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Expression of the Cholinergic Signal-Transduction Pathway Components During Embryonic Rat Heart Development

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ABSTRACT **Background:** Previous studies showed that acetylcholinesterase (AChE) activity is present in the downstream (arterial) part of the embryonic chick and rat heart, but its functional significance was unclear. To establish whether other components of a cholinergic signal-transduction pathway are present in the embryonic heart, we localised the mRNAs encoding choline acetyltransferase (ChAT), acetylcholinesterase (AChE), and the muscarinic receptor isoforms (mAChRs; m1–m5).

Methods: Messenger RNA detection and localisation by in situ hybridisation and reverse transcriptase-polymerase chain reaction were employed.

Results: Expression of ChAT and AChE mRNAs was observed from 15 embryonic days onward in the neural tissue covering the dorsocranial wall of the atria. Muscarinic receptors (m1, m2, m4) were observed at the same localisation as AChE and ChAT mRNAs, both during embryogenesis and after birth. In addition, m1 and m4 mAChRs showed a low level of expression in the atrial myocardium during the fetal period. No expression of the m3 or the m5 mAChRs was observed in or near the embryonic hearts. ChAT, AChE, and mAChRs (m1, m2, m4) mRNAs always colocalised in the cardiac ganglia. However, none of these mRNAs was found at a detectable level in the outflow tract and/or the ventricular trabeculations.

Conclusons: The AChE activity in the arterial part of the embryonic heart is probably synthesised elsewhere and subserves a function different from the hydrolysis of locally produced acetylcholine. Anat. Rec. **248:110–120, 1997.** © 1997 Wiley-Liss, Inc.

Key words: **acetylcholinesterase; choline acetyltransferase; muscarinic acetylcholine receptors; in situ hybridisation; heart; rat; development**

Key proteins in the cholinergic signal-transduction pathway are the enzyme choline acetyltransferase (ChAT) that synthesises acetylcholine (ACh), the enzyme acetylcholinesterase (AChE) that hydrolyses ACh, and the acetylcholine receptors. ACh signals through two types of unrelated membrane receptors referred to as nicotinic (nAChR) and muscarinic (mAChR) acetylcholine receptors (see, e.g., Hosey, 1992). Muscarinic receptors mediate several actions of ACh in the central and peripheral nervous systems and in cardiac tissue. Muscarinic receptors are pharmacologically classified into three types designated as M1–M3, although five different genes (m1–m5) have been identified in rodents (Mei et al., 1989; Hulme et al., 1990). The M1, M2, and M3 types correspond to the m1, m2, and m3 molecular isoforms, respectively. In general, it is accepted that m2- and m4-encoded gene products are coupled to inhibition of adenylate cyclase, whereas m1-, m3-, and m5-encoded gene products are acting via stimulation of inositol triphosphate and diacylglycerol production (Hulme et al., 1990). In the adult heart, ACh released from parasympathetic nerve terminals acts on mAChR to decrease both the rate and the force of contraction by mediating the inhibition of adenylate cyclase (Tang and Gilman, 1992) and the stimulation of inositol triphosphate and diacylglycerol production (Barnett et al., 1990; Masters et al., 1985).

ChAT is the enzyme that catalyses the formation of the ACh. ChAT localisation is always restricted to neural tissue and, hence, is a more reliable marker to assess cholinergic innervation than is AChE (Eckenstein and Sofroniew, 1983; Appleyard, 1992). ChAT activity has been demonstrated in the rat heart from 19 embryonic days (ED) onward (Marvin et al., 1980), but no information is available concerning its cardiac localisation.

AChE activity has been detected in early embryonic rat (Lamers et al., 1987; Nakamura et al., 1994) and

chicken (Lamers et al., 1990) hearts, where it is expressed in the myocardial layer of the outflow tract and in the ventricular trabeculations. Its expression declines in the fetal period and disappears shortly after birth. Because these parts of the heart are characterised by a long duration of the contraction (De Jong et al., 1992; Moorman and Lamers, 1994), an ACh-dependent calcium mobilisation has been suggested to play an important role in those areas expressing high AChE activity (Lamers et al., 1990). In vitro experiments in embryonic chicken cardiac cells showed that ACh stimulation of mAChRs results in intracellular accumulation of inositol triphosphate (Oettling et al., 1989, 1992; Lohmann et al., 1991). These data suggest that these mAChRs belong to the m1, m3, or m5 subtype. Inositol triphosphate accumulation results in an increased intracellular calcium content that triggers contraction.

In this study we wished to test whether such a mechanism is effectively present in the embryonic rat heart. For that purpose, we studied the mRNA expression patterns of AChE, ChAT, and the five mAChRs isoforms described so far.

MATERIALS AND METHODS
Embryos

Wistar rat embryos were obtained from timed-pregnant rats (12–20 embryonic days; ED). Neonatal hearts (3, 8, and 10 days old) and adult hearts (3 months old) and adult spinal cord were also processed. Samples were fixed for 4 hr in 4% freshly prepared formaldehyde in phosphate-buffered saline (PBS) at room temperature (in situ hybridisation) or overnight in methanol:acetone:water (2:2:1; immunohistochemistry). The embryos and tissues were dehydrated in a graded series of ethanol and embedded in paraplast. Serial sections were cut at 7 μm thickness and mounted on RNAse-free 3-aminopropyltriethoxysilane (AAS)-coated slides (in situ hybridisation) or on polylysine-coated slides (immunohistochemistry).

In Situ Hybridisation

Complementary RNA probes of rat ChAT mRNA (Brice et al., 1989); human AChE mRNA (Soreq et al., 1990); rat β-myosin heavy chain (β-MHC) mRNA; sarcoplasmic reticulum calcium ATPase (SERCA2) (Moorman et al., 1995); glutamine synthetase (GS) mRNA (van der Zande, 1989); and human m1, m2, m3, m4, and m5 mAChRs (Peralta et al., 1987) mRNAs were radiolabelled with ^{35}S-UTP by in vitro transcription according to standard protocols (Melton et al., 1984). ChAT cDNA [EcoRI-NcoI; nucleotides (nt) 1–1870] was subcloned into pBluescript (Stratagene). AChE cDNA was linearised with BstXI (nt 1484–2227), BstEII (nt 822–2227), or Asp718 (full length) to assess the specificity of the probe. Linearisation of AChE cDNA with BstXI gave the lowest background signal and therefore was eventually used. Human m1 (StuI-SacI; nt 1259–1410), m2 (BamHI-RsaI; nt 995–1204), m3 (AvaI-AvaI; nt 1586–1975), m4 (HindIII-PstI; nt 867–1114), and m5 (AccI-TaqI; nt 1038–1308) fragments were subcloned into pBluescript (Stratagene) to obtain probes that were specific for each subtype.

