Tyrosine hydroxylase is expressed during early heart development and is required for cardiac chamber formation

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Aims

Tyrosine hydroxylase (TH) is the first and rate-limiting enzyme in catecholamine biosynthesis. Whereas the neuroendocrine roles of cathecolamines postnatally are well known, the presence and function of TH in organogenesis is unclear. The aim of this study was to define the expression of TH during cardiac development and to unravel the role it may play in heart formation.

Methods and results

We studied TH expression in chick embryos by whole mount in situ hybridization and by quantitative reverse transcription-polymerase chain reaction and analysed TH activity by high-performance liquid chromatography. We used gain- and loss-of-function models to characterize the role of TH in early cardiogenesis. We found that TH expression was enriched in the cardiac field of gastrulating chick embryos. By stage 8, TH mRNA was restricted to the splanchnic mesoderm of both endocardial tubes and was subsequently expressed predominantly in the myocardial layer of the atrial segment. Overexpression of TH led to increased atrial myosin heavy chain (AMHC1) and T-box 5 gene (Tbx5) expression in the ventricular region and induced bradyarrhythmia. Similarly, addition of L-3,4-dihydroxyphenylalanine (L-DOPA) or dopamine induced ectopic expression of cardiac transcription factors (cNkx2.5, Tbx5) and AMHC1 as well as sarcomere formation. Conversely, blockage of dopamine biosynthesis and loss of TH activity decreased AMHC1 and Tbx5 expression, whereas exposure to retinoic acid (RA) induced TH expression in parallel to that of AMHC1 and Tbx5. Concordantly, inhibition of endogenous RA synthesis decreased TH expression as well as that of AMHC1 and Tbx5.

Conclusion

TH is expressed in a dynamic pattern during the primitive heart tube formation. TH induces cardiac differentiation in vivo and it is a key regulator of the heart patterning, conferring atrionic identity.

Keywords

Tyrosine hydroxylase • Cardiogenesis • L-DOPA • Dopamine

1. Introduction

Catecholamines are hormones/neurotransmitters known to influence cardiovascular and endocrine physiology postnatally and processes such as movement, learning, and emotional behaviour. Tyrosine hydroxylase (TH) is the first and rate-limiting enzyme in catecholamine biosynthesis. TH catalyses the conversion of the amino acid L-tyrosine to L-3,4-dihydroxyphenylalanine (L-DOPA), which generates dopamine by the action of aromatic amino acid decarboxylase (AAADC). Subsequently, dopamine can be converted to noradrenaline, by the dopamine beta hydroxylase (DBH), and adrenaline in the mature nervous system and adrenal glands (Figure 1A).3

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Despite the well-characterized biological functions of catecholamines in postnatal organisms, very little is known about their function in embryonic development prior to neuronal differentiation. Early reports of the presence of the pathway members, and their pharmacological and genetic interference, revealed roles in gastrulation and early organogenesis. Indeed, null-mutations of TH and DBH caused embryonic lethality due to apparent heart failure. Although studies of the TH-null mice revealed a functional role of TH in maintaining oxygen homeostasis at mid-gestation stages, the role of TH in early cardiogenesis remains unexplored.

In vertebrates, the heart is initially formed by the fusion of the two bilateral endocardial tubes arising in the lateral plate splanchnic mesoderm. The resulting primitive heart tube, located at the ventral midline of the organism, undergoes a complex series of movements and tissue remodelling events that leads to the formation of the mature chambered organ. Positional information for the formation of the atria and ventricles at specific sites within the heart tube, comes from the integration of the anterior–posterior, dorso-ventral, and left–right patterning at early stages. One of the first features of anterior–posterior patterning is the restriction of the ventricular myosin heavy chain (VMHC1) and atrial myosin heavy chain (AMHC1) to the anterior and posterior pole, respectively, of the heart tube. In this study, we show that TH mRNA is enriched in the cardiac field of stage (st.) 5 chick embryos; by st. 8, TH expression was restricted to the splanchnic mesoderm of both endocardial tubes. Subsequently, TH localized predominantly to the myocardial layer of the atrio-ventricular region and the atrio-ventricular canal. Treatments with L-DOPA and dopamine induced ectopic expression of cardiac transcription factors and the contractile protein AMHC1, as well as sarcomere formation. Overexpression of TH led to the expansion of AMHC1 and Tbx5 expression into the anterior region, and induced bradyarrhythmia. Inhibition of dopamine biosynthesis or knockdown of TH decreased AMHC1 and Tbx5 expression, and perturbed correct cardiac looping. Exposure to retinoic acid (RA) induced and expanded TH expression, as well as that of AMHC1 and Tbx5, whereas blockage of endogenous RA synthesis inhibited TH expression. Our results show that TH is expressed in a dynamic pattern during the formation of the primitive heart tube. TH induces cardiac differentiation in vivo and it is a key regulator of the heart patterning, conferring atrio-genic identity. 

