Sympathetic Innervation Alters Growth and Intrinsic Heart Rate of Fetal Rat Atria Maturing in Oculo

Diane C. Tucker and Richard Gist

The influence of sympathetic innervation on the growth and intrinsic rate of beating established by fetal rat heart was studied by culturing fetal atrial tissue in sympathetically innervated and denervated anterior eye chambers of adult Sprague-Dawley rats. One anterior eye chamber in each host rat was sympathetically denervated by removing the ipsilateral superior cervical ganglion. In oculo, atrial grafts were vascularized by blood vessels sprouting from the iris and innervated by sympathetic and parasympathetic fibers from the ground plexus of the iris. Innervation was assessed by light-activated efferent nerve stimulation to the grafts that changed their rates of beating. The norepinephrine contents of 16 atria cultured for 2.5 months in sympathetically innervated and denervated eye chambers were 5.7 ± 1.1 ng/implant vs. 0.2 ± 0.07 ng/implant (mean ± SEM), indicating permanent sympathetic denervation of the anterior eye chamber and the implanted atria. By 8 weeks in oculo, atria maturing in sympathetically innervated anterior eye chambers were 86% larger than those in denervated eye chambers (2.22 ± 0.29 vs. 1.19 ± 0.13 mm²); the weight of innervated transplants was over 3 times that of noninnervated grafts (2.35 ± 0.75 vs. 0.76 ± 0.21 mg). After implanted atria had ceased growing rapidly (2.5 months in oculo), bipolar electrodes were implanted adjacent to the cornea to record impulses from atrial grafts while host rats were unanesthetized. The dark-adapted baseline heart rates of sympathetically innervated and noninnervated atria were virtually identical (289 vs. 290 bpm). Graft intrinsic heart rate was estimated by combined β-adrenergic and muscarinic receptor blockade with atenolol (1.0 mg/kg) and methylatropine (10 μg/kg). Sympathetically innervated transplants had lower intrinsic heart rates than noninnervated atria (134 ± 25 vs. 213 ± 12 bpm). These data suggest that sympathetic innervation of the developing heart influences both growth and intrinsic rate of beating. (Circulation Research 1986;59:534-544)

CONTACTORY exists over the role of sympathetic stimulation in maturation of the heart. Individual differences in tonic sympathetic stimulation of the heart are observed in newborn animals and may contribute to subsequent differences in heart size and function1-3 (and Clubb et al., unpublished manuscript). Since cardiac function and autonomic innervation each mature rapidly during fetal and perinatal development, it is difficult to separate inherent from innervation-induced changes in the heart. In vivo and in vitro experiments suggest that sympathetic stimulation exerts a trophic effect on the heart and alters its intrinsic pacemaker activity.14-9 However, other investigations argue against this hypothesis.10-18 Because sympathetic innervation of the heart occurs prior to birth,19-21 resolution of this controversy requires a developmental model in which sympathetic innervation of the heart can be manipulated beginning early in cardiac development and in which direct consequences of sympathetic innervation can be observed. To this end, we compared the growth and intrinsic rate of beating of fetal rat heart tissue cultured in rat anterior eye chambers that were sympathetically innervated or denervated.

Sympathetic influences on cardiac growth depend on the developmental stage of the heart. In fetal and newborn rodents, increases in cell number contribute to cardiac growth. During the second postnatal week, cardiac cells stop dividing, become binucleated, and the heart begins to grow by cellular hypertrophy.22 The transition of the heart to growth by hypertrophy coincides with functional maturation of sympathetic cardiac innervation.23-24 β-Adrenergic receptor stimulation of newborn rat hearts through a bolus injection of isoproterenol inhibits further cell division and DNA synthesis, suggesting that catecholamine stimulation of the developing heart may influence its mechanism of growth. In hearts which have made the transition to growth by hypertrophy, isoproterenol treatment results in cardiac hypertrophy which is mediated by an increase in cell size.25 In young rabbits whose hearts are presumably postmitotic, chronic blockade of β-adrenergic receptors inhibited cardiac growth.8,9 In vitro, nondividing cells isolated from neonatal hearts showed hypertrophy when incubated with β-adrenergic6 or α-adrenergic receptor antagonists.7

Sympathetic stimulation of developing heart is also reported to influence cardiac function. Nayler and colleagues9 found that prolonged β-adrenergic receptor blockade resulted in a faster pacemaker rate in young
rabbit hearts, as measured in vitro while perfusing with the Langendorff technique. Ishii and colleagues reported that immunological or chemical sympathectomy of neonatal rats caused postjunctional inotropic supersensitivity to norepinephrine in both atrial and ventricular myocardium. Beating of quiescent myocytes isolated from newborn rat ventricle was induced by combined \( \beta \)-adrenergic and \( \alpha \)-adrenergic receptor stimulation. Thus, in vivo and in vitro experiments suggest that sympathetic innervation may influence both the growth and function of the developing heart.