The hybridisation conditions were as detailed elsewhere (Moorman et al., 1995). Briefly, the sections were deparaffinised, rinsed in absolute ethanol, and dried in an air stream. Pretreatment of the sections was as follows: 20 min 0.2 N HCl, 5 min bidistilled water, 20 min 2× SSC (70°C), 5 min bidistilled water, 2–20 min digestion in 0.1% pepsin dissolved in 0.01 N HCl, 30 sec in 0.2% glycine/PBS, twice for 30 sec each in PBS, 20 min of postfixation in 4% freshly prepared formaldehyde, 5 min in bidistilled water, 5 min in 10 mM EDTA, 5 min in 10 mM dithiothreitol (DTT), and finally drying in an air stream. The prehybridisation mixture contained 50% formamide, 10% dextransulphate, 2× SSC, 2× Denhardt's solution, 0.1% Triton X-100, 10 mM DTT, and 200 ng/μl heat-denatured herring sperm DNA. The sections were hybridised overnight at 52°C and washed as follows: a rinse in 1× SSC; 30 min at 52°C in 50% formamide dissolved in 1× SSC; 10 min in 1× SSC; 30 min in RNAse A (10 μg/ml); 10 min in 1× SSC; 10 min in 0.1× SSC; and dehydration in 50%, 70%, and 90% ethanol containing 0.3 M ammonium acetate. The sections were dried and immersed in nuclear autoradiographic emulsion (Ilford). The exposure time ranged from 10 to 24 days and the development time from 4 to 8 minutes as indicated. Photographs were taken with a Zeiss Axiophot microscope, using Agfa 25ASA films.

Reverse Transcriptase-Polymerase Chain Reaction (RT-PCR)

Embryos ranging from 13ED to 20ED were used to isolate total RNA according to Chomszynski and Sacchi (1987). Embryos were excised from the uterus in ice-cold sterile PBS, and the hearts were removed and the atria and ventricles were dissected separately. Total RNA from embryonic ventricles at different stages, 16ED rat spinal cord (16SP), adult spinal cord (SP), adult ventricles (AV), and adult liver (L), were isolated. 13ED and 14ED embryos from two different litters were pooled. RNA samples were stored (−20°C) in TE containing 0.01% sodium dodecyl sulfate (SDS). The single-step RT-PCR reaction according to Aatsinki et al. (1994) was performed in 50 μl total volume containing 3 μg total RNA, 100 pmol of each oligonucleotide, 100 μM dNTPs, 100 mM Tris HCl buffer (pH 8.2), 0.01% bovine serum albumin (BSA), 1.5 mM MgCl$_2$, 10U AMV-RT (Promega), and 2.5 U Taq polymerase (Eurogentec, Berse, Belgium). Briefly, an initial annealing step (15 min, 65°C) was performed. Subsequently, the reaction mixture was incubated at 42°C (1 hr, RT step) and heated for 5 min at 95°C (96°C for AChE oligonucleotides; RT denaturation step). Thirty-five PCR cycles were performed as follows: 1 min at 95°C (denaturation step), 2 min at 52°C (60°C for AChE oligonucleotides; annealing step), 10 min at 72°C (elongation step), with a final elongation step of 5 min at 72°C. Specific 30-mer oligonucleotides as designed by Soreq et al. (1994) were used to distinguish between AChE and butyrylcholinesterase mRNA. These sequences were adapted to the rat AChE cDNA (Brice et al., 1989). Specific oligonucleotides complementary to AChE and GS mRNAs, respectively, were designed to distinguish amplified mRNA product from amplified genomic (DNA) product (an intron was always present between the upstream and downstream oligonucleotides; see Table 1). Amplifica-

TABLE 1. Oligonucleotides sequences used for amplification of acetylcholinesterase and glutamine synthetase mRNA sequences after reverse transcription[1]

rache01	5′ AAGACTGCCTTTATCTTAATGTGTGGACACC 3′ (nt 384–414)
rache02	5′ GCTTCAGCAAAGACAATGAATCTCTCATCAG 3′ (nt 1127–1157)
gs1	5′ CCACCTCAGCAAGTTCCC 3′ (nt 36–53)
gs2	5′ CAGCACCCCTGGTTTGG 3′ (nt 510–526)

[1]Oligonucleotide sequences for the rat acetylcholinesterase (rache01 and rache02) and rat glutamine synthetase (gs1 and gs2). The numbers in parentheses indicate the corresponding mRNA annealing sequence.

tion of GS mRNA resulted in a fragment 555 nt in length, whereas the corresponding genomic fragment (gDNA) was 4 kilobases (kb) in length. Amplification of AChE mRNA resulted in a fragment of 786 nt length, whereas its corresponding genomic fragment was 1186 nt. RT-PCR products were loaded and run on a 1% agarose gel and transferred to nylon membranes (Hybond N; Amersham, Amersham, U.K.). Southern blots were performed according to Sambrook et al. (1989) and hybridised to full-length AChE cDNA or an 800 bp EcoRI fragment of the rat GS cDNA (van der Zande, 1989). ^{32}P labelling of the cDNAs was by the random-primed method. Unincorporated dNTPs were removed on Sephadex G-50 columns. Probes were quantified in a liquid scintillation counter (1900CA Tri-Carb; Packard) and hybridised overnight at 52°C. Membranes were washed in 40 mM sodium phosphate 1% SDS buffer (52°C, 2 × 15 min; RT, 1 × 30 min). A final wash with buffer only was performed. The membranes were then exposed in a phosphorimage analyser (Molecular Dynamics) for 2–3 hr and analysed.

