Neuropeptide Y in Rat Sympathetic Neurons Is Altered By Genetic Hypertension and By Age

Chandan J. Gurusinghe, Peter J. Harris, David F. Abbott, and Christopher Bell

We have used immunocytochemistry to quantitate neuronal neuropeptide Y in superior cervical ganglia of a strain of normotensive Wistar-Otago rats and a related genetically hypertensive strain over the age range 1–60 weeks. The numbers of neuropeptide Y–immunoreactive cells and total ganglionic cell numbers were both greater in ganglia of young normotensive than in those of hypertensive rats. Between 10 and 60 weeks of age, peptide immunoreactivity and total cell numbers both fell in normotensive rat ganglia but remained constant in ganglia from hypertensive rats. Densitometric analysis showed that the concentrations of neuropeptide Y were similar in neurons of age-matched individuals of both strains, but during aging there was a substantial decline in neuronal peptide content that was similar in both strains and that was not accompanied by any decline in neuronal immunoreactivity for tyrosine hydroxylase. Our results suggest that there is a developmental abnormality of neuropeptide Y in sympathetic neurons of this strain of genetically hypertensive rat and that, furthermore, the aging process is accompanied by a selective loss of neuronal neuropeptide Y that is independent of blood pressure status. (Hypertension 1990;16:63–71)

In adult animals of both the Otago (GH) and the Kyoto (SHR) strains of rat with a hereditary predisposition to hypertension, there is biochemical and functional evidence that peripheral sympathetic activity is inappropriately high.1–7 Furthermore, neonatal destruction of the peripheral sympathetic nervous system retards the development of hypertension and reduces the absolute extent to which both blood pressure elevation and the associated cardiovascular hypertrophy are seen in adulthood.8–14 Also, studies in the GH strain have shown that hypertension can be reversed completely by ganglion blocking agents until the rats are about 4 weeks of age.15 There are, therefore, several lines of evidence to suggest that elevated sympathetic drive may be an etiologic factor in the genesis of high blood pressure in these animal models for essential hypertension.

In the sympathetic nervous system, neuropeptide tyrosine or neuropeptide Y (NPY) is jointly localized with noradrenaline in some neurons, predominantly those supplying cardiovascular tissues.16,17 The intraneuronal levels of NPY are raised by manipulations known to increase sympathetic vasomotor drive,18,19 and because the peptide is vasoconstrictive in action and is released from sympathetic nerve terminals by electrical stimulation,17,20–22 it has been suggested that the peptide is a sympathetic cotransmitter involved in physiological mediation of cardiovascular neural tone.22,23 Therefore, we compared the patterns of NPY immunoreactivity in sympathetic neurons of normotensive and GH hypertensive rats from the neonatal period through late adulthood.

Methods

Animals

The rats used in the present study were raised in our animal house from a normotensive white Wistar line originating in the Otago Medical School and from the GH line that was bred from the same stock.24 Breeding was from brother-sister matings, and all rats were fed a proprietary rat chow (quoted sodium content 0.5%) with water ad libitum. Periodic checks of blood pressures in conscious, prewarmed adult rats using an occlusive tail cuff and a pulse transducer confirmed that resting systolic pressures were less than 140 mm Hg in normotensive rats and at least 170 mm Hg in hypertensive rats.
Tissue Preparation

Age-matched rats from each strain (1–60 weeks old) were overdosed with sodium pentobarbitone and both superior cervical ganglia were removed. The tissues were freeze-dried at −40°C and 10⁻³ mm Hg for 40 hours, fixed in paraformaldehyde vapor at 37°C for 1 hour and paraffin embedded (melting point 56°C). We have previously documented that this preparative procedure results in good preservation both of tissue structure and of antigenicity of enzymes and neuropeptides. For all animals, the ganglia were serially sectioned at 8 μm in the longitudinal direction and mounted on gel-coated slides. Sections of right-hand side ganglia were stained for NPY. Those from left-hand side ganglia were stained for tyrosine hydroxylase (TH) or with cresyl violet.