In contrast, other investigators suggest that inherent developmental processes determine the growth and functional characteristics of the young heart. Kirby and Stewart produced "sympathetically aneurial hearts" by ablating the portion of the neural crest containing presumptive sympathetic neurons in embryonic chicks and observed normal cardiac morphology. Similarly, destruction of sympathetic innervation of the embryonic chick heart by injection of either reserpine or 6-hydroxydopamine in ovo altered neither the subsequent growth of the heart in vivo nor the developmental changes in its inotropic sensitivity to \( \beta \)-adrenergic receptor stimulation. Postnatal sympathectomy of rats with either 6-hydroxydopamine or antiserum to nerve growth factor did not prevent cardiac hypertrophy in two strains of genetically hypertensive rats (i.e., SHR, New Zealand GHR). These data argue against a role for sympathetic innervation in determining the growth of the young heart.

Developmental changes in intrinsic rate of beating have been argued to be inherent to the maturing heart. Heart rate increases gradually during fetal and early postnatal development. Because neither acute electrical nor pharmacological manipulations altered heart rate significantly during this period, Adolph concluded that this gradual increase in the resting heart rate of fetal and neonatal rodents was due to changes to "intrinsic controls" rather than autonomic influences. Vlk and Vincenzi corroborated this observation through in vitro study of hearts isolated from newborn, 1-week-old and adult mammals (i.e., rats, guinea pigs, and rabbits). Wekstein reported that the baseline heart rate of 1-week-old rats was not altered by treatment with antiserum to nerve growth factor or by chronic blockade of sympathetic influences with reserpine. These data support the hypothesis that changes in the pacemaker rate of the heart during early development are independent of sympathetic stimulation.

Catecholamine fibers innervate the heart prior to birth. In the rat, catecholamine-synthesizing enzymes are first detected in the primordia of the thoracic sympathetic ganglia of rat embryos on the 11th day of gestation (i.e., 12 days postconception, 6-mm crown-to-rump length). Catecholamine-induced fluorescence was observed by de Champlain and colleagues in the stellate ganglia of 13-day-postconception rat embryos and fluorescent axon bundles were visualized in the heart at 18 days after conception. Thus, the rat heart is not innervated by sympathetic neurons prior to 12–13 days after conception.

To test the hypothesis that sympathetic innervation influences the growth and intrinsic rate of beating of the developing mammalian heart, it is necessary to manipulate sympathetic innervation of the heart beginning in midgestation. We tested this hypothesis by culturing heart tissue dissected prior to innervation in situ in the anterior eye chamber of a host rat.

Culture of heart tissue in oculo has several advantages over alternative methods. While fetal hearts cultured in vitro steadily lose weight and are a "failing preparation," embryonic or fetal hearts cultured in oculo become vascularized by vessels from the ground plexus of the iris, grow and continue to beat for over a year. Sympathetic and parasympathetic fibers that normally control pupil size sprout and innervate the transplanted fetal heart. One anterior eye chamber can be sympathetically denervated in host rats by ipsilateral superior cervical ganglionectomy. Using this procedure, we were able to compare the growth and controls of pacemaker activity of fetal hearts innervated by the sympathetic nervous system with those never innervated. As the in oculo heart does not pump against a pressure gradient, hemodynamic consequences of manipulating sympathetic innervation during development do not confound interpretation of the data.

The present study examined two questions. (1) Does sympathetic innervation exert a trophic effect on the growth of fetal heart tissue? (2) Does sympathetic innervation influence the intrinsic rate of beating established by the pacemaker of fetal heart tissue maturing in oculo?

**Materials and Methods**

Heart tissue was dissected from Sprague-Dawley rats at 12 days postconception. Dams and sires were purchased from Taconic Farms and mated in our laboratory. Successful mating (day 0 of gestation) was judged by the presence of a vaginal plug. Host rats were 34–4-week-old Sprague-Dawley males (Taconic Farms). All rats were maintained on a 12-hour light-dark cycle.