Immunohistochemistry

Specific primary monoclonal antibodies against rat 68 kD neurofilament protein (Dako Inc.), Leu-7 (Becton-Dickinson Co.), α-smooth muscle actin (Sigma), and β-myosin heavy chain (Wessels et al., 1991) were used to visualise the neural tissue and the myocardium. After deparaffination and hydration, the sections were washed in PBS and treated with hydrogen peroxide (3% in PBS, 30 min) to reduce endogenous peroxidase activity. Subsequently, an incubation in TENG-T (10 mM Tris, 5 mM EDTA, 150 mM NaCl, 0.25% gelatin, 0.05% Tween-20, pH 8.0; 30 min) was performed to avoid nonspecific binding. After this pretreatment, the sections were incubated overnight with the specific primary antibody. Binding of the first antibody was detected using a rabbit antimouse immunoglobulin, followed by a goat antirabbit immunoglobulin and finally a rabbit peroxidase antiperoxidase (PAP) complex. Each incubation lasted 2 hr and was followed by three washes in PBS (5 min each). All sera were diluted in PBS. The visualisation of the PAP complex was performed by incubation with 0.5 mg/ml 3,3′-diaminobenzidine and 0.02% hydrogen peroxide in 30 mM imidazole, 1 mM EDTA (pH 7.0) buffer. Sections were dehydrated and mounted in Entellan (Merck).
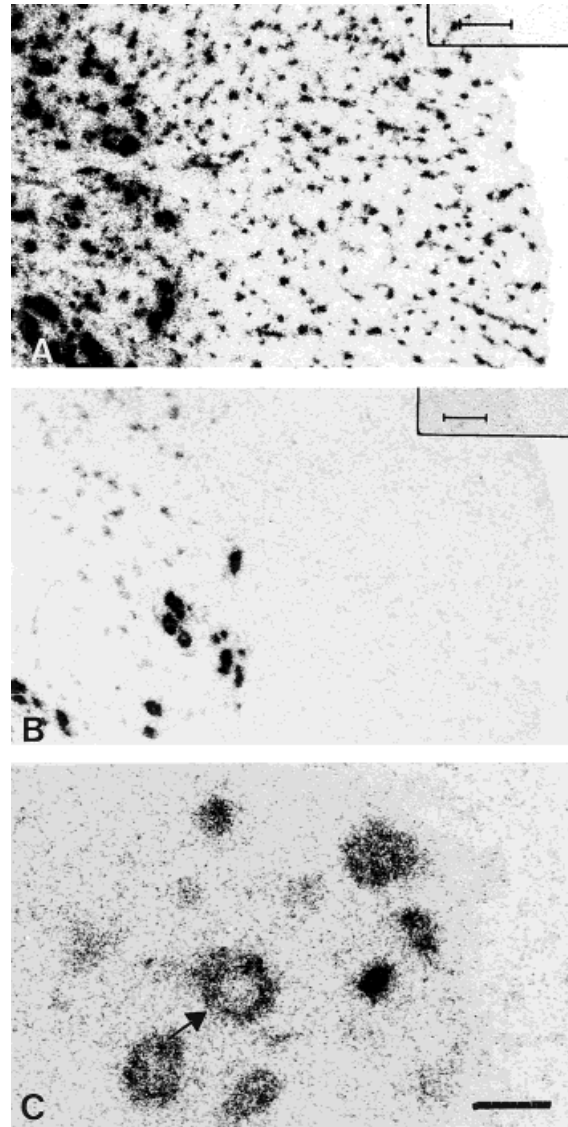

Fig. 1. Adult neural tissue. Expression of AChE (**A**) and ChAT (**B, C**) mRNAs in the spinal cord. A and B are transverse sections through the spinal cord. **C** shows a high-magnification view of neurons with perinuclear expression of ChAT mRNA (arrow). Scale bars = 70 µm in A, B, 50 µm in C.

RESULTS
In Situ Hybridization

In the present study, adult brain and spinal cord and embryonic spinal cord were used to assess the specificity of AChE, ChAT, and mAChR mRNA localisation. GS (neural tissue and myocardium), SERCA2, and β-MHC mRNAs were used as internal controls of embryonic and adult hearts. The expression pattern of the respective mRNAs is first described for control tissues and subsequently for the heart.

Adult neural tissue

The specificity of the ChAT, AChE, and mAChRs mRNAs was evaluated using adult rat spinal cord and
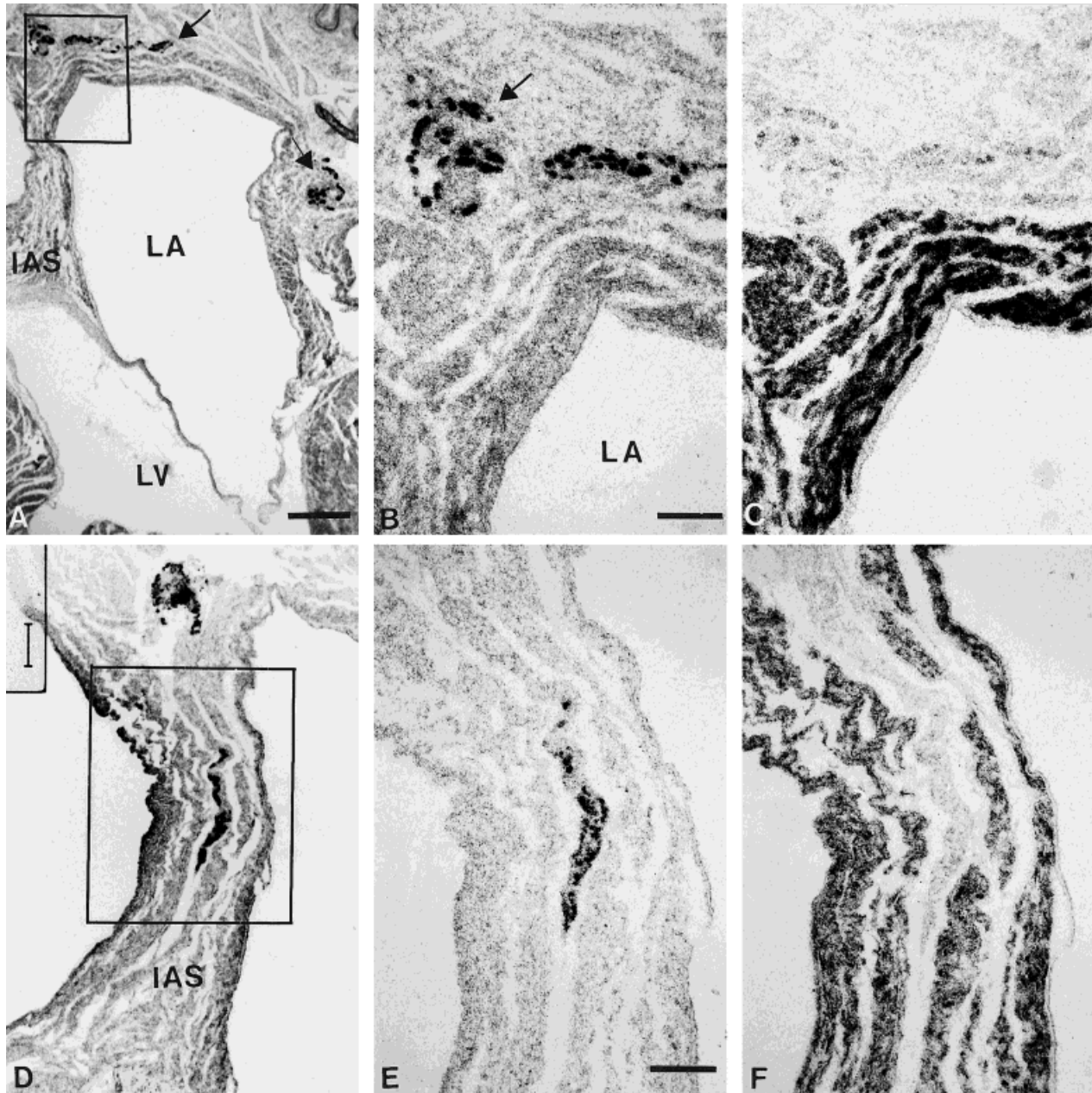
Fig. 2. Adult heart. Expression of ChAT (**A, B, E**), SERCA2 (**C, F**), and AChE (**D**) mRNAs in transverse serial sections of an adult rat heart. B, C and E, F are high magnifications of the rectangles in A and D, respectively. The cardiac ganglia are located on the dorsal wall of the atria (arrows) and, more caudally, in the atrioventricular groove (D, F). Expression of SERCA2 identifies the myocardium. IAS, inter-atrial septum; LA, left atrium; LV, left ventricle. Panels B and C and panels E and F have the same magnification. Scale bars = 400 μm in A, 100 μm in B, E, 110 μm in D.

