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REVIEW

Arrhythmias in the developing heart

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Abstract

Prevalence of cardiac arrhythmias increases gradually with age; however, specific rhythm disturbances can appear even prior to birth and markedly affect foetal development. Relatively little is known about these disorders, chiefly because of their relative rarity and difficulty in diagnosis. In this review, we cover the most common forms found in human pathology, specifically congenital heart block, pre-excitation, extrasystoles and long QT syndrome. In addition, we cover pertinent literature data from prenatal animal models, providing a glimpse into pathogenesis of arrhythmias and possible strategies for treatment.

Keywords anti-arrhythmic drugs, cardiac development, chick embryo, conduction system, hypoxia, mouse.

Disturbances of cardiac rhythm in the human foetus

During routine obstetric examination, foetal rhythm disturbances may be detected in at least 2% of pregnancies (Copel *et al.* 2000, Jaeggi & Nii 2005). Foetal arrhythmias account for about 10–20% of referrals for foetal cardiology assessment (Srinivasan & Strasburger 2008). Due to a number of limitations, a foetal electrocardiogram (cardiotocogram) is not the ideal method for assessment of arrhythmias. A relatively novel and efficient method for foetal heart electrical activity recording is foetal magnetocardiography (Strasburger *et al.* 2008, Strasburger & Wakai 2010). However, this method is not widely available and is preferred only after the 20th week of gestation because it is less reliable in the earlier stages of pregnancy. Thus, echocardiography remains the principal method in evaluation of heart rhythm disturbance in the foetus. In addition to heart rhythm analysis, echocardiography may reveal other signs associated with prolonged or persistent foetal rhythm disturbances, such as hydrops (pleural or pericardial effusion, ascites) in its early as well as more advanced stages. The severity of foetal heart failure can be then monitored using the 'heart failure score' presented by Huhta (2005).

Using all three standard echocardiographic modalities (B-mode, M-mode and Doppler), we can assess atrial and ventricular contraction frequencies and their time relations. An equivalent for P wave on electrocardiogram is the A wave detected by pulse wave Doppler in mitral inflow or atrial wall motion detected by M-mode. Similarly, the beginning of retrograde flow in the superior vena cava indicates the beginning of atrial systole. Atrioventricular (AV) valve closure, semilunar valve opening and positive Doppler flow in the aorta are equivalents of the beginning of QRS complex. Simultaneous Doppler recording in the superior vena cava and the aorta shows the time correlation between the atrial and ventricular systole – times corresponding to the P wave and QRS complex on the ECG. These parameters allow us to calculate the heart rate, AV delay and diagnose different types of arrhythmias by measuring the mechanical response of the heart chambers to the electrical stimulus.

The relationship between foetal arrhythmia and structural heart disease is not clearly established. Stewart and Copel found no clear relationship between foetal arrhythmia and structural heart disease

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(Stewart *et al.* 1983, Copel *et al.* 2000). In the observational study published by Stewart and associates, only two foetuses from 17 with documented ectopic beats had structural heart disease. No structural heart disease was found in five foetuses with tachycardia (heart rate over 180 bpm), but four of eight foetuses with documented bradycardia had severe structural heart disease. Copel and colleagues reported only two of 10 foetuses diagnosed with significant arrhythmia – one with supraventricular tachycardia and one with a second-degree AV block – associated with structural heart disease of all 614 foetuses with irregular heart rhythm. On the other hand, there is evidence that foetal arrhythmia may be associated with structural heart disease. Schmidt *et al.* (1991) reported that 53% of foetuses (of a total of 55) with complete AV block had concomitant structural heart disease (left atrial isomerism, discordant AV connection). Vergani *et al.* (2005) reported structural heart anomalies in five of six foetuses with bradycardia from a total cohort of 114 infants with foetal arrhythmias. Only two of four foetuses with AV block survived. Eronen reported 12 foetuses (three supraventricular and three ventricular ectopic activities, four AV blocks and two sinus bradycardias) with significant arrhythmia associated with structural heart disease from a total of 125 foetuses with significant arrhythmia (Eronen 1997). She also found 95% survival in foetuses with sole significant arrhythmia compared to a 75% mortality in those with arrhythmia associated with structural heart disease. Interestingly, the total mortality in the group of foetuses with structural heart disease was only 67%. Based on these two observational studies, it could be speculated that bradyarrhythmias are more frequently associated with structural heart disease and have a worse outcome than tachyarrhythmias or irregular heart rhythm, which are frequently curable or might resolve spontaneously during development.

For simplicity, we may divide foetal arrhythmias into the three groups (Fig. 1): ectopic beats, mostly originating in atrial ectopic foci; tachyarrhythmias, which are defined as heart rates over 180 bpm; and bradyarrhythmias, defined as heart rates below 110 bpm (Jaeggi & Nii 2005).

Of these three types, extrasystoles typically have the best outcomes (Reed 1989). Vergani *et al.* (2005) reported that 38% of cases with extrasystoles (in 87 foetuses) resolved *in utero* and 49% at birth. Only one neonate required postnatal therapy, and in nine neonates, the arrhythmia was still present at 1-year follow-up without need for therapy. Two foetuses with extrasystoles converted to supraventricular tachycardia *in utero* and were successfully treated pharmacologically with no impact on their further development.

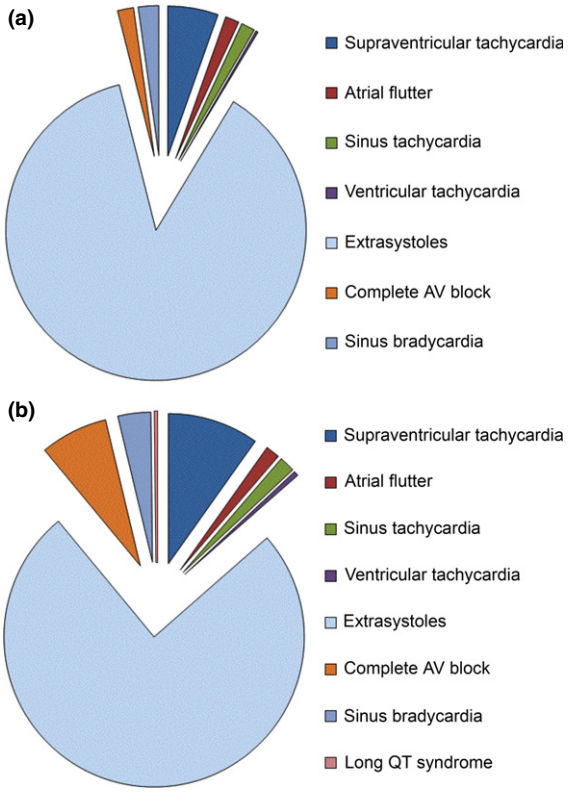

Figure 1 Epidemiology of foetal arrhythmias in humans. (a) Incidence of various types of arrhythmias in non-selected population ($N = 406$, collated from references (Copel *et al.* 2000), (Vergani *et al.* 2005)). (b) Incidence in highly selected population ($N = 591$, collated from references (Stewart *et al.* 1983), (Reed *et al.* 1990), (Eronen 1997), (Vergani *et al.* 2005), (Zhao *et al.* 2006)).

None of these were associated with structural heart disease.

Prolonged foetal tachycardia is usually a serious condition often leading to foetal hydrops or even death. Simpson & Sharland (1998) reported hydrops occurrence in 41% of 127 foetuses diagnosed with tachycardia. Seventy-five non-hydropic foetuses from this cohort responded well to transplacental treatment (mostly with digoxin) with an excellent survival to birth (96%). Conversely, only two-thirds of hydropic foetuses with tachycardia responded to transplacental treatment, and of these, only 73% survived till birth. Thus, foetal hydrops is a negative prognostic sign suggesting severe hemodynamic consequences from the underlying causes – for example, arrhythmia and/or structural heart disease.

