

Characterization of serotonergic cells in fetal heart tissue

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Abstract

Background: We undertook this study to present biochemical and morphological characterization of serotonergic cells during fetal heart development.

Methods: Wistar rats (10, 12, 16 and 20 days of gestation) were used. After obtaining the fetuses by Cesarean section, the hearts were removed and fixed for immunohistochemical assay of tryptophan-5-hydroxylase (Tph), in addition to Western blot for enzyme. Serotonin concentration and Tph were also evaluated with high-performance liquid chromatography. Results were analyzed using Mann-Whitney U test with a significance level of $p < 0.05$.

Results: Metachromatic cells immunoreactive for Tph were observed from day 10 of gestation. Nerve fibers were also labeled, apparently making contact with serotonergic cells. Tph activity was measurable and serotonin levels increased with gestational age. The presence of Tph protein was confirmed by Western blot on day 16.

Conclusions: The present results support the existence of cells located in the fetal myocardium, capable of producing serotonin whose phenotype belongs to cardiac mast cells. Their presence in this tissue strongly suggests that serotonin may play a key role in normal and abnormal development of cardiac tissue.

Key words: fetal heart, serotonin, tryptophan-5-hydroxylase, serotonergic cells.

Introduction

Congenital cardiac diseases are the leading cause of children morbimortality in children.¹ They frequently start from interaction of environmental and genetic susceptibility factors.^{2,3} In order to better understand the pathophysiology of congenital cardiac

diseases, it is important to identify factors that regulate myocardial proliferation and differentiation.⁴⁻⁶ Evidence suggests 5-hydroxytryptamine (5-HT, serotonin) regulates cardiovascular functions during embryogenesis and adulthood.⁷⁻¹⁰ Serotonin, its receptors as well as serotonin transporter (SERT), have been identified in neuroepithelium and myocardium in mice at 8 days of gestation.^{7,11-18} Also, miscellaneous congenital malformations in head, neural tube and heart have been observed in mice embryos cultured in the presence of 1 μ M ritanserin (a serotonergic antagonist).^{8,12,13,19} The most frequently observed heart malformations are absence of myocardial trabeculation, thin ventricular wall, alterations in sarcomere organization, epicardial dilation, reduced cardiocyte proliferation, alterations in cardiac mesenchyme and vascular endothelium.^{8,9} These malformations have also been observed in embryos cultured in the presence of serotonin reuptake inhibitors.^{8,12,15} Likewise, they have been observed in transgenic animals lacking the gene that codes 5-HT_{2B} receptor, which binds to Gq proteins and RAS signaling pathway and activates protein kinase c and MAP protein kinase.^{8,12,15} These findings, as a whole, strongly suggest that serotonin actively participates in cardiocyte proliferation and differentiation during embryonic heart development.^{7,11-18} However, even though it has been demonstrated that serotonin and its receptors are present during cardiogenesis, it has not been identified when and where their production starts. It has been suggested that serotonin is obtained from maternal bloodstream during early gestation stages

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or that it is produced in neural crest cells that migrate to peripheral tissues, especially to digestive tube, suprarenal glands, kidneys and possibly to lungs. Serotonin would be released into the interstitial tissue, taken up, stored and transported by platelets into peripheral tissues. Platelets would then release it through an active transport mechanism and immediately there would be reuptake of serotonin by endothelial cells from blood vessels and neurons in cardiac tissue.^{8,9,16} We propose that, during embryonic development of the heart, serotonergic cells appear in cardiac structures and serotonin synthesized there participates in proliferation and differentiation of myocardiocytes. This is our hypothesis, and we evaluate it in the current investigation immunocytochemically and biochemically, characterizing serotonergic cells during fetal development of cardiac tissue.

Materials and Methods

We used Wistar rats (males and nulliparous females) provided by the Unit of Production and Experimentation of Laboratory Animals (UPEAL) from CINVESTAV-IPN in Mexico City. Rats were maintained under controlled environmental conditions at $22 \pm 2^\circ\text{C}$, with 12 h light/darkness cycles (7:00-19:00 h and 19:00-7:00 h, respectively) and 50%-60% relative humidity. Rats were fed with a standard balanced diet and water ad libitum. Lab Diet 5P14 for rodents (PMI Feeds Inc., Richmond, IN) contains 23% proteins, 49% carbohydrates, 4.5% fat, 6% and 8% ash, yeast, vitamins and minerals (lysine, thiamine, α -tocopherol, calcium, ferrous sulfate, manganese, and other micronutrients) and provides a caloric density of 4.05 kcal/g.