**Sympathetic Denervation of the Anterior Eye Chamber.** Two days prior to implantation of heart tissue, one anterior eye chamber of each host rat was sympathetically denervated by removal of the ipsilateral superior cervical ganglion (SCG). Both the preganglionic and postganglionic nerve trunks were severed and the entire ganglion was removed to prevent regrowth of sympathetic fibers into the anterior eye chamber. Other targets of the SCG receive bilateral innervation (e.g., pineal and thyroid glands) and unilateral superior cervical ganglionectomy does not compromise their function.

**Dissection and Implantation of Atrial Tissue.** At 12 days postconception, both uterine horns were removed aseptically from 8 ether-anesthetized dams. Dissections were done in a sterile Tris-Tyrode's solution (pH 7.40) at room temperature. The beating atria were dissected from each fetus (10–12 fetuses per dam) using an Aus Jena surgical microscope. Measurements of fetal crown-to-rump length, heart size,
and atrial size were made using a calibrated micrometer in the microscope eyepiece. Crown-to-rump length of fetuses from the 8 litters ranged from 5.9 to 8.4 mm (mean 6.3 ± 0.3 mm). Fetuses had both forelimb and hindlimb buds. Hearts were four-chambered and 1.6 ± 0.09 mm² in size; heart size was measured as the product of length and width.

Atria rather than whole fetal hearts were cultured to avoid confusing slow pacemaker activity by the sinoatrial node with rates controlled by the atrioventricular node. In a pilot study, 7 whole fetal hearts and 7 atria were cultured in the same host rats. While whole hearts were larger than atria after 6 weeks in oculo (3.61 ± 0.50 vs. 1.73 ± 0.22 mm²; mean ± SEM), the rate of beating did not differ (219 ± 45 vs. 270 ± 28 bpm), suggesting that chronotropic characteristics of atrial grafts reproduce those of the whole fetal heart grafts.

Prior to implantation of fetal atria, host rats were anesthetized with ether and the iris was constricted by topical application of methylatropine (1.0 mg/ml) to the cornea. Implantation was accomplished by gently drawing spontaneously beating atrial tissue into a beveled pipette and injecting it into the anterior eye chamber of a host rat through a small cut made in the cornea. Atrial tissue rapidly adhered to the iris over which it was positioned. Neosporin ophthalmic ointment was placed on the eye after surgery to prevent infection. Healing of the cornea began almost immediately, and no scar was evident within a week in most cases. The behavioral competence of the host rat was not compromised by the implantation of heart tissue into the anterior eye chamber for 20 minutes. Baseline in oculo heart rates were collected from dark-adapted host animals. Heart rate responses to the onset and offset of a 5-second light stimulus (40-foot candles) were then measured. Pronounced bradycardia (greater than 150 bpm fall) in response to light onset was observed from in oculo atria but not from the host rat, verifying that the electrograms recorded from in oculo heart leads originated from the transplanted heart and not the host heart. The light stimulus was repeated once during the baseline period and during subsequent pharmacological treatments. Failure to observe light-induced bradycardia after methylatropine treatment confirmed successful blockade of muscarinic receptors. Drugs were administered via a subcutaneous catheter. The intrinsic heart rate recording protocol comprised sequential pharmacological blockade of parasympathetic influence with atropine methyl nitrate (10 μg/kg) and sympathetic influence with atenolol (1.0 mg/kg). Dose–response curves were generated for each drug during preliminary studies; these doses were selected as the lowest which produced a maximal blockade to challenge with agonists. Maximal heart rate response to β-adrenergic receptor stimulation was determined by administering isoproterenol sulfate (1.0 μg/kg). Determination of maximal heart rate was separated from the intrinsic heart rate protocol by 2–3 days to assure clearance of the receptor blockers from the host rat.

Catecholamine content of implanted atria. To confirm prevention of sympathetic innervation of atria implanted into sympathetically denervated anterior eye chambers, norepinephrine (NE) and epinephrine (E) content of implanted atria were measured by high pressure liquid chromatography with electrochemical detection. Host rats were killed by decapitation and the eyes rapidly removed. The implanted atria were dissected from the anterior eye chamber in ice cold saline, the iris was trimmed from around the graft, and the...
implanted atria were placed in a microcentrifuge tube and frozen in liquid nitrogen. Implants from both eyes were dissected simultaneously.

