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The innervation of the human myocardium at birth

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ABSTRACT

In order to delineate the type and distribution of autonomic nerves within the atrial and ventricular myocardium of the neonatal human heart, numerous samples of atrial and ventricular myocardium from 4 neonatal human hearts with no cardiac anomaly, freshly obtained at necropsy, were processed and studied using immunohistochemical and enzyme histochemical techniques. The antisera included those used to demonstrate protein gene product (PGP) 9.5 as a general neural marker, dopamine β-hydroxylase (DBH) and tyrosine hydroxylase (TH) as indicators for presumptive sympathetic neural tissue, and neuropeptide Y (NPY). A histochemical technique was used to reveal tissue cholinesterase activity. Numerous PGP-immunoreactive (PGP-IR) nerves were seen in the atrial myocardium, forming perivascular plexuses and lying in close apposition to myocardial cells. Fewer PGP-IR nerves were found amongst the myocardium of the ventricles. Both DBH-IR and TH-IR nerves demonstrated a similar pattern of distribution as that of PGP-IR nerves; in the atria, however, they were less numerous, while in the ventricles, their density approximated to that of PGP-IR nerves. Relatively few NPY-IR nerves were observed either in the atrial or the ventricular myocardium. The density of acetylcholinesterase (AChE) positive nerves in the walls of the atria was less than that of PGP-IR nerves although their distribution patterns were similar. In the ventricles, AChE positive nerves were rarely observed. It is concluded that the neonatal human heart possesses a rich supply of autonomic nerves. The atria possess at least two populations of nerves, presumably sympathetic and vagal, whereas the walls of the ventricles are innervated principally by presumptive sympathetic nerves.

Key words: Heart; protein gene product 9.5; dopamine β-hydroxylase; tyrosine hydroxylase; neuropeptide Y; acetylcholinesterase.

INTRODUCTION

With the recent development of sensitive immunohistochemical methods, cardiac innervation may now be visualised with better precision and specificity than was previously possible (Weihe et al. 1984; Sternini & Brecha, 1985; Dalsgaard et al. 1986). These techniques have allowed the determination of the different patterns of distribution of various nerve subtypes in the hearts of several mammalian species (Ursell et al. 1990, 1991*a*, *b*; Choate et al. 1993; Slavikova et al. 1993; Zhang et al. 1993). There have been a number of such studies on the human heart (Rechardt et al. 1986; Wharton et al. 1988, 1990; Chow et al. 1993; Gordon et al. 1993; Crick et al. 1994), although most of these were performed on surgically excised cardiac tissue obtained from diseased adult hearts (Rechardt et al. 1986; Wharton et al. 1988, 1990). Thus, knowledge about the pattern of innervation of the human heart, especially in the neonatal period, is far from complete. Recently we have studied the innervation of the conduction system in the newborn human heart, employing a combination of enzyme and immunohistochemical techniques (Chow et al. 1993). In the present study, the same techniques were employed to investigate the innervation of the neonatal human atrial and ventricular myocardium.

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MATERIALS AND METHODS

The hearts were obtained during autopsy performed within 18 h of death from 4 fullterm newborn infants with no evident congenital cardiac anomalies. Relevant clinical information is summarised in Table 1.

Tissue preparation

In order to study the pattern of innervation of the atria, 3 blocks of full-thickness transmural tissue were taken from each of the following regions: the anterior, lateral and posterior walls of each atrium, the interatrial septum, and the atrial appendages. For the ventricles, the areas selected for study included the anterior, lateral and posterior walls of each ventricle together with the interventricular septum. Again, 3 blocks of full-thickness transmural tissue were taken from the superior, middle and inferior regions of each of the aforementioned areas.

The tissue blocks were immediately frozen in isopentane previously cooled in liquid nitrogen and serially sectioned in a cryostat at 10 μm. Each section was mounted on a gelatin-subbed glass slide and stored at −70 °C. For every block of tissue, 30 such serial sections were prepared. The first and last sections from each block were stained using Masson's trichrome technique and used for routine histology. As appropriate, intermediate sections were processed using immunohistochemical or enzyme histochemical techniques.