2. Methods

Experimental protocols with animals were performed in agreement with the Spanish law in application of the EU Guidelines for animal research, and conformed to the Guide for the Care and Use of Laboratory Animals. The methods for TH expression and activity in developing chick embryos are described in detail in Figure 1. Tyrosine hydroxylase expression and activity were measured by whole-mount in situ hybridization (ISH) for TH at stages 8–12. RT-qPCR was used to quantify TH mRNA levels in whole embryos at stages 5, 8, and 10 or from their corresponding cardiac regions. L-DOPA was measured by high-performance liquid chromatography (HPLC) in extracts from one pool of 30 st. 8 embryos, two pools of 15 st. 10 embryos and two pools of 10 st.12 embryos. The results represent the mean ± SD of three experiments. Our results show that TH is expressed in a dynamic pattern during the formation of the primitive heart tube. TH induces cardiac differentiation in vivo and it is a key regulator of the heart patterning, conferring atrio-genic identity.

Figure 1 Tyrosine hydroxylase expression and activity in developing chick embryos. (A) The catecholamine biosynthesis pathway. (B) Whole-mount ISH for TH at st. 8–12. a, b, and c correspond to transverse paraffin sections at the levels indicated by the corresponding lines. Note that at st. 8 TH mRNA is restricted to the splanchnic mesoderm of the endocardial tubes, and later it is predominantly expressed in the myocardial layer of the atrio-ventricular region (arrows in a and c). (C) RT-qPCR of RNA from whole embryos at st. 5, 8, and 10 or from their corresponding cardiac regions. The levels of TH mRNA were normalized to GAPDH mRNA levels. The results represent the mean ± SD of three experiments. (D) L-DOPA was measured by HPLC in extracts from one pool of 30 st. 8 embryos, two pools of 15 st. 10 embryos and two pools of 10 st.12 embryos. The results represent, except in st. 8, the mean ± SD.
3. Results

3.1 Early TH expression is localized to the developing heart and is active prior to its appearance in the nervous system

In postnatal organisms, TH is predominantly expressed in discrete areas of the brain, in the peripheral nervous system, and in the adrenal gland. However, our identification of a dynamic pattern of TH expression in the early chick (EC) embryo led us to search for an as yet uncharacterized, preneuronal, function for TH. Whole mount ISH demonstrated that TH mRNA was expressed in st. 8 embryos, in the heart forming regions overlying the left and right endocardial tubes (Figure 1B and see Supplementary material online, Figure S1). Transverse sections showed that TH mRNA was restricted to pre-cardiac splanchnic mesoderm (Figure 1Bo). At st. 9, TH mRNA was found specifically in the fusing cardiac tubes and, once the primitive heart tube was formed, TH displayed a graded pattern of expression. TH transcripts were concentrated in the posterior part of the looping heart, the prospective atrial region (Figure 1B, st. 12). Whereas TH expression was restricted to the myocardial layer at the posterior pole (Figure 1Bc), no transcripts were evident at the anterior pole of the looping heart (the prospective ventricular region; Figure 1Bb). This pattern of gene expression is remarkably similar to that of other genes that become progressively restricted to the posterior heart tube (e.g. AMHC110–12 and Tbx5,14–16 see Supplementary material online, Figure S1 for better comparison). Moreover, the distribution of TH transcripts coincided with that of TH protein (data not shown).

