR-QTL on chromosome 3

As genetic variation underlying a QTL may act on the trait through effects on gene expression [11] we tested the presence of such variability in the F2 mice. We measured genome-wide gene expression profiles of cardiac tissue in 109 previously genotyped F2 mice using microarrays. Normalized log-transformed intensities of individual transcripts were used as quantitative inputs for eQTL mapping. Considering a multiple-comparison corrected significance threshold of \(p<1.83 \times 10^{-6}\) (LOD>6.83), we uncovered 16 eQTLs within the 1.5 LOD drop for the PR interval chromosome 3 QTL (Table 1). Of these 16 eQTLs, seven were deemed cis-regulated (cognate gene and eQTL SNP physically map to the same genomic region) indicating a possible direct effect of the underlying genetic variation on transcript levels; whereas nine eQTLs were labeled as trans-regulated (cognate gene physically maps to a different genomic region than the eQTL SNP) suggesting indirect effects such as transcription factor levels influencing the transcript level.

To determine whether any of the identified eQTLs could be a candidate for the effect of the Chr3 PR-QTL we tested correlation of the transcript levels with the PR interval duration. Only four transcripts showed significant correlation to the PR interval, namely, Eif4e (rho -0.246, \(p<0.01\)), Gipc2 (rho -0.300, \(p<0.001\)), Socs2 (rho 0.279, \(p<0.001\)) and Tnni3k (rho 0.263, \(p<0.01\)). Data from the GEO database indicates that of these only Tnni3k displays a high cardiac specific expression pattern [13,14]. We therefore investigated Tnni3k further as the prime candidate for the effect on the chromosome 3 PR-QTL.

Inspection of the genome-wide LOD plot for Tnni3k (Figure 1) shows a very sharp LOD peak with a maximum LOD score of 56.5 at rs13477506. The allele effect-size plot for the chromosome 3 QTL (Table 1). Of these 16 eQTLs, seven were deemed cis-regulated (cognate gene and eQTL SNP physically map to the same genomic region) indicating a possible direct effect of the underlying genetic variation on transcript levels; whereas nine eQTLs were labeled as trans-regulated (cognate gene physically maps to a different genomic region than the eQTL SNP) suggesting indirect effects such as transcription factor levels influencing the transcript level.

In silico haplotype analysis in a panel of inbred mice

We next examined the haplotypes at the locus of interest in a panel of inbred mouse strains using the mouse phenome database [12]. Further validation was performed by testing the correlation of Tnni3k expression level with PR interval in 6 inbred mouse strains harboring 3 independent haplotypes at the Tnni3k genomic locus. Finally, the role of Tnni3k in modulation of the PR interval was validated in vivo in (i) congenic mice harboring the high-Tnni3k expression haplotype of the AKR/J strain in the DBA/2J (low expression of Tnni3k) genetic background and (ii) in DBA/2J mice overexpressing human TNNI3K.
To determine whether these different genomic backgrounds in the different mouse strains were related to the PR interval we first looked at the published PR interval in publicly accessible data in the mouse phenome database [12,17,18]. Interestingly, this uncovered a significantly longer PR interval in inbred strains with the green (similar to 129P2) haplotype compared to strains with the red (DBA/2J) or blue (FVBN/J) haplotype (P, 0.001) (www.phenome.jax.org). Since Tnni3k expression levels have been shown to be low in DBA/2J (red haplotype) and FVBN/J (blue haplotype) mice (which both have short PR intervals), and high in 129/P2 and AKR/J strains (both green haplotype and both having long PR interval durations) (Figure 2A and Wheeler et al. [16]), PR interval duration in these various mouse lines appears to correspond to the (predicted) Tnni3k expression levels.