Immunofluorescence reactivity

An indirect immunofluorescence procedure using the avidin-biotin technique was employed and the details of the primary antisera are summarized in Table 2. Briefly, cryostat sections were air-dried at room temperature for 1 h and fixed in 0.4% parabenzoquinone for 3 min. They were then sequentially incubated at room temperature with the diluted primary antisera for 16 h, biotinylated antirabbit immunoglobulin for 30 min, and finally fluorescein avidin D for 30 min, with thorough washing in phosphate buffered saline between each step. The sections were mounted in glycerol mixed 1:1 with buffered saline and examined with a microscope equipped for epi-illumination.

For positive controls, tissue sections from a segment of adult human small intestine, freshly obtained during surgical operation, were used. The autonomic nerves in the wall of the intestine showed positive immunostaining with all the antibodies. For negative control sections, the primary antisera were either omitted, replaced with preimmune serum, or pre-

Table 1. *Clinical summaries of the* 4 *newborn infants*

Patient	Sex	Age	Heart weight	Cause of death
1	M	Day 2 (18 h)*	16.7 g	Short-limbed dwarfism (thanatophoric dysplasia)
2	F	Still birth (15 h)*	18.0 g	Cord round neck twice
3	F	Day 10 (6 h)*	17.1 g	Severe asphyxia neonatorum (undiagnosed breech presentation)
4	M	Day 1 (12 h)*	12.0 g	Tracheal agenesis

* Time intervals between death and autopsy in hours indicated in parenthesis.

Table 2. *Characterisation of antisera for immunohistochemistry*

Antisera to:	Dilution	Source
Protein gene product (PGP) 9.5 —polyclonal (rabbit)	1:400	Ultraclone, UK
Dopamine β-hydroxylase (DBH) —polyclonal (rabbit)	1:200	Eugene Tech International, USA
Tyrosine hydroxylase (TH) —polyclonal (rabbit)	1:500	INCSTAR, USA
Neuropeptide Y (NPY) —polyclonal (rabbit)	1:800	UCB Bioproducts, Belgium

Table 3. *Pattern of innervation by immunofluorescence**

Region	PGP 9.5	DBH	TH	NPY
Right atrium	++++	++	++	+
Left atrium	++++	++	++	+
Right ventricle	++	++	++	+
Left ventricle	++	++	++	+
Coronary artery				
Epicardial	+	+	±	±
Myocardial	+++	++	++	+

* Relative density of immunoreactive nerves graded arbitrarily. 0, no immunoreactivity detected; ±, extremely sparse immunoreactive nerves; +, sparse nerve fibres; ++, moderate number of fibres; +++, numerous nerve fibres; ++++, very numerous nerve fibres. PGP 9.5, protein gene product 9.5; DBH, dopamine β-hydroxylase; TH, tyrosine hydroxylase; NPY, neuropeptide Y.

absorbed with their respective antigens. In every instance, immunofluorescent staining was negative.

The relative number of immunostained nerves was assessed visually and graded subjectively from 0 to ++++. The details of the method of grading are given in the footnote to Table 3.

Cholinesterase reactivity

Tissue sections were fixed in formol calcium at 4 °C for 20 min. After washing in tap water, they were incubated in Gomori (1948) stock solution, containing 2 mg/ml of acetyl thiocholine iodide as substrate, at 37 °C and pH 6.0 for 16 h. Sections were then washed again in tap water, developed for 60 s in freshly prepared 1 % ammonium sulphide solution in 20 °C and counterstained in haematoxylin for 2 min. The sections were dehydrated, cleared and mounted in Canada balsam dissolved in tetrachlorethylene. In the positive control sections, the autonomic nerves in the segment of intestine showed positive cholinesterase activity. The negative control sections, incubated in the absence of substrate, showed negative staining.

As appropriate, sections were preincubated at 20 °C with tetra-isopropylpyrophosphoramide (Sigma, USA) as an inhibitor of pseudocholinesterase. Other sections were incubated in the presence of 1,5-bis (4-allyldimethyl-ammoniumphenyl)-pentan-3-1-dibromide (Sigma, USA) as an inhibitor of acetylcholinesterase.

The intensity of the staining reaction was also assessed and graded subjectively from 0 to ++++ (Table 4), with examples illustrated by the figures and their legends.

RESULTS

The patterns of immunofluorescence and enzyme histochemistry of the innervation of the myocardium of the 4 hearts were similar and are summarised in Tables 3 and 4.