Immunohistochemistry

The NPY antibody was raised in rabbits against synthetic NPY and purchased from Amersham International, Bucks, UK. The TH antibody was raised in rabbits against purified bovine adrenal medullary TH and purchased from Eugene Tech, Allendale, N.J. Sections were preincubated in sheep serum diluted 1:5 with phosphate-buffered saline (PBS) and then incubated overnight at room temperature with the primary antibody diluted 1:800 (NPY) or 1:200 (TH) in PBS. After washing in PBS, they were incubated with 1:50 horseradish peroxidase-labeled sheep anti-rabbit immunoglobulin (Silenus Laboratories, Hawthorn, Victoria, Australia) for 45 minutes, rinsed, and placed in the dark in a stirred PBS bath containing 0.05% diaminobenzidine, 0.1% hydrogen peroxide, and 0.1% nickel ammonium sulphate for 4–6 minutes. The slides were then dehydrated and coverslipped with DPX.

Counting Cell Numbers

Total ganglionic cell numbers were estimated by counting all nucleated neuron profiles from serial-sectioned ganglia stained with cresyl violet with use of Abercrombie's correction to allow for double counting of split cells.

In alternate serial sections of each ganglion stained for NPY, nucleated, stained neuron profiles were counted using a square ocular grid that, at the magnification used, enclosed a section area of 0.4 mm². This grid was initially positioned over the longitudinal direction of a grid outlined two further, adjacent areas that represented central and rostral thirds of the ganglion. For each rat, the number of stained neurons per grid area was totaled for all sections of the ganglion examined and expressed in terms of mean number of neurons per square millimeter of section.

Image Analysis

Sections were viewed by transmitted light using a Leitz Orthoplan microscope fitted with a 60 W tungsten light source. The illumination level was stabilized with the aid of a constant current power supply (GW Instruments, Sydney, Australia). Images were televised via a video camera (model 68000, Dage-MTI, Inc., San Diego) fitted with a Newvicon tube and external gain and black level controls. Spatial and densitometric calibrations were initially carried out to confirm linear operation of the camera over the range of lighting levels used. Digitization of images, as 512×512 pixels with 8-bit resolution providing 256 gray levels, was performed using an image processing subsystem (Series 151, Imaging Technology, Woburn, Mass.) with images passed from the analog-to-digital interface to a frame buffer. An arithmetic logic unit enabled pipeline processing to be used in image averaging. Image processing cards were housed in an expansion rack that extended one slot of a SUN 3/110c workstation. Output images and a mouse-driven cursor were displayed on a Barco CD233 monitor to allow operator selection of areas in the visual field.

The software used was written in the C language and operated in a Sunview ("windows") environment under the SUN UNIX Operating System version 3.2. An in-house, menu-driven program enabled the operator to select image acquisition, averaging, subtraction, and editing functions based on procedures from the itex 151 subroutine library (Imaging Technology, Woburn, Mass.). In a typical session the following steps were performed: 1) The specimen illumination was set to a standard level using a thresholding procedure. 2) An unstained area of section was viewed to provide a background image and 50 successive frames of this image were digitized and subjected to weighted averaging. The final, averaged image was stored in one of the frame buffers. 3) The sample image, also averaged from 50 frames, was stored in a second frame buffer. During this process, the background image was subtracted, providing an image corrected for nonuniform lighting intensity and with a light level normalized to a maximum value of 255 on the 256 gray level scale. 4) The cytoplastic area of each cell to be analyzed was delineated by using the mouse to outline cell and nuclear boundaries, which were displayed in an overlay plane on the display monitor. The number of pixels in the area and their grey level values were then stored for computation of cell cytoplastic areas and immunoreactivity, respectively.

Transmittance (t) was calculated as:

\[ t = \frac{\text{grey level} + 1}{\text{maximum grey level} + 1} \]

and optical density (OD) was then derived as:

\[ \text{OD} = -\log_{10} t \]

The values calculated gave optical densities for each pixel and consequently, when values for all
pixels in the selected area were added, integrated optical density was obtained. This was then divided by the number of pixels comprising the area to give a value of average optical density (AOD) that was independent of cell size.

Although it can be predicted that the amount of antibody binding in a tissue section will be proportional to antigen concentration, this is reflected in proportional changes of immunocytochemical optical density only when the conditions for formation of reaction product are optimal. To ensure that this was so, we stained nontissue sections containing known concentrations of antigen using conditions for second antibody binding and horseradish peroxidase visualization that were identical to those used for tissue. Because the information to be obtained from this calibration procedure was independent of the antigen visualized, we chose for reasons of practicality to use dopamine β-hydroxylase (DBH) rather than NPY or TH. A solution of purified bovine adrenal medullary DBH25 in 2.5% gelatin and 2.5% bovine serum albumin was prepared so as to give final enzyme concentrations of between 50 and 800 ng/ml. Aliquots of these solutions were pipetted into 10×15×8 mm wells cut in rubber molds and frozen in liquid nitrogen. They were then freeze-dried, fixed in formaldehyde vapor, and paraffin embedded, as described above for tissue preparation. The blocks were cut at 10 μm, and sections were stained with 1:20 rabbit anti-bovine DBH antiserum (Eugene Tech) for 3 hours and subsequently with 1:50 horse-radish peroxidase-labeled sheep anti-rabbit immunoglobulin (Silenus Laboratories) for 30 minutes. Diaminobenzidine was used as the chromogen, as described above.

By using the same magnification as for tissue analysis, we measured the AOD from five areas of each antigen concentration. Mean AODs were calculated and a standard curve of AOD versus log10 antigen concentration was plotted.

For morphometric and densitometric analysis of tissue sections, three adjacent areas representing the rostral, central, and caudal thirds of the ganglion were selected at random in at least four sections from each animal. Cell areas and cytoplasmic AOD values were collected until the standard errors for mean AOD and for mean cytoplasmic area were both less than 10% of the means.29

Statistical Analysis

Comparisons of population means for cell numbers and AOD values were made using two-tailed, unpaired Student’s t tests for single comparisons and analysis of variance for multiple comparisons (Num ber Cruncher Statistical System, Kaysville, Utah). Comparisons of size distributions between NPY-immunoreactive (NPYI) cells and total ganglionic neuron populations were made using a two-tailed Kolmogorov-Smirnov test for large samples and the Behrens-Fisher Statistic.30 All data are reported as mean±SEM.

Results

Neurons containing NPY-like immunoreactivity were scattered throughout the superior cervical ganglion, making up about 35% of the total cell numbers (Figure 1A). These NPYI cells were scattered throughout the ganglion and showed no apparent topographic localization with respect either to the rostrocaudal axis or to specific areas within the ganglion. No appreciable gender difference existed in the distribution of NPYI cells. All neurons in the ganglion were stained for TH-like immunoreactivity, confirming that they were all catecholaminergic (Figure 1B).

Quantitation of Neuropeptide Y-Containing Neuron Numbers

Tissues from between three and five rats of each strain were examined at each age. In both strains, the absolute numbers of NPYI cells rose substantially between 1 and 3 weeks of age (p<0.05) before returning by 8 weeks to a value close to that seen in the 1-week-old rats (Figure 2). However, appreciable differences were observed between normotensive and GH rats in numbers of NPYI cells. At 1 week, there were approximately twice as many NPYI cells in ganglia of normotensive rats as were present in the GH strain (p<0.05). A significant, but smaller, strain difference was maintained at 2, 3, and 8 weeks (p<0.05) (Figure 2). Between 8 and 60 weeks, the number of NPYI cells in normotensive ganglia declined by about 40%. However, there was no reduction in NPYI cells in ganglia of hypertensive rats during this period, with the result that, at 60 weeks, the numbers of stained neurons were similar in both strains (Figure 2).

To determine whether the strain and age differences observed for NPYI cells was paralleled by differences in total numbers of neurons in the ganglia, neurons were counted in a series of normotensive and GH rats at 8–10 weeks and 50–60 weeks of age. To avoid any variation due to sexual dimorphism,31 all rats used were females. The results (Table 1) showed that there were about 25% more neurons in ganglia of young normotensive than young GH rats. During aging, ganglionic cell numbers declined in normotensive but not in GH ganglia, resulting in similar population sizes for each strain at 50–60 weeks. Thus, the general pattern was similar to that seen for the subpopulation of neurons that exhibited NPY immunoreactivity.

Size Distributions of Neuron Populations

To determine whether NPY was restricted to a subpopulation of neurons with a specific size profile, we compared the size distributions of the neurons sampled for measurement of NPY staining with those of neurons randomly sampled from the contralateral ganglia of the same rats at 2 weeks, 8–10 weeks, and 50–60 weeks of age, for each strain. The results are shown in Figure 3. As expected, cellular dimensions of the total neuron population increased with growth, from a mean cross-sectional area of about 200 μm² at
FIGURE 1. Photomicrograph showing typical neuronal patterns of staining for (panel a) neuropeptide Y-like (NPY) and (panel b) tyrosine hydroxylase-like (TH) immunoreactivity in superior cervical ganglion of a normotensive rat. Note that NPY is restricted to a subpopulation of neurons, whereas every cell contains TH. Calibration bar represents 50 μm.

2 weeks to 320 μm² at 50–60 weeks. There was no significant strain difference in general cell size distributions at any age. However, application of the Kolmogorov-Smirnov test showed that the size distributions of NPYI cells were different from those of the general population at all ages for the normotensive rats (2 and 8 weeks, p<0.01; 50 weeks, p<0.05), whereas there was no difference between NPYI and general distributions in GH rats of any age (p>0.05). The basis of the distribution difference was analyzed more precisely by application of the Behrens-Fisher test. This showed that the NPYI cells had significantly smaller mean sizes than the general population in 2-week-old and 8-week-old normotensive rats (p<0.01) but that this size difference had disappeared by 50 weeks (p>0.05). By contrast, in rats of the GH strain NPYI cells were no different in size from the general population (p>0.05) at any age (Table 2).

Quantitation of Neuropeptide Y Immunoreactivity

Densitometric analysis of immunoreactivity in sections containing known concentrations of antigen demonstrated that our immunocytochemical protocol produced a linear increase in staining density with increasing antigen concentration over the range 50–800 ng/ml. To confirm that the range of densities of stain produced covered the variation likely to be seen in tissue sections, we compared the calibration curve with the maximal and minimal densitometric values obtained in sections of ganglion stained for NPY and for TH. The results of this comparison (Figure 4) showed that linearity was maintained over the whole range of staining densities found with either of these antigens.

Comparison of NPY staining in sections of ganglia from rats of different ages (Figure 5) showed that the
amount of immunoreactivity was somewhat greater in normotensive ganglia than in ganglia from hypertensive rats at 1 week but, from 2 weeks onward, neurons in growing rats of each strain showed closely similar levels of NPY. Between 8 weeks and 60 weeks, the levels of immunoreactivity declined by about 30% in neurons of both normotensive and GH rats (p<0.05).

To ascertain whether the age-related fall in NPY was associated with depression of catecholamine synthesis, we also compared densitometrically the levels of TH in randomly sampled neurons of 2-week-old and 50-week-old rats (Figure 6). As with NPY, the amounts of TH immunoreactivity were closely similar in age-matched rats of each strain. However, by contrast with the decline in neuronal NPY immunoreactivity seen during aging, TH levels showed no reduction with age in either normotensive or GH rats.

**Discussion**

With few exceptions, the sympathetic neurons that contain NPY appear to project to cardiovascular tissues.16-18 This peptide therefore appears to be a general marker for neurons involved in cardiac and vasomotor control. In the present study, we found that the numbers of cells that exhibited NPYI were substantially lower in the superior cervical ganglia of young genetically hypertensive rats than in the ganglia of a closely related normotensive strain. One explanation for this finding would be that low amounts of peptide in some cells of the hypertensive rat ganglia were not detected by our staining technique. However, this seems unlikely because the measured density of staining, which we demonstrated to be proportional to antigen concentration, was similar in neurons of both strains. Furthermore, counts of total ganglionic cells in young adult rats also showed smaller populations in GH individuals. Thus, there appears to be a real difference in the numbers of ganglionic NPYI neurons in the two strains.

We saw a progressive decrease in the numbers of NPYI cells in ganglia of normotensive rats between 3 and 50–60 weeks of age, whereas the numbers in ganglia of the GH rats remained relatively constant over the same period. In normotensive rats, the decline in NPYI neurons with age was paralleled by a fall in total ganglionic cell numbers but, in ganglia from GH rats, total population size did not change with age. As well, analysis of the size distribution of neurons showed that NPY in young normotensive rats was restricted to a subpopulation of relatively small cells, whereas there was no size difference between the NPYI and the overall populations in older normotensive rats or in either young or old GH rats. Overall, these data are consistent with the hypothesis that aging in the rat is normally accompanied by loss of some NPY-containing sympathetic neurons, which is absent from ganglia of the hypertensive strain. It is of interest that Dhir and colleagues35 found that total NPY in superior cervical ganglia, as measured by radioimmunoassay, fell during maturation in normal Wistar rats but not in SHR. These workers did not detect any significant strain difference in ganglionic NPY at the end of that period, but it is not certain whether the normotensive rats used were from the Kyoto or another line, and therefore whether they represented an appropriate control for the SHR.

