Sympathetic and Nonsympathetic Neuropeptide Y–Containing Nerves in the Rat Myocardium and Coronary Arteries

Laura A. Corr, Judy A. Aberdeen, Pamela Milner, Jill Lincoln, and Geoffrey Burnstock

We have examined the neuropeptide Y–containing intrinsic nerves of the heart in young (6-week-old) and adult (4-month-old) rats to determine whether they project to the coronary arteries or are capable of doing so if the neuropeptide Y–containing extrinsic nerves are removed. Chronic treatment of neonates with guanethidine was used to permanently destroy the sympathetic nerves. In the young treated animals, 33–54% of the neuropeptide Y remained in the heart despite a 90–99% reduction in norepinephrine; these proportions did not change in the animals that were allowed to develop to adulthood. The level of neuropeptide Y in the right atrium of young animals was unexpectedly high (252±28.7 pmol/g) compared with adults (75.4±18.8 pmol/g). The coronary arteries in the control rats received a moderately dense supply of neuropeptide Y–containing nerves; after guanethidine, all neuropeptide Y–containing nerves innervating the large coronary arteries disappeared, but some were still seen in association with small resistance vessels. No compensatory proliferation of the intrinsic neuropeptide Y–containing neurons occurred in the adult sympathectomized animals, and the intrinsic nerves did not reinnervate the large coronary arteries. These results are discussed in relation to the clinical syndrome of coronary artery spasm. (Circulation Research 1990;66:1602–1609)

Neuropeptide Y is a 36–amino acid peptide, described in 1982,1 which has been shown to be coexist with norepinephrine in peripheral nerves2 and to potentiate the actions of norepinephrine in peripheral blood vessels.3,4 It is a major cardiac neuropeptide5 and is most abundant around the coronary arteries6; recently, it has also been shown to be present in the intrinsic neurons of the heart where it is not coexist with catecholamines.7,8 The projection and function of these neurons are unknown.

Neuropeptide Y has been implicated as a cause of coronary artery spasm,9 a clinical syndrome known to involve the large epicardial coronary arteries, rather than the small resistance vessels,10,11 in which intense spasm of these arteries causes cardiac ischemia in the absence of any fixed coronary artery obstruction or increase in myocardial oxygen demand. The condition responds poorly to antiadrenergic therapy with α-receptor blocking agents,12,13 while calcium channel blocking agents such as nifedipine are effective in preventing the disorder. Furthermore, coronary artery spasm can occur spontaneously or be provoked even after cardiac transplantation,14,15 indicating that extrinsic cardiac innervation is not essential for the vasospasm to occur.

Neuropeptide Y is attractive as a candidate for the cause of this syndrome, because it has been shown to have a potent direct constrictor effect on the coronary arteries of several species including rabbits,16 pigs,17 dogs,18 guinea pigs,19 and humans.20 Furthermore, it is known to act postsynaptically via a calcium-dependent mechanism,19 and its actions can be blocked by calcium antagonists such as diltiazam or verapamil,21,22 but not by adrenoceptor blockade.17,23 In view of the fact that extrinsic innervation is not essential, the neuropeptide Y–containing intrinsic neurons would have to project to the large coronary arteries, or at least be capable of doing so under conditions of extrinsic denervation, for this peptide to be implicated in coronary artery spasm.

The aim of this study was to examine the intrinsic neuropeptide Y–containing nerves to determine whether they project to the large coronary arteries or can proliferate and send out collateral fibers to these arteries after long-term sympathectomy. We used the sympathetic neurotoxin guanethidine sulfate, which has been shown to cause a profound and long-lasting

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Received April 6, 1989; accepted January 19, 1990.
destruction of sympathetic neurons when given chronically in high doses to neonatal rats.24

Materials and Methods

Neonatal Sprague-Dawley rats were injected with guanethidine sulfate (50 mg/kg s.c., Ismelin, CIBA Laboratories, Horsham, UK) for 5 days of every 7 days from day 8 to day 28 of life according to the method of Johnson et al;24 age-matched controls were injected with the same volume of saline. The animals were examined at 6 weeks (when they would be considered juvenile) or 4 months (adults) of age. We have demonstrated plasticity of the peptidergic nerves in the heart in these young animals,25 and allowing the rats to grow to maturity without their sympathetic nerves should provide sufficient time for reinnervation of the coronary arteries to occur through any proliferation of intrinsic neurons. The animals were killed by inhalation of carbon dioxide and the hearts were rapidly removed. The free walls of the left and right atria and ventricles and the septal branch of the left coronary artery were dissected out and examined individually by using histochemical and biochemical techniques.