Validation of in silico data in inbred mice of diverse genetic background

We next validated the correlation between Tnni3k expression levels and PR interval duration in a diverse panel of inbred mice with the three different haplotypes (red, green and blue) and a further two wild-derived inbred lines for which no haplotype Table 1. eQTLs overlapping the 1.5 LOD drop region of the PR QTL.

| Gene symbol | Refseq ID | Gene name | Max LOD | SNP marker | eQTL cis/ trans | Spearman’s Corr. to PR rho | pvalue |
|-------------|-----------|-----------|---------|------------|-----------------|--------------------------|--------|
| Elf4e       | NM_007917 | eukaryotic translation initiation factor 4E | 21.58   | rs6407142   | cis            | -0.246                   | 0.01   |
| Gbp1        | NM_010259 | guanylate binding protein 1 | 9.3     | rs6407142   | cis            | 0.128                    | 0.19   |
| Ccbl2       | NM_173763 | cysteine conjugate-beta lyase 2 | 19.77   | rs13477494  | cis            | -0.001                   | 0.99   |
| Cyr61       | NM_010516 | cysteine rich protein 61 | 7.54    | rs13477494  | cis            | -0.076                   | 0.43   |
| Myoz2       | NM_021503 | myozin 2 | 12.1    | rs13477494  | trans         | -0.104                   | 0.28   |
| Gipc2       | NM_016867 | GIPC PDZ domain containing family, member 2 | 8.05  | rs13477494  | cis            | -0.300                   | 0.00   |
| Abca4       | NM_007378 | ATP-binding cassette, sub-family A (ABCA1), member 4 | 12.63  | rs13477494  | trans         | 0.105                    | 0.28   |
| Shmt1       | NM_009171 | serine hydroxymethyltransferase 1 (soluble) | 6.94  | rs13477506  | trans         | 0.093                    | 0.33   |
| Bcl6        | NM_009744 | B-cell leukemia/lymphoma 6 | 9.71    | rs13477506  | trans         | -0.218                   | 0.02   |
| Socs2       | NM_00116865 | suppressor of cytokine signaling 2 | 25.31  | rs13477506  | trans         | 0.279                    | 0.00   |
| Igfals      | NM_008340 | insulin-like growth factor binding protein, acid labile subunit | 21.43  | rs13477506  | trans         | 0.184                    | 0.06   |
| Esrb        | NM_001159500 | estrogen related receptor, beta | 7.17  | rs13477506  | trans         | 0.102                    | 0.29   |
| 3110057012Rik | NM_026622 | Riken cdna 3110057012 gene | 10.34  | rs13477506  | trans         | 0.233                    | 0.02   |
| Tnni3k      | NM_177066 | TNNI3 interacting kinase | 56.46  | rs13477506  | cis            | 0.263                    | 0.01   |
| Extl2       | NM_001163514 | exostoses (multiple)-like 2 | 8.01   | rs13477506  | trans         | 0.044                    | 0.65   |
| Gpr177      | NM_026582 | G protein-coupled receptor 177 | 7.93   | CEL_3_159340478 cis | -0.175 | 0.07                |

Transcripts with a significant linkage overlapping the 1.5 LOD drop region of the PR QTL are listed with their genomic location (build NCBI37), specific LOD score, marker with the highest LOD score, Spearman’s correlation to PR (Rho and uncorrected p value). Transcripts with a significant correlation are marked in bold; Tnni3k is marked in bold and underlined.

doi:10.1371/journal.pgen.1003113.t001

To determine whether these different genomic backgrounds in the different mouse strains were related to the PR interval we first looked at the published PR interval in publicly accessible data in the mouse phenome database [12,17,18]. Interestingly, this uncovered a significantly longer PR interval in inbred strains with the green (similar to 129P2) haplotype compared to strains with the red (DBA/2J) or blue (FVBN/J) haplotype (P<0.001) (www.phenome.jax.org). Since Tnni3k expression levels have been shown to be low in DBA/2J (red haplotype) and FVBN/J (blue haplotype) mice (which both have short PR intervals), and high in 129/P2 and AKR/J strains (both green haplotype and both having long PR interval durations) (Figure 2A and Wheeler et al. [16]), PR interval duration in these various mouse lines appears to correspond to the (predicted) Tnni3k expression levels.

Validation of in silico data in inbred mice of diverse genetic background

We next validated the correlation between Tnni3k expression levels and PR interval duration in a diverse panel of inbred mice with the three different haplotypes (red, green and blue) and a further two wild-derived inbred lines for which no haplotype

Figure 1. LOD plot of PR interval (black, left y-axis) and Tnni3k (purple right y-axis) on chromosome 3.
doi:10.1371/journal.pgen.1003113.g001

Figure 2. Genotype effects at rs13477506.
(A) Tnni3k expression (Illumina probe ILMN_3023962) as a function of genotype at rs13477506 in F2 mice; homozygous 129P2: AA, green; 129P2-FVBN/J heterozygous: AB, turquoise; homozygous FVBN/J: BB, blue; the darker shades represent the independent validation of the Tnni3k transcription levels by Q-PCR (right y-axis). (B) PR interval as a function of genotype at rs13477506 in F2 mice. Error bars indicate standard errors.
doi:10.1371/journal.pgen.1003113.g002
In these lines we determined cardiac expression levels of \textit{Tnni3k} by qRT-PCR and measured surface ECGs for assessment of PR interval duration. As shown in Figure 4B, \textit{Tnni3k} expression levels significantly correlate to PR interval indices ($\rho = 0.475$, $p = 0.012$), thus validating the correlation observed in silico.

**ECG analysis in congenic DBA.AKR-Hrtfm2 and TNNI3K overexpression mice**

To exclude effects from loci that differ between the parental inbred strains but which map elsewhere in the genome, we measured ECGs in congenic DBA.AKR-Hrtfm2 mice. These mice harbor approximately 20 Mb of the AKR/J (green, ‘high-\textit{Tnni3k} expression’) haplotype at the \textit{Tnni3k} locus in the genetic background of the DBA/2J inbred mice; pure DBA/2J mice have short PR intervals and low \textit{Tnni3k} levels (red haplotype) (Figure 3 and Figure 4B). In DBA.AKR-Hrtfm2 mice the level of \textit{Tnni3k} is indistinguishable from that in AKR/J mice while the rest of the genetic background is the same as that of DBA/2J. Strikingly, DBA.AKR-Hrtfm2 mice completely recapitulate the long PR interval of the AKR/J mice, suggesting that the difference in the level of \textit{Tnni3k} expression is a major cause of the difference in PR interval duration between these lines (Figure 5). An overview of all the ECG parameters measured is given in Table 2.

In order to exclude the contribution of any of the 117 other genes present in the congenic region to the observed effects in the DBA.AKR-Hrtfm2 mice we measured ECGs in DBA/2J mice overexpressing human \textit{TNNI3K}. As expected, the PR interval in these overexpression mice (with $20\times$ overexpression of h\textit{TNNI3K} [16]) was extremely prolonged (Figure 5).

**Western blot analysis of Tnni3k**

As prolonged PR interval indicates a possible role for \textit{Tnni3k} in atrial and/or atrio-ventricular conduction we next investigated whether \textit{Tnni3k} protein is also present in the atria besides its known presence ventricle [16]. We therefore performed Western Blotting on atrial (A) and ventricular (V) protein lysates from AKR/J (high \textit{Tnni3k} expression) and DBA/2J (low \textit{Tnni3k} expression). \textit{Tnni3k} protein was detected in both atrial and ventricular lysates in AKR/J and as expected was undetectable in DBA/2J hearts (Figure 6). In AKR/J hearts expression appeared higher in atria compared to ventricle.
Figure 4. In silico and in vivo correlation of Tnni3k levels and PR interval duration. (A) In silico analysis: mouse inbred lines are colored based on the haplotypes in the minimal region of overlap; PR interval duration obtained from the mouse phenome database (http://phenome.jax.org/) FVB/NJ (blue) (low expression) and green (high expression), inbred lines with the reddish colors carry rs49812611 (associated with nonsense mediated decay). (B) In vivo analysis of Tnni3k expression levels (y-axis) and PR interval duration (x-axis) in six inbred mouse lines, colors again denote the haplotype (gray is unknown haplotype).

doi:10.1371/journal.pgen.1003113.g004

Discussion

We here dissect the PR interval QTL on mouse chromosome 3 identifying the causal gene. We used eQTL mapping to identify genes whose expression is genetically regulated by variation within the QTL region. We tested the correlation of these genes to PR interval and, based on its in vivo expression pattern, selected Tnni3k as candidate gene for the effect. We subsequently validated the role of Tnni3k by in silico haplotype-phenotype correlation and by assessing the relation between Tnni3k expression levels and PR interval in 6 inbred mouse lines. Finally, the causality of Tnni3k was proven by in vivo studies in (i) congenic mice harboring a “high-TNNI3K–long-PR-interval” haplotype in a “low-Tnni3k-short PR” genetic background, and (ii) mice overexpressing Tnni3k. These in vivo studies provided unequivocal evidence for the involvement of Tnni3k in regulation of PR interval duration.

Genetic variation is known to affect a phenotype by altering the transcriptional activity of genes [20]. Thus, eQTL mapping, integrating genome-wide transcript profiling and genetic data in a hybrid population as carried out here, provides a powerful tool for the identification of causal genes at QTLs impacting on complex traits. Moreover, coupling eQTL detection with transcript-trait correlation analysis as performed here further aids prioritization of physiologically relevant genes within the QTL region [21].

Online databases providing genotypic and phenotypic information on diverse inbred mouse lines have been instrumental in the identification and validation of the causal gene underlying the PR interval QTL. Expression pattern data deposited in the Gene Expression Omnibus (GEO) database [13,22] aided our selection of Tnni3k as the prime candidate among the 4 identified eQTLs whose transcripts correlated to the PR interval. In silico validation of the possible influence of Tnni3k expression levels on the PR interval was made possible by the mouse phylogeny viewer (http://msub.csbio.unc.edu/) [19] and the mouse phenome database (http://phenome.jax.org/) [12]. Furthermore, we used the information in the phylogeny viewer to select the panel of 6 inbred mouse lines in such a way that they represented all possible independent haplotypes at the Tnni3k locus.

To functionally validate the role of Tnni3k in the in vivo regulation of PR interval we studied the DBA.AKR.hrtfm2 congenic mouse line harboring the high-Tnni3k expression AKR/J QTL region in the low-TNNI3K expression DBA/2J genetic background as well as transgenic mice overexpressing human TNNI3K. Strikingly, the congenic mice recapitulate perfectly the PR interval of the high-Tnni3k expression donor line; furthermore, the hTNNI3K overexpression mice, as expected, display an extremely prolonged PR interval. Taken together this implies a dose-dependent effect of Tnni3k on atrio-ventricular conduction. Of note, hTNNI3K overexpression mice displayed longer QRS duration in comparison to DBA/2J wild-type mice. This concurs with our previous observation of a QTL for QRS

Table 2. Overview of the ECG results mean (st.err) in DBA/2J, DBA.AKR.hrtfm2 and transgenic hTNNI3K mice.

| Genotype            | PR    | QRS   | QTC   | HR    | N   |
|---------------------|-------|-------|-------|-------|-----|
| DBA                 | 32.8  (0.68) | 11.1 (0.31) | 51.5 (1.5) | 481.0 (11.6) | 12  |
| DBA.AKR.hrtfm2      | 40.2*  (1.2) | 12.8 (0.26) | 51.4 (1.6) | 498.0 (14.4) | 11  |
| Congenic            |       |       |       |       |     |
| Transgenic hTNNI3K  | 51.8*  (2.4) | 19.5 (1.5)* | 49.2 (2.3) | 417.0 (17.0)* | 8   |
| ANOVA               | p = <0.0001 | p = <0.0001 | p = 0.62 | p = 0.0018 | n.a. |

*denotes the values significantly different from DBA/2J in the Bonferroni corrected post hoc analysis at the 0.05 significance level.

doi:10.1371/journal.pgen.1003113.0002
duration post-flecainide overlapping the PR-QTL on mouse chromosome 3 [8].

Tnni3k was recently identified as a genetic modifier of disease progression in the Cgq7 mouse model of cardiomyopathy [16]. In this model, with cardiac-specific overexpression of Calsequestrin (Csq), Tnni3k was shown to be the causative gene underlying the heart failure modifier 2 (Hrtfm2) locus, with high levels of Tnni3k accelerating the progression of the cardiomyopathic phenotype.

Little is known thus far about the physiological role of Tnni3k. Tnni3k encodes for cardiac Troponin I-interacting kinase 3, initially identified as a cardiac-specific protein kinase interacting with cardiac Troponin I in a yeast-two hybrid assay [23]. Its phosphorylation target(s) remain unknown [16]. The protein structure is predicted to contain an Ankyrin repeat domain besides the the Serine/Threonine-Tyrosine universal kinase domain, most likely involved in extensive protein-protein interactions.

The molecular mechanism whereby Tnni3k impacts on atrio-ventricular conduction requires further in-depth studies. Cardiac conduction slowing may stem from multiple mechanisms affecting cardiomyocyte depolarization, cell-cell electrical communication via gap-junctional coupling, or fibrosis, processes which may not necessarily be mutually exclusive. It is tempting to speculate that Tnni3k influences atrioventricular conduction.

In conclusion, we identified Tnni3k as the causal gene for a mouse PR interval QTL. Our findings provide the first evidence for Tnni3k in modulation of the electrocardiographic PR interval, indicating a previously unknown role for Tnni3k in atrio-ventricular conduction.

**Methods**

**Mouse breeding and husbandry**

The transgenic 129P2-Scn5a1798insD+/+ (129P2-MUT) and FVBN/J-Scn5a1798insD+/+ (FVBN/J-MUT) mice were generated as previously described [24]. 129P2xFVBN/J-Scn5a1798insD+/+ F1 mice (F1-MUT) were reared from these mice, and subsequently intercrossed to produce 120 F2 mice from each parental strain were genotyped across the 19 autosomes and X chromosome by means of an Illumina Golden Gate mouse medium density (768 SNP) panel. This genotyping was carried out at Harvard Partners Center for Genetics and Genomics (HPCGG, Cambridge MA, USA). Mice with call rates <95% and single nucleotide polymorphisms (SNPs) with a call rate <95% and a minor allele frequency (MAF) <0.45 were removed from the analyses. Genotyping errors were identified using error LOD scores [13].

All analysed strains were assessed for the presence of

**ECC measurements**

ECC analysis of F2-MUT and inbred mice at 12 to 14 weeks of age (n = 109) was performed as previously described [10]. Briefly, mice were weighed, lightly anesthetized by isoflurane inhalation (4.0% v/v induction; 0.8–1.2% v/v maintenance) with 800 ml/min oxygen and allowed to acclimatize for 5 minutes. The ambient temperature within the ECC recording hood was kept warm by means of a heat lamp. A 3-lead surface ECC was acquired digitally from subcutaneous 23-gauge needle electrodes at each limb of mice in the prone position using the Powerlab acquisition system (ADInstruments). Each channel was amplified and sampled at a rate of 1 kHz and a high-pass filter setting of 15 Hz. Baseline surface ECC traces were recorded for the duration of 5 minutes. A 3 minute ECC trace was analyzed for HR, and the signal average ECC (SAECC) calculated from each of leads I and II, aligned at QRS maximum, was analyzed for PR duration using the LabChartPro software (ADInstruments) and utilized for subsequent QTL mapping. We excluded mice that exhibited ECC parameter standard deviations greater than 1.5 ms between leads.

**Harvest and dissection of heart samples**

Mice were sacrificed at 12–14 weeks by CO2-O2 asphyxiation followed by cervical dislocation. Hearts were excised, washed in 1×PBS, and dissected left ventricular (LV) free-wall flash-frozen in liquid N2.

**RNA preparation and microarray analysis**

Total RNA was extracted from LV samples (n = 109) using the Qiagen RNEasy mini kit 50 protocol for isolating total RNA from animal tissue using spin technology (Qiagen Inc.) according to manufacturer’s protocol. Total RNA yield (μg) and purity (260 nm:280 nm) were determined spectrophotometrically using the NanoDrop spectrophotometer (USA). The integrity (RIN>9.0) of the re-suspended total RNA was determined using the RNA Nano Chip Kit on the Bioanalyzer 2100 and the 2100 Expert software (Agilent technologies). Synthesis, amplification and purification of anti-sense RNA was performed by using the Illumina TotalPrep RNA Amplification Kit (Ambion art. No. AM-IL1791) following the Illumina Sentrix Array Matrix expression protocol at ServiceXS (Leiden, The Netherlands). A total of 750 ng biotinylated cRNA was hybridized onto the MouseRef-6v2 Expression BeadChip (Illumina).

The raw scan data were read using the beadarray package (version 1.12.1) [14], available through Bioconductor [15]. Quality control using the function calculateBeadLevelScores showed no evidence of low-quality arrays. Illumina’s default pre-processing
steps were performed using beadarray. In short, estimated background was subtracted from the foreground for each bead. For replicate beads, outliers greater than 3 median absolute deviations (MADs) from the median were removed and the average signal was calculated for the remaining intensities. A variance-stabilization transformation [19] was applied to the summarized data in order to remove the mean-variance relationship in the intensities. Resulting data was then quantile normalized [17].

eQTL mapping
eQTL mapping was performed using the R/eqtl package based on the R-statistical program, as previously described [18]. Briefly, for each transcript probe (n = 26,529) a genome-wide scan was performed with genotype and the covariates sex, weight and age as main-effects. The logarithm-of-odds (LOD) scores were calculated by interval mapping using the expectation-maximization (EM) algorithm. A multiple-transcript genome-wide significance threshold (p<1.88×10⁻⁶) Bonferroni correction for 26,529 transcript traits) was applied. Corresponding empirical LOD threshold was determined using 10,000 permutations (swapping phenotypes for replicate beads, outliers greater than 3 median absolute deviations (MADs) from the median were removed and the background was subtracted from the foreground for each bead. The 10 Mb [19].

Quantitative trait transcript analysis
Transcripts for which we identified cis-eQTLs co-localizing with ECG trait QTL were tested for correlations with the respective ECG indices for conduction. Spearman’s correlation coefficients (rho) were generated with a commercial program (SSP software, ver. 16.0; SPSS, Chicago, IL).

qRT–PCR
mRNA expression levels of Tnni3k were validated in LV tissue samples (n = 10 in each group) by quantification using the LightCycler system for real-time RT-PCR (Roche Applied Science). Quantitative PCR data was analyzed with the LinRegPCR program [2]. All samples were processed in triplicate and expression levels were normalized to GAPDH.

Protein isolation and Western blotting
Protein was isolated from left ventricular and atrial tissue from a snap frozen mouse heart using standard procedures. In short: the tissue was homogenized in ice-cold RIPA buffer (50 mM Tris HCl pH 7.6, 150 mM NaCl, 1% NP-40, 0.5% sodium deoxycholate, 0.1% SDS) supplemented with protease inhibitors (Complete Mini; Roche) and Sodium Orthovanadate (final concentration 0.5 mM) using magnasyler ceramic beads (Roche scientific, 03558941001) for 60 sec. The unsoluble parts were spun down (30 sec at 4°C, 13000 rpm), the supernatant was transferred to a fresh tube and protein concentrations were determined using a BCA protein assay kit (Thermo Fisher Scientific). Protein lysates were run on a 8% acrylamide gel and blotted onto a pre-equilibrated PVDF Immobilon-P membrane (Millipore) by means of a semidry system. Blots were cut at appropriate heights and probed with primary antibodies (1:2000 Tnni3k rbt [16] 1:10 000 0-ILK1 (4G9) (3856; Cell Signalling) as loading control. HRP conjugated secondary antibodies were detected with ECL-Plus (Amersham). Chemiluminescent signals were visualized using a digital image analyzer (LAS-4000 Lite; Fujifilm).

In silico validation and haplotype analysis
We analyzed the haplotype structure in the 1.5 LOD drop region of the Tnni3k eQTL using the mouse phenology viewer [19]. PR interval data for 9 strains with known haplotype structure was downloaded from the mouse genome database [12,18] and analysed by Student’s T-test (after testing for normal distribution) for differences between the strains harboring the red versus the green haplotype. FVBN/J was excluded from this test, as no data for other strains with the same haplotype was available.

Data access
Expression data was deposited in the public gene expression omnibus (GEO) database of NCBI GEO database: GSE19741.

Author Contributions
Conceived and designed the experiments: EML BPS AM AYS HT CAR. Performed the experiments: EML BPS AM AYS HT CAR. Analyzed the data: EML BPS AM AYS HT CAR. Contributed reagents/materials/analysis tools: GSP DAM. Wrote the paper: EML BCRB.

References
1. Benson D (2004) Genetics of atrioventricular conduction disease in humans. Anat Rec A Discov Mol Cell Evol Biol 280: 934–939.
2. Cheng S, Kayes MJ, Larson MG, McBride EL, Newton-Cheh C, et al. (2009) Long-term Outcomes in Individuals With Prolonged PR Interval or First Degree Atrioventricular Block. JAMA 301: 2571–2577.
3. Smith JG, Magnani JW, Palmer C, Meng YA, Soliman EZ, et al. (2011) Genome-wide association studies of the PR interval in African Americans. PLoS Genet 7: e1001304. doi:10.1371/journal.pgen.1001304
4. Heeringa J, Conwaym D, van der Kuip D, Hofman A, Kors J, et al. (2006) Prevalence incidence and lifetime risk of atrial fibrillation: the Rotterdam study. Eur Heart J 27: 949–953.
5. Havlik R, Garrison R, Fabsitz R, Feinleib M (1980) Variability of heart rate, P-R, QRS and Q-T durations in twins. J Electrocardiol 13: 45–48.
6. Smith J, Lowe JK, Kovvali S, Maller JB, Salit J, et al. (2009) Genome-wide association study of electrocardiographic conduction measures in an isolated founder population: Korase. Heart Rhythm 6: 634–641.
7. Newton-Cheh C, Guo CY, Wang TJ, O’Donnell C J, Levy D, et al. (2007) Genome-wide association study of electrocardiographic and heart rate variability traits: the Framingham Heart Study. BMC Med Genet 8 Suppl 1: S7.
8. Scicluna BP, Tanck MW, Remme CA, Beckman I, Cosentino R, et al. (2011) Quantitative trait loci for electrocardiographic parameters and arrhythmia in the mouse. J Mol Cell Cardiol 50: 380–389.
9. Remme CA, Scicluna BP, Verkerk AO, Amin AS, van Brummelhuis S, et al. (2009) Genetically determined differences in sodium current characteristics modulate conduction disease severity in mice with cardiac sodium channelopathy. Circ Res 106: 1293–1292.
10. Scicluna BP, Wilde AA, Bezzina CR (2008) The primary arrhythmia syndromes: same mutation, different manifestations. Are we starting to understand why? J Cardiovasc Electrophysiol 19: 443–452.
11. Hubner N, Wallace CA, Zindahl H, Petretto E, Schulz H, et al. (2005) Integrated transcriptional profiling and linkage analysis for identification of genes underlying disease. Nat Genet 37: 243–253.
12. Bogue MA, Grubb SC (2004) The Mouse Phenome Project. Genetica 122: 71–74.
13. http://www.ncbi.nlm.nih.gov/sites/GoDShowere/acce?GDS3142.
14. Thourer L, Van Deun K, Tranchevent LC, Van Lommel L, Engelen K, et al. (2008) Using ribosomal protein genes as references: a tale of caution. PLoS ONE 3: e1654. doi:10.1371/journal.pone.0001654
15. http://cshlcbio.unc.edu/
16. Wheeler FC, Tang H, Marks OA, Hadnott TN, Chu PL, et al. (2009) Tnni3k modifies disease progression in murine models of cardiomyopathy. PLoS Genet 5: e1000647. doi:10.1371/journal.pgen.1000647
17. http://phenome.jax.org/.
18. Berthonneche C, Peter B, Schupfer F, Hayoz P, Kutalik Z, et al. (2009) Cardiovascular response to beta-adrenergic blockade or activation in 23 inbred mouse strains. PLoS ONE 4: e6610. doi:10.1371/journal.pone.0006610
19. Yang H, Wang JR, Dillen JP, Buss RJ, Bell TA, et al. (2011) Subspecific origin and haplotype diversity in the laboratory mouse. Nat Genet 43: 648–655.
20. Schadt EE, Lamb J, Yang X, Zhu J, Edwards S, et al. (2005) An integrative genomics approach to infer causal associations between gene expression and disease. Nat Genet 37: 710–717.

21. Petretto E, Sarwar R, Grieve I, Lu H, Kumaran MK, et al. (2008) Integrated genomic approaches implicate osteoglycin (Ogn) in the regulation of left ventricular mass. Nat Genet 40: 546–552.

22. Barrett T, Troup D, Wilhite S, Ledoux P, Evangelista C, et al. (2011) NCBI GEO: archive for functional genomics data sets—10 years on. Nucleic Acids Res 39: D1005–1010.

23. Zhao Y, Meng X, Wei Y, Zhao X, Liu D, et al. (2003) Cloning and characterization of a novel cardiac-specific kinase that interacts specifically with cardiac troponin I. J Mol Med 81: 297–304.

24. Remme CA, Verkerk AO, Nuyens D, van Ginneken AC, van Brunschot S, et al. (2006) Overlap syndrome of cardiac sodium channel disease in mice carrying the equivalent mutation of human SCN5A-1795insD. Circulation 114: 2584–2594.