Rats were mated after an adaptation period, two females (2500 ± 10 g body weight) for each male (250 g body weight). After mating, the presence of a vaginal plug and measurement from head to sacrum allowed determination of gestational age with a 6 h variation. During gestation, rats were kept under the same environmental and nutritional conditions. Fetuses were obtained through Cesarean section at 10, 12, 16 and 20 gestation days. Heart was removed from fetuses immediately to perform biochemical and immunocytochemical assays. Management of experimental animals was carried out at UPEAL from CINVESTAV-IPN according to sanitary and ethical norms from the Internal Committee for Care and Use of Laboratory Animals (CICUAL) according to Mexican norm (NOM-062-ZOO-1999) published in the Official Journal of the Federation (August 22, 2001).

Biochemical Assays

Tph immunocytochemistry

Fetal hearts were obtained at 10, 12, 16 and 20 gestation days. They were fixed with 10% formol diluted in PBS, 0.1 M, 7.4 pH. Hearts were then dehydrated and placed in paraffin to obtain

4- μm sections and these were placed on 10% poly-L-lysine-coated slides. Once paraffin was removed, antigenic unmasking was done through a citrate buffered solution (0.1 M), pH 6.0 (Declere, Cell Marque Corp., Rocklin, CA) using a microwave oven and three 2-min cycles. Endogenous peroxidase activity was inhibited immediately with 5% H_2O_2 for 15 min. Once sections were washed, these were incubated with specific monoclonal Tph primary antibody at a 1:500 dilution (Monoclonal Anti-tryptophan hydroxylase Clone WH-3, Sigma, St. Louis, MO) in a PBS solution (0.1 M), pH 7.4, Triton X-100 at 0.3 and 3% horse serum for 18 h at 30°C . The following day, sections were washed and incubated with secondary antibody diluted in PBS and Triton X 100 (biotinylated antibody against goat IgGs) for 30 min. After that, sections were incubated with avidin-biotin complex for 15 min at room temperature. They were washed and peroxidase activity was revealed using a commercial kit with 3,3'-diaminobenzidine and H_2O_2 according to the protocol by Vector Laboratories (Burlingame, CA).²⁰ Photomicrographs were taken using a digital camera (Coolpix 995, Nikon, Tokyo, Japan).

Tph activity from high-performance liquid chromatography (HPLC)

Tph activity was evaluated by determining formed 5-hydroxytryptophan through HPLC (Waters S.A de C.V, Mexico City) using a fluorometric detector (Waters 474 scanning fluorescence detector).^{21,22} In short, this method consist of incubating 300 μg of enzymatic protein in the presence of a buffering solution: 50 mM Tris-HCl, pH 7.40, 1.0 mM EGTA, 15 μg catalase, 200 μM 2-amino-4-hydroxy-1-methyl tetrahydrobiopterin. Reaction was incubated at 37°C for 10 min and was stopped by the addition of HClO_4 6 M > 5 mM EDTA and 0.1% ascorbic acid. Twenty milliliters of reaction media was injected for HPLC. A C_{18} symmetrical column was used (5- μm particle size, 3.9×150 mm) (Waters S.A de C.V). Mobile phase was prepared using 40 mM sodium acetate, pH 3.30 and acetonitrile in a 95:5 ratio, respectively, and was run at 1 ml/min. Excitation and emission wavelengths used to detect formed 5-hydroxytryptophan were 280 nm and 340 nm, respectively. Retention time for 5-hydroxytryptophan was 2.3 min and 5.5 min for L-tryptophan.