brain. Figure 1A,B illustrates the expression patterns of AChE and ChAT mRNAs, respectively. These mRNAs were found to be specifically localised in the perinuclear area of the motor neurons of the spinal cord (see, e.g., high magnification of ChAT mRNA in Fig. 1C). These findings are in accordance with previously published data (Oh et al., 1992; Vilaro et al., 1992; Lauterborn et al., 1993; Landwehrmeyer et al., 1993; Hoover et al., 1994), demonstrating that we could reliably visualise these rare mRNAs.

Neonatal and adult heart

The expression pattern of the different mRNAs studied in neonatal and adult specimens did not show significant differences, so no further distinction is made in the description. The expression of ChAT (Fig. 2A,B,E) and AChE (Fig. 2D) mRNAs was found to be restricted to the cardiac ganglia. These ganglia were found on the dorsal wall of the atria and around the pulmonary veins, reaching laterally as far as the caval veins. Some

neurons were found in the atrioventricular groove (Fig. 2D–F). The mRNAs of the muscarinic receptor types m1, m2, and m4, but not m3 and m5, were localised in the same structures as AChE and ChAT mRNAs (Fig. 3). None of the mRNAs studied was observed at detectable levels within the working myocardium or in the conduction system (i.e., sinoatrial and atrioventricular nodes and ventricular conduction system).

Embryonic and fetal neural tissue

ChAT and AChE mRNAs were observed in the ventral horns of the spinal cord from 13ED onward. Only muscarinic receptors type m1, m2, and m3 (but not m4 or m5) were very weakly expressed in the spinal cord during early embryogenesis (13ED–15ED; data not shown). None of the mRNAs studied was expressed at detectable levels in the peripheral vagal nerves or in the intracardiac nerves during the fetal period.

Embryonic and fetal heart

The myocardium of the outflow tract (OFT) and the ventricular trabeculations do not contain detectable levels of AChE, ChAT, or mAChR mRNAs during any stage of development (Fig. 4B–D). The first cells containing AChE and ChAT mRNAs were observed in the dorsal wall of the atrium at 15ED (Fig. 5B), but those cells were not myocardial cells, as demonstrated by comparison with the expression of β-MHC mRNA (Fig. 5A). From 16ED onward, AChE and ChAT mRNAs showed a well-defined pattern, which, based on immunohistochemistry with monoclonal antibodies against neurofilament proteins and Leu-7 antigens, corresponded to the cardiac ganglia. The cardiac ganglia developed several branches towards the superior caval vein and the interventricular groove and around the pulmonary veins (Fig. 5E–G). Their final distribution pattern was achieved around 20ED (Fig. 6).

Muscarinic receptors (m1, m2, and m4) mRNAs could be clearly demonstrated from 18ED onward in the cardiac ganglia (Fig. 7A–C). The m1 and m4 subtypes were also observed in the vascular smooth muscle cells and lung buds (Fig. 7A,C). The m1 and m4 subtypes were slightly more abundant than the m2 subtype in the cardiac ganglia during the fetal period (Fig. 7B). On the other hand, no detectable level above background was observed for the m3 and m5 mAChRs. The atrial myocardium shows a just detectable hybridisation signal for the m1 and m4 mAChR mRNAs, but this was not the case for the ventricular myocardium. No differences regarding the mAChR mRNAs were found in the developing conduction system (SA and AV nodes, and ventricular conduction system) compared to the surrounding myocardium. The pattern of expression of the mAChRs during the fetal and adult periods is summarised in Table 2.

RT-PCR

AChE is an enzyme with an extremely high turnover rate, so, potentially, only very low levels of protein, and hence mRNA, are necessary to generate detectable activity levels. To obtain more definitive data about the absence or presence of AChE mRNA during early rat heart development, we applied RT-PCR to total RNA extracts from embryonic ventricles. Embryonic ven-
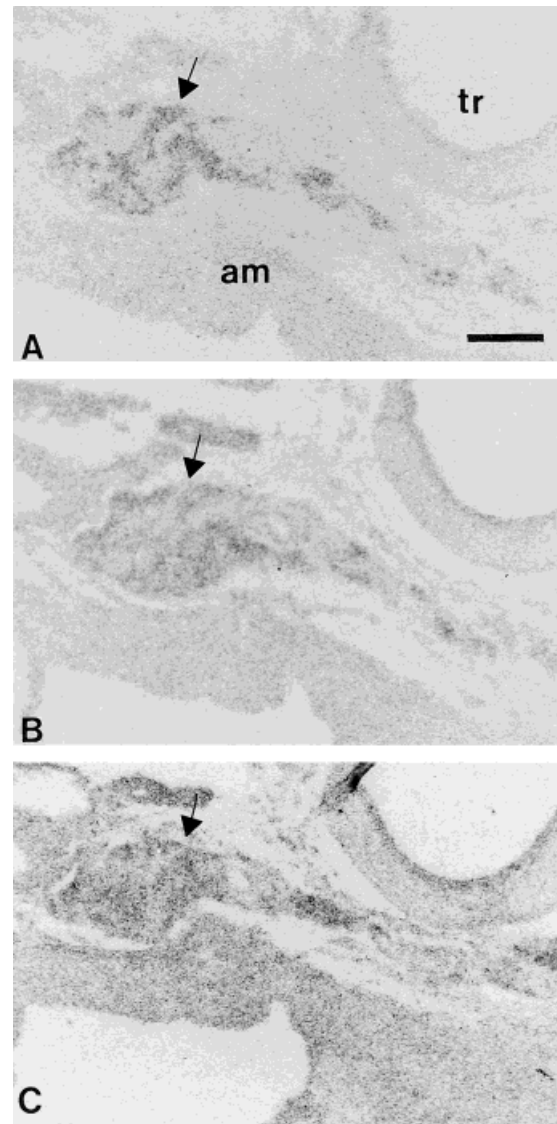