Immunofluorescence (Table 3)

Numerous protein gene product 9.5 immunoreactive (PGP-IR) nerve fascicles and fibres were present in both the right and left atrium (Fig 1 *a*). Large PGP-IR nerve fascicles were seen in the epicardium, extending with the coronary vessels into the myocardium where they ramified into smaller fascicles. Epicardial and large intramyocardial coronary arteries were accompanied by large nerve fascicles, although these vessels possessed relatively few nerve fibres at their adventitial–medial borders. Within the myocardium proper, PGP-IR nerve fibres were seen forming perivascular plexuses while other nerves ran in close relationship with atrial myocardial cells (Fig. 1 *b*). The latter were unrelated to vascular structures and many of the nerves possessed numerous varicosities along their lengths. In the ventricles, a similar pattern and distribution of perivascular and myocardial PGP-IR nerves were noted, although by comparison with the

Table 4. *Pattern of cholinesterase activity**

Region	Uninhibited	Acetylcholinesterase inhibitor	Pseudocholinesterase inhibitor
Right atrium	++	±	++
Left atrium	++	±	++
Right ventricle	±	0	±
Left ventricle	±	0	±
Coronary artery			
Epicardial	±	0	±
Myocardial	+	0	+

* Relative density of enzyme positive nerves graded subjectively. 0, no nerves; ±, extremely sparse stained nerves; +, sparse nerve fibres; ++, moderate number of stained fibres; +++, numerous stained nerve fibres; ++++, very numerous nerve fibres.

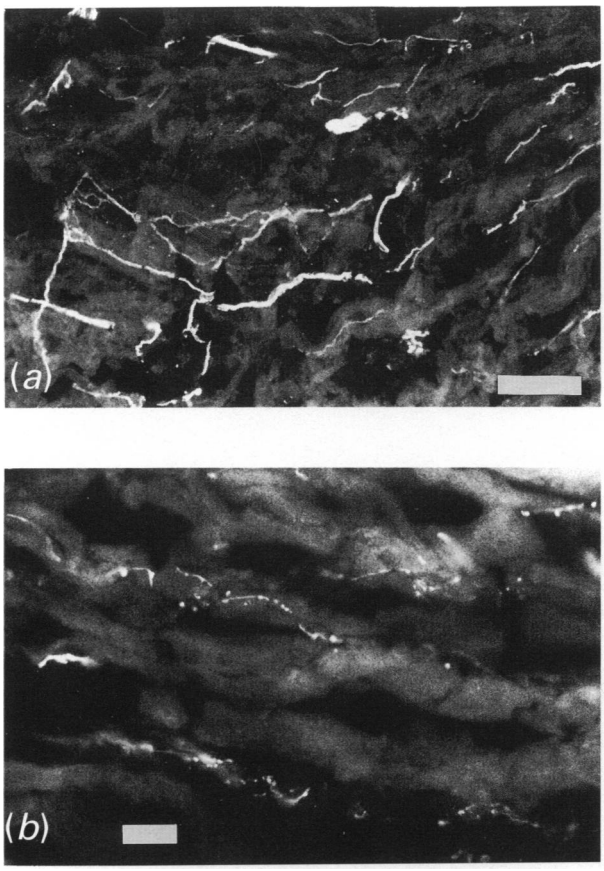

Fig. 1. (*a*) Immunofluorescence micrograph of a section of right atrium, showing very numerous protein gene product 9.5 immunoreactive (PGP-IR) nerves (+ + + +) which appear as white wavy lines. Bar, 250 μm. (*b*) High power magnification to show the close apposition of nerve fibres to atrial myocardial cells. Bar, 50 μm.