The selective cardiac expression of TH was confirmed by RT–qPCR and TH mRNA was detected in gastrulating st. 5 embryos, prior to the time specific staining can be observed by whole mount ISH. TH transcript levels were two-fold higher in the heart field of st. 5 embryos than in the total embryo (Figure 1C and, overall, TH transcript levels rose as development continued (approximately two-fold between st. 5 and 8 and four-fold between st. 8 and 10)). This increase was more noticeable in the cardiac region, particularly between st. 5 and 8, in accordance with the distribution observed by ISH.

TH activity depends on allosteric factors and on the phosphorylation state of the enzyme, as well as on the presence of co-factors.1,17 To assess whether TH was active at these early embryonic stages, we measured catecholamine levels by HPLC in whole embryos at the endocardial, linear, and looped tube stages (st. 8, 10, and 12, respectively). We chose not to dissect cardiac tissue to avoid material losses. L-DOPA was detected at st. 8 and their levels increased throughout cardiac development (Figure 1D). Dopamine was detected only occasionally at st. 8, 10, and 12, and noradrenaline and adrenaline were not found at these embryonic stages. To explain the absence of catecholamines downstream of dopamine, we analysed the expression of DBH. We could only detect DHB mRNA by RT–qPCR, and no specific signal by ISH was obtained. At all the stages analysed, much lower levels of DHB mRNA than of TH mRNA were found (~9-, 80-, and 190-fold lower at st. 5, 8, and 10, respectively). These differences were even greater (from 12- to 900-fold) in the cardiac region, indicating that the precursor L-DOPA and small amounts of dopamine are the principal, or perhaps the only, catecholamines present in the embryo prior to neuronal differentiation. The facts that dopamine is an unstable metabolite and the HPLC...
Figure 2. Effect of L-DOPA and dopamine and their inhibition, on cardiac development. (A) Effect of L-DOPA and dopamine on the expression of cardiac genes. Left drawing corresponds to a st. 5 embryo with a bead implanted lateral to one of the bilateral heart fields (yellow bead). Beads were soaked in either PBS (vehicle), or a solution of 10 μmol/L L-DOPA or dopamine. St. 10–12 embryos were subjected to whole-mount ISH for Nkx2.5, Tbx5, and AMHC1, or immunohistochemistry for MF20. Ectopic tissue adjacent to the bead coated with L-DOPA or dopamine (arrow) expressed all markers. Labelling was not detected around the control bead (arrowhead). (B) Ultrastructure of the ectopic tissue induced by dopamine. Semi-thin sections of the bead area and ectopic tissue (a: 400×). Transmission electron microscopy of cells adjacent to the dopamine bead (b: 20 000×) or of cardiomyocytes in the primitive heart tube (c: 10 000×). The white arrowheads indicate the Z bands, and the purple and yellow lines delineate the I and A bands, respectively. (C) Effect of the inhibition of L-DOPA or dopamine synthesis on AMHC1 expression. The position of the bead implanted medial to one of the bilateral heart fields of a st. 5 embryo is shown in (A) (red bead). Beads were soaked in either 3I-Tyr or mHBH, or the vehicle solution (NaOH and PBS, respectively). Embryos at st. 8 were analysed by whole-mount ISH for AMHC1. Note that AMHC1 expression is inhibited in the endocardial tube ipsilateral to the bead (arrow), but not by the control bead (arrowhead).
method is less sensitive for dopamine than for l-DOPA detection may account for the irregular detection of the former. These results extend previous findings, using less sensitive assays, where TH activity in the chick embryo was detected on the first day of incubation (st. 8), yet DBH activity was not evident until st. 19–20.18

### 3.2 l-DOPA and dopamine induce cardiac differentiation

The pattern of TH expression and the presence of catecholamines suggested that TH, l-DOPA, and possibly dopamine may play a role in cardiac development. We thus implanted heparine-acrylamide beads soaked in either l-DOPA (10 μmol/L) or dopamine (10 μmol/L) lateral to one of the bilateral heart fields in embryos cultured at st. 5 (Figure 2A, yellow bead). These embryos were allowed to develop until the linear or looped heart stage (st. 10 and 12, respectively), and were subsequently analysed by whole mount ISH. St. 12 was the furthest developmental stage analysed because the EC culture interferes with normal development beyond this stage.