Fig. 3. Neonatal heart. Expression of m1 (**A**), m2 (**B**), and m4 (**C**) mAChR mRNAs in transverse sections of a 10-day-old rat heart. Observe that the expression of the mAChRs is restricted to the cardiac ganglia (arrows) and is not observed in the myocardial cells. am, atrial myocardium; tr, trachea. A–C have the same magnification. Scale bar = 100 μm.

tricles were used to avoid possible contamination with the cardiac ganglia. Furthermore, the OFT was removed to exclude nerves in the arterial pole of the heart. In effect, we therefore determined whether or not the trabeculations contain AChE mRNA. AChE and GS primers were tested in adult rat spinal cord, adult basal nuclei, and 16ED spinal cord, total RNA generating the expected fragments.

No AChE mRNA could be detected in extracts of ventricles between 13ED and 18ED (Fig. 8A). RT-PCR of GS mRNA, which served as a control of the RT procedure, showed that the RNA was of sufficient quality to allow the detection of low-abundance mRNAs (Fig. 8B). Therefore, both the in situ hybridisation and the RT-PCR data show that no mRNA coding for the
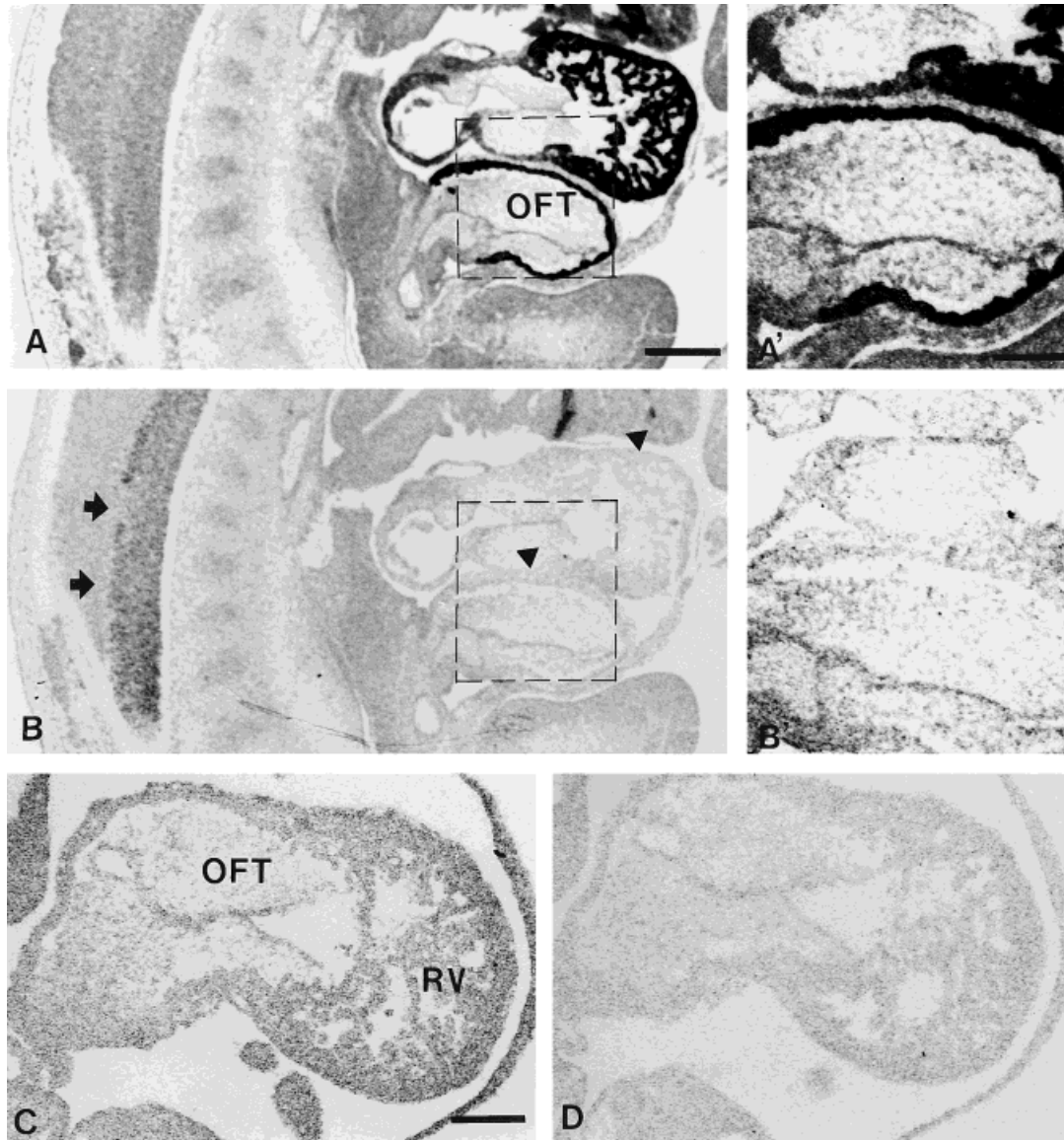
Fig. 4. Embryonic and fetal heart. Expression of β-MHC (**A,A′**), ChAT (**B,B′**), AChE (**C**), and m3 mAChR (**D**) mRNAs in sagittal (A,A′,B,B′) and transverse (C, D) sections of 13ED rat hearts. A′ and B′ are high-magnification views of the rectangles in A and B, respectively. Observe the localisation of ChAT mRNA (B) in the spinal cord (arrows), whereas it is absent in the outflow tract myocardium (OFT) and ventricular trabeculations (arrowheads). AChE and m3 mAChR mRNA were nor observed in the ventricular trabeculations. Panels A and B, panels A′ and B′, and panels C and D have the same magnification. Scale bars = 330 μm in A, 200 μm in A′, 120 μm in C.

AChE is present in the myocardium of the embryonic rat.