Sustained or prolonged bradycardia (heart rates <100 bpm) or tachycardia (heart rates over 180 bpm) are of clinical significance and might have a significant impact on further foetal development *in utero*; even later postnatal development might be affected. Jaeggi

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and Nii reported foetal tachycardia as the most frequent arrhythmia in the foetus and was present in 57% of 66 foetuses examined with proven serious arrhythmia (Jaeggi & Nii 2005). Supraventricular tachycardia was present in 40% of cases, atrial flutter accounted for 11%, and sinus tachycardia was present in 6%.

Diagnosis, classification and management of foetal arrhythmias

The 12-lead ECG that is so useful in newborn or adult cardiology suffers from major limitations in the foetus. Echocardiography is typically the only way to diagnose tachyarrhythmia in the foetus, and it is not easy to differentiate between different types of tachyarrhythmias. Supraventricular tachycardia with mostly 1 : 1 AV conduction can be distinguished from atrial flutter, with mostly 2 : 1 AV conduction block, due to excessive atrial frequency in flutter (about 440–480 bpm) translating into a 220–240 bpm ventricular rate. In AV re-entry, the time interval between the ventricular and atrial activity would be short, while in atrial tachycardia originating from ectopic foci, this time interval is usually prolonged. Ventricular tachycardia with typical dissociation of ventricular and atrial rhythm or conducted 1 : 1 from ventricles to atria is extremely rare in the foetus as most tachyarrhythmias originate in the atria. In such cases, it is clear that only an experienced physician trained in echocardiography can make the correct diagnosis.

The most frequent foetal tachyarrhythmia is supraventricular tachycardia represented by three different types: AV re-entrant tachycardia, permanent junctional reciprocating tachycardia and atrial ectopic tachycardia. The second most frequent foetal tachyarrhythmia is atrial flutter caused by a macro-re-entry circuit located in the atria. The final differentiation is often made only after birth when the arrhythmia persists or reoccurs, or a delta wave typical for the accessory pathway is present on the 12-lead ECG. The treatment strategy for most types of tachyarrhythmias is based on transplacental digoxin administration in non-hydropic foetuses. Sotalol, flecainide or amiodarone is mostly reserved for hydropic foetuses or more resistant tachyarrhythmias. Treatment is required for pure sinus tachycardia with typical heart rates of 180–200 bpm usually caused by foetal distress, foetal thyrotoxicosis, anaemia etc.

Sustained or prolonged bradycardia is present in 43% of significant foetal arrhythmia cases, as presented by Jaeggi & Nii (2005). Complete AV block accounts for 38%, and only 5% manifest as sinus bradycardia cases. The treatment of foetal bradycardia is limited. For significant number of foetuses with complete heart block caused by maternal autoantibodies, transplacental treatment with beta-receptor-stimulating agents, corticosteroids or immunosuppressives is recommended. In principle, foetal pacemaker implantation (Liddicoat *et al.* 1997) should be considered using minimally invasive techniques (Sydorak *et al.* 2001, Eghtesady *et al.* 2011, Nicholson *et al.* 2012).

During sinus bradycardia, there is 1 : 1 AV coupling with a slow frequency of atrial contractions (<100 bpm). Simple sinus bradycardia may be caused by foetal distress with episodes of hypoxia and blood flow redistribution, while brain and heart are supplied preferentially. Sinus bradycardia can be a manifestation of foetal long QT syndrome, and all newborns with a history of foetal heart rate below the 3rd percentile should be assessed for this entity early after birth (Mitchell *et al.* 2012). Sinus bradycardia may be a rare manifestation of sinus node dysfunction. Supraventricular bigeminy or trigeminy with AV block must always be excluded when assessing the foetus for bradycardia. The telltale sign would be an atrial frequency above that of the ventricle and an irregular heart rhythm. The outcome is usually benign and this arrhythmia mostly does not require treatment.

Foetal AV block

The most frequent cause of bradycardia is congenital AV block. First-degree AV block is characterized by prolonged AV conduction with 1 : 1 AV coupling. It is necessary to realize that AV conduction time increases during gestation and the exact numbers also differ for various ECHO modalities. Normal values for 30–34 weeks of gestational stage are 122.7 ± 11.1 ms by left ventricle inflow/outflow Doppler method, 116.5 ± 8.8 ms by Doppler method in the superior vena cava/aorta, 142.4 ± 14.2 by atrial contraction/ventricular systole as measured via Tissue Doppler Imaging (TDI) of the basal right ventricular free wall (Nii *et al.* 2006).

We distinguish two types of second-degree AV block. Wenckebach type (Mobitz I) second-degree AV block is characterized by the gradual lengthening of AV conduction time terminated by a dropped ventricular contraction. Mobitz type (Mobitz II) of second-degree AV block is typified by sudden loss of ventricular contraction, while AV conduction time remains unchanged. A specific type of Mobitz II AV block is 2 : 1 conduction when every second atrial beat is not conducted to the ventricles.

The third-degree AV block (complete heart block) has the most serious impact on further foetal development leading frequently to foetal demise. Atrial and

ventricular electrical and mechanical activities are completely independent in this type of AV block. This always leads to significant and prolonged bradycardia. The only physiological pathway to compensate the decrease in cardiac output caused by bradycardia is the Frank-Starling mechanism which might be limited at early stages according to data from animal experiments (Kockova *et al.* 2013). When increased stroke volume fails to compensate severe foetal bradycardia, heart failure occurs leading to foetal hydrops.

Foetal complete heart block occurs more frequently in conjunction with various congenital structural heart diseases (Stewart *et al.* 1983, Jaeggi & Nii 2005, Vergani *et al.* 2005). Schmidt reported that 53% of foetuses diagnosed with complete heart block had associated complex congenital heart disease (Schmidt *et al.* 1991). Another major reason for congenital complete heart block is maternal autoimmune disease such as lupus erythematosus, Sjögren syndrome, rheumatoid arthritis or unclassified systemic rheumatoid disease. Elevated titres of anti – Ro/SSA and anti – La/SSB antibodies are typically found in mothers affected by the above-mentioned autoimmune diseases. The risk of developing foetal complete heart block in pregnant women with positive anti-Ro/SSA antibodies is about 2% (Brucato *et al.* 2001). These antibodies cause myocardial inflammation specifically affecting the AV node leading to various degrees of AV conduction impairment, which usually occurs around 20–24 gestational weeks. This might also present as endomyocardial fibrosis in the foetus or newborn. Because complete heart block has been shown to be associated with very high mortality rates ranging between 18% and 43% (Jaeggi & Nii 2005), there has been a major effort to prevent this autoimmune disease. Corticosteroids were administrated to pregnant women with positive titres of autoantibodies intravenously or orally (Reinisch *et al.* 1978, Friedman *et al.* 2009), but major side effects were noticed afterwards including oligohydramnion, foetal adrenal suppression, intrauterine grow retardation and so on. Corticosteroid treatment is recommended only for advanced heart block with significant and prolonged bradycardia with a high risk of hydrops development. Isolated prolongation of AV conduction only rarely leads to progressive AV block, as shown by Jaeggi *et al.* (2011) in anti-Ro and anti-La positive mothers, and corticosteroid treatment is therefore not recommended. Recently, current recommendations of the American Heart Association regarding diagnosis and management of foetal heart disease, including prenatal arrhythmias, were summarized in a form of Scientific Statement (Donofrio *et al.* 2014).