l-DOPA and dopamine induced expression of the cardiac transcription factors Nkx2.5 and Tbx5 in the ectopic tissue adjacent to the bead (Figure 2A; see Supplementary material online, Table S1). Moreover, expression of AMHC1, a sarcomeric protein and marker of terminal cardiac differentiation,16,17 was also induced, as seen by ISH and by immunohistochemistry with the MF20 antibody. The cells of the induced ectopic tissue adjacent to the bead developed myofibrils organized into sarcomeres, similar to those found in the cardiomyocytes of the primitive heart tube (Figure 2B). No ectopic tissue or ectopic expression of cardiac genes was provoked by control beads (Figure 2A), ruling out the possibility that implantation of the bead alone triggered ectopic cardiogenesis. This also argues against the possibility that the bead acts as a physical barrier for an inhibitory signal, and suggests that exogenous l-DOPA and dopamine can stimulate cardiomyocyte differentiation. Preliminary studies show that dopamine beads also induce the expression of Bmp2 (see Supplementary material online, Figure S2), linking TH to early cardiac differentiation programmes.19

We further confirmed this hypothesis by blocking the endogenous synthesis of l-DOPA and dopamine. Accordingly, beads soaked in either 3-iodo-tyrosine (3I-Tyr, 1 mmol/L), an inhibitor of TH, or in meta-hydroxybenzylhydrazine (mHBH, 1 mmol/L), an inhibitor of l-DOPA decarboxylase (Figure 1A), were implanted medial to one of the bilateral heart fields of cultured embryos at st. 5 (Figure 2A, red bead). Blockage of l-DOPA, and more clearly of dopamine biosynthesis, led to an ipsilateral decrease of AMHC1 expression in the endocardial tube at st. 8 (Figure 2C; see Supplementary material online, Table S2). These results suggest that dopamine synthesis is necessary for the correct expression of AMHC1.

### 3.3 Overexpression and knock-down of TH modified the limits of the cardiac chambers

To gain further insight into the role of TH in cardiac differentiation, we performed gain-of-function experiments. A bicistronic construct containing the chicken TH cDNA and the GFP cDNA (pCAGs-TH-I-GFP) was injected and electroporated into the cardiac progenitor cells that were migrating through the primitive streak of st. 3 embryos. As a control, we used a vector containing the GFP cDNA alone (pCAGs-I-GFP). Analysis of TH expression in pCAGs-TH-I-GFP electroporated embryos confirmed that TH mRNA and protein coincided with GFP expression (see Supplementary material online, Figure S3). In addition, there was an increase in l-DOPA levels measured by HPLC in embryos overexpressing TH (data not shown). Consistent with the myocardigenic stimulatory effect of the l-DOPA and dopamine beads (Figure 2A), the embryos overexpressing TH displayed a marked increase in AMHC1 expression when compared with control electroporated or non-electroporated embryos, particularly at the stages of heart tube fusion and looping (st. 10 and 12, respectively; Figure 3A). In addition, the domain of AMHC1 expression at the looped heart tube stage (st. 12) had expanded abnormally towards the anterior pole of the primitive heart tube (Figure 3A). To further characterize the changes in the developing heart, we analysed the expression of Tbx5. This T-box family transcription factor is expressed in cardiac progenitors and, as development proceeds, it is found in a graded fashion along the heart tube with the highest levels in the sino-atrial region.14–16 Significantly, the pattern of Tbx5 expression in these tissues very closely resembles that of TH and AMHC1 (see Supplementary material online, Figure S1). Like AMHC1 expression, Tbx5 expression expanded abnormally towards the anterior region of the heart tube in TH-overexpressing embryos (Figure 3A, see Supplementary material online, Table S3). Moreover, RT-qPCR analysis of dissected ventricles showed a six-fold increased of AMHC1 levels in TH overexpressing embryos when compared with control electroporated ventricles (Figure 3B). Tbx5 expression increased more modestly (not statistically significant) similarly to what was also observed by ISH.