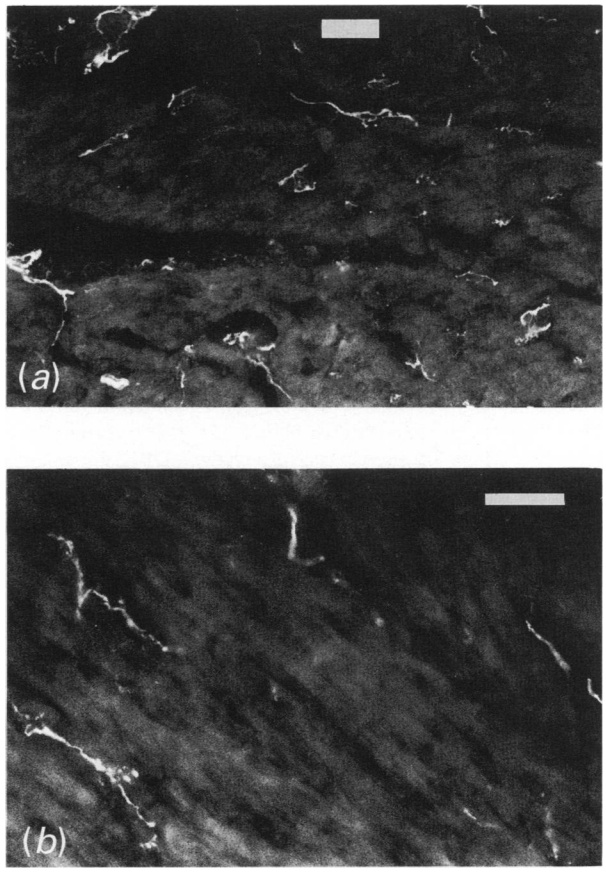

Fig. 2. (*a*) Immunofluorescence micrograph of a section of left ventricle, showing moderate number of PGP-IR nerves (+ +). Bar, 250 μm. (*b*) Close apposition of varicose PGP-IR nerves to ventricular myocytes is noted. Bar, 100 μm.

atria, they were relatively sparse (Fig. 2*a*). Nevertheless, close apposition of varicose PGP-IR nerves to ventricular myocardial cells could be identified (Fig. 2*b*). For each cardiac chamber, the density of PGP-IR nerves was uniform throughout the myocardium, with no significant difference in the subepicardial and subendocardial areas and in the various regions of the same chamber. Overall, using PGP 9.5 as a general neural marker, immunoreactive nerves in the atria were more numerous than those in the walls of the ventricles (Figs 1, 2).

When the innervation of the atria and ventricles was compared, the distribution and density of dopamine β-hydroxylase immunoreactive (DBH-IR) nerves were similar. Large epicardial nerve fascicles together with those accompanying the large intramyocardial coronary arteries were found to be devoid of DBH immunoreactivity. In the myocardium, however, the pattern and distribution of DBH-IR nerves were similar to those observed using PGP. In the atria, BDH-IR nerves were relatively fewer than those demonstrated with PGP, approximately half of their density (Fig. 3*A*). In the ventricles, on the other hand, the density of DBH-IR nerves approximated that of PGP-IR nerves (Fig. 3*b*). DBH-IR nerves were found mainly in the perivascular regions, with extension into the adjacent myocardium. An inverse relation was also observed between coronary arterial diameter and the number of perivascular DBH-IR nerves. In the small intramyocardial arteries and arterioles, numerous DBH-IR nerves were found to encompass the vessels, forming distinct perivascular plexuses. In contrast, the epicardial and large intramyocardial coronary arteries possessed relatively few nerve fibres at the adventitial-medial borders. Although relatively fewer DBH-IR nerve fibres were present among the myocardial muscle fibres, close apposition of varicose DBH-IR nerves to myocardial cells was observed in both the atria and the ventricles (Fig. 3).

The pattern and distribution of tyrosine hydroxylase immunoreactive (TH-IR) nerves were similar to those of DBH-IR nerves. In contrast, NPY-IR nerves were sparse (Fig. 4).