Tissue samples were weighed on a Metler H54 AR analytical balance and placed in a microhomogenizer (Kontes Glassware, Vineland, N.J.). The tissue was homogenized in 400 μl of 0.1 M perchloric acid containing 0.5 mM glutathione as an antioxidant and 5 ng/ml 3,4-dihydroxybenzylamine (DHBA; Sigma Chemical, St. Louis, Mo.) as an internal standard. Protein determination was by a modification of the method of Bradford from 100 μl of the homogenate. The remaining homogenate was centrifuged at 12,000 rpm for 10 minutes in an Eppendorf 5414 centrifuge. Supernatant (200 μl) was added to 1 ml of 0.1 M phosphate buffer (pH 7.0) and 50 mg of washed alumina (BioAnalytical Systems, West Lafayette, Ind.). Tris buffer (1 ml of 1.5 M, pH 8.6) was added, the tubes were capped and shaken by hand for 10 minutes. After the alumina had settled, the liquid was aspirated off and the alumina was washed with distilled water three times. The alumina was transferred to micro-

Table 1. Protocols for Determining Intrinsic and Maximal Heart Rate

<table>
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<tr>
<th>Heart rate</th>
<th>Interpretation</th>
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<tr>
<td><strong>Intrinsic</strong></td>
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<tr>
<td>I. Dark-adapted baseline heart rate</td>
<td>Rate under conditions of high sympathetic tone</td>
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<td>Light stimulus (5-second pulse, repeated once)</td>
<td>Functional innervation</td>
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<td>II. Muscarinic receptor blockade (10 μg/kg methylatropine s.c.)</td>
<td>Tonic parasympathetic control</td>
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<td>Light stimulus</td>
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<td>III. β-adrenergic receptor blockade (1.0 mg/kg atenolol s.c.)</td>
<td>Infer sympathetic component of response to light stimulus</td>
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<tr>
<td>Light stimulus</td>
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<tr>
<td><strong>Maximal</strong></td>
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<tr>
<td>I. Dark-adapted baseline</td>
<td>Rate under conditions of high sympathetic tone</td>
</tr>
<tr>
<td>II. Isoproterenol (1.0 μg/kg)</td>
<td>Maximum heart rate to direct β-adrenergic receptor stimulation</td>
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filters (BioAnalytical Systems) and centrifuged to remove the water. A new recovery cap was placed on the filter and 200 µl of 0.1 M perchloric acid added to the alumina and vortexed. The acid/alumina slurry was allowed to stand for 5 minutes, vortexed again and centrifuged at 3000 rpm for 5 minutes. A 50 µl sample of the acidic extract was injected into the HPLC system. The HPLC system consisted of an LDC minipump (Milton Roy, Rivera Beach, Fla.), an IBM C-18 column (IBM Instruments, Danbury, Conn.), LC4B electrochemical detector (BioAnalytic Systems) and a Hewlett Packard HP3390A integrator (Hewlett-Packard Instruments, Atlanta, Ga.). Mobile phase conditions were 0.02 M citrate/0.02 M Na2HPO4/acetoni-trile (650:300:40) pH 3.75 with 1.5 mM TEA and octyle sodium sulfate 115 mg/L. All chemicals were analytical grade (Sigma Chemical, St. Louis, Mo.).

**Data analysis.** Developmental changes in the transplant size and rate were analyzed using multivariate profile analysis. Univariate analyses of variance were used to follow up significant multivariate results. The dark-adapted heart rate, light response and response to drug treatments of sympathetically innervated and noninnervated atria were compared by analysis of variance. Transplant weight and catecholamine content were compared using Student’s t test. Follow-up comparisons were made using Newman-Kuel’s tests. Data are reported throughout as mean ± standard error (SEM).

**Results**

**Observations of fetal atrial tissue in oculo.** Within 24 hours after implantation, sinuses of blood were evident on implanted atria, with the tissue surface becoming well vascularized within 1 week. In oculo, fetal heart tissue does not grow to the size it would achieve in situ and does not retain distinct chambers. Instead blood-filled sinusoids are observed. The coro-nary arterial system does not develop in oculo. Blood vessels grow in from the ground plexus of the iris and are relatively uniform in their distribution.