HPLC serotonin determination

5-HT in hearts was determined through HPLC according to the method of Peat and Gibb.²³ Hearts were deproteinized using a 0.1 N HClO_4 solution plus 4 mM sodium metabisulfite with a 1:3 weight/volume ratio. Hearts were homogenized using a Teflon plunger (Glas-Col Instruments, Terre Haute, IN) at $1000 \times \text{g}$ for 30 sec. Homogenized solution was centrifuged at $10,000 \times \text{g}$ for 15 min at 4°C . The supernatant was then removed and filtered in a nylon membrane with a 0.45- μm pore size. Afterwards, 20- μl from filtrate was injected into HPLC using a C_{18} reverse-phase

symmetry column with 5 μm particle size, 3.9×150 mm in length. We used a binary system with 2 mM monobasic potassium phosphate solution, pH 3.5, plus 1 g/L heptanesulfonic acid solution and a methanol/water mix with a 3:2 v/v ratio applying 1 mL/min. 5-HT determination was carried out using a fluorescence detector (Waters 474) at 290 nm excitation and 330 nm emission. Response was obtained through an analogic system (Matrox, Millennium 32, Quebec, Canada). 5-HT concentration was considered as the maximum signal height according to a standard curve of known 5-HT quantities (expressed as ng/g tissue).

Tph Western blot immunotransference

Fetal hearts were homogenized for 30 sec at 4°C in a 50 mM Tris-HCl (pH 7.4) solution. Samples were then centrifuged at $29,000 \times g$ for 15 min at 4°C . Protein concentration was determined using the method of Lowry et al.,²⁴ placing 30 μg protein in each channel of a 10% SDS-polyacrylamide gel with 1-mm thickness. Electrophoresis was carried out at 100 V for 1 h at 5°C . For electrotransference of proteins, gel was mounted in nitrocellulose membranes (0.45 μm), and transference was carried during the night at 25 V.²⁵ Nitrocellulose membranes with transferred proteins were placed in a blocking solution based on 1% skimmed milk diluted in 0.1 M PBS, pH 7.4, plus 0.1 M Tween-20 for 30 min. Membranes were then incubated with specific monoclonal Tph primary antibody at a 1:500 dilution (Monoclonal Anti-tryptophan hydroxylase Clone WH-3, Sigma) in the same blocking solution. The following day, membranes were incubated with secondary anti-mouse antibody (Chemicon, Temecula, CA) at a 1:5000 dilution in the same blocking solution. Soon afterwards, membranes were incubated with ABC Elite system (Vector Laboratories) for 1 h. After washing membranes with PBS, they were revealed through chemiluminescence with Amersham ECL system (Amersham, Buckinghamshire, UK).

Protein determination through spectrophotometry

Fetal heart proteins were determined by the method of Lowry et al.²⁴ Homogenized solution of the heart was diluted 40 times with bidistilled water, and 0.1 ml was taken from this dilution. Reaction began when adding 0.5 ml of reactive A, which contained 0.5 ml Cu_2SO_4 at 1% and 0.5 ml of potassium sodium tartrate at 2% in 50 ml Na_2CO_3 at 2%, diluted in NaOH at 0.1 N. The mixture was vigorously stirred and left to rest at room temperature for 10 min. Then, 0.05 ml reactive B was added, which contained Folin reactive at a 1:1 dilution with bidistilled water. Samples were stirred and 30 min later we measured absorption in a Beckman spectrophotometer at 700 nm wavelength. We included a standard curve of known albumin concentrations in each test. Protein concentration in heart was expressed as mg/g tissue.

Statistical Analysis

To compare results of specific Tph activity and serotonin concentration in heart tissue at 16 and 20 days of gestation, we first obtained averages and standard deviations from each of them. We then compared Tph activity expressed as fmol 5-HTP/mg protein/h at 16 days of gestation vs. values obtained from samples of 20 days of gestation. Likewise, we analyzed serotonin concentration in cardiac tissue using Mann-Whitney U test because values for enzyme activity and serotonin concentration did not present a normal distribution; $p < 0.05$ was accepted as statistically significant.

Results

To confirm antibody specificity against Tph, we used brainstem sections from adult rats as control, where serotonergic cells are located. Figure 1 shows these neurons and enzyme-immunoreactive nerve fibers stained with 3,3'-diaminobenzidine.

Fetal heart sections presented Tph immunoreactive cells between endocardium and myocardium with intracytoplasmic enzyme granules from the 10th day of gestation (Figures 2A and 2B). Figures 2C and 2D show Tph immunoreactive cells at 16 and 20 days of gestation that present fusiform morphology and are located around myocardiocytes. These Tph immunoreactive cells are observed with higher definition in endocardium at 20 days of gestation (Figure 2D).