DISCUSSION

Within the embryonic heart, five different compartments can be distinguished according to their electrophysiological characteristics. These segments are a slow-conducting inflow tract, the fast-conducting atria, a slow-conducting atrioventricular canal, the fast-conducting ventricles, and a slow-conducting outflow tract (Arguello et al., 1986; De Jong et al., 1992). In addition, the upstream venous part of the heart is characterised by short contractions, whereas the downstream arterial part is characterised by long-lasting contractions (De Jong et al., 1992; Moorman and Lamers, 1994). Each of these compartments therefore acquires a distinct molecular phenotype that reflects this functional heterogeneity (Moorman and Lamers, 1994).

The characterisation of the mechanisms underlying the differences in the contraction-relaxation cycle in the arterial and venous poles of the heart is far from complete. Changes in the free intracellular calcium concentrations are an important effector regulating contraction-relaxation states within myocytes. The group of Drews (Oettling et al., 1989, 1992; Lohmann et al., 1991) has identified the transient presence of a muscarinic, inositiol triphosphate-dependent calcium-mobilising pathway in isolated cardiomyocytes of embry-
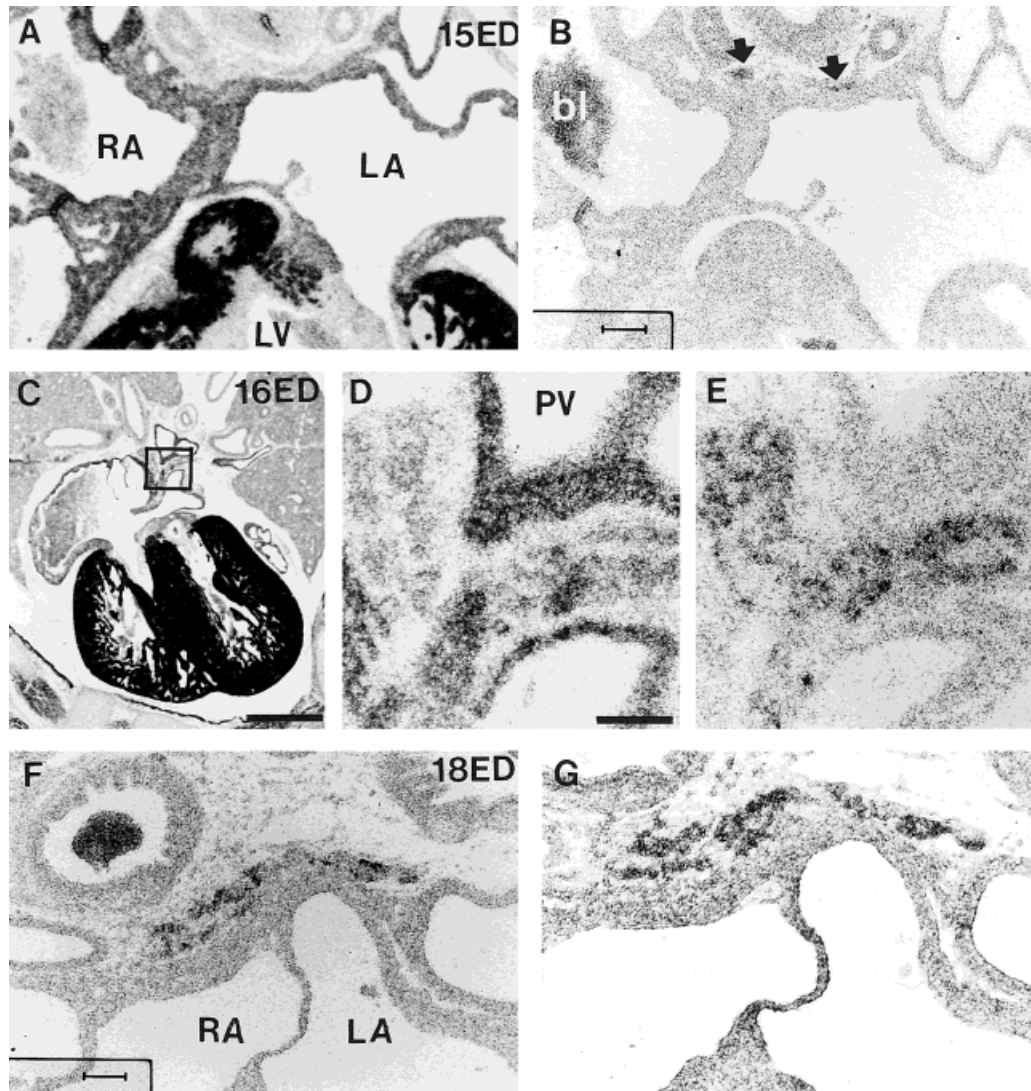
Fig. 5. Embryonic and fetal heart. Expression of β-MHC (**A, C, D**), ChAT (**B, E, F**), and AChE (**G**) in sections through the heart of 15ED (A, B), 16ED (C–E), and 18ED (F, G) rat embryos. β-MHC mRNA (A, C, D) demonstrates the atrial and ventricular myocardium. At 15ED, ChAT-positive cells were seen on the roof of the atrium (arrows in B). D and E are high-magnification views of the area indicated in C, showing that these ChAT-positive cells do not belong to the myocardium (compare D and E). Later in development, the ChAT-positive cells are located on the dorsal wall of the atrium (F) and around the pulmonary veins. AChE mRNA shows the same pattern of expression (G). bl, Blood; RA, right atrium; LA, left atrium; LV, left ventricle; PV, pulmonary veins. Panels A and B, panels D and E, panels F and G have the same magnification. Scale bars = 100 μm in A, 330 μm in C, 40 μm in D, 200 μm in F.

onic chickens. These findings appeared to provide a functional role for the AChE activity that we (Lamers et al., 1987, 1990) and others (Coraboeuf et al., 1970; Nakamura et al., 1994) have observed in the myocardium of the ventricular trabeculations and the outflow tract of embryonic and fetal hearts of rats and chickens. The use of appropriate inhibitors excluded the possibility that a nonspecific cholinesterase or esterase activity was involved. Furthermore, Paff et al. (1966) identified a less well-defined ACh-dependent regulation of cardiac rhythmicity in early chicken embryos that was localised at the atrioventricular junction.