**Catecholamine content of atrial grafts.** Figure 2 presents norepinephrine content of atria cultured in sympathetically innervated and noninnervated anterior eye chambers. Measurement of norepinephrine content of atria implanted into sympathetically denervated eye chambers confirmed nondetectable to very low catecholamine levels (range, 0-0.550 ng/implant, mean = 0.17 ± 0.07 ng/implant compared to range 1.4-11.9 ng/implant, mean = 5.7 ± 1.1 ng/implant for innervated implants). As care was taken to remove the iris not directly adherent to the atrial graft, the norepinephrine content of atria maturing in sympathetically innervated eye chambers suggests that the expected ingrowth of sympathetic fibers into the grafted atria did, in fact, occur. Measurable epinephrine content was found in 2 noninnervated and one innervated implant (1.6 and 1.4 ng/implant in noninnervated and 7.8 ng/implant in innervated implants). The very low to nondetectable catecholamine content of noninnervated atrial implants suggests that noninnervated implants do not sequester catecholamines from the circulation. This finding is consistent with Potter’s report that the capability of denervated myocardium to take up and store catecholamines is minimal (6% of capacity of innervated myocardium).

**Growth of atria maturing in oculo.** Sympathetically innervated grafts showed significantly greater growth than grafts into denervated eye chambers [F (3, 22) = 5.25, p < 0.007]. Subsequent analyses indicated differential growth between weeks 2 and 4 and between weeks 4 and 6. By 8 weeks after implant, atria maturing in sympathetically innervated anterior eye chambers were 86% larger than atria maturing in sympathetically denervated eye chambers (2.22 ± 0.29 vs. 1.19 ± 0.13 mm²). Figures 3 and 4 show the differential growth of atria in sympathetically innervated and noninnervated eye chambers. Because the implants are three dimensional structures, surface dimensions are likely to underestimate actual differences in growth as a function of experimental manipulations. To examine this possibility, implanted atria from 8 host rats were weighed. Sympathetically innervated atrial grafts weighed three times more than noninnervated atria (2.35 ± 0.75 mg vs. 0.76 ± 0.21 mg). When viewed through the surgical microscope, sympathetically innervated implants were dark red in color and appeared to be thicker than the more amber-colored noninnervated implants.

**Rate of atria maturing in oculo.** Atria maturing in sympathetically innervated eye chambers maintained a higher rate than innervated implants when measurements were made with the host rats anesthetized with ether and using the white light source on the surgical microscope (see Figure 5). These measurement conditions produce high parasympathetic tone to the anterior chamber (white light) and high levels of
Growth of Atria In Oculo

![Graph showing growth of fetal rat atria in oculo](image)

**FIGURE 3.** Effects of sympathetic innervation on growth of fetal rat atria transplanted into the anterior chamber of the eye. Atria placed into anterior chambers denervated by removal of the ipsilateral superior cervical ganglion did not differ in size at the time of implantation (i.e., they were from the same litter of fetuses and implanted at the same time), yet by 6 weeks in oculo atrial grafts into sympathetically innervated anterior eye chambers had more than doubled in size, while atria in denervated eyes failed to grow ($t(55) = 2.3, p < 0.05$). Data are mean ± SEM.

Circulating catecholamines (ether anesthesia). Thus, the higher basal rates in sympathetically noninnervated atria could reflect an increased intrinsic heart rate, reduced parasympathetic tone and/or an increased sensitivity to circulating catecholamines in sympathetically noninnervated transplants.

After two months in oculo, intrinsic heart rate and autonomic controls were estimated from ether-anesthetized hosts using white microscope light (Figure 6). Parasympathetic tone was estimated by the heart rate increase after intraperitoneal injection of atropine methyl nitrate (10 μg/kg). Sympathetic tone was estimated by the heart rate decrease after injection of atenolol (1.0 mg/kg). Intrinsic heart rate was estimated after combined muscarinic and β-adrenergic receptor blockade. There was no evidence of reduced functional parasympathetic control in sympathetically noninnervated transplants. Instead, the intrinsic heart rate of transplants not receiving sympathetic innervation was increased ($167 ± 6$ vs. $111 ± 18$ bpm, $p < 0.01$). A possible enhanced sensitivity to circulating catecholamines was suggested by the significant fall in heart rate after blockade of β-adrenergic receptors with atenolol in both sympathetically innervated and denervated transplants ($−30 ± 13$ and $−58 ± 10$ bpm, respectively).

To obviate concerns about the effects of anesthesia of the host and measurement under white light, recordings of heart rate were also made from chronic recording electrodes. On the first day of testing, a dark-adapted baseline heart rate was obtained simultaneously from atria implanted into sympathetically innervated and denervated eye chambers in each host. Baseline heart rates of sympathetically innervated and noninnervated atria were virtually identical (289 vs. 290, see Figure 7). Adaptation to a darkened chamber produces high sympathetic and low parasympathetic tone to the anterior eye chamber.