Histochemistry

Six treated and six control animals of each age were examined for the presence of both neuropeptide Y and catecholamines.

Fluorescence Histochemistry

The catecholamine-containing nerves in the periadventitia of the septal coronary arteries were examined by using the glyoxylic acid method of Lindvall and Björklund;26 the arteries were processed as whole mounts and counterstained with pontamine sky blue to reduce background staining.27 The atria and ventricles were rinsed, blotted dry, and embedded in cryoprotectant (OCT compound, BDH Ltd., Poole, UK) before being frozen in isopentane cooled in liquid nitrogen. Subsequently, 10-μm sections were cut with the use of a −30°C microtome cryostat and mounted on microscope slides. The sections were treated with glyoxylic acid according to the method of De La Torre and Surgeon.28 Both whole mounts and cryostat sections were mounted in liquid paraffin under coverslips for subsequent examination.

Immunohistochemistry

After fixation in 4% paraformaldehyde for 1–1.5 hours, the myocardial specimens were immersed in 7% sucrose in physiological buffered saline overnight; they were then rinsed, blotted dry, embedded in OCT cryoprotectant, and frozen in cooled isopentane before storage in liquid nitrogen. Subsequently, 10-μm sections were cut as above. The coronary arteries were pinned onto Sylgard (Dow Corning, Seneffe, Belgium) as whole mounts, fixed in 4% paraformaldehyde, and dehydrated in 80% alcohol. After they were washed in physiological buffered saline containing 1% Triton-X, the cryostat sections and whole mounts were incubated at room temperature for 12–18 hours with antibodies to neuropeptide Y raised in rabbits (Immunodiagnostic Systems Ltd., Washington, UK); the dilutions used were 1:200 for whole mounts and 1:400 for sections. A second-layer antibody, goat anti-rabbit IgG conjugated to fluorescein isothiocyanate (IDS), was subsequently applied at a dilution of 1:50. All specimens were counterstained with pontamine sky blue as above and mounted under coverslips in buffered glycerol (Citifluor; City University, London, UK) before being examined.

All histochemical specimens were examined by using a fluorescence microscope (Carl Zeiss, Thorn.

FIGURE 1. Sections through the right atrium of 6-week-old rats treated to show catecholamine-containing nerves. Panel a: Brightly fluorescent noradrenergic nerve fibers can be seen in the control animals in the myocardium and around blood vessels (for examples of nerve fibers, see arrows). Panel b: There is a faint background staining, but no noradrenergic nerve fibers can be seen in animals treated with guanethidine sulfate. Asterisks show a small arteriole in transverse section. Calibration bar represents 50 μm.
wood, New Jersey) equipped with a filter optimized for fluorescein.

**Assay**

Once dissected, the tissues were rapidly frozen in liquid nitrogen until assay. Each area of myocardium was assayed for both norepinephrine and neuropeptide Y. Norepinephrine levels were determined by high-performance liquid chromatography with electrochemical detection as previously described. Neuropeptide Y was extracted and assayed with an inhibition enzyme-linked immunosorbent assay as previously described. Control and treated tissues were assayed at the same time.

**Statistics**

Differences in tissue content of norepinephrine and neuropeptide Y at 6 weeks and 4 months of age after sympathectomy were assessed using Student’s

**FIGURE 2.** Whole-mount stretch preparations of the septal branch of the left coronary artery of the rat treated to show catecholamine-containing nerves. Panel a: Coronary artery from control rat at 6 weeks of age showing plexus of nerve fibers. Panel b: Coronary artery from 6-week-old rat treated with guanethidine sulfate. No fibers are seen. Panel c: Coronary artery from 4-month-old rat treated with guanethidine sulfate. No reinnervation with catecholamine-containing nerve fibers can be seen. Calibration bar represents 50 μm.

**FIGURE 3.** Norepinephrine levels in the myocardium of control and treated rats at 6 weeks of age (panel a) and at 4 months of age (panel b). RA, right atrium; LA, left atrium; RV, right ventricle; LV, left ventricle; n, number of animals.
two-tailed unpaired *t* tests. Results are expressed as mean±SEM; *p*<0.05 was considered significant.

**Results**

These were minor differences in the physical characteristics of the treated and control rats, similar to those previously described,24,31 in that the treated rats developed a transient bilateral ptosis during the injection period and showed slightly retarded growth. The ptosis had, however, disappeared by the time the rats were studied at 6 weeks and, although the treated rats were marginally lighter than the controls at 6 weeks, there was no difference in the weight of the rats at 4 months. The results for both histochemistry and biochemical assay within each group of animals studied were consistent.