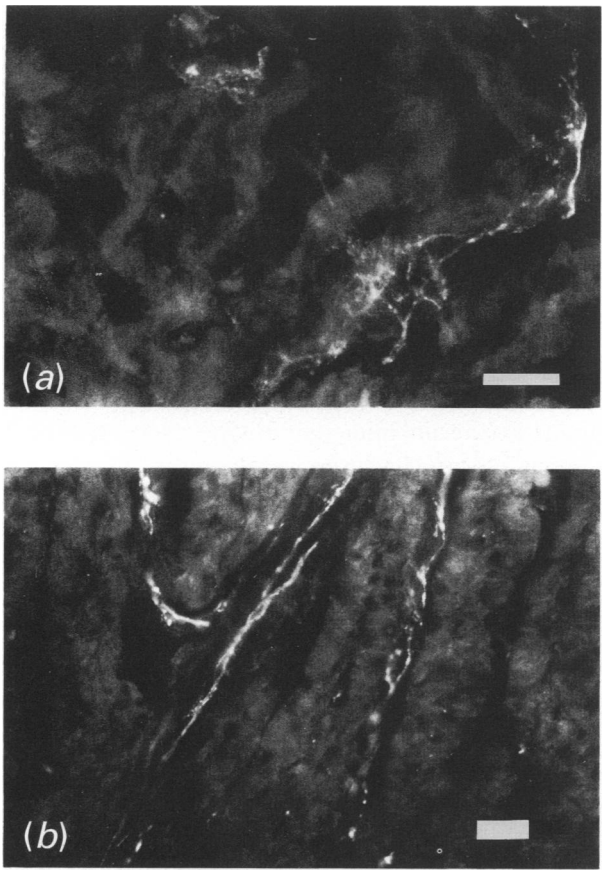

Fig. 3. (*a*) Moderate numbers of dopamine β-hydroxylase immunoreactive (DBH-IR) nerves (+ +) are present in the right atrium. Bar, 100 μm. (*b*) The density of DBH-IR nerves in the left ventricle is similar to that of PGP-IR nerves; they are present in moderate numbers (+ +). Bar, 100 μm.

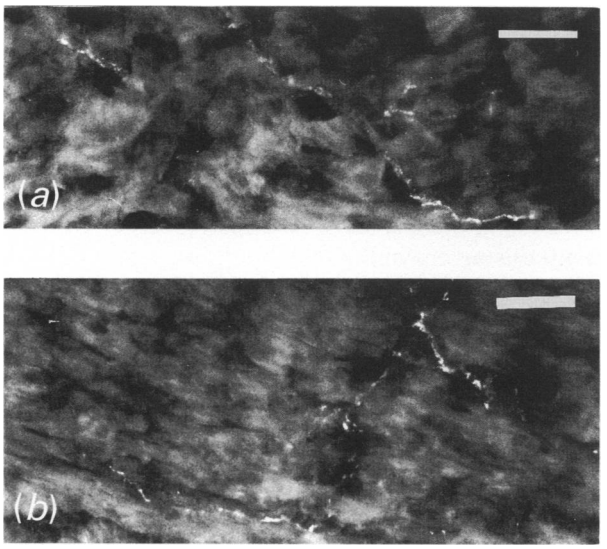

Fig. 4. Sparse neuropeptide Y immunoreactive (NPY-IR) nerves (+) are found in both the right atrium (*a*) and right ventricle (*b*). Bar, 100 μm.

Cholinesterase activity (*Table* 4)

The pattern and distribution of nerves showing positive cholinesterase activity were similar in the left and right atria. Large nerve fascicles demonstrating positive cholinesterase activity were seen in the epicardium and accompanying the large intramyocardial coronary vessels. In the atrial myocardium, their distribution was similar to that of PGP-IR nerves, seen both in the perivascular regions and in close apposition to myocardial fibres, but they were fewer in number, the density being approximately half that of PGP-IR nerves (Fig. 5a). Nerve fascicles showing positive cholinesterase reactivity were rarely encountered in the walls of the ventricles (Fig. 5*b*). In both the atria and ventricles, the staining pattern remained similar with the addition of an inhibitor of pseudocholinesterase, but decreased significantly in intensity after inhibition of acetylcholinesterase. Thus the atrial myocardium was associated with nerves which contained acetylcholinesterase, whereas such nerves were sparse in the ventricles.

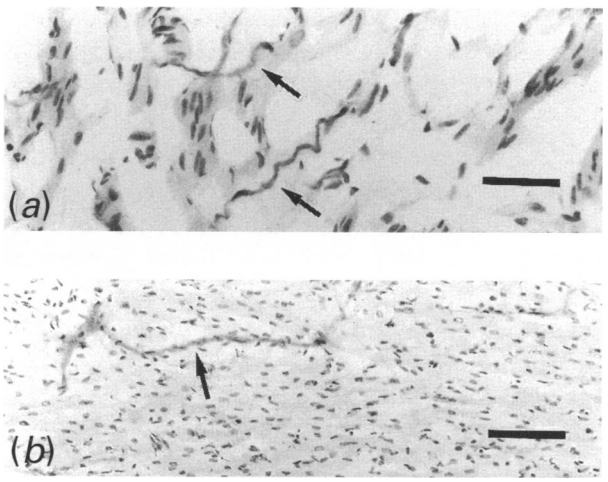

Fig. 5. (*a*) Moderate numbers of nerves (+ +) showing positive cholinesterase staining are seen in the left atrium (arrows), some of which are in close apposition to atrial myocytes. Bar, 100 μm. (*b*) In the ventricles, nerves positive for cholinesterase staining are extremely sparse (arrow)(±). Bar, 500 μm.

