0270-6474/85/0502-0450$02.00/0
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The Journal of Neuroscience
Vol. 5, No. 2, pp. 450–458
February 1985

EVIDENCE FOR DIFFERENTIAL LOCALIZATION OF NORADRENALINE AND NEUROPEPTIDE Y IN NEURONAL STORAGE VESICLES ISOLATED FROM RAT VAS DEFERENS[1]

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Received April 11, 1984; Revised July 9, 1984; Accepted July 23, 1984

Abstract

Using the technique of homogenization and subsequent density gradient centrifugation combined with ultrastructural analysis, the subcellular localization of noradrenaline and neuropeptide Y (NPY) was studied in vas deferens of castrated male rats. Noradrenaline showed two peaks in the gradient: one major peak at low density and another at high density. Only one NPY peak was seen, which coincided with the high density peak of noradrenaline. Electron microscopic analysis revealed high proportions of small and large vesicles in the light and heavy fractions, respectively. The present results indicate a differential subcellular localization of noradrenaline and NPY in the noradrenergic nerve endings of vas deferens. Thus, small vesicles seem to contain only noradrenaline, whereas the large vesicles may contain both noradrenaline and NPY.

Evidence has recently been obtained that neurons in both the PNS and CNS contain, in addition to its classical transmitter, a small biologically active peptide (Hökfelt et al., 1980). For some of these peptides a transmitter role has been suggested (Otsuka and Takahashi, 1977; Snyder, 1980). Thus, it is possible that neurons may release more than one messenger at their synapses. In the PNS classical transmitter and peptide in some instances may cooperate to cause a physiological response (see Lundberg and Hökfelt, 1983). In this model the classical transmitter causes a rapid response of short duration, whereas the peptide seems to evoke a long-lasting response with a slow onset. It has been speculated that there is a differential, frequency-dependent release of the two compounds (see Lundberg and Hökfelt, 1983). For the latter hypothesis it would be important to know whether or not transmitter and peptide are stored at the same place, since separate storage sites would provide a morphological basis for differential release.

Immunohistochemical studies have shown that a population of peripheral sympathetic nerves contain noradrenaline (NA) and neuropeptide Y (NPY) (Lundberg et al., 1982b). NPY is a recently isolated polypeptide with a C-terminal amide, structurally and biologically similar to a number of peptides belonging to the pancreatic polypeptide family (Tatemoto, 1982; Tatemoto et al., 1982).

Immunohistochemical studies have shown NPY-like immunoreactivity in peripheral neurons (Lundberg et al., 1982b, 1983, 1984; Edvinsson et al., 1983; Gu et al., 1983; Sundler et al., 1983; Terenghi et al., 1983), and some of these neurons are noradrenergic (Lundberg et al., 1982a, 1983, 1984), such as ganglion cells in the superior cervical stellate, and celiac ganglia as well as nerve terminals in, for example, vas deferens and heart auricles.

NA in peripheral nerves is stored in two types of vesicles, large (diameter, 70 to 100 nm) and small (diameter about 50 nm) dense-core vesicles (Pellegrino de Iraldi and DeRobertis, 1961; Grillo and Palay, 1962; Richardson, 1962, 1964; Taxi, 1965; Bloom and Barrnett, 1966; see also von Euler and Hillarp, 1956). Small vesicles make up about 95% and large vesicles about 5% of the vesicles in sympathetic terminals on small rodents such as rats and guinea pigs (Hökfelt, 1969; Fillenz, 1971; Fried, 1981; Thureson-Klein, 1983). The intraneuronal storage site for NPY so far is unknown. Therefore, we have analyzed the subcellular distribution of NPY in relation to NA in rat vas deferens, an organ with dense sympathetic innervation (Sjöstrand, 1965; Norberg et al., 1966) that has previously been used as a source for isolation of noradrenergic vesicles (Bisby and Fillenz, 1971; Fried et al., 1978; Fried, 1981).

Materials and Methods

Immunohistochemistry. Normal male and castrated (6 to 8 weeks) male rats (body weight, 200–250 gm; pathogen-free strain; Anticimex, Stockholm, Sweden) were anesthetized with mebumal (40 mg/kg, i.p.) and perfused via the ascending aorta with 100 ml of 0.9% sodium chloride followed by ice-cold 10% formalin (Pease, 1962) for 30 min. The vasa deferentia were dissected out, immersed in the same fixative for 90 min, rinsed in 0.1 M phosphate buffer with 10% sucrose added

[1] This work was supported by Swedish Medical Research Council Grants 14X-07164, 04X-2887, 19X-0534, and 13X-1010; Magnus Bergvalls Stiftelse; Lars Hiertas Minne; Knut och Alice Wallenbergs Stiftelse; The Swedish Society for Medical Sciences, Karolinska Institutets Forskningsfonder; National Institute for Neurological and Communicative Disorders and Stroke Grant 06801; and National Institute of Mental Health Grant 02714. For expert technical and secretarial assistance we thank Mrs. Ingrid Dahlin, Mrs. Waldtraut Hiort, and Mrs. Elisabet Björklund.