In the myocardium of the control rats studied at 6 weeks of age there was a rich sympathetic innervation, with adrenergic fibers running parallel to the myocardial bundles and surrounding the large and small coronary arteries at the adventitial-medial border (Figure 1a); the innervation was most marked in the atria and was sparser in the ventricles. A dense plexus of adrenergic fibers was seen surrounding the septal artery in the whole-mount preparation (Figure 2a). However, in animals treated with guanethidine, no adrenergic fibers could be seen in the myocardium (Figure 1b) or surrounding the coronary arteries of the 6-week-old animals (Figure 2b), indicating that the regime used successfully removed all of the adrenergic neuronal fluorescence. The profound sympathectomy affected all areas of the myocardium and was confirmed by the results of biochemical assay, which showed a 90–97% reduction in the norepinephrine concentration in the treated animals compared with the controls (Figure 3a, Table 1).

The density and pattern of adrenergic innervation was unchanged in the control rats examined at 4 months compared with those at 6 weeks of age, suggesting that adrenergic innervation of the young animals is essentially already fully developed. Furthermore, in the treated animals allowed to develop to adulthood, there were still no adrenergic fibers in the myocardium or around the septal artery (Figure 2c). This indicated that there was no regeneration of the sympathetic nerves. Biochemical assay confirmed this, with a 95–99% reduction in norepinephrine in the myocardium in the treated rats compared with the controls (Figure 3b, Table 1).

Immunohistochemical staining of the control tissues for neuropeptide Y showed a dense innervation of the myocardium (Figure 4a) and septal coronary artery (Figure 5a) with neuropeptide Y-containing nerve fibers. The distribution of these nerve fibers in the control animals was similar to that of the noradrenergic fibers, and again the atria were more densely innervated than the ventricles at both 6 weeks and 4 months. However, unlike noradrenergic fibers, the density of innervation of neuropeptide Y–containing nerves in the right atrium of the rats at 6 weeks of age appeared greater than that in the 4-month-old rats. Biochemical assay confirmed the immunohistochemical findings (Figure 6). In all the animals, the concentration of neuropeptide Y was higher in the

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<th>Table 1.</th>
<th>Percent Reduction in Concentration of Norepinephrine (NE) and Neuropeptide Y (NPY) in Guanethidine Sulfate–Treated Rats Examined at 6 Weeks and at 4 Months of Age</th>
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<tr>
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<td>6 Weeks</td>
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<td>Right atrium</td>
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**Figure 4.** Sections through the right atrium of 6-week-old rats treated to show neuropeptide Y-containing nerves (examples indicated by arrows). Panel a: In the control animals, neuropeptide Y–containing nerves can be seen as brightly fluorescent fibers. Panel b: In animals treated with guanethidine to remove the sympathetic nerves, many fibers positive for neuropeptide Y can still be seen. Calibration bar represents 50 μm.
right atrium than in the other areas of the myocardium, but an unexpected finding was the exceptionally high levels of neuropeptide Y in the right atrium of the young animals. In the control animals, the concentration of neuropeptide Y in the right atrium was significantly higher at 6 weeks (252±28.7 pmol/g) than at 4 months (75.4±18.8 pmol/g, p<0.001), while the concentration of neuropeptide Y in the other areas of the myocardium showed no change between the control 6-week-old and control 4-month-old animals. Even after sympathectomy, the neuropeptide Y remaining in the right atrium of the young animals was exceptionally high (136.4±24.0 pmol/g).

In both the young and adult animals after sympathectomy, there was a substantial reduction in the density of the neuropeptide Y-containing nerve fibers on immunostaining, but fibers could still be seen in all regions of the heart (Figure 4b). Biochemical assay confirmed that, unlike norepinephrine, which was reduced by 90–99%, neuropeptide Y was not totally depleted from the myocardium after sympathectomy, but was reduced to 34–54% of the

FIGURE 5. Whole-mount stretch preparations of the septal branch of the left coronary artery treated to show fibers containing neuropeptide Y. Panel a: Coronary artery from 6-week-old animal showing plexus of fibers. Panel b: Coronary artery from 6-week-old rat treated with guanethidine sulfate. No neuropeptide Y-positive nerve fibers can be seen. Panel c: Coronary artery from 4-month-old rat treated with guanethidine sulfate. There is no evidence of reinnervation of the vessel from the neuropeptide Y-containing intrinsic nerve fibers remaining in the myocardium. Calibration bar represents 50 μm.