We then analysed the effect of TH on the expression of the ventricular myosin heavy chain, VMHC1, initially expressed throughout the entire heart tube and down-regulated in the atria after the looping stage (st. 12–13).11,12,20 VMHC1 became restricted to the anterior region of the heart tube of TH-overexpressing embryos (Figure 3C, see Supplementary material online, Table S4). Likewise, the expression of the transcription factor Irx4, which is involved in specifying the prospective ventricular region,20 regressed anteriorly (Figure 3D), suggesting that TH confers a posterior character on the looping heart tube.

Overexpression of TH also had major functional consequences, since TH-electroporated embryos displayed characteristics compatible with bradyarrhythmia (see Supplementary material online, movie online). When recorded in culture, the heart rate of control electroporated embryos at st. 11 was 100 beats per minute (bpm), compared with 53 bpm in TH-overexpressing embryos (Table 1). In addition, TH-electroporated embryos displayed arrhythmic heart beats (see Supplementary material online, movie online).

To further confirm that TH is a fundamental element in cardiac development, we knocked down TH expression. A TH MO or luciferase, control MO were electroporated into the cardiac progenitor cells migrating through the primitive streak of st. 3 chick embryos. The TH morphants displayed a decrease in the expression of the atrial markers AMHC1 and Tbx5 (Figure 4A). Conversely, the expression of VMHC1 in the most affected embryos was increased (Figure 4A). In parallel, the silencing of TH expression diminished the atrial segment area and oversized the ventricular segment (Figure 4B, see Supplementary material online, Table S5). Similar anterior–posterior heart patterning disruption was observed when RA signalling was inhibited.21 Additionally, the progress of cardiac morphogenesis was very limited: TH morphants displayed a globular cardiac tube that did not form a fully looped tube (Figure 4B), and the dysmorphic hearts barely beat (data not shown).
Figure 3  Effect of TH on anterior–posterior heart tube patterning and specification of chambers. (A) Effect of TH gain-of-function on sino-atrial gene expression. Whole-mount ISH for AMHC1 and Tbx5 in non-electroporated embryos, and in embryos electroporated with either the control construct (pCAGs-I-GFP) or with the TH expressing construct (pCAGs-TH-I-GFP). The anterior expanded expression of AMHC1 and Tbx5 in the heart tube is indicated by the black arrows. Visualization of GFP expression for the embryos processed for ISH is shown in the corresponding left panels. (B) RT–qPCR of RNA from ventricles (V in scheme) of st. 12 embryos electroporated with either the control construct (pCAGs-I-GFP) or with the TH expressing construct (pCAGs-TH-I-GFP). The levels of AMHC1 and Tbx5 mRNA were normalized to GAPDH mRNA levels. The results represent the mean ± SEM of three pools of four ventricles each. *P < 0.01 with respect to pCAGs-I-GFP electroporated embryos. (C) Effect of TH gain-of-function on ventricular gene expression. Whole-mount ISH for VMHC1 and Irx4 in embryos as in (A). The posterior regression of VMHC1 and Irx4 expression is indicated by the red arrows. In supplementary materials this figure is reproduced including GFP expression (see Supplementary material online, Figure S4).
Table 1  Effect of TH overexpression on heart rate

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<th>Non-electroporated (n = 10)</th>
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<th>pCAGs-TH-I-GFP (n = 10)</th>
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<td>100.6 ± 3.53</td>
<td>100.5 ± 3.02</td>
<td>53.1 ± 2.68</td>
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BMP, Beats per minute. The results represent the mean ± SD.

Figure 4  Effect of TH knock-down on anterior–posterior heart tube patterning and specification of chambers. (A) Whole-mount ISH for AMHC1, Tbx5, and VMHC1 in embryos electroporated with either luciferase MO or with TH MO. Note also the decrease in AMHC1 and Tbx5 expression in the TH MO treated (arrow) versus the luciferase MO control (arrowhead). On the contrary, VMHC1 expression did not change or showed a mild increase. (B) Light microscopy of st. 10 and 12 non-electroporated or electroporated embryos with either luciferase MO or with TH MO. The heart tube of the TH morphants show abnormal morphogenesis displaying an atrophic sino-atrial region and oversized ventricular region (in embryos with comparable number of somites and similar prosencephalon development). The ventricular segment is outlined in blue and the sino-atrial segment in yellow.
3.4 TH action is linked to RA patterning effect

To integrate these findings into what is already known about heart morphogenesis; we turned to the effect of RA known to be involved in cardiac patterning. In vertebrates, the anterior–posterior identity of the primitive heart tube is established by RA signalling. The documented posteriorization of RA and that of TH described here, together with the fact that TH gene expression and activity are positively regulated by RA in the nervous system and adrenal gland, suggested that TH might be a putative downstream target of RA activity in establishing the anterior–posterior heart tube axis.