Although blood pressure in rats with hereditary hypertension rises most dramatically between 5 and 8 weeks postnatally,33 data from both GH and SHR strains indicate that, at least under some conditions, blood pressure is significantly elevated relative to age-matched control rats from within a few days after birth.33-36 It is therefore not possible to be certain whether the neuronal deficit that we observed in GH rats preceded or followed the onset of hypertension. However, it seems most likely not to have represented a response to chronically raised blood pressure as it was as pronounced 1 week after birth, when pressure is only about 115% of normal, as it was 9 weeks later, when there is a 25% elevation above normal.15,24,34

Several published studies have suggested that there is elevated sympathetic drive in both GH6,7,24

**TABLE 1. Total Neuron Numbers in Superior Cervical Ganglia of Normotensive and Genetically Hypertensive Rats at Different Ages**

<table>
<thead>
<tr>
<th>Age</th>
<th>Normotensive</th>
<th>Hypertensive</th>
</tr>
</thead>
<tbody>
<tr>
<td>8–10 weeks</td>
<td>14,713±570†</td>
<td>11,176±477</td>
</tr>
<tr>
<td>50–60 weeks</td>
<td>12,059±880†</td>
<td>11,208±897</td>
</tr>
</tbody>
</table>

All values represent counts of nucleated cell profiles and are corrected for double-counting of split cells. n=4 rats in each group.

†Significantly greater than value for age-matched hypertensive rats (p<0.01).

*Significantly less than value for normotensive rats at age 8–10 weeks (p<0.05).
Normotensive

Hypertensive

2 weeks

8 weeks

50-60 weeks

FIGURE 3. Bar graphs showing comparisons of size distributions (expressed as mm² cytoplasmic area) for randomly sampled neuropeptide Y-containing neurons (hatched columns) and cells representative of the general population of ganglionic neurons (open columns) in normotensive and genetically hypertensive rats of different ages. Data for the population means are given in Table 2.
and SHR-5 hypertensive strains. Furthermore, there is histochemical evidence that blood vessels in the SHR receive a denser sympathetic innervation than do those of normotensive animals.32,37,39 Taken together with observations that regional sympathectomy reduces medial thickness in arterial vessels,40-42 these data are compatible with the involvement of sympathetic nerve activity in development of genetic hypertension. In contrast with these published reports, our findings imply that sympathetic vascular innervation in young GH rats is sparser than that in normotensive controls, at least with regard to the numbers of postganglionic neurons involved. The situation clearly requires further analysis before this apparent paradox can be resolved. It should also be noted that, regardless of whether the functional neural control of arterial tone is elevated or reduced in the rat strains with genetic hypertension, neural influences appear not to be the sole initiator of increased blood pressure. Both SHR and GH rats that have been chemically sympathectomized soon after birth still have elevated blood pressures when adult when compared with similarly treated normotensive rats.14,15,24,43-44

Sympathetic neuronal NPY has been reported to correlate with sympathetic drive. Thus, exposure of rabbits to cold environments leads to elevation of NPY in the superior cervical ganglion, in parallel with enhanced cutaneous vasoconstrictor activity,19 whereas reserpine treatment of rats, which increases noradrenaline synthesis rate by withdrawal of feedback inhibition,45 also increases ganglionic NPY levels.20 By contrast, our densitometric measurements indicated no elevation of NPY content in individual sympathetic neurons of GH rats compared with normotensive rats. Thus, our data do not suggest that there is chronically elevated activity in the vasomotor system of the GH strain, although this does not exclude the possibility that the level of activity is inappropriate to the circulatory status of the rats or that exaggerated reflex responses to

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**TABLE 2. Comparison of Cytoplasmic Areas for Neuropeptide Y Immunoreactive Neurons and General Ganglionic Neuronal Population in Superior Cervical Ganglia of Normotensive and Genetically Hypertensive Rats**

<table>
<thead>
<tr>
<th>Age</th>
<th>Cell class</th>
<th>Normotensive</th>
<th>Hypertensive</th>
</tr>
</thead>
<tbody>
<tr>
<td>2 weeks</td>
<td>NPYI</td>
<td>169±8</td>
<td>166±13</td>
</tr>
<tr>
<td></td>
<td>all</td>
<td>217±8*</td>
<td>189±13</td>
</tr>
<tr>
<td>8–10 weeks</td>
<td>NPYI</td>
<td>252±6</td>
<td>289±21</td>
</tr>
<tr>
<td></td>
<td>all</td>
<td>353±13*</td>
<td>336±3</td>
</tr>
<tr>
<td>50–60 weeks</td>
<td>NPYI</td>
<td>293±11</td>
<td>291±14</td>
</tr>
<tr>
<td></td>
<td>all</td>
<td>324±6</td>
<td>318±8</td>
</tr>
</tbody>
</table>

All data are expressed as mean cytoplasmic area (μm²)±SEM and represent means of the neuron populations illustrated in Figure 3. NPYI, neuropeptide Y immunoreactive neurons.

*Significantly greater than age-matched NPYI data by Behrens-Fisher test (p<0.01)

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**FIGURE 5.** Bar graph showing densitometric readings of neuropeptide Y (expressed as average optical density) within individual neurons from normotensive (norm) and genetically hypertensive (hyp) rats of different ages (n=5 for each group). Vertical bars represent SEM. *Indicate significant differences between group means from same strain at different ages. ▲Indicates significant difference between group means for normotensive and hypertensive rats at the same age (p<0.05).

**FIGURE 6.** Bar graph showing densitometric readings of tyrosine hydroxylase (expressed as average optical density) within individual neurons from normotensive (norm) and genetically hypertensive (hyp) rats at 2 weeks and 50 weeks of age (n=5 for each sample). Vertical bars represent SEM.
specific stimuli may occur. Furthermore, we cannot rule out the possibility that differences may exist in the postganglionic terminal axons originating from these neurons. It has been reported that, in the SHR, the density of NPY perivascular axons projecting from the superior cervical ganglion to the cerebral vasculature was considerably greater than in normotensive controls, despite the fact that the ganglionic content of NPY, as measured by radioimmunoassay, was similar for both strains.32

Aging was associated with a decrease in NPY within individual neurons, as well as with decreased numbers of NPYI neurons. However, these two phenomena appear to have been independent, as the intensity of NPY staining decreased similarly in neurons of normal and of GH rats. It is of interest that NPY levels have also been reported to decrease during aging in nuclei of the rat brain associated with blood pressure control, but not in other nuclei containing catecholaminergic neurons.46 As the decline in neuronal NPY that we saw was not accompanied by decreased immunoreactivity for TH, which is the rate-limiting enzyme for catecholamine production,47 it does not seem to be secondary to generalized depression of cellular pathways related to neurotransmitter production. However, we have no way of knowing whether the differences seen are due to differences in neuronal synthesis or storage of NPY or to differences in its cellular transport or use. Furthermore, we cannot be certain whether the effect seen is specific to NPY or involves some other cellular constituents as well. In view of the reported correlation of neuronal NPY and activation rate,19-20 the loss of NPY may reflect a general decrease in sympathetic drive with age. As far as we know, there is no information on whether sympathetic drive does decline during aging in the rat, but this would be consistent with clinical data that show decreased effectiveness of sympathetic reflexes in the aged.48-50

Although all sympathetic noradrenergic neurons share a common neurotransmitter that acts only by activation of either α- or β-adrenergic receptors regardless of the effector cell supplied, different subpopulations of noradrenergic neurons are known to contain many different peptides, either singly or in combination.51,52 This wide variety of peptide profiles seems excessive if the function of jointly localized peptides is solely one of junctional equal transmission. In other parts of the nervous system, there is evidence that peptides that are localized to specific neuron populations, including calcitonin gene-related peptide, melacortin, somatostatin, vasoactive intestinal peptide, thyroxin-releasing hormone, and adrenocorticotropic, may exert anterograde trophic actions that help to maintain the functional integrity of the relevant target cells.53-56 In view of this, it is possible, within the sympathetic nervous system, that the peptides localized to different neuron pools also subserve specific anterograde trophic roles. The status of NPY as a potential trophic factor for the cardiovascular system therefore warrants further investigation.

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