Unexpectedly, we have shown in the present study that neither ChAT nor AChE mRNA can be detected in the prenatal rat myocardium. A similar paradox, i.e.,

the presence of AChE activity and the absence of its mRNA, was recently reported for embryonic rabbit hearts by using the Northern blotting technique (Jbilo et al., 1994). Because AChE is an enzyme with an extremely high turnover rate, potentially only very low levels of mRNA are necessary to generate AChE protein and, hence, detectable activity levels. On the other hand, our in situ hybridisation study did show that mRNAs of AChE, ChAT, and mAChRs are present from 15ED (AChE, ChAT) and 18ED (mAChRs) onward in the cardiac ganglia on the dorsal wall of the atria. This demonstrates that we were able to localise these mRNAs specifically in embryonic tissues. Furthermore, only a very low level of m1 and m4 mAChR mRNA is present in the atria of prenatal rats. None of these mRNAs
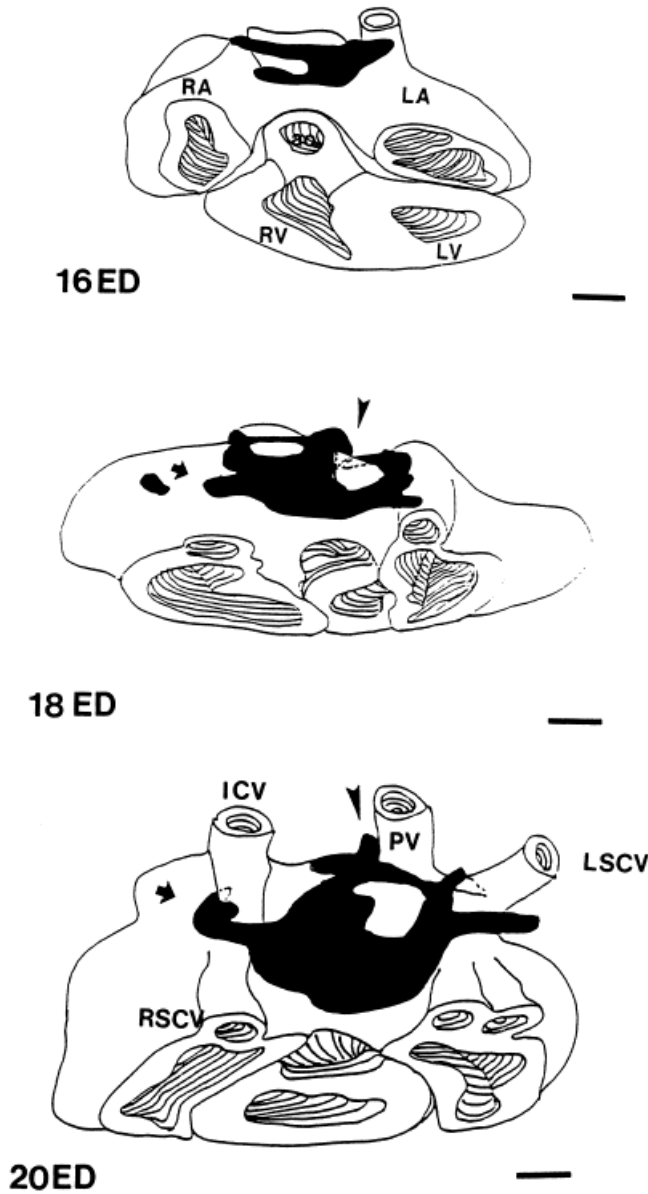
Fig. 6. Localisation of the cardiac ganglia. Schematic drawings indicating the location of the cardiac ganglia in 16ED, 18ED, and 20ED rat embryos. Observe the branching towards the superior caval veins (arrows) and the atrioventricular groove (arrowheads). ao, Aorta; ICV, inferior caval vein; LA, left atrium; LSCV, left superior caval vein; LV, left ventricle; PV, pulmonary veins; RA, right atrium; RV, right ventricle; RSCV, right superior caval vein. Scale bars = 330 μm for 16ED, 300 μm for 18ED, 375 μm for 20ED.

Fig. 7. Embryonic and fetal heart. Expression of m1 (**A**), m2 (**B**), and m4 (**C**) mAChR mRNAs in transverse serial sections of a 16ED rat heart. The m1 and m4 mAChR mRNAs showed a high level of expression in the vascular smooth muscle cells (asterisks) and in the cardiac ganglia (solid arrows), whereas they are barely detectable in the atrial myocardium (arrowheads) and are absent in the ventricular myocardium (open arrows). The m2 mAChR mRNA is observed only in the cardiac ganglia (B). A–C have the same magnification. Scale bar = 165 μm.

could be visualised by in situ hybridisation in the ventricular trabeculations or in the outflow tract myocardium. Moreover, no AChE mRNA could be demonstrated in the ventricles by the very sensitive RT-PCR technique. On the other hand, this method revealed the presence of GS mRNA, which also could not be visualised by in situ hybridisation in these embryonic hearts.

As stated, the presence of true AChE activity in the ventricular trabeculations and the outflow tract was demonstrated by the use of specific inhibitors. Unfortu-
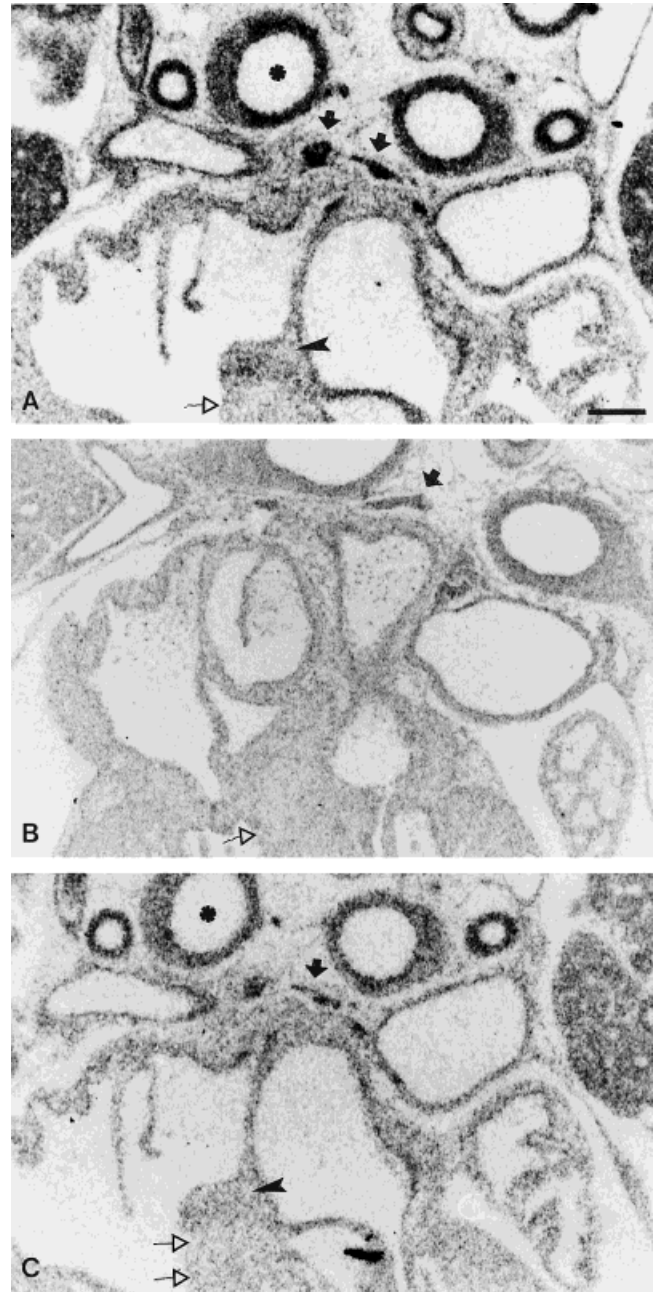
nately, AChE protein has not yet been demonstrated in the heart. This is because the AChE activity measurement requires only a very low AChE protein concentration and because the specific monoclonal antibodies that were raised against the human (Brimijoin et al., 1983) and the chicken (Chatel et al., 1994) AChE