FIGURE 6. Neuropeptide Y levels in the myocardium of control and treated rats at 6 weeks of age (panel a) and at 4 months of age (panel b). RA, right atrium; LA, left atrium; RV, right ventricle; LV, left ventricle; n, number of animals.
controls in the young animals. Importantly, there was no increase in concentration of neuropeptide Y, or in the proportion of the peptide remaining after sympathectomy compared with control, in the adult treated animals (Table 1). Thus the population of neuropeptide Y–containing nerves in the rat heart that are intrinsic rather than sympathetic do not appear to proliferate after sympathectomy.

Despite the substantial population of neuropeptide Y–containing fibers remaining in the myocardium after sympathectomy, no fibers could be seen around the larger coronary vessels, although fibers could occasionally be seen in association with the smaller arteries in some sections. In the whole-mount preparation of the septal artery, neuropeptide Y–containing fibers could not be seen in the treated animals at 6 weeks or at 4 months (Figures 5b and 5c).

Thus, the intrinsic neuropeptide Y–containing nerve fibers that remain after sympathectomy do not project to the large coronary arteries in the rat, nor do they appear to reinnervate the large coronary arteries after denervation of the sympathetic nerves.

**Discussion**

Chronic high-dose guanethidine treatment of rats results in the selective and long-lasting destruction of postganglionic sympathetic neurons32–34 in both neonatal and adult rats35,36, although qualitatively similar, the destruction in neonates is more rapid and more complete.37 The treatment regime that produces neuronal destruction (confirmed at the ultrastructural level) uses doses of guanethidine well in excess of those required to produce depletion of catecholamine stores from the tissues and has been well characterized.38

Thus, our study was not confounded by the differential rate of depletion of catecholamines and peptides, which may occur with acute or low-dose regimes of guanethidine; the reduction in norepinephrine indicates destruction of the sympathetic nerves and hence all the associated transmitters contained therein. In agreement with Johnson et al.24 we obtained profound sympathectomy with a greater than 90% reduction in norepinephrine in all areas of the heart on assay and abolition of the catecholamine fluorescence on histochemistry.

The mechanism of action of guanethidine as a neurotoxin is not clear, but it appears to be immune mediated and specific for rats and has been shown to be ineffective in guinea pigs,39 hamsters and rabbits,40 mice,41 cats,42,43 and pigs.43 Variability in the response between different strains of rat, and even between different suppliers of the same strain, is recognized.38 and there have been reports of increases in the atrial content of norepinephrine in Wistar rats treated with the same regime and examined after 26 weeks.44 However, we saw no evidence of sympathetic reinnervation of the myocardium in our rats examined after 4 months, indicating that the Sprague-Dawley rats used were sensitive to the treatment and that we had produced an effectively permanent sympathectomy.

This enabled us to assess the potential for reinnervation of the myocardium by the remaining intrinsic neurons of the heart.

It is well recognized that there are intrinsic neuropeptide Y–containing nerves in the rat heart, and the reduction in neuropeptide Y we observed in the adult animals by using immunohistochemistry and assay agrees closely with reports by other workers, who used different methods to produce either acute depletion or destruction of sympathetic neurons. Using reserpine, Allen et al.45 found a reduction in neuropeptide Y concentration to 44–47% of controls in adult Wistar rats; Maccarrone and Jarrott46 carried out bilateral surgical sympathectomy in Sprague-Dawley rats and produced a 98% depletion of norepinephrine in the right atrium, while neuropeptide Y levels were reduced to only 50% of controls. Thus far, our study supports the evidence that in the rat approximately 50% of the neuropeptide Y in the heart does not come from sympathetic nerves and is likely to represent neuropeptide Y contained in intrinsic neurons.

However, all such studies reported to date have examined only the adult rat. The finding in the young rats of the remarkably high levels of neuropeptide Y in the right atria (both before and after sympathectomy), but not of norepinephrine, was unexpected and interesting. In our control 6-week-old animals, the mean level of neuropeptide Y was over three times higher than the level in our control 4-month-old animals and the level found by others in Sprague-Dawley adults (81.5±5.0 pmol/g)46 and Wistar adults (24.8±2.9 pmol/g in combined atria).45 After sympathectomy, the levels in the young animals were still greater than the control levels in older animals.