We tested this hypothesis by implanting beads soaked in RA (10 µg/mL) into the heart field of cultured embryos at st. 5. RA expanded the expression of TH mainly in the ipsilateral inflow tract, as well as rostrally within the heart tube. A parallel effect was observed on the expression of AMHC1 and Tbx5 (Figure 5A, see Supplementary material online, Table S6). To further support that TH expression is under RA control, we inhibited endogenous RA synthesis with citral. Accordingly, cultured embryos treated at st. 5 with citral (10 mmol/L) displayed a dramatic decrease in TH expression parallel to diminished AMHC1 and Tbx5 expression (Figure 5B, see Supplementary material online, Table S7).

**Figure 5** TH, AMHC1, and Tbx5 expression are controlled by RA. (A) Increased TH, AMHC1, and Tbx5 expression by RA. St. 5 embryos with a bead soaked in either DMSO (vehicle) or RA implanted lateral to one of the bilateral heart fields (yellow bead) were then analysed by whole-mount ISH at st. 12. The expanded expression of TH, AMHC1, and Tbx5 in the heart tube and in the inflow tract are indicated by arrows, and their normal expression domains are outlined by yellow dotted-lines. (B) Decreased TH, AMHC1, and Tbx5 expression by RA synthesis inhibition. St. 5 embryos were treated with ethanol (vehicle) or 10 mmol/L citral and analysed by ISH at st. 10–11. (C) Scheme of the interplay of RA and TH in chamber-specific genes regulation.
4. Discussion

The results presented here indicate that TH/ L-DOPA/dopamine are involved in the network of signals that drive cardiac precursor cells to a sino-atrial fate, specifically by regionalizing Tbx5 and AMHC1 expression to the posterior part of the heart tube.

In st. 5 chick embryos, TH mRNA was enriched in the cardiac field; by st. 8, TH expression localized to the splanchnic mesoderm of endocardial tubes and it progressively became restricted to the sino-atrial region. Perturbing graded TH expression altered atrial and ventricular myosin segregation. Thus, whereas an increase in TH levels resulted in expansion of the AMHC1 and Tbx5 atrial domains and regression of the VHMC1 and Irx4 ventricular domains, loss of TH activity decreased AMHC1 and Tbx5 expression, and occasionally increased VMHC1 expression. The less widespread VMHC1 expansion phenotype indicates that other factors are probably involved in restricting VMHC1 expression. Significantly, it has been shown that the graded expression of Tbx5 is crucial for the correct cranio-caudal patterning of the primitive heart tube, since disruption of this pattern results in abnormalities in heart chamber formation. Indeed, the Holt–Oram syndrome in humans (with structural cardiac and conduction anomalies) is associated with mutations in the Tbx5 gene.

The graded expression of Tbx5 and the posterior identity of the primitive heart tube are maintained by RA. An excess of RA causes expansion towards the anterior region of genes that are normally concentrated to the posterior region (i.e. Tbx5 and AMHC1), together with hyperplasia of the sino-atrial region. Conversely, inhibition of RA signalling impairs development of the sino-atrial region and enlarges that of the ventricular region. In our model system, we uncover a link between RA and TH action as suggested by the parallelism in the effect of blockage of endogenous TH expression and inhibition of RA signalling, together with the fact that RA controlled TH expression. Thus, according to these results, TH appears to reinforce the genetic program of the sino-atrial region, in a scenario where RA modulates the graded TH expression in the heart tube. In concert, TH activity favours the restriction of Tbx5 and AMHC1 expression to the posterior heart tube, whereas suppressing Irx4 and VMHC1 in the prospective atria.