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for at least 24 hr and processed for indirect immunofluorescence according to the method of Coons and collaborators (see Coons, 1958). Briefly, adjacent sections were incubated for 18 to 24 hr at 4°C with rabbit antisera to NPY (102 B; see Lundberg et al., 1984) diluted 1:400 or to tyrosine hydroxylase (TH) (see Markey et al., 1980) diluted 1:400, rinsed in phosphate-buffered saline (PBS), incubated in fluorescein isothiocyanate-conjugated swine anti-rabbit antibodies (Dakopatts, Copenhagen, Denmark) diluted 1:10, rinsed in PBS, mounted in glycerine:PBS (3:1), and examined in a Zeiss Standard fluorescence microscope equipped with proper filter combinations.

Electron microscopy of intact tissue. Rats of the same groups as described above were anesthetized with mebumal (40 mg/kg, i.p.) and small pieces of vas deferens were immersed in 3% potassium permanganate (Richardson, 1966) for 2 hr, rinsed in Ringer's solution, contrasted *en bloc* with 2% uranyl acetate in Ringer's solution for 4 to 6 hr, and embedded and examined as described below. For further details on the technique, see Hökfelt (1968).

Rats (as above) were perfused under mebumal anesthesia (as above) via the ascending aorta with 50 ml of Tyrode's buffer followed by 1% glutaraldehyde (Merck, Darmstadt) in 0.1 M phosphate buffer (pH 7.2 to 7.4) for 30 min. Small pieces of vas deferens were taken out and immersed in the same fixative for 30 to 90 min, followed by immersion in 0.2 M sodium chromate buffer (pH 6.0) for 2 to 18 hr (Tranzer and Richards, 1976), postfixed in 2% osmium tetroxide, dehydrated, embedded, and examined as described below.

Subcellular fractionation. Vasa deferentia (0.7 to 1.0 gm) from about 20 rats (body weight, 200 gm; pathogen-free strain; Anticimex), castrated 6 to 8 weeks before killing, were used in each experiment. Organs were homogenized in 10 vol of ice-cold 0.25 M sucrose with 10 mM potassium phosphate, pH 7.3. The homogenate was subjected to differential and density gradient centrifugation using procedures similar to those described previously (Fried, 1981).

Briefly, the homogenate was centrifuged at 800 × g for 10 min in a Sorvall refrigerated centrifuge. The pellet (P1) containing nuclei and cell debris was removed, and the supernatant (S1) was centrifuged at 20,000 × g for 30 min. The second pellet (P2), containing most of the mitochondria, was removed and the second supernatant (S2) was put on a linear density gradient (0.25 to 1.2 M) and centrifuged at 280,000 × g_{max} for 90 min in a Beckman L62B ultracentrifuge using an SW40 rotor. After centrifugation the gradient was divided into 7 to 12 equal fractions, which were removed from above using hooked Pasteur pipettes (Fig. 1). For biochemical analysis, fractions were diluted to isotonicity and centrifuged at 140,000 × g_{max} for 45 min in order to separate sedimentable from soluble material. Pellets were resuspended in ½ to 1 vol of ice-cold water. NA was determined by high pressure liquid chromatography with electrochemical detection (Hjemdahl et al., 1979). NPY was determined by radioimmunoassay (Lundberg et al., 1984).

The density gradient fractions as well as the starting material (S2) were analyzed in the electron microscope. The fractions (volume, 700 to 1000 μl) were diluted up to 2 ml with ice-cold 5% glutaraldehyde in 0.1 M phosphate buffer (pH 7.2 to 7.4) in polycarbonate tubes (Beckman Instruments, Palo Alto, CA) and centrifuged at 145,000 × g_{max} for 30 min. After fixation for 2 to 4 hr, the pellets were rinsed in 0.01 M phosphate buffer (pH 7.2), immersed in 2% osmium tetroxide for 2 hr, rinsed in Ringer's solution, dehydrated in increasing concentrations of ethanol, cleared in propylene oxide, and embedded in Epon. Ultrathin sections were cut in a Reichert OmU3 ultratome, contrasted by uranyl acetate and lead citrate (Reynolds, 1963), and examined in a Philips 300 electron microscope. The diameters of vesicular structures were studied on micrographs taken at a magnification of × 57,000 with a Zeiss TGZ3 particle size analyzer.