DISCUSSION

Previous studies used tissue from diseased and transplanted hearts, as well as fetal tissues to investigate the type and distribution of peptidergic cardiac nerves (Rechardt et al. 1986; Wharton et al. 1988, 1990; Gordon et al. 1993). In the present study, we examined the autonomic innervation of normal human neonatal myocardium, using a combination of enzyme and immunohistochemical techniques applied to postmortem specimens. Furthermore, we have chosen to use fresh frozen tissue in our study because

it has the theoretical advantage over immersion fixation in that tissue enzymes are less susceptible to damage and loss, thereby rendering the tissue useful for a wide range of enzyme and immunohistochemical studies. We have also performed a comparative study on the pattern and intensity of staining of cardiac nerves showing positive immunoreactivity for PGP, DBH, TH and NPY, using fresh frozen postmortem cardiac tissue compared with cardiac tissue fixed in modified Bouin's solution (Wharton et al. 1990; Gordon et al. 1993; Crick et al. 1994). For both methods of tissue preparation, the results were similar, endorsing the validity of results obtained using fresh frozen material (unpublished observations).

One of the likely reasons for the relative paucity of published studies on the innervation of the human heart using immunohistochemical techniques lies in the difficulty of obtaining tissue sufficiently fresh for use in such investigations. This is particularly true for the neonatal human heart, since there is no reasonable alternative, apart from obtaining recipient congenitally malformed hearts at transplantation, other than to use postmortem material for such an investigation. The validity of employing relatively fresh autopsy tissue has been justified by previous workers (Kent et al. 1974; Wharton et al. 1990) who have shown that both surgical and postmortem human cardiac tissue obtained within 24 h of death gave comparable results on immunohistochemical and enzyme histochemical analysis. In this context, the results of our present study were consistent in that comparisons of the findings from each heart were similar even though they were obtained at different times after death.

The overall distribution of intramural nerves supplying the neonatal human heart has been demonstrated in the present study using PGP 9.5, a sensitive and specific marker for neural tissue (Thompson et al. 1983; Rode et al. 1985). Our results tally with the findings in adult and fetal human hearts as recently described by Wharton et al. (1990) and Gordon et al. (1993) respectively. The former have demonstrated that, in the adult human heart, an abundant network of PGP-IR nerves was present in the walls of the atria. In the ventricles, numerous nerves were also observed but were relatively fewer than in the atria. In the fetus, Gordon et al. showed that perivascular PGP-IR nerves first appeared in the atrial and then in the ventricular epicardium at the 7th and 9th weeks of gestation respectively. Thereafter, nerves extended with the coronary vessels into the myocardium and endocardium. The density of innervation then progressively increased until the 24th week of gestation, when the distribution pattern resembled that observed in the adult. Thus, at all stages of development, the atria of the human heart possess a relatively greater innervation than the ventricles.

Since DBH and TH are enzymes involved in the synthesis of catecholamines, they have been used as useful markers for locating presumptive sympathetic neural tissue (Molinoff & Axelrod, 1971). Recent studies by Hardebo et al. (1992), however, have shown that some nonadrenergic neural tissue also contain these enzymes. Their presence should thus be taken as supportive rather than conclusive evidence that the tissue is catecholaminergic in type. Nevertheless, the present study has shown that the density and distribution of DBH-IR and TH-IR nerves were similar in both the atria and ventricles (Fig. 3). Our findings contrasts with those of Gordon et al. (1993) and Wharton et al. (1990), as they described more TH-IR nerves in the atria than the ventricles in both fetal and adult hearts. However, our finding is in agreement with the fact that a sympathetic neural influence on the ventricles is readily and unequivocally demonstrable, as it is for the atria (James, 1967, 1980). Thus it is suspected that the density gradient between atrium and ventricle for TH-IR nerves, if present, would be small or minimal so that it may well be functionally insignificant.

Furthermore, it is interesting to note that, in the atria, the density of DBH-IR and TH-IR nerves was approximately half that of PGP-IR nerves (Figs 1, 3*a*). In the ventricles, in contrast, the density and distribution pattern of DBH-IR and TH-IR nerves approximated closely those of PGP-IR nerves (Figs 2, 3*b*) (Table 3). Thus, while the ventricles were predominantly innervated by these presumptive sympathetic nerves, the atria possessed, in addition, a second population of nerves devoid of catecholamine-synthesising enzymes.