**TABLE 2. Distribution of mAChR mRNAs during cardiac morphogenesis[1]**

mAChR	Central nervous system		Cardiac ganglia		Atria	
	SP	Hi	Fetal	Adult	Fetal	Adult
m1	+	+++	+++	+	+/−	+/−
m2	++	+/−	++	+++	−	+/−
m3	+	++	−	−	−	−
m4	+++	+++	+++	++	+/−	−
m5	−	+	−	−	−	−

[1]Summary of the distribution pattern of mAChRs in adult spinal cord (SP) and hippocampus (Hi), cardiac ganglia, and atria.

Glutamine synthetase mRNA

subunits do not cross-react well with the rat protein. Nevertheless, the paradoxical presence of AChE activity has to be reconciled with the absence of its encoding mRNA. We can hypothesise that AChE protein is derived from other sources, via diffusion or the circulation. Innervation of the heart has not yet developed at 13ED (Gomez, 1958), when AChE activity is already demonstrable (Lamers et al., 1990). It is well documented that AChE can be present in different molecular forms, being either soluble (globular forms) or insoluble (attached to membranes; asymmetrical forms; see, e.g., Massoulie and Bon, 1982). Interestingly, both molecular forms of AChE are present in adult rat heart. The asymmetrical isoforms are homogeneously distributed, whereas globular isoforms are more highly expressed in the atria than in the ventricles (Nyquist-Battie and Trans-Saltmann, 1989, 1990). So far, no data are available about the embryonic heart in this respect.

Even if the AChE of the arterial pole of the embryonic heart was synthesised elsewhere, for it to be part of an ACh-dependent signal-transduction pathway would require the presence of specific receptors. In adult rats, muscarinic receptors are present in higher concentrations in the atria and the conduction system than in ventricles (Hancock et al., 1987) as revealed by autoradiographic studies. More recently, a comparison between radiolabeled binding assays and immunohistochemical localisation of mAChRs in the embryonic mouse pointed out that both protein and binding activity colocalised in the ventricular myocardiocytes (Lammerding-Koppel et al., 1995). However, in situ hybridisation studies have shown that no mAChRs mRNA is observed in the ventricular myocardium and that only m2 mRNA is expressed in adult rat atria. Furthermore, no differences were seen between conduction system (nodal and ventricular) and working myocardium (Hoover et al., 1994; this study). Within the cardiac ganglia, m2 mRNA was also found as the predominant subtype, but lower amounts of m1 and m4 were present (Hoover et al., 1994). These data support the ACh sensitivity of the atrium but not of the more downstream myocardium. The presence of ACh binding activity in the conduction system and the absence of local mRNA concentration, as has also been discussed by other authors (Hoover et al., 1994), is paradoxical. It implies that only a few mRNA molecules, below the delection level of in situ hybridisation, are capable of generating enough mAChRs to be visualised by means
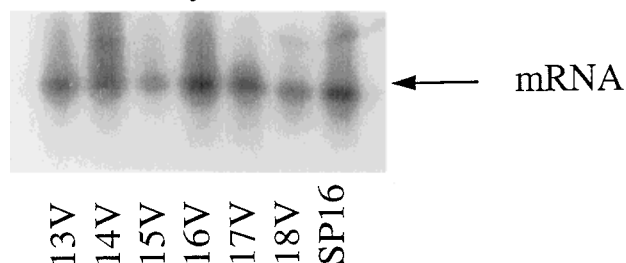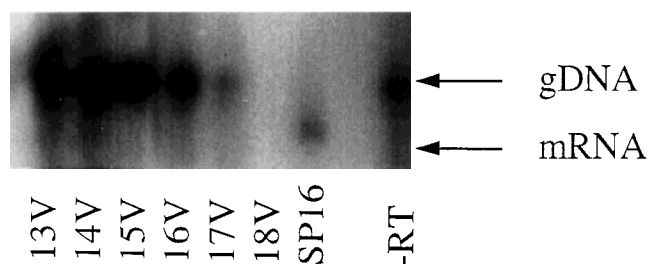
Acetylcholinesterase mRNA

Fig. 8. Reverse transcriptase-polymerase chain reaction. RT-PCR analysis of the expression of glutamine synthetase and AChE of ED13–18 embryonic rat ventricles and ED16 spinal cord. PCR results were visualised after Southern blotting by hybridisation with the respective cDNAs. Observe that no AChE mRNA is found in embryonic ventricular extracts, whereas amplification is observed in 16ED spinal cord. GS mRNA was amplified in all embryonic tissues studied.

of immunohistochemistry and autoradiographic binding assays. Current studies aim to address this point.

Even though the best defined role of the AChE is to hydrolyse ACh (Small, 1989, 1990; Appleyard, 1992), AChE activity has been found in other locations where other components of the cholinergic signal-transduction pathway are lacking, such as migrating neural crest-derived cells (Miki and Mizoguti, 1982; Cochard and Coltey, 1983), adult placenta, erythrocytes, platelets, and lymphocytes (see, e.g., Small, 1989, 1990). Furthermore, AChE activity is not always colocalised with ChAT in adult brain (Levey et al., 1983). These data suggest that AChE can play a role other than hydrolysis of ACh (see Small, 1989, 1990). AChE sequence analysis has revealed that some domains share similarities with exopeptidases, such as the trypsinlike serine protease family. Therefore, roles as a protease (Chubb et al., 1980), zymogen (i.e., an inactive protease precursor; Small, 1989), and morphogenetic factor (Miki and Mizoguti, 1982; Drews and Mengis, 1990; Lammerding-Koppel et al., 1995) have been hypothesised for the AChE. Our results support the notion that AChE plays such a role during embryonic heart development.

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