Results

Immunohistochemistry. After incubation of adjacent sections of vas deferens with antibodies to NPY and TH, dense overlapping networks of strongly immunoreactive fibers were observed in the muscle layers (Fig. 2, *a* and *b*). In addition, fluorescent NPY- and TH-positive fibers were seen around blood vessels around vas deferens (Fig. 2, *a* and *b*). Adjacent axon bundles were strongly TH and moderately strongly NPY positive (Fig. 2, *a* and *b*). In addition, a loose plexus of NPY-immunoreactive fibers was seen in the connective tissue under the epithelium (Fig. 2*c*). No corresponding TH-positive networks could be seen (Fig. 2*d*).

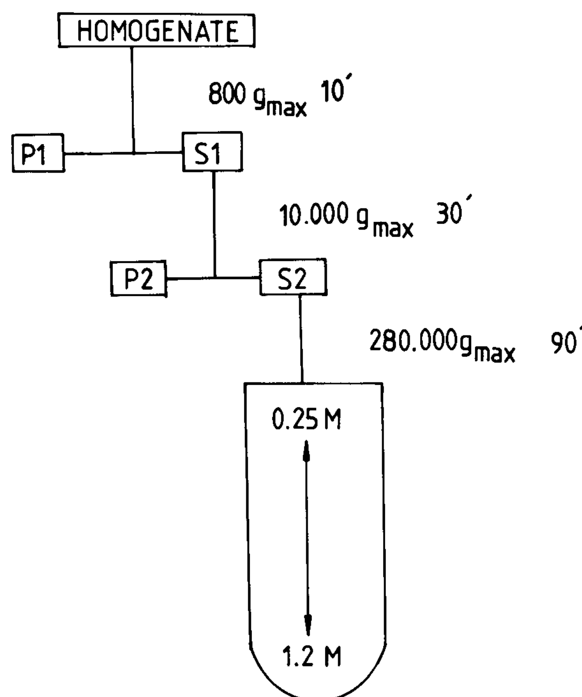


Figure 1. Fractionation procedure used in the studies on vas deferens of castrated rat. The homogenate was centrifuged at 800 × g_{max} for 10 min; the supernatant was centrifuged at 10,000 × g_{max} for 30 min, and the supernatant of the centrifugation (S2) was layered on linear sucrose density gradients (0.25 to 1.2 M), which were centrifuged at 280,000 × g_{max} for 90 min. The gradient was divided into 7 to 12 equal fractions and analyzed.

Electron microscopy of intact tissue. In glutaraldehyde/OsO_4-fixed material, nerve endings containing small and large dense-core vesicles were observed. Whereas the density and size of the cores in the large vesicles mostly were similar in all vesicles, the small ones often had a core of varying size, and some small vesicles lacked a core. In permanganate-fixed material the smallest vesicles had an electron-dense core (Fig. 3, *a* and *b*). The large vesicles had a homogeneous interior of either a moderate or low electron density (Fig. 3*a*) but could also contain a strongly electron-dense core of varying size (Fig. 3*b*).

Biochemistry of subcellular fractions. The homogenate contained 20.6 ± 7.4 pmol of NPY/mg of progein and 1596 ± 152 pmol of NA/mg of protein. When related to organ weight, the values were 0.29 ± 0.16 nmol of NPY/gm of wet weight and 27.2 ± 3.9 nmol of NA/gm of wet weight (n = 3; values ± SEM). The molar ratio of NA to NPY was 77:1 in the homogenate.

After differential centrifugation, 14.1% of the NPY remained in the P1 pellet and 16.5% remained in the P2 pellet as compared to 11.6% and 7.6% of the NA. In S2, 69.4% of the NPY remained, as compared to 78.4% of the NA (Table I). Of the total amount of NPY that was put on the gradient, 61% was recovered in a sedimentable form after centrifugation at 140,000 × g_{max} for 45 min. The corresponding figure for NA was 39%.

In the density gradient, NPY showed a single peak in a high density fraction (corresponding to 0.86 M sucrose). This fraction contained 11.9 ± 3.9 pmol of NPY/ml. The specific activity was 35.9 ± 11.2 pmol/mg (Fig. 4).