We also observed Tph immunoreactive cells at 20 gestation days (Figure 3A). These are ovoid cells and some present a higher quantity of intracytoplasmic granules located along lines that follow

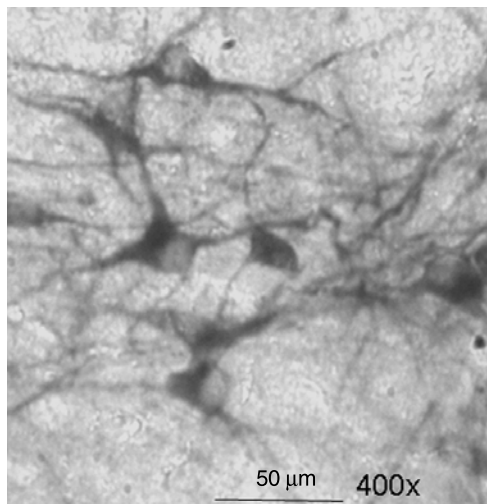


Figure 1. Tph immunoreactive serotonergic neurons in brainstem nuclei from adult rat. Stained with 3,3'-diaminobenzidine.

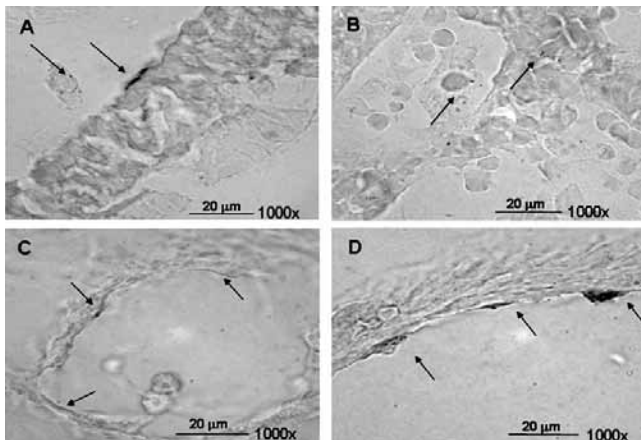


Figure 2. Tph immunoreactive cells in rat fetal heart. Longitudinal sections correspond to (A) 10, (B) 12, (C) 16, and (D) 20 gestation days. Dates show enzyme immunoreactive cells located in fetal myocardium and endocardium.

blood vessels and myocardiocyte pathways. Figure 3B shows cells located around blood vessels in cardiac interstices. It is interesting to observe that when counterstaining Tph immunoreactive cells with toluidine blue, they present metachromasia with multiple intracytoplasmic granules (Figure 3C). Note that in addition to Tph immunoreactive cells there are also Tph immunoreactive nervous fibers that follow blood vessels and myocardiocyte pathways, giving the impression of making contact between the aforementioned cells and nerve fibers.

Specific Tph activity in fetal heart at 16 days of gestation is shown in the representative chromatogram (Figure 4A, blue line). Standard L-tryptophan (L-Trp) and 5-hydroxytryptophan (5-HTP) values are shown with a black line. Enzymatic activity expressed in fmol 5-HTP/mg protein/h showed an ascending developmental pattern of 14 ± 2.8 fmol at 16 days of gestation and of 62 ± 7.6 fmol at 20 days of gestation ($p < 0.001$) (Table 1). In

Table 1. Serotonergic activity in rat fetal heart

Fetal age (days)	Tph	5-HT
16	14 ± 2.8	$50 \pm 10^*$
20	62 ± 7.6	$110 \pm 15^*$

Tph, tryptophan-5-hydroxylase activity in nmol of 5-HTP/mg protein/hr; 5-HT, serotonin concentration in ng/mg of tissue.

Each value represents the average \pm SD of four experiments performed twice. Statistical difference between 16 and 20 gestational days of each biochemical parameter was calculated using Mann-Whitney U test.

* $p < 0.001$.

order to evaluate Tph expression in fetal heart, we carried out Western blot using brainstem tissue from adult rats as control. Brainstem sections showed only one 56-kDa band of Tph (Figure 4Ba), whereas fetal heart at 16 days of gestation showed two immunoreactive enzyme bands of 53 and 56 kDa (Figure 4Bb).