Although our study does not allow us to explain the significance of this finding, other workers have recently shown that the parasympathetic nerve fibers in the ventricles of the dog arise from ganglion cells in the atria; the long postganglionic axons of these cells cross the atioventricular groove.47 Thus it is possible that the intrinsic neuropeptide Y–containing nerve fibers throughout the heart arise from the ganglia in the interatrial septum, which would allow for a greater concentration of neuropeptide Y in the right atrium of the rat. However, this does not explain the reduction in concentration of neuropeptide Y with age. Previous studies have shown a difference in the development of peptidergic and adrenergic nerves,48 and it has been suggested that neuropeptides may act as trophic factors in developing animals,49 Clearly, more studies are needed to ascertain the role of neuropeptide Y in the developing rat.

The proportion of intrinsic neuropeptide Y–containing neurons in the heart may vary between different species. For example, Sternini and Brecha50 found no neuropeptide Y–containing nerves in the guinea pig heart that did not also contain tyrosine hydroxylase and noted parallel but incomplete deple-
tion of the two substances after treatment with 6-hydroxydopamine. In other studies in the guinea pig, reserpine depleted the right atrium of neuropeptide Y by 71% to 77% compared with controls, while 6-hydroxydopamine caused a reduction to 53% of control. This discrepancy seems likely to represent a difference in the mechanism of action of the two drugs. When norepinephrine levels were reduced to around 10% of control by bilateral stelllectomy in the guinea pig, the levels of neuropeptide Y were also reduced to the same degree. These studies imply a less substantial population of intrinsic neurons containing neuropeptide Y in the guinea pig compared with the rat.

In our older animals, the fact that there was no increase in the concentration of neuropeptide Y on immunohistochemistry or assay and no increase in the proportion of neuropeptide Y remaining in the treated rats compared with the controls indicates that the neuropeptide Y–containing intrinsic neurons do not show compensatory proliferation after removal of the sympathetic nerves. Since we have shown an increase in other, extrinsic, peptide-containing neurons in the heart after sympathectomy, this suggests the possibility that intrinsic neurons may be under different trophic influences than other peptidergic nerves. A study of the intrinsic neuropeptide Y–containing nerves in other organs after sympathectomy is currently under way.

Despite the evidence for the existence of the intrinsic neuropeptide Y–containing nerves in the rat heart, there are no reports in the literature of their projections or possible functional role. We examined the possibility that they innervate the large coronary arteries by using whole-mount preparations of the septal branch of the left coronary artery. This preparation demonstrates the innervation of the artery more clearly than would be possible in transverse sections of the myocardium where individual fine nerve fibers can be difficult to visualize.

We found that in the septal artery the immunoreactivity to neuropeptide Y in the young rats was completely abolished after the catecholamine-containing nerves were removed, which implies that the intrinsic neurons do not innervate this large coronary artery. We could only examine the innervation of the smaller arteries by using transverse sections of the myocardium, since any attempt to dissect out these vessels tended to disrupt the adventitia. However, while the immunoreactivity to neuropeptide Y around the smaller arteries appeared reduced after sympathectomy, it was not abolished in all areas.

Reinecke and Forssman have also reported neuropeptide Y–containing fibers in the denervated dog myocardium that were seen in contact with the coronary vasculature by using electron microscopy. In this regard, it is interesting to note some studies that suggest that the dominant vasoconstrictor action of neuropeptide Y in the coronary circulation is at the level of the smaller resistance vessels. However, these are not the vessels that are involved in the clinical syndrome of coronary artery spasm.

In the rats left until 4 months of age after guanethidine treatment, there were still no neuropeptide Y–containing nerves seen around the septal coronary artery, indicating that the intrinsic neurons are not capable of innervating this large coronary artery even after long-term destruction of the sympathetic nerves. In view of the persistence of coronary artery spasm after transplantation, a similar finding in humans would suggest that neuropeptide Y does not play a primary role in coronary artery spasm causing variant angina.

In summary, we have shown that the neuropeptide Y–containing intrinsic nerves do not innervate the large septal coronary artery, even under conditions of long-term sympathetic denervation, but may innervate the smaller resistance vessels. Furthermore, there appears to be a very high concentration of both intrinsic and extrinsic neuropeptide Y–containing nerves in the young rat compared with the mature animal. The role of neuropeptide Y in intrinsic nerves supplying the smaller resistance vessels and in the developing animal deserves further investigation.

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KEY WORDS • neuropeptide Y • noradrenaline • innervation • heart • coronary arteries
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doi: 10.1161/01.RES.66.6.1602

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