Apart from the discrepancy concerning the atrioventricular gradient, the pattern of distribution of DBH-IR and TH-IR nerves in the neonatal human heart, as delineated by the present study, is similar to that described for the adult by Wharton et al. (1990). This is in marked contrast to the findings in rabbit as described by Friedman et al. (1968), who demonstrated a marked paucity of sympathetic nerves and low stores of noradrenaline in the fetal and newborn rabbit heart as compared with the adult. In their study, the adult pattern of sympathetic innervation was not achieved until the 4th week of postnatal life. In this regard, our findings may be of particular clinical relevance, as conventional cardiac drug therapy in the neonate is largely based on the concept espoused by

Friedman et al. of relatively late maturation of adrenergic nerves.

NPY, a peptide found in certain sympathetic nerves, has been shown to be released with noradrenaline in response to sympathetic stimulation in humans and other mammals (Pernow, 1988; Lundberg et al. 1989; Warner and Levy, 1990*a*). It appears to be an important messenger in sympathetic neurotransmission both at presynaptic and postsynaptic sites (Franco-Cereceda et al. 1985; Warner & Levy, 1990*b*; Corr, 1992). Correspondingly we have found that, in all the cardiac chambers, the pattern and distribution of NPY-IR and DBH-IR, as well as TH-IR nerves, were similar. NPY-IR nerves, however, were less numerous than DBH-IR and TH-IR nerves (Fig. 4, Table 3). In contrast, NPY-IR nerves were found to be comparable in density to TH-IR nerves in both fetal and adult heart (Gordon et al. 1993; Wharton et al. 1990). This discrepancy may be due to the differences in the specimen fixation protocols and antibodies employed in our investigation compared with their studies. Nevertheless, irrespective of these differences, it is possible to conclude that the pattern of NPY innervation of human heart is already well established at birth.

Nerve fascicles and fibres showing positive cholinesterase activity were seen in moderate number in the atria but were scanty in the ventricles (Fig. 5, Table 4). Most of these nerves were rich in acetylcholinesterase (AChE), their enzymic reactivity being unaffected by preincubation with inhibitors of pseudocholinesterase. As compared with PGP-IR nerves, the density of these AChE-positive nerves in the atria was significantly less, and was approximately half that of PGP-IR nerves (Figs 1, 5*a*). In contrast, AChE-positive nerves were exceedingly scarce in the walls of the ventricles (Fig. 5*b*). These findings are especially intriguing in the context of the nonadrenergic component of the innervation of the atria alluded to previously. Approximately half of the atrial PGP-IR nerves contain DBH, while the remainder correspond qualitatively with the density of AChE-positive nerves demonstrated in the atria. It seems reasonable to postulate, therefore, that the AChE-positive nerves represent a population of atrial nonadrenergic nerves which are presumably cholinergic in type and vagal in origin. This hypothesis is in keeping with the fact that, in marked contrast to the sympathetic system, vagal influences on both the mechanical and electrical properties of the atria are much more profound than on the ventricles (James, 1967, 1980).

In the study of Wharton et al. (1990), however, cholinesterase-positive nerves were found to be similar in density in the adult human heart to the populations of both TH-IR and NPY-IR nerves, both in the atria as well as in the ventricles. This contrasts with our finding of few cholinesterase-positive nerves in the ventricles of the neonatal heart. In this regard, it is important to note that the pattern of distribution of AChE positive nerves in the myocardium in the newborn human heart, as delineated by our present study, besides being supported by physiological studies (James, 1967, 1980), is also in agreement with the findings of Kent et al. (1974), who have shown that, in the adult human heart, AChE positive nerves are abundant in the atria but sparse in the ventricular myocardium.

In conclusion, our study shows that, at birth, the human heart possesses a rich supply of autonomic nerves. The atria are supplied by at least two populations of autonomic nerves, presumably sympathetic and vagal, and presumably influencing directly the myocardium. The ventricles, in contrast, are predominantly innervated by presumptive sympathetic nerves. These findings indicate that, other than NPY-IR nerves, the intrinsic innervation of the chambers of the newborn human heart correspond closely with the pattern and distribution seen in the adult.

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