Serotonin in fetal heart at 16 days of gestation is shown in the representative chromatogram (Figure 5, red line). Standard values for L-Trp, 5-HT and 5-hydroxyindole acetic acid (5-HIAA) are represented in black. Note there was a significant increase in serotonin quantity in fetal hearts between 16 and 20 days of gestation ($p < 0.001$) (Table 1).

Discussion

Our study hypothesis was to demonstrate the presence of serotonergic cells during cardiac tissue development. In fact, morphological findings confirm that, during cardiogenesis, there are immunoreactive cells for tryptophan-5-hydroxylase (serotonin synthesis-limiting enzyme) from day 10 of gestation. These Tph immunoreactive cells are distributed in all fetal heart layers and mainly around blood vessels. Because of their location and presence of multiple intracytoplasmic granules during the period between days 16 and 20 of gestation, we considered the possibility that

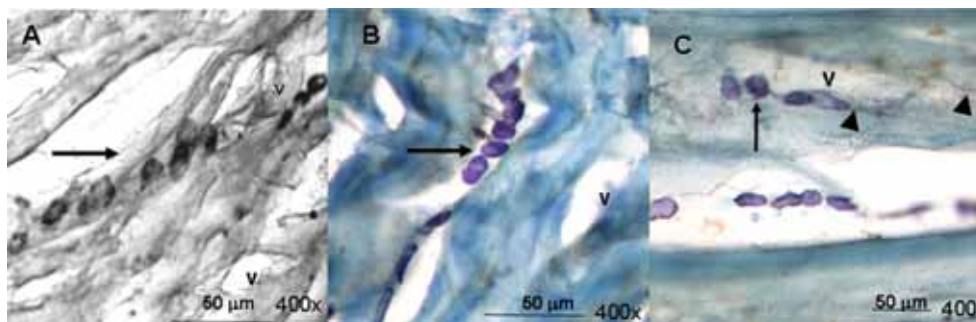


Figure 3. Tph immunoreactive cells in rat heart at 20 days of gestation. (A) Tph immunoreactive cells stained with 3,3'-diaminobenzidine. (B,C) Immunoreactive cells stained with 3,3'-diaminobenzidine and toluidine blue. Note that Tph immunoreactive fibers are in contact with immunoreactive cells (arrowhead) and follow the same pathway as blood vessels (v).

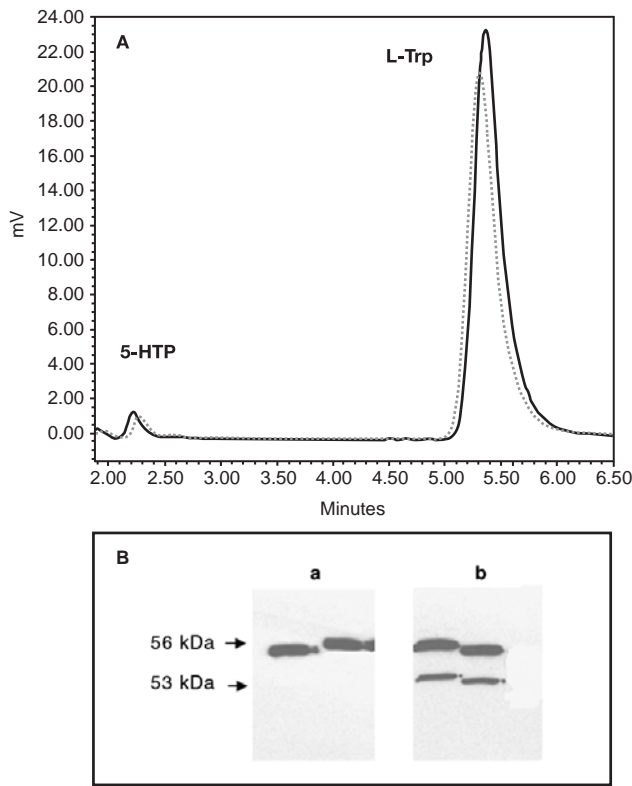


Figure 4. Tph specific activity and enzymatic protein expression from Western blot in rat fetal heart. (A) Representative chromatogram of standard 5-HTP and L-Trp (black line) and Tph specific activity in fetal heart at 16 days of gestation (blue line). (B) Tph expression detected through electrotransference and immunodetection from Western blot with enzyme-specific antibodies. (a) Adult rat brainstem with only one 56-kDa band. (b) Fetal rat heart at 16 days of gestation with two bands (53 kDa and 56 kDa).