NA showed two peaks in the gradient, one at lower (0.68 M) and one at higher (0.86 M) density. The high density peak coincided with the NPY peak (Fig. 4). The light NA peak

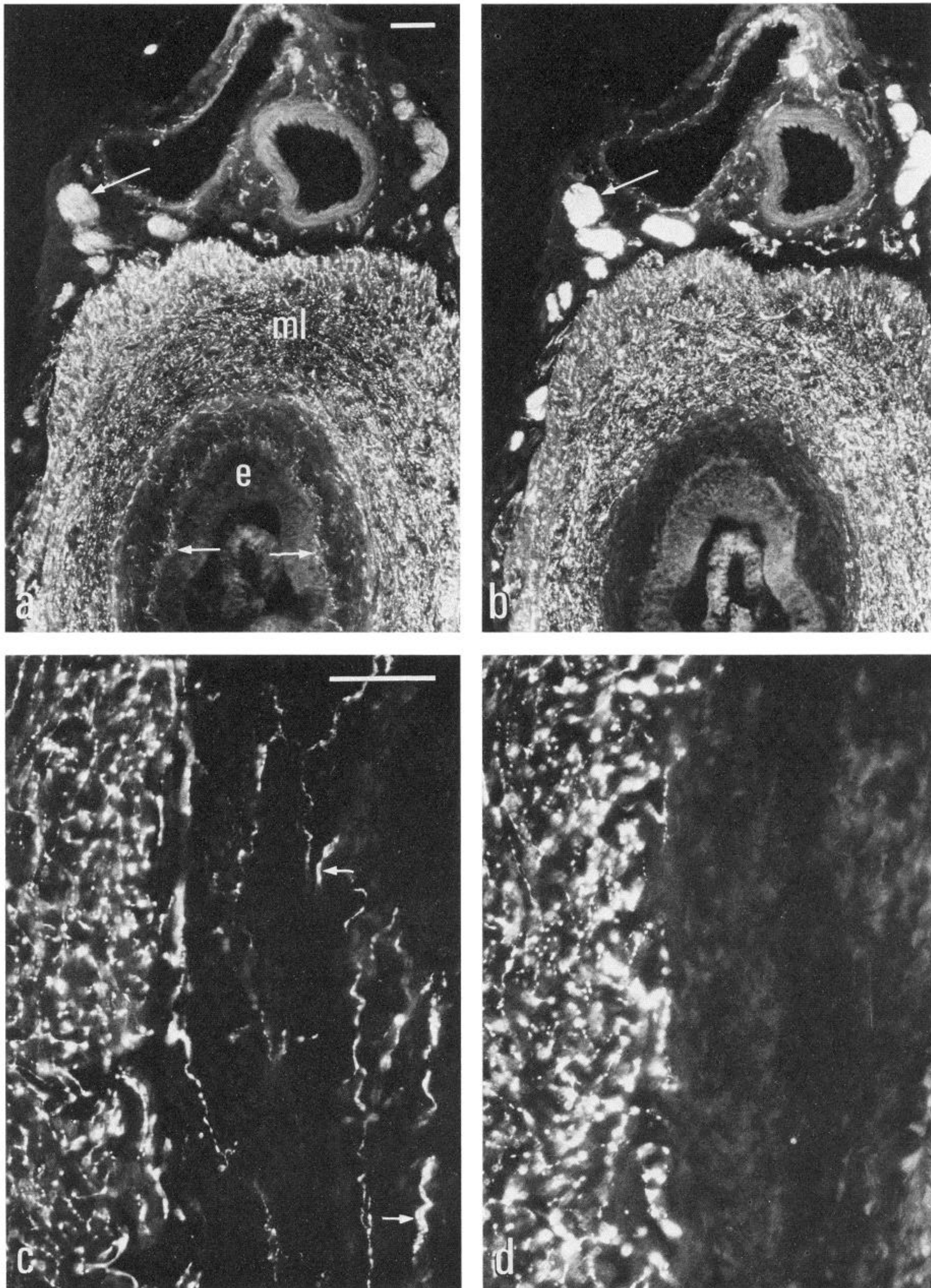


Figure 2. Immunofluorescence micrographs of the vas deferens after incubation with antiserum to NPY (*a* and *c*) and to TH (*b* and *d*). *a* and *b* as well as *c* and *d* show adjacent sections, respectively. Dense overlapping networks of NPY- and TH-immunoreactive fibers can be seen in the muscle layers (*ml*). Axon bundles (*long arrows*) surrounding vas deferens are immunoreactive to both substances and positive fibers can also be seen around blood vessels. Note that NPY-immunoreactive fibers (*short arrows* in *a* and *c*) can be seen just under the epithelium (*e*), and no corresponding TH-immunoreactive fibers are present (*b* and *d*). *Bars* indicate 50 μm.