Importance of the cardiac conduction system for the origin of arrhythmias

It is widely recognized in clinical practice that the cardiac conduction system (CCS) can be a focal point of arrhythmogenesis (Braunwald *et al.* 2001). This propensity was extensively analysed from developmental perspective by Jongbloed and associates (Jongbloed *et al.* 2004) using CCS-LacZ transgenic mouse model. Detailed analysis of the developing CCS was performed on hearts at embryonic day (ED) 9.5–15.5 stained for beta-galactosidase activity and co-stained with the myocardial marker HHF35 followed by three-dimensional reconstruction. CCS-lacZ expression detected by X-gal staining was observed in the sinoatrial node, left and right venous valves, septum spurium, right and left AV ring, His bundle, bundle branches, moderator band, Bachmann's bundle, left atrial posterior wall surrounding the pulmonary venous orifice and later on in the pulmonary vein wall. These data supported the idea that areas derived from the developing CCS may form the arrhythmogenic substrate in adult hearts.

A comparative study between patients with left atrial tachycardia originating from the junction of mitral annulus and aortic ring and mouse embryos demonstrated the presence of the developing specialized conduction system in this region starting at embryonic age 11.5 (Gonzalez *et al.* 2004).

Particular attention was focused on the developmental origin of pulmonary vein myocardium (Mommersteeg *et al.* 2007a), which is derived from the second heart field. The area around the pulmonary veins entrance is in humans a frequent site of origin of atrial fibrillation, so its electrical insulation by catheter intervention is a frequent procedure during clinical intervention for ablation of this increasingly prevalent human arrhythmia. A recent study based on HCN4-Cre mouse line with LacZ or eGFP reporter (Liang *et al.* 2013) precisely delineated relative contributions of first and second heart lineages to the CCS and provided a time line of developmental expression of this CCS marker in concert with other markers during its formation.

Genetic and epigenetic determination of the CCS

To better appreciate the developmental potential of CCS to generate arrhythmias, one needs to consider the mechanisms governing its induction and patterning (reviewed in (Gourdie *et al.* 2003), (Christoffels *et al.* 2010). Lineage tracing experiments performed by the Mikawa lab have shown that cardiac pacemaker cells

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are physically segregated and molecularly programmed in a tertiary heart field prior to the onset of cardiac morphogenesis, and this process depends on Wnt signalling (Bressan *et al.* 2013). Recently, the genetic cascade governing specification of cardiac pacemaking tissues was elucidated by the Amsterdam group (Mommersteeg *et al.* 2007b). Restricted expression pattern of the homeodomain transcription factor Shox2 in the sinus venosus myocardium, including the sinoatrial nodal region and the venous valves, was found to be important for the recruitment of these cells to the pacemaking fate (Blaschke *et al.* 2007). The authors furthermore demonstrated aberrant expression of gap junction proteins connexin 40 and 43 as well as the transcription factor Nkx2.5 specifically within the sinoatrial nodal region, leading to embryonic lethality between ED11.5 and ED13.5 in $Shox2^{-/-}$ mice. Finally, they showed that Shox2 deficiency interferes with pacemaking function in embryonic zebrafish *in vivo*. Particular attention was also devoted to specification of pulmonary venous myocardium (Mommersteeg *et al.* 2007a), which is a significant source of atrial fibrillation. Genetic labelling reveals that atrial cells do not contribute to this specific population, characterized by Nkx2.5 expression distinguishing it from the systemic venous return. Maintenance of this phenotype is dependent on Pitx2c, which prevents it from adopting the Cx40-negative, Hcn4-positive pacemaking phenotype of the right-sided sinoatrial node.

Embryonic pacemaking differs in details from the mechanisms operating in the adult sinoatrial node. The early stages are crucially dependent on the calcium clock, as demonstrated by Wakimoto *et al.* (2000) who studied the functional importance of sodium-calcium exchanger (NCX) for heartbeat initiation and maintenance. To address this question, they generated Ncx1-deficient mice by gene targeting to determine the *in vivo* function of the exchanger. The hearts of $Ncx^{-/-}$ embryonic deficiency in Ncx, embryos did not beat, and cardiomyocytes frequently underwent apoptosis leading to embryonic lethality between ED9 and ED10.

To study cardiac physiology near the onset of the heartbeat in embryonic mouse hearts, Chen and associates performed dual optical mapping of membrane voltage and intracellular calcium (Chen *et al.* 2010). Action potentials and calcium transients were detected in approx. 50% of mouse embryo hearts at ED8.5 and 100% at E9.0, indicating that the heartbeat starts between ED8 and ED9. Cardiac activity was abolished by calcium channel blocker nifedipine and the I (f) blocker ZD7288, suggesting that both HCN4 and voltage-dependent calcium channels are important for embryonic pacemaking. The role of sodium channels and intracellular calcium cycling is of lesser importance at this early stage.

From the functional side, endothelin signalling was shown to be necessary not for specification like in birds (Gourdie *et al.* 1998), but normal function of the embryonic pacemaker in mammals (Karppinen *et al.* 2013). Stimulation with endothelin-1 increased beating frequency of ED9–ED11 cardiomyocytes. Inhibition by receptor antagonist tezosentan led to dose-dependent bradycardia *in vitro* as well as *in utero*, but only during the early (ED12.5) and not late (ED18.5) embryonic stages. Irregular rhythm was also observed, and use of specific antagonists indicated that the effects are mediated via endothelin receptor B.

Location of the first activation site in the rat embryonic heart was investigated by the Kamino group (Hirota *et al.* 1985). At the time of heartbeat initiation, the first pacemaking activity was located in the left side of the sinus venosus, but within a few hours migrated to the right side, where the definitive pacemaker is located. A similar situation was reported also in avian embryos; remnants of this initial left-sided activity were reported in a small proportion of normal avian hearts at later stages of development (Sedmera *et al.* 2006); under normal conditions, no such left-sided activity was reported in a large series of embryonic mice (Leaf *et al.* 2008, Ammirabile *et al.* 2012, Benes *et al.* 2014).

Considerably less is known about the mechanisms regulating specification of the remaining components of the CCS. Neuregulin was proposed as a factor influencing differentiation of the ventricular myocytes towards the conduction phenotype (Rentschler *et al.* 2002), but this secreted molecule has many important functions in the embryonic cardiomyocytes, such as their survival (Liu *et al.* 2010). The neuregulin/Erb signalling cascade could function in concert with endothelin signalling, which was shown to be important in Purkinje fibre differentiation in the chick (Gourdie *et al.* 1998, 2003, Takebayashi-Suzuki *et al.* 2000, Sedmera *et al.* 2008). Other important factors participating in formation of the His bundle and its branches include Nkx2.5 (Jay *et al.* 2004), Irx3 (Zhang *et al.* 2011) and T-box transcription factors (Jerome & Papaioannou 2001, Moskowitz *et al.* 2004, Hoogaars *et al.* 2007, Aanhaanen *et al.* 2009, Frank *et al.* 2012).

Differentiation of embryonic myocytes into the conducting phenotype is governed also by the epigenetic factors, of which mechanical loading is of the most critical importance. *In vitro* unloading of chick embryonic hearts (Sankova *et al.* 2010) led to de-differentiation of the ventricular conduction system that could be rescued by ventricular stretching using a droplet of silicone oil. These experiments resolved the issue

arising from previous *in vivo* studies using altered haemodynamics models (Reckova *et al.* 2003, Hall *et al.* 2004) that showed that increased hemodynamic loading accelerated, while reduced ventricular preload inhibited ventricular CCS differentiation by attributing the stimulus to myocyte stretching, rather than to shear stress-induced signalling from the endocardium.

Studies on chick embryos *in vivo* showed that hypoxia can accelerate maturation of the AV junction and lead to earlier appearance of mature (apex-to-base) ventricular activation patterns (Nanka *et al.* 2008), possibly through increased apoptosis of the AV myocardium. Another player in developing proper fibrous insulation of the AV junction is the developing epicardium (Kolditz *et al.* 2007, 2008), and perturbations of this process may lead to ventricular pre-excitation. Electrical insulation of the His bundle is also dependent on immigrating cardiac neural crest cells (Gurjarpadhye *et al.* 2007).