they were mast cells. We counterstained fetal sections with toluidine blue to confirm this possibility and we observed that Tph immunoreactive cells showed metachromasia, just as in adult mast cells.²⁶⁻²⁸ These findings support the existence of mast cells that produce serotonin during cardiogenesis and that possibly participate actively in myocardiocyte differentiation, which we are evaluating in our laboratory. On the other hand, we do not know if during early cardiogenesis stages, serotonergic phenotype is expressed first, followed by mast cell differentiation, or if both phenotypical characteristics appear simultaneously, which would require further study.

Mast cells uptake, synthesize, store and release several neuropeptides and neurotransmitters such as neurotensin, somatostatin, substance P, serotonin and acetylcholine.²⁸⁻³⁰ Also, mast cells have a close relationship with peripheral nerve fibers. These interactions have been demonstrated in diaphragm, brain, bladder, cecum, mesentery and skin.³¹ It is important to highlight that, in the

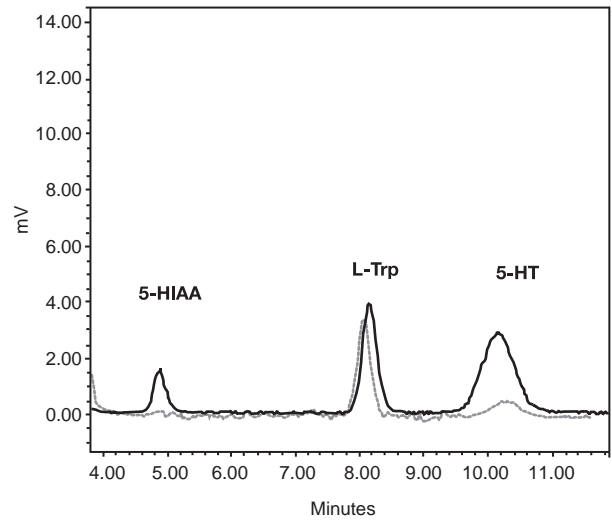


Figure 5. Serotonin concentration in fetal rat heart. Chromatogram shows a representative run of standard 5-hydroxyindole acetic acid (5-HIAA), L-L-Trp and 5-HT (4 ng/μL) (black line) compared to 5-HT (red line) concentration obtained from fetal rat heart at 16 days of gestation, which shows the same retention time as standard 5-HT.

present study, we observed such interactions between nerve fibers and Tph immunoreactive mast cells. This strongly suggests that interactions between serotonergic mast cells and nerve fibers may be actively participating in myocardiocyte proliferation and differentiation. In fact, higher embryonic and neonatal mortality has been observed in transgenic animals that lack 5-HT_{2B} receptor because they present multiple congenital heart malformations. Animals that survive have thinned ventricular walls because of damage during myocardiocyte proliferation and sarcomere disorganization at compact myocardium, as well as myocardial trabeculation defects.^{8,12,15} As a whole, these results suggest that 5-HT participates in regulation of myocardiocyte proliferation and differentiation through 5-HT_{2B} receptor activation.^{8,12,15} Also, several pharmacological studies have associated cardiovascular function with the following receptors: 5-HT_{1A}, 5-HT_{1B}, 5-HT_{2A}, 5-HT₃, 5-HT₄, and 5-HT₇.^{32,33} These receptors are located in nerve terminals or on myocardium and their role remains to be investigated.^{18,32,33} In the current study we were able to confirm Tph activity as well as the presence of serotonin in fetal cardiac tissue. We also identified two bands from enzyme proteins (53 and 56 kDa) using Western blot, in contrast to brainstem from adult animals (control) where only one 56-kDa band was found. This finding has also been demonstrated by other authors.³⁴ This suggests that fetal heart presents two Tph isoforms that could match the two isoforms described in adult tissue.

In conclusion, these results demonstrate the presence of serotonergic cells during cardiac tissue development, appearing to be cardiac mast cells.

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