Alteration of TH expression (gain or loss) had also important functional consequences. Increased TH expression led to low rate and arrhythmic heart beating whereas loss of TH activity caused partial looping and discontinuous beating of the heart tube. The cause of this abnormal functionality is an open question. Previous studies have shown that restricting expression of contractile proteins to the ventricular and atrial chamber, including that of the myosin heavy and light chains, is essential for normal cardiac function. Given that TH overexpression alters the distribution of atrial and ventricular myosin heavy-chain isoforms, this could be at least partially responsible for some of the heart function abnormalities found in the TH-overexpressing embryos. Nevertheless, we are tempted to speculate that TH might be involved in specifying the cardiac pacemaker of the embryonic heart. In the chick, the pacemaker differentiates at around st. 9–10 in the posterior most segment of the primitive heart tube, assuring the rhythmic propagation of the action potential with posterior–anterior polarity. The bradyarrhythmia of the TH-overexpressing embryos, together with the restriction of TH expression to the sino-atrial region, are compatible with the interpretation that the gradient of TH activity in the primitive heart tube may be part of the signals leading to the dominance of the pacemaker in the posterior tube. This phenomenon could also be responsible for the development of the prospective sino-atrial node responsible for the pacemaker activity in the adult heart. This hypothesis is consistent with Pollack's proposal several decades ago (reviewed by Ebert) that catecholamines are essential for cardiac pacemaker specification. Future experiments analysing the expression of early pacemaker genes, among others, should clarify the role of TH in the pacemaker specification.

Generation of the catecholamine depleted mice models (TH and DBH knockouts) have more recently shown that catecholamines are essential for embryo survival beyond mid-gestation. The catecholamine deficient mice die from apparent heart failure starting at E11.5–12.5, and the heart of surviving embryos is able to beat autonomously with slight bradycardia. Albeit the penetrance of the lethal phenotype was variable and some null-mice survived to term, recent reports have shown that catecholamines, in particular noradrenaline, mediate foetal survival by maintaining oxygen homeostasis in mid-gestation. Moreover, restoration of noradrenaline, although not of dopamine, synthesis in the noradrenergic cells is sufficient to prevent lethality. Using the chick embryo as a maternally independent vertebrate model, we show that catecholamines, in particular dopamine, also have an earlier role in cardiac morphogenesis. Similar to our results in the chick embryo, in the study by Thomas et al., dopamine was the only catecholamine detected in E9.5 mouse embryos (long before the lethality occurred). In fact, we could detect TH expression in the whole mouse embryo since gastrulation stage at E6.5 and in the heart since E8.5 (the earliest stages analysed) (see Supplementary material online, Figure S3). Our results are compatible with the findings in mice: dopamine plays an important role in early cardiac tube formation, whereas noradrenaline can be essential for mid-gestation foetal survival. Moreover, the apparent absence of major histological alterations in the catecholamine null-mice could be due to the presence of compensatory factors, including a potential contribution of maternal catecholamines or to species variation. A detailed characterization of the phenotype in mouse organogenesis would be required to unravel the role of all catecholamines in mouse cardiogenesis.

Here, we have shown a strong concordant phenotype in TH-gain and loss-of-function experiments in chick embryos. The remarkable change in the pattern of electrical activation in the TH-overexpressing hearts is also consistent with the identification of intrinsic catecholamine-synthesizing cardiac cells, in human and rodent hearts, prior to inervation. In mice and rats, cardiac catecholaminergic cells appear to be associated with the pacemaker and conduction system in four-chamber hearts. The three-dimensional requirements of cardiac development need complex signalling networks to lead cells through the appropriate fate decisions and morphogenetic movements. In addition, antero-posterior polarity plays a role in the coupling between heart and blood vessels. Here, we reveal a novel function of TH in cardiac development, and suggest that TH may be a key player acting in concert with additional factors to define multiple aspects of chamber identity, including pacemaker specification. These results are consistent with the paradigm whereby molecules that act as intercellular signalling mediators, with well-defined restricted roles in postnatal organisms, are present at embryonic stages, when they participate in diverse functions often unrelated to their later roles (reviewed in ). The relevance of TH in human cardiogenesis and the possible significance
of TH pathway alterations in cardiac syndromes may be a field deserving further studies.

Supplementary material
Supplementary material is available at Cardiovascular Research online.

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