Spontaneously occurring arrhythmias in embryos

This area of embryonic arrhythmias is not well investigated for numerous reasons. First, there are the methodological difficulties inherent to all observational studies of mammalian embryos that are shielded *in utero* by maternal tissues. The most significant breakthrough in this respect was availability of high-resolution ultrasound (Phoon *et al.* 2002, Phoon 2006, Nomura-Kitabayashi *et al.* 2009, Lo *et al.* 2010), paralleling the advances in human embryonic echocardiography (Maeno *et al.* 1999, Pedra *et al.* 2002). The second obstacle is the relative rarity of such events (compare with the situation in clinical settings, discussed above) necessitating the examination of large numbers of embryos. Therefore, most arrhythmias detected in the embryonic hearts could be at least in part be due to 'gentle' alterations of physiological conditions, as it is close to impossible to monitor the embryonic mammalian heart in a completely non-invasive manner.

Various arrhythmias in the isolated mouse embryonic heart were revealed using simultaneous voltage and calcium optical mapping (Valderrabano *et al.* 2006). The focus of this study was on AV conduction during transition from immature base-to-apex to mature apex-to-base ventricular activation pattern. The authors hypothesized that after this transition, the remnants of the myocardial AV ring remain transiently able to conduct, providing a possible substrate for arrhythmias. They noted that arrhythmias were rare under normal conditions, with only occasional AV blocks (4%) and junctional rhythms in four of 309 embryonic hearts analysed. The frequency notably increased after isoproterenol stimulation with 6% incidence of ventricular ectopic rhythms. Addition of carbachol after isoproterenol caused dissociated antegrade and retrograde AV ring conduction in almost 10% of ED10.5–ED11.5 hearts. Re-entry persisting for multiple beats was also observed, but none occurred at ED9.5. Rare cases of irregular rhythm (sinoatrial and AV block, alternating patterns of ventricular activation) were also observed in our large mouse series (Sankova *et al.* 2012), while ectopics originating from the outflow tract myocardium were seen exclusively in ED10.5–ED11.5 hearts cultured for 24 h (Vostarek *et al.* 2014). AV re-entry was observed as a rarity in one ED4 chick heart (Fig. 2).

Genetic mouse models of arrhythmias

As noted above, spontaneous arrhythmias are very rare in normal embryonic hearts, thus facilitating analysis of results in experimental perturbation models. The importance of catecholaminergic signalling in development and function of the CCS was recently reported by Steve Ebert's group (Baker *et al.* 2012). These results are in good agreement with previous studies showing lethality of mouse embryos deficient in a component of adrenergic signalling, beta-adrenergic receptor kinase (Jaber *et al.* 1996).

Studies by Collin Phoon validated ultrasound biomicroscopy as a prime tool for *in vivo* identification of abnormal mouse embryonic heart function, including arrhythmias. Using this technique, they studied longitudinally embryonic ED10.5–ED14.5 NFATc1$^{-/-}$ embryos and control littermates (Phoon *et al.* 2004). The null embryos, lacking the outflow valves, die prior to completion of ventricular septation from presumed heart failure. The authors showed that abnormal blood flow was present at E12.5 when outflow valves normally first develop. Reduced cardiac output and diastolic dysfunction contributed to heart failure, but contractile function remained unexpectedly normal. The only arrhythmia detected prior to embryonic demise was progressive bradycardia, indicating that embryonic heart failure occurs rapidly in this mouse model.

Mutations in TBX3 cause congenital anomalies in patients with ulnar-mammary syndrome (Frank *et al.* 2012). Data from both mice and humans suggest multiple roles of this transcription factor in morphogenesis and function of the CCS. Disruption of Tbx3 function in different regions of the developing heart caused discrete phenotypes and lethal arrhythmias. Sinus pauses (normally present at low frequency in adult mice) and bradycardia indicated sinoatrial node dysfunction; pre-excitation and AV block revealed problems in the AV junction. These arrhythmias were accompanied by perturbed expression of several ion

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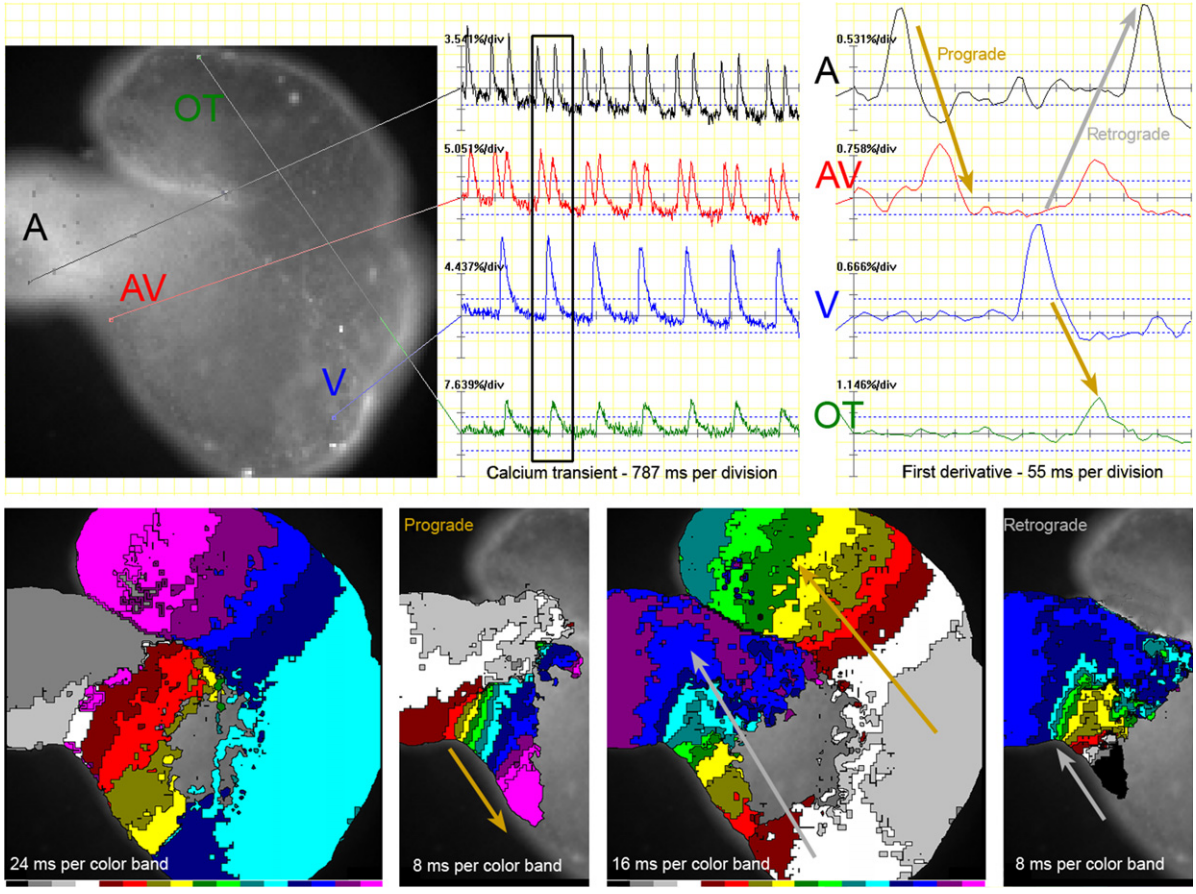

Figure 2 Atrioventricular re-entry in ED4 chick embryonic heart. Top panels show the embryonic heart from the back and time course of calcium transients. The first derivative panel shows both prograde activation (from the atrium to the ventricle to the outflow tract, orange arrow) and retrograde activation from the ventricle back to the AV canal and atrium (grey arrow). Activation maps in the bottom depict this phenomenon at different temporal scales. Note that the activation pattern of the atrium differs between prograde and retrograde activation. A, atrium; AV, atrioventricular canal; V, ventricle; OT, outflow tract. For better visualization of the activation sequence, see the Movie S1.

channel components (e.g. upregulation of Kcne3, Chac1, Kcnj4 and downregulation of Scn7a), despite normal expression of previously identified CCS markers, raising the possibility of functional disturbances in apparently morphologically normal CCS.

The Notch signalling cascade was found to be important in regulation of AV conduction in the mouse (Rentschler *et al.* 2011), and activation of Notch signalling during development consistently led to accessory AV pathways and pre-excitation. On the other hand, inhibition of this cascade led to AV node hypoplasia and loss of expression of slowly conducting connexin 30.2 gap junction channels, resulting in shortened AV delay.

Drug-induced arrhythmias in mammalian models

A significant worry of every clinician taking care of women of childbearing age is the teratogenic potential of prescribed medicines. This does not only impact overt morphological anomalies, but also more subtle functional alterations, such as mild neurological defects, or indeed, embryonic arrhythmias that in extreme cases can lead to embryonic or foetal death.

As mentioned above, propensity to arrhythmia depends considerably on developmental stage. At the earliest stages, where the heart is small and conduction generally slow, the only 'allowed' arrhythmias are alterations of heart rate of which bradycardia is the most dangerous as it can lead to reduced cardiac output and embryonic death. Once the cardiac chambers are formed (Moorman *et al.* 2010), alternating regions of fast and slow conduction develop, creating a heterogeneity in conduction that can lead to unidirectional or bidirectional blocks, re-entry and more complex arrhythmias (Valderrabano *et al.* 2006). With further development, the heart becomes more complex with the establishment of the coronary vascular network and autonomic innervation (Hildreth

et al. 2009). In humans, sensitivity to bradycardia in premature infants suggests that the heart rate response to cholinergic stimulation may change during development (Maurer 1979). This hypothesis was tested on isolated intact foetal mouse hearts (ED13–ED22). Acetylcholine led to a marked (−50%) heart rate decrease in the micromolar range in ED13–ED14 hearts, but the decrease was progressively blunted with increasing age with a mere 3% drop at ED21–ED22 with the same dose. Physostigmine significantly enhanced the cholinergic response in older hearts, suggesting that the effect is at least in part due to increasing intrinsic cholinesterase activity with gestational age.

Unlike the adult heart, whose energy needs are mostly met by fatty acid oxidation, the developing myocardium relies mostly on glycolysis. Chen *et al.* (2007) investigated how inhibition of glycolysis affects membrane voltage and calcium transients in embryonic mouse hearts. Glycolysis inhibition by 10 mM 2-deoxyglucose or 0.1 mM iodoacetate decreased significantly heart rate and induced (unspecified) arrhythmias in over 50% of the treated hearts. Similar effects were noted when oxidative phosphorylation was blocked by 500 nM p-(trifluoromethoxy)phenylhydrazone. During experiments aimed at elucidation of pacemaking mechanisms in early mouse heart (Chen *et al.* 2010), the investigators observed various arrhythmias, including AV re-entry induced by adenosine (ADO). This occurred at stages that had already differentiated fast-conducting atrial and ventricular chamber myocardium and slowly conducting AV canal.

Anti-epileptic drugs frequently act on ion channels regulating membrane potential in excitable tissues; some of these channels are present also in the developing heart. This could be one explanation for the known teratogenic potential of these substances. Danielsson *et al.* (1997) investigated the capacity of phenytoin, a hERG channel blocker inhibiting the IKr that is critical for embryonic heart function, to induce embryonic hypoxia via adverse effects on the embryonic heart using a whole embryo culture model. In these mouse embryo studies, phenytoin caused a concentration-dependent decrease in embryonic heart rate, with temporary or permanent cardiac arrest at the highest dosage. The exact concentration, as well as incidence of other arrhythmias, was strain-dependent. Similar results were obtained in rat embryos.

Arrhythmogenic properties of phenytoin were examined in mouse (Azarbayjani & Danielsson 2002). Between ED9 and ED13, a dose-dependent bradycardia and other unspecified arrhythmias such as AV block were observed at maternal plasma concentrations in the micromolar range. Patch-clamp recording on HERG-transfected cells demonstrated that phenytoin inhibits the inward rectifier potassium current. The authors attributed these effects to reactive oxygen species (ROS) generated at reoxygenation after resumption of normal rhythm, as an antioxidant agent alpha-phenyl-*N*-tert-butyl-nitrone showed protective effects. A similar mechanism was proposed for another teratogenic anti-epileptic drug, trimethadione and its pharmacologically active metabolite dimethadione. The same group of investigators then followed up by showing that these effects were exacerbated in combination with several anti-epileptics (phenytoin, phenobarbital, dimethadione and carbamazepine), supporting the idea that the increased risk for malformations following polytherapy is linked to an increased risk for cardiac rhythm disturbance (Danielsson *et al.* 2007).

Almokalant, a class III anti-arrhythmic drug, caused embryotoxicity in the mouse (Skold & Danielsson 2000), most likely secondary to its adverse effects on the embryonic heart, as dose-dependent bradycardia and periods of cardiac arrest were observed in whole embryo culture at ED10. Thus, all drugs capable of causing embryonic bradycardia should be regarded as potentially embryotoxic and used during pregnancy with extreme caution.

Chick embryonic model

The cardiac electrical activity in the chick embryo has been investigated in pioneering works, *in vivo* (Van Mierop 1967, Rajala *et al.* 1984, Tazawa *et al.* 1989, Sugiyama *et al.* 1996), in the intact embryo (Hoff & Kramer 1939, Paff *et al.* 1964), the dissected heart (Paff *et al.* 1968, Paff & Boucek 1975, Kasuya *et al.* 1977, Hirota *et al.* 1987), isolated cardiac chambers (Boucek *et al.* 1959, Arguello *et al.* 1986) and in cultured cardiomyocytes (Shrier & Clay 1982). In particular, ECG of the whole heart displays characteristic P, QRS and T components which allows assessment of the beating rate from PP or RR interval, AV conduction from PR interval, duration of the ventricular activation from QT interval and intraventricular conduction from of the QRS complex width. The spatio-temporal interpretation of the ECG is facilitated by the fact that ventricular activation occurs in a 'base-to-apex' fashion, and there is no differentiated conduction system at early developmental stages (Chuck *et al.* 1997, Reckova *et al.* 2003).

The principal types of arrhythmias observed in the validated 4-day-old embryonic chick heart model under various stresses (e.g. anoxia–reoxygenation) or exposed to pharmacological agents are transient atrial tachycardia (range 180–300 bpm) and bradycardia (range 110–140 bpm), atrial ectopy, first-degree atrioventricular blocks (AVB), second-degree AVB (2 : 1 to

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8 : 1), Wenckebach phenomenon (Mobitz type I), third-degree AVB (ventricular escape beats) and bursts of irregular activity followed by intermittent atrial arrest (cardioplegia) as previously documented (Sarre *et al.* 2006) and presented in Figure 3. Some of these arrhythmias resemble those observed in the human foetus (Strasburger & Wakai 2010).

Effects of drugs inducing arrhythmias can be conveniently studied in the chick embryo *in vivo*. Paff and collaborators described heart blocks after digoxin treatment (Paff *et al.* 1964), defining the stage of chamber formation as critical for induction of conal (40 h of incubation) and AV block (42 h). Before these stages, the only reaction of the embryonic heart to drug treatment was complete cardiac arrest. The authors noted similarity between this AV block and the situation in humans, including the Wenckebach phenomenon. The Rochester group studied effects of

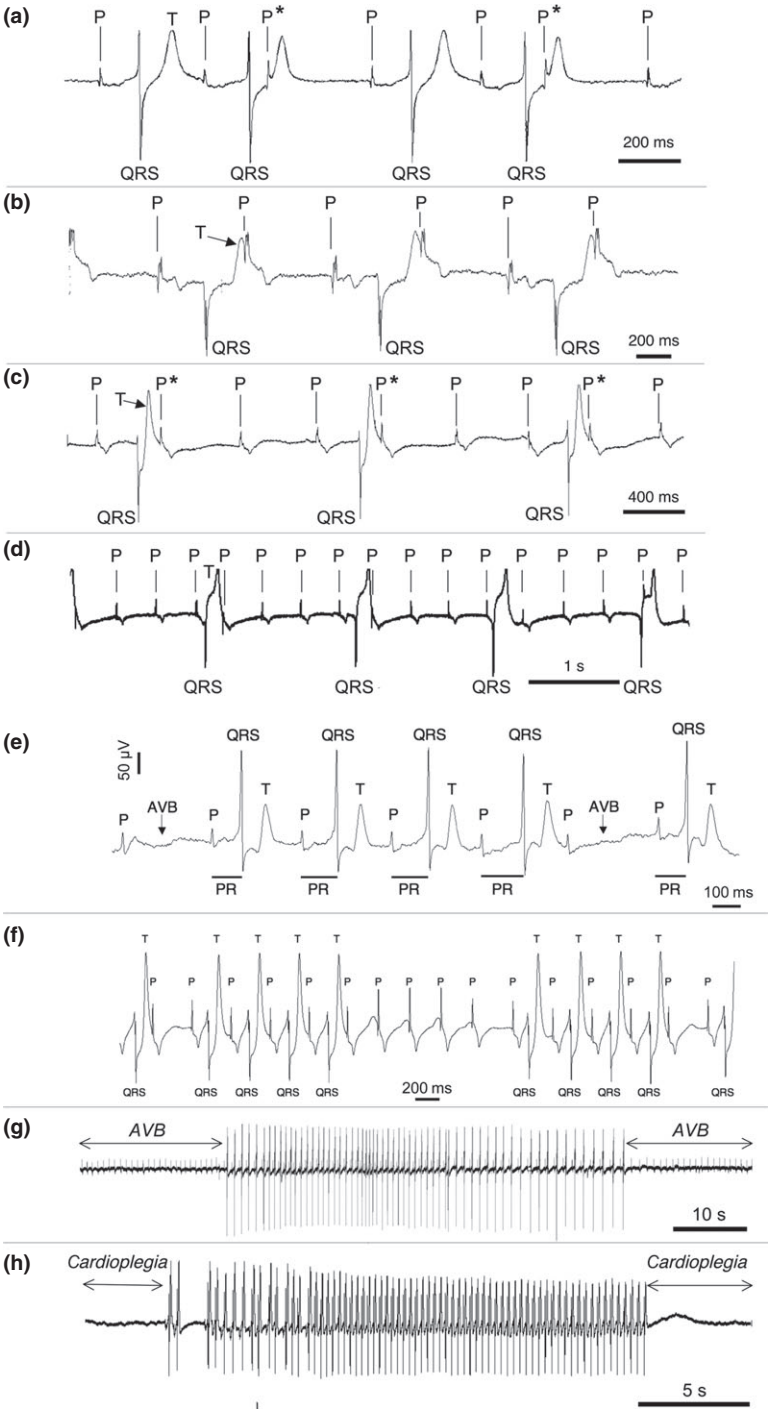

Figure 3 Examples of major types of arrhythmias observed in the 4-day-old embryonic chick heart. ECG of the isolated heart displays characteristic P wave, QRS complex and T wave components. (a) Atrial ectopy, (b) 2 : 1 AVB, (c) atrial ectopy + 3 : 1 AVB, (d) ventricular escape beats (third-degree AVB), (e) Wenckebach phenomenon (Mobitz type I), (f) episode of heart block + Wenckebach, (g) third-degree AVB + bursting activity and (h) intermittent sinoatrial arrest (cardioplegia) + bursting activity. AVB, atrioventricular block. Asterisk indicates atrial premature beat.

various cardiac drugs on the developing cardiovascular system. Isoproterenol at a teratogenic dose induced increased vascular resistance and reduced cardiac output (Clark *et al.* 1985), suggesting the presence of a functional adrenergic signalling system in the 4-day-old embryonic heart. On the other hand, chronic verapamil (calcium antagonist) infusion decreased both cardiac and embryonic growth through decreased cardiac performance (Clark *et al.* 1991, Sedmera *et al.* 1998) and led to delayed ventricular morphogenesis (increased trabeculation, decreased compact layer thickness). Recently, Kockova and colleagues studied the effects of beta blockers and ivabradine on cardiac function and embryonic survival (Kockova *et al.* 2013). High doses led to mortality through decreased cardiac output, based upon bradycardia and insufficient Frank-Starling compensation. Partial AV blocks were also observed in both early (day 4) and later (day 8) embryos.

Arrhythmias during anoxia–reoxygenation

In the 4-day-old embryonic chick heart model (Raddatz *et al.* 1997, Sarre *et al.* 2006), the chrono-, dromo- and inotropic disturbances and the ultrastructural modifications (e.g. mitochondrial and nuclear swelling) induced by 30-min anoxia followed by 60-min reoxygenation are reversible within a period of time depending on the developmental stage; the older the embryo, the lower the reversibility (Sedmera *et al.* 2002). Anoxia induces bradycardia, atrial ectopy, first-, second-, and third-degree AVB and transient cardioplegia. Reoxygenation provokes also the Wenckebach phenomenon and ventricular escape beats. At the onset of reoxygenation, PR, QT and ventricular electromechanical delay (reflecting excitation-contraction (EC) coupling) significantly increase, whereas atrial EC coupling remains unchanged. Ventricular contractility at the apex and intraventricular conduction are also significantly reduced by anoxia–reoxygenation (Fig. 4), but no fibrillations, no re-entry and no ventricular ectopic beats are observed. At reoxygenation, arrhythmias and conduction disturbances are associated with a burst of ROS production (Sarre *et al.* 2005, Raddatz *et al.* 2011) and reduced by the antioxidant ascorbic acid (Fig. 5). Although the presence of glucose at the physiological concentration of 8 mM prolongs cardiac activity during anoxia, it enhances the reoxygenation-induced ROS production and arrhythmias relative to glucose-free conditions (Tran *et al.* 1996, Raddatz *et al.* 2011). This observation underscores the role that alterations of glycolytic activity may play in arrhythmogenesis associated with ROS. Nitric oxide (NO) at supraphysiological concentration delays post-anoxic recovery of AV propagation, and sinoatrial pacemaker cells are less responsive to NO (Terrand *et al.* 2003). An NO synthase inhibitor (L-NAME) prolongs the ventricular electromechanical delay during anoxia and delays its recovery during reoxygenation, while an NO donor (DETA-NONOate) has opposite effects (Maury *et al.* 2004). Thus, a NO-dependent pathway appears to contribute to regulation of ventricular excitation–contraction coupling in the anoxic–reoxygenated embryonic heart.