TABLE I

Differential centrifugation of rat vas deferens

The distribution of NA and NPY in fractions P1, P2, and S2 is shown, expressed as both percentage and absolute value of the total amount recovered in P1, P2, and S2. The mean of three experiments is given as values ± SEM. Recovery for NA was 98%, for NPY, 87%, and for protein, 83%.

	P1	P2	S2	P1 + P2 + S2
NA				
Percentage	11.6 ± 0.7	7.6 ± 1.3	78.4 ± 4.0	100
Picomoles	4,522 ± 759	2,857 ± 410	30,209 ± 4,739	38,242 ± 5,175
NPY				
Percentage	14.1 ± 4.9	16.5 ± 1.6	69.4 ± 6.2	100
Picomoles	23.4 ± 7.8	28.7 ± 4.2	118.7 ± 59	170.8 ± 8.6

contained 2100 ± 412 pmol/ml and the heavy peak contained 500 ± 104 pmol/ml.

Corresponding values for specific activity were 22,443 ± 3,625 pmol/mg and 3,095 ± 1,747 pmol/mg. The molar ratio of NA to NPY in the heavy fraction was 46:1.

The relative enrichment in the fractions, expressed as ratio of specific activity (picomoles per milligram) in the fractions over that of the homogenate, is given in Table II. NA is enriched about 30-fold in fraction 5. NPY is enriched about 2-fold in fractions 6 to 8. The recoveries in the gradient were 70% for NA, 65.1% for NPY, and 87% for protein.

Electron microscopy. The S2 pellet contained a heterogenous collection of vesicles of various sizes and of various densities as well as ribosomes, lysosome-like structures, membrane fragments, and mitochondria (Fig. 3*c*). Fraction 1 (0.27 M) was characterized by high numbers of ribosome-like structures (Fig. 3*d*) and, in its upper part, small numbers of vesicles of varying size. Also in fraction 2, numerous ribosomes were still observed but with an increasing number of vesicles (Fig. 3*e*). Fractions 3 to 8 all contained numerous vesicles of various sizes and varying electron density as well as membrane fragments often forming rounded structures (Fig. 3, *f* to *i*). In fraction 8 an increasing number of club-shaped, elongated, electron-dense structures, as well as lipid droplets and mitochondria, were found (Fig. 3*i*). The quantitative analysis revealed that the vast majority of particles in fraction 1 had a diameter of about 150 to 200 nm. In fractions 3 and 4 most vesicles had a diameter of about 500 to 550 nm. Fraction 5 also contained numerous vesicles in this size range; many of them, about 50%, had a diameter between 440 and 560 nm (Fig. 5), and of these vesicles about 10% had an electron-dense core. The heavier fractions had a higher proportion of larger vesicles (Fig. 5). For example, in fraction 7 most vesicles had a diameter of about 625 to 750 nm, but also, many vesicles with a diameter ranging between 800 and 1100 nm were seen (Fig. 5). In fraction 7 about 20% of the vesicles had a dense core.

TABLE II

Relative enrichment in fractions expressed as ratio of specific activity (picomoles per milligram of protein) in fraction to that of the homogenate

For description of fractions, see "Materials and Methods" and Figure 1. The molarity (M) of the fractions is given within parentheses. In the third column, the molar ratio of NA to NPY is given. Values are mean of three experiments ± SEM.

Fraction (M)	NA	NPY	NA:NPY (mol/mol)
Homogenate	1	1	77
P1	0.5	0.08	229
P2	0.7	0.3	92
S2	1.9	0.6	125
1 (0.27)	1.2	0.1	430
2 (0.34)	2.6	0.2	590
3 (0.49)	5.0	0.2	892
4 (0.61)	16.4	0.6	1107
5 (0.68)	28.1	1.2	905
6 (0.73)	14	1.8	300
7 (0.86)	3.9	1.7	86
8 (0.96)	2.2	1.9	46
9 (1.04)	1.2	0.8	61
10 (1.2)	1.1	1.0	96

Discussion

The present findings confirm that the vast majority of the noradrenergic nerve endings in the vas deferens contain an NPY-like peptide (Lundberg et al., 1982b). In addition, a small population of nerves under the epithelium may contain NPY-like immunoreactivity but no catecholamines. Their number is very small when compared to the NA/NPY-immunoreactive fibers in the muscle layers. Therefore, NPY-like immunoreactivity present in these fibers in all probability do not influence the major conclusions drawn from the subcellular fractionation experiments in this study.

Many earlier electron microscopic studies on intact tissue, as well as the present one, have demonstrated the presence of two types of vesicles in peripheral noradrenergic nerve endings including vas deferens containing, respectively, small and large dense-core vesicