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The role of connexin40 in developing atrial conduction

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ABSTRACT

Connexin40 (Cx40) is the main connexin expressed in the murine atria and ventricular conduction system. We assess here the developmental role of Cx40 in atrial conduction of the mouse. Cx40 deficiency significantly prolonged activation times in embryonic day 10.5, 12.5 and 14.5 atria during spontaneous activation; the severity decreased with increasing age. In a majority of Cx40 deficient mice the impulse originated from an ectopic focus in the right atrial appendage; in such a case the activation time was even longer due to prolonged activation. Cx40 has thus an important physiological role in the developing atria.

1. Introduction

Intercellular connections in the heart via gap junctions play an important role in impulse propagation in the myocardium. Cardiac gap junctions are composed of proteins called connexins (Cxs) that form low resistance channels, which enable electrical coupling of adjacent myocytes allowing intercellular electrical communication. Many studies have shown that alterations in the localization and expression of connexin proteins in the heart may cause abnormal activation spreading through the myocardium, thus leading to arrhythmias [1–3].

In mammalian hearts, mRNA for connexins 30.2, 40, 43 and 45 has been detected [4]. The expression of these connexins is dynamic in different parts of the heart, and the expression pattern is well conserved among species [5]. Each connexin forms a channel with unique electrophysiological properties [6]. The main connexin in murine atria is Cx40, but Cx43 and Cx45 are also expressed in smaller amounts [7]. Nevertheless, as a differentiation marker of the chamber myocardium [8], Cx40 is absent in the sinoatrial node.

The role of Cx40 in human arrhythmias was studied extensively [9,10]. The most common supraventricular arrhythmia in humans is atrial fibrillation. The studies investigating the relation between this arrhythmia and Cx40 have yielded rather conflicting results [11]. Simon et al. studied cardiac conduction abnormalities in mice lacking Cx40 and found no case of atrial fibrillation [3]. Verheule et al. described predisposition to tachyarrhythmias in adult Cx40 deficient mice after atrial burst pacing [12]. Recent studies in humans have discovered a polymorphism in the *Cx40* gene that is connected with a higher risk of developing atrial fibrillation [13]. Recently, Yang et al. [14] described a null mutation of Cx40 significantly associated with atrial fibrillation onset in humans.

Despite this fact, most of the studies performed in Cx40 deficient mice were focused on the ventricular conduction system, where several conduction pathologies such as right bundle branch block or slowed atrioventricular conduction were described [12,15]. In the adult mouse atria, prolongation of P wave and ectopic foci were reported [16]. Probably the most comprehensive study of the role of Cx40 deficiency on the developing atria was made by Greg Morley's group [17]. These authors described conduction velocity heterogeneity of the paced beat between the left and right atria in adult mice, which is lost in Cx40 deficiency. At the prenatal stages – embryonic day (ED)13.5 and ED15.5 – they found impaired SAN impulse initiation with ectopic sites of

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activation. The main objective of our study was to assess the role of Cx40 in impulse generation and spontaneous propagation in murine atria during early embryonic development.

2. Methods

2.1. Animals

Cx40:GFP knock-in mice developed by Lucile Miquerol [18] were used. As the knock-in of the *GFP* disrupts the endogenous *Cx40* gene, the homozygotes are functional nulls. *Cx40* null mice were maintained in homozygous state, as these animals are viable and fertile (albeit with a reduced life span, a smaller number of embryos per litter and a non-Mendelian ratio of nulls in heterozygous matings). Heterozygous embryos were obtained from timed matings of a Cx40-deficient male and a wild type (WT) female; for WT, mixed genetic background related to the Swiss strain and *Cx40:GFP* mice was used [19]. The experimental protocol was approved by the local animal committee and conforms to the Guiding Principles in the Care and Use of Vertebrate Animals in Research and Training.

2.2. Optical mapping of embryonic hearts

The animals were caged together overnight, and the noon of the day of vaginal plug detection was considered ED0.5. Pregnant females were killed by cervical dislocation on ED10.5, 12.5 and 14.5 and the embryos were rapidly dissected in ice-cold (to prevent ischemic damage to the heart) Tyrodes solution (composition: NaCl 145 mmol/l, KCl 5.9 mmol/l, $CaCl_2$ 1.1 mmol/l, $MgCl_2$ 1.2 mmol/l, glucose 11 mmol/l, HEPES 5 mmol/l; pH = 7.4). These stages were selected to cover the early period of cardiac development prior to septation. The hearts with adjacent posterior body wall structures were isolated and stained in 2.5 mmol/l di-4-ANEPPS (Invitrogen) for 10 min. Motion control was achieved by addition of 0.1 μM blebbistatin (Sigma) and pinning the torso to the bottom of the dish. Because of different focal planes for atria and ventricles (Fig. 1), our standard imaging protocol had to be modified slightly as we were unable to use the recordings performed for the analysis of ventricular activation patterns in a separate study [19]. To avoid any perturbation of the pacemaking region, we chose the antero-superior view (Figs. 1 and 2). Despite the overall transparency of the heart, the outflow region obscured the central area, where the pacemaker is normally located; for this reason, we removed the outflow tract at the ventricular border and at its distal connection to pharyngeal arch arteries. Data acquisition and analysis were performed using the Ultima L high-speed camera and bundled software as described recently [20]. To better appreciate the dynamics of atrial activation, the percentile activation over time method, recently validated by the Gourdie lab [21], was used (Fig. 2). The area of each color band was measured and expressed as a percentage of the whole atrial area. We then interpolated the data in the resulting graph to obtain times, at which 20%, 40%, 60%, 80%, and 100% of the atria was activated. Times across these intervals were then averaged among groups. Differences in quantitative parameters among groups were analyzed by ANOVA and Scheffe' test for multiple comparisons. Values between left and right atrium were compared using paired two-tailed Student's *t*-test. In all cases, values of $P < 0.05$ were considered statistically significant.

2.3. Immunohistochemistry

To obtain a morphological view of the atrial myocardium and SAN position, six WT embryos and ten embryos of the *Cx40*−/− line at ED12.5 were fixed in 4% paraformaldehyde in PBS overnight at 4 °C, and transferred into OCT medium through ascending saccharose gradients. Ten-micron sections were cut on a cryomicrotome,

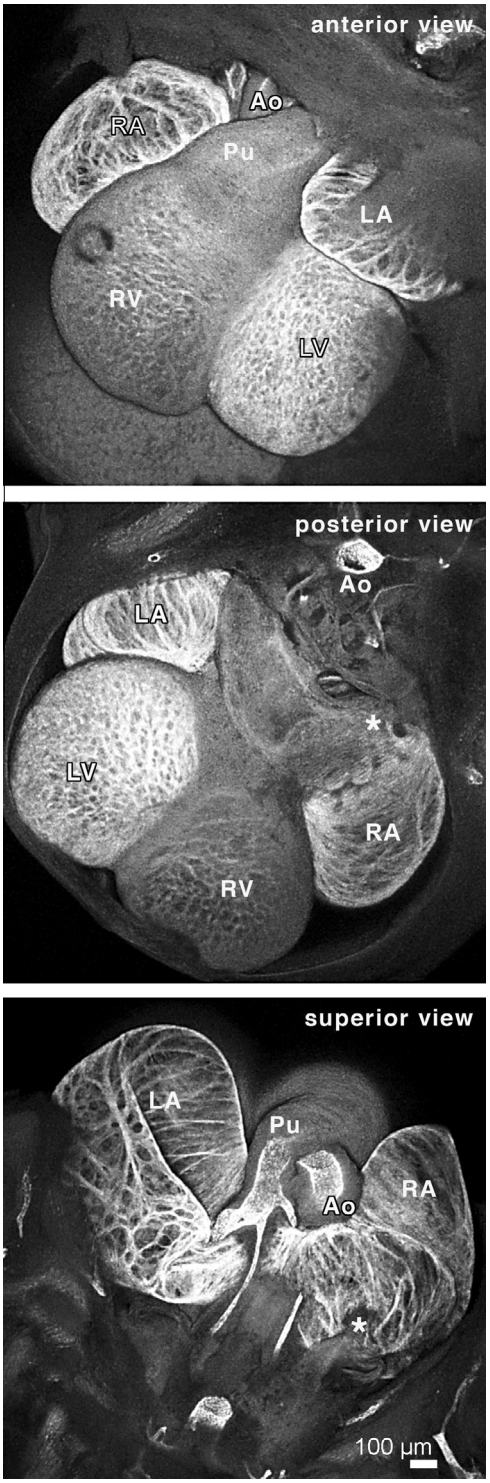

Fig. 1. Morphology of an ED12.5 mouse heart. The heart of a *Cx40*+/− mouse embryo at ED12.5 in confocal projections (GFP fluorescence) in three complementary views. In the anterior view, it is evident how the outflow tract covers the central portion of the atria. In the posterior view, the same area is hidden by the veins entering the right atrium. In the superior view the extensive antero-posterior dimension of the atria is clearly visible, and a fine network of pectinate muscles in both atria can be appreciated. RA – right atrium; LA – left atrium; RV – right ventricle; LV – left ventricle; Ao – aorta; Pu – pulmonary trunk; asterisks mark the putative site of the SAN, distinguished from the surrounding GFP-positive atrial myocardium by its darkness.