A profound bradycardia after presentation of a light stimulus ($−205 ± 33$ bpm for sympathetically innervated and $−207 ± 35$ bpm for noninnervated transplants) confirmed that electrograms were from in oculo atria rather than the host rat. No significant change in rate of the in oculo atria was observed after atropine treatment (see Figure 7). This result was expected, given the low parasympathetic tone to the anterior chamber when host rats are adapted to a darkened environment. However, muscarinic blockade attenuated substantially the bradycardic response to light stimulation (to $−40 ± 16$ for sympathetically innervated and $−38 ± 19$ for noninnervated transplants). Substantial sympathetic control of in oculo atria when host rats are in a darkened environment was confirmed by

**FIGURE 4.** Photographs of sympathetically innervated (Panel A) and noninnervated (Panel B) atrial grafts that illustrate the difference in size observed as a function of sympathetic innervation.
the heart rate decrease after β-adrenergic receptor blockade with atenolol (−135 ± 16 and −88 ± 15 bpm in innervated and noninnervated transplants). In noninnervated atrial grafts, the bradycardia in response to atenolol treatment represents functional control of in oculo heart rate by circulating catecholamines. In contrast, both circulating catecholamines and high sympathetic neural tone to the anterior eye chamber contribute to adrenergic stimulation of atria in sympathetically innervated eye chambers.

The intrinsic heart rates estimated after combined β-adrenergic and muscarinic receptor blockade were 134 ± 25 for sympathetically innervated atria and 213 ± 12 for noninnervated atria (p < 0.05; see Figures 7 and 8). This difference suggests that innervation by the sympathetic nervous system can affect the intrinsic pacemaker rate established by developing myocardium.

Maximal heart rates estimated after injection of 1 µg/kg isoproterenol did not differ between sympathetically innervated and noninnervated atrial grafts (i.e., 317 ± 18 vs. 334 ± 17 bpm; see Figure 8).

Discussion

Sympathetic innervation exerted a trophic effect on the growth of fetal rat atria cultured in the anterior chamber of the rat eye and depressed the intrinsic rate at which the atrial grafts beat. These findings suggest that differences in sympathetic stimulation during development may contribute to variability among individuals in heart size and rate.

Advantages of in oculo culture for studying the effects of sympathetic innervation on cardiac development. In oculo, atria from fetal rat hearts grow, become vascularized, and are functionally innervated. In contrast, atrial strips from adult
Intrinsic and maximal heart rates (mean ± SEM) of atria cultured in sympathetically innervated and denervated anterior eye chambers. Recordings were made after atria had been in oculo for 3 months from chronically implanted electrodes. Intrinsic rate was determined after combined blockade with atenolol and metyl-xatrpopine. Maximal heart rate was determined after injection of 10 μg/kg isoproterenol into a subcutaneously implanted catheter. Maximal heart rate did not differ between sympathetically innervated and noninnervated grafts (p < 0.05).

The influence of innervation on cardiac development can also be studied in other model systems. Fetal hearts placed in organ culture fail to grow, remaining viable for only 2–3 weeks. Cells dispersed from fetal and newborn rat hearts can be grown in culture. Their environment can be precisely specified but is very different from their milieu in situ (e.g., myocytes are isolated from rather than in close contact with other heart cells). Culture of fetal heart in oculo is a pace-maker-driven model of heart development33 that preserves many aspects of heart tissue organization yet allows the neural and hormonal environment to be manipulated. Different questions about heart development are best examined in cell culture, in organ culture, in oculo, and in intact animals. Within these model systems degree of experimental control is balanced against disruption of normal conditions for cardiac development.

Atria cultured in oculo become innervated at approximately the same time after conception as do atria in situ. Olson and colleagues30,31 reported innervation of fetal rat hearts cultured in the anterior eye chamber to occur during the second and third weeks after implantation. For atria grafted at 12 days postconception in our study, this would correspond to innervation in oculo between 20 and 26 days after conception (E-20 to P-3), the period during which sympathetic innervation of the heart undergoes final maturation in situ.23 Prior to growth of catecholamine fibers into the grafted atria, catecholamine stimulation would come both from circulating catecholamines and, in sympathetically innervated eye chambers, by diffusion of norepinephrine released from the sympathetic nerves innervating the iris. The consequences of this early catecholamine stimulation of hearts developing in oculo remain to be determined.