It should also be mentioned that a cycle of cooling (4 °C, 30 min)/rewarming (37 °C, 60 min) under normoxia is less arrhythmogenic than anoxia (30 min) followed by reoxygenation (60 min). However, between 15 and 20 min of rewarming, when temperature rises from 27 to 31 °C, the beating rate

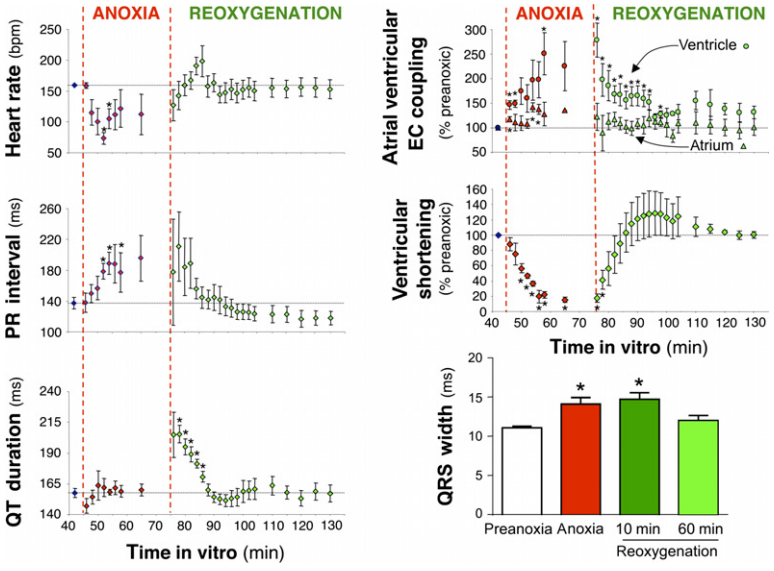

Figure 4 In a 4-day-old chick embryonic heart, heart rate, atrioventricular (AV) propagation (PR interval), QT duration, atrial and ventricular excitation-contraction (EC) coupling, contractility (apical ventricular shortening) and intraventricular conduction (QRS width) are markedly altered during anoxia and reoxygenation, but fully recover after 30–40 min. Mean ± SEM; $n = 4$; $n = 20$ for QRS determination; bpm, beats per minute. $^*P < 0.05$ vs. preanoxic values.

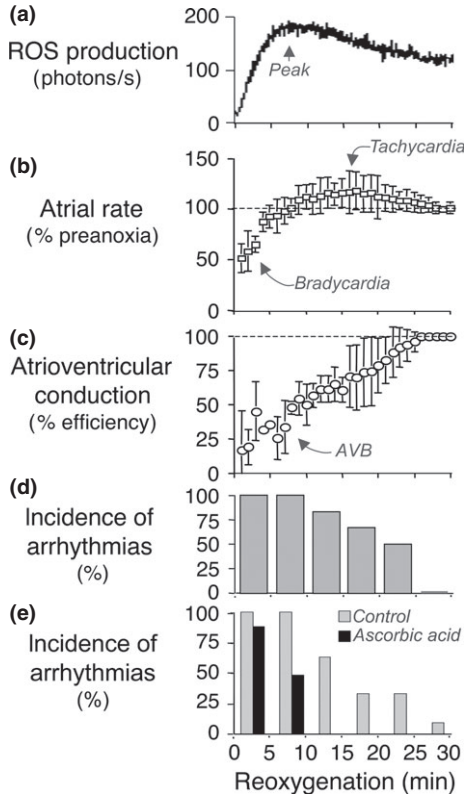

Figure 5 Functional recovery of the 4-day-old embryonic chick heart during the first 30 min of reoxygenation after preceding 30 min of anoxia. (a) typical reactive oxygen species (ROS) production determined by lucigenin-induced chemiluminescence peaking after about 8 min (arrow). (b) atrial rate reported as percentage of the pre-anoxic value. (c) efficiency of the atrioventricular (AV) propagation calculated as the ratio of the ventricular to the atrial electrical activity duration and expressed as a percentage, 100% representing one-to-one AV conduction. (d) highest incidence of arrhythmias is associated with the burst of ROS. (e) antioxidant ascorbic acid (Vit C, 10 mM) reduces incidence of arrhythmias. Mean ± SD; horizontal dashed lines represent basal pre-anoxic levels; b and c: $n = 3$; d and e: $n = 6$.

transiently accelerates, the PR interval is prolonged, and the rate of recovery of QT decreased, clearly indicating that this range of temperature is critical for the return to normal rhythmicity (Sarre *et al.* 2006).

Acidosis (transition from pH 7.4 to 6.5), which can occur under prolonged anoxia, has negative chrono-, dromo- and inotropic effects, essentially characterized by intermittent atrial and ventricular activity (bursts). At pH 6.5, heart rate and AV conduction velocity remain significantly decreased, whereas ventricular shortening and contractility recover after 5 min. Under acidotic anoxia and during reoxygenation, inactivation of HCO_3-dependent mechanisms increases the incidence of arrhythmias. This indicates that in the anoxic–reoxygenated embryonic heart pH regulation appears to depend predominantly on HCO_3 availability and transport. The Na^+/H^+ exchange (NHE) appears to be protective only under anoxia (Meiltz *et al.* 1998).

Under normoxia, H_2O_2 differentially modulates ERK, p38 and JNK pathways in atria, ventricle and outflow tract (Gardier *et al.* 2010). Only exposure to a rather high concentration of H_2O_2 (>500 μM) leads to cardioplegia and markedly increased phosphorylation of ERK2 and p38 specifically in atria and outflow tract, without modifying the level of JNK phosphorylation. Moreover, during the post-anoxic reoxygenation, the phosphorylation level of ERK2 and p38 is altered specifically in the ventricle. During the early phase of post-anoxic reoxygenation, the Janus Kinase 2/Signal Transducer and Activator of Transcription 3 (JAK2/STAT3) pathway is activated by ROS, interacts with Reperfusion Injury Salvage Kinase (RISK) proteins [(PI3K, Akt, Glycogen Synthase Kinase 3beta (GSK3beta)), Extracellular signal-Regulated Kinase 2 (ERK2)] and reduces arrhythmias (Pedretti & Raddatz 2011).

The hyperpolarization-activated cyclic nucleotide-gated (HCN) channels are expressed very early during cardiogenesis and play an important role in the control of the rate of diastolic depolarization in pacemaker cells of atria, ventricle and outflow tract (Sarre *et al.* 2010). Inhibition of the HCN channels by ivabradine has a negative chronotropic effect in all these cardiac regions (characterized by a decreasing AV-conotruncal gradient of intrinsic beating rate) and stabilizes the PR interval under normoxia but does not alter the types and duration of arrhythmias during anoxia–reoxygenation.

Pharmacological opening of the mitochondrial KATP channel by diazoxide selectively improves recovery of the PR interval and ventricular E-C coupling during reoxygenation, via NO-, ROS- and PKC-dependent pathways (Sarre *et al.* 2005) and reduces reoxygenation-induced JNK activity in the ventricle (Sarre *et al.* 2008). Furthermore, the open-state of the sarcolemmal L-Type Ca^{2+} channel, mitochondrial Ca^{2+} uniporter and mitochondrial KATP channel can be a major determinant of JNK activity and anoxia–reoxygenation-induced arrhythmias.

The TRPC1, 3, 4, 5, 6 and 7 isoforms of the voltage-insensitive cationic transient receptor potential canonical (TRPC) channels are expressed in the heart of 4-day-old chick embryos and can form a macromolecular complex with the alpha1C subunit of the L-type voltage-gated calcium channel (Cav1.2). Under normoxia, inhibition of TRPCs by SKF96365 leads to negative chrono-, dromo- and inotropic effects, prolongs QT interval and triggers Wenckebach phenomenon, clearly indicating that inactivation of these

channels is implicated in arrhythmias. Blockade of the TRPC3 isoform by Pyr3 affects AV conduction specifically, whereas inhibition of all TRPCs by SKF combined with that of Cav1.2 by nifedipine results in severe arrhythmias and finally in cardioplegia (Sabourin *et al.* 2011).