In oculo, sympathetically innervated and noninnervated atrial grafts are perfused by the same circulating catecholamine and hormonal stimulation. By comparing sympathetically innervated and noninnervated grafts, we can determine the effects of sympathetic innervation per se on cardiac growth and rate. Growth of sympathetically innervated atrial grafts may have been enhanced because they received a greater quantity of norepinephrine stimulation than noninnervated grafts or because they were exposed to some other trophic substance released from sympathetic nerves. In oculo, beating heart tissue does not pump against a blood pressure gradient so the increased growth of sympathetically innervated atrial grafts cannot be attributed to differential hemodynamic load.

Trophic influences of sympathetic innervation on heart growth. This experiment demonstrated that sympathetic innervation promotes the growth of fetal rat atria grafted into the anterior eye chamber. At the time the atria were grafted, cell division was contributing to cardiac growth.22 Sympathetic innervation may have increased graft size by promoting ongoing cell division, increasing the size of individual myocytes, or both. Claycomb’s report4 that isoproterenol treatment of newborn rats inhibited subsequent cell division argues against the possibility that sympathetic innervation of grafted fetal atria would promote cell division. Since catecholamine stimulation is known to induce hypertrophy of myocytes,6,25 it seems likely that sympathetically innervated grafts had larger cells. However, determination of whether cell size or number or both were increased by sympathetic innervation awaits empirical verification.

Our findings are consistent with the report of Nayler and colleagues8 that pharmacological blockade of sympathetic stimulation of young rabbits reduced heart size and inconsistent with the reports of Kirby and Stewart14 and of Higgins and Pappano15 that sympathectomy of the embryonic chick heart did not alter its growth. In the present study, the surface dimensions of sympathetically innervated atrial grafts were not significantly increased over noninnervated grafts for the first 4 weeks in oculo. If Kirby and Stewart14 and Higgins and Pappano15 had been able to follow their sympathectomized chick hearts for a longer period of time, growth differences might have been detected. Alternatively, the immature sympathetic innervation of the fetal heart may exert minimal control of cardiac growth, with more substantial sympathetic influence on heart size occurring after birth.

Trophic influence of sympathetic innervation on other cardiovascular target organs. Sympathetic innervation has also been reported to exert a trophic influence on developing blood vessels. Bevan and Bevan39,40 demonstrated that sympathetic innervation promotes the growth of blood vessels in the ear of young rabbits using a strategy similar to that used in the present experiment. They produced unilateral denervation by removing the superior cervical ganglion.
which supplies sympathetic innervation to the ipsilateral ear. In young rabbits (4 weeks of age), sympathetic innervation resulted in decreased vascular smooth muscle proliferation, fewer vascular smooth muscle cells and stiffer blood vessel walls.\textsuperscript{50-52} Sympathetic denervation of the arteries of older rabbits did not alter the number of smooth muscle cells. However, in all age groups, sympathetic denervation reduced force of vascular contraction and produced postjunctional hypersensitivity to depolarizing stimuli.

Aprigliano and Hermansmeyer\textsuperscript{43} produced sympathetic denervation of the portal vein by 6-hydroxydopamine treatment of newborn rats. Postjunctional alterations were observed in the sympathectomized portal veins which Aprigliano and Hermansmeyer\textsuperscript{43} attributed to removal of a trophic influence of the sympathetic nervous system. Specifically, a partial depolarization was observed in denervated portal veins. They hypothesized that the associated ionic changes were components of the trophic interaction between sympathetic nerves and blood vessels.

In spontaneously hypertensive rats (SHR), the hypertrophy of blood vessel walls and increased vascular reactivity are at least partially dependent on sympathetic innervation. Unilateral surgical denervation of cerebral arteries in 3-week-old stroke-prone SHR attenuated the wall thickening expected in intraparenchymal vessels 1 year later.\textsuperscript{44} Abel and Hermansmeyer\textsuperscript{45} demonstrated that sympathetic innervation may be necessary for development of the membrane properties which underly vascular hyperreactivity in SHR rats. They transplanted tail arteries from 2-week-old SHR and WKY control pups into the anterior eye chamber of SHR or WKY host rats. In sympathetically innervated eye chambers, the blood vessels underwent interconversion to membrane properties (resting potential and NE sensitivity) characteristic of the host strain. However, if cultured in sympathetically denervated eye chambers, the arteries developed membrane properties characteristic of the donor strain.

Both increased sympathetic nervous system activity and increased size of the heart and blood vessel walls are observed in neonatal rats of the genetically hypertensive SHR strain. Because of technical difficulties in selectively preventing sympathetic innervation of the heart and blood vessels during fetal development, the role of sympathetic stimulation in the early growth and differentiation of cardiac and vascular smooth muscle in situ has yet to be determined. By 4–5 days of age, pharmacological blockade of sympathetic stimulation produces a larger bradycardia\textsuperscript{1} and a greater fall in blood pressure\textsuperscript{46} in SHR than in WKY pups. By 3 weeks of age, norepinephrine in plasma and dopamine \(\beta\)-hydroxylase in blood vessels are both increased in SHR rats.\textsuperscript{47,48} Measurements of heart weight, ventricular weight to body weight ratios, ventricular wall thickness and myocardial DNA and RNA content indicate that the hearts of newborn SHR are larger than WKY, contain more cells, and are synthesizing more proteins.\textsuperscript{1,4,49,51} Similarly, the earliest measurements of the wall to lumen ratios and medial wall thickness of blood vessels in SHR indicate that hypertrophy begins during the first 2 postnatal weeks.\textsuperscript{52,53} Thus, there is considerable circumstantial evidence linking enhanced sympathetic activity with increased growth of cardiac and vascular smooth muscle. However, the increased hemodynamic demand placed on the heart and blood vessels of young SHR by the higher mean arterial pressure\textsuperscript{2,54-56} could also account for the cardiac and vascular hypertrophy.

**Effects of Sympathetic INNERVATION ON INTRINSIC HEART RATE.** In contrast to the tachycardic response observed to phasic sympathetic stimulation, the results of the present study indicate that tonic sympathetic stimulation of the developing heart reduces the intrinsic rate of firing of its pacemaker. Little is understood of how pacemaker rate is determined during development. Specialization of the sinoatrial region as pacemaker occurs very early in embryonic development.\textsuperscript{57,58} Electrical activity is initiated in the atrial region of the embryonic chick heart.\textsuperscript{57-59} Rhythmic electrical activity precedes mechanical activity and histologic identification of pacemaker cells.\textsuperscript{57,60} There is a gradual increase in the rate at which the embryonic heart beats during fetal and early postnatal development.\textsuperscript{1,10,11,61} An increased pacemaker rate in the maturing heart may result from changes inherent in the developing heart, from the changes in the fetal and postnatal heart’s neurohumoral milieu or from an interaction between intrinsic and neurohumoral factors. The current data indicate that sympathetic innervation can influence the pacemaker rate established by the embryonic heart.

Individual differences in functional sympathetic maturation and tonic activity exist early in development.\textsuperscript{1,4} Individual differences in sympathetic nervous system function may be genetically based (e.g., SHR rats)\textsuperscript{40} or induced by environmental factors.\textsuperscript{62} The present results suggest that sympathetic innervation of developing cardiovascular target organs may be one effector system through which genetically determined and environmentally induced differences influence subsequent cardiovascular function.

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**References**

26. Teitelman G, Baker H, Joh TH, Reis DJ: Appearance of cate-
22. Clubb FJ, Bishop SP: Formation of binucleated myocardial
21. Pappano AJ: Ontogenetic development of autonomic neuroef-
12. Vlk J, Vincenzi FF: Functional autonomic innervation of mam-
11. Adolph EF: Ontogeny of heart-rate controls in hamster, rat,
18. Clark DW, Jones DR, Phelan EL, Devine CE: Blood pressure
16. Cutilletta AF, Benjamin M, Culpepper WS, Opari S: Myocardia-
19. DeChamplain J, Malmors T, Olson L, Sachs C: Ontogenesis of
7. Simpson P: Stimulation of hypertrophy of cultured neonatal rat
6.. Simpson P, McGrath A, Savion S: Myocyte hypertrophy in
30:153-157

28. Ingwall JS, Goldhaber SZ, Roeseke WR, Wildenthal K: Mouse
29. Ingwall JS, Roeseke WR, Wildenthal K: The fetal mouse heart
growth and intrinsic rate of hearts maturing in oculo. Circulation
25. Zapk R, Rabinowitz M: Molecular aspects of cardiac hyper-
24. Seidler FJ, Slotkin TA: Presynaptic and postsynaptic contribu-
22. Clubb FJ, Bishop SP: Formation of binucleated myocardial
18. Clark DW, Jones DR, Phelan EL, Devine CE: Blood pressure
17. Clark DW, Jones DR, Phelan EL, Devine CE: Blood pressure


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D C Tucker and R Gist

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