Proarrhythmic Ca^{2+} overload can result from Ca^{2+} entry through sarcolemmal voltage-dependent L-type and T-type Ca^{2+} channels (Cav1.2 and Cav3.1, respectively) and voltage-independent cation channels (TRPC), as well as Ca^{2+} release from the sarcoplasmic reticulum after anomalous activation of ryanodine receptor (RyR2) channels by ryanodine (Tenthorey *et al.* 1998) and/or inhibition of sarco/endoplasmic reticulum Ca2+-ATPase (SERCA) by thapsigargin (Sabourin *et al.* 2012). The L-type Ca^{2+} channel agonist Bay-K-8644 induces atrial tachycardia and tends to prolong arrhythmias during reoxygenation (Bruchez *et al.* 2008). ROS and reactive nitrogen species (RNS) are known to alter the redox state and increase activity of RyR2 leading to a potentially proarrhythmic Ca^{2+} release. Pharmacological opening of RyR channels with ryanodine (10 nM) triggers severe arrhythmias (mainly bursting activity) under normoxia and during anoxia and reoxygenation, whereas verapamil (10 nM), an antagonist of L-type Ca^{2+} channel at this concentration, affords protection against reoxygenation-induced arrhythmias (Tenthorey *et al.* 1998). Furthermore, relative to the Cav3.1 channel (T-type), the Cav1.2 channel (L-type) plays a major role in spontaneous electrical activity of the embryonic chick heart. Indeed, inhibition of Cav1.2 with nifedipine induces a progressive and significant shortening of QT and prolongs the ventricular electromechanical delay, whereas specific inhibition of Cav3.1 with mibefradil has only a slight effect (Sabourin *et al.* 2011).

Adenosine is a crucial regulator of the developing cardiovascular system, derives from intra- and extracellular ATP degradation and accumulates in the myocardial interstitial fluid under hypoxia or ischaemia. In the embryonic heart, developing normally in an environment poor in oxygen, the physiological concentration of ADO is much higher than in the adult normoxic heart and ADO metabolism relies on ecto-nucleoside triphosphate diphosphohydrolase (CD39), ecto-5′-nucleotidase (CD73), adenosine kinase (AdK) and ADO deaminase (ADA). CD39 and CD73 sequentially convert ATP to ADO and ADA convert ADO into inosine (INO). ADO is transported by equilibrative (ENT1,3,4) or concentrative (CNT3) transporters and interacts with the four subtypes of ADO receptors (AR), A_1AR, $A_{2A}AR$, $A_{2B}AR$ and A_3AR (Robin *et al.* 2011, 2013). ADO or A_1AR activation transiently provokes bradycardia, second-degree AVB and Mobitz type I second-degree AVB (Wenckebach phenomenon). These transient pacemaking and AV conduction disturbances are induced by A_1AR activation through concomitant stimulation of NADPH oxidase and phospholipase C (PLC), followed by downstream ROS-dependent activation of ERK2 and ROS-independent activation of PKC with Cav1.2 channel as a possible target (Robin *et al.* 2011). Furthermore, A_1AR activation mediates also a proarrhythmic Ca^{2+} entry through the TRPC3 channel functioning as a receptor-operated channel, via the stimulation of the PLC/DAG/PKC cascade in atrial and ventricular cardiomyocytes (Sabourin *et al.* 2012). Inactivation of ENTs (i) increases myocardial ADO level, (ii) provokes atrial ectopy and AVB, (iii) prolongs P wave and QT interval and (iv) increases ERK2 phosphorylation. Inhibition of CD73, MEK/ERK pathway or A_1AR prevents these arrhythmias. Exposure to INO also causes arrhythmias associated with AVB and ERK2 phosphorylation, which are prevented by A_1AR or $A_{2A}AR$ antagonists exclusively or by MEK/ERK inhibitor. Thus, disturbances of nucleoside metabolism and transport can lead to interstitial accumulation of ADO and INO and provoke arrhythmias in an autocrine/paracrine manner through A_1AR and $A_{2A}AR$ stimulation and ERK2 activation (Robin *et al.* 2013).

Pathogenesis of autoimmunity-caused congenital heart block

It is well recognized that maternal antibodies causing lupus pass the placenta and can induce prenatal or congenital heart block (CHB, reviewed in (Buyon & Clancy 2003)). Current understanding of its pathogenesis was obtained from animal models that were instrumental in revealing its mechanisms. It is believed that the foetal cardiomyocytes undergoing apoptosis such as those in the AV canal (Cheng *et al.* 2002) expose the originally intracellular Ro and La antigens to their surface, where they can be bound by circulating maternal autoantibodies (anti-SSA/Ro-SSB/La). The macrophages then phagocytose these 'opsonized' cells, leading to the secretion of pro-inflammatory and pro-fibrotic cytokines, leading to fibrosis (which does not normally occur during prenatal tissue healing), differentiation of myofibroblasts and scarring. This overshoots the normal process of reduction of the originally broad myocardial connection between the atria and ventricles to His bundle and leads in extreme cases to a complete ventricular block. More recent studies (Kamel *et al.* 2007) linked the AV bundle and sinoatrial node dysplasia in autoimmune lupus to antiserotonin (5-HT4) receptor antibodies in mice.

One of the problems with analysing this human disease is its incomplete penetrance and far from 100%

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recurrence rate in the same mother. Some of these features also complicate the murine models of lupus erythematosus, where the frequency of CHB is low as well (Suzuki *et al.* 2005). The authors' assessment of heart block incidence in murine maternal lupus models by measuring AV conduction in neonatal offspring is potentially confounded by loss by death *in utero* of the most severely affected foetuses. However, prenatal embryonic Doppler echocardiography employed in mice immunized with 60 kDa Ro, 48 kDa La or recombinant calreticulin autoantigens revealed a significant decrease in foetal heart rate and 18% incidence of lower degrees of AV block in all groups relative to controls from ED13. However, the number of pups born with an overt block was lower. This shows that a lot of potentially significant events that may even lead to foetal demise, such as foetal bradycardia and temporary conduction deficits, could be missed if one concentrates only on postnatal stages.

Qu *et al.* (2001) identified L-type calcium channel as a potential target for maternal antibodies inducing CHB in the foetus. *In vitro* studies showed the binding of these antibodies to the sarcolemma and *in vivo* demonstrated lower channel density in myocytes isolated from neonatal mice born to immunized dams. Deletion of the neuroendocrine alpha1D Ca channel in mice resulted in significant sinus bradycardia and AV block, a phenotype reminiscent to that seen in CHB. Another study by this group (Qu *et al.* 2005) confirmed expression of the alpha1D Ca channel in human foetal heart, showed the inhibitory effect of anti-Ro/La antibodies on this channel and direct cross-reactivity with this protein. This inhibition was rescued by a Ca channel activator, Bay K8644, opening a potential therapeutic avenue in this disease.

Conclusions

Despite recent advances in developmental cardiology, foetal medicine and genomics, little is known regarding the dysfunction of the developing human heart. This review shows the importance of the correct detection, characterization and diagnosis of cardiac rhythm disturbances as early as possible during *in utero* life. Experimental and transgenic animal models (e.g. sheep, mouse, chick and zebrafish) can help to decipher the cellular and molecular mechanisms underlying embryonic and foetal arrhythmias and assist in the identification of novel therapeutic targets. Such approaches also allow the investigation of the potentially deleterious short- and long-term effects of early intra-uterine stress-, drug- and mutation-induced cardiac dysrhythmias. Research in this field provides complementary scientific data to make possible the treatment of the « foetal patient » before their birth, limiting possible detrimental consequences in adulthood.

Conflict of interests

None.

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Supporting Information

Additional Supporting Information may be found in the online version of this article:

Movie S1. Example of atrioventricular re-entry in ED4 isolated chick embryonic heart visualized by optical mapping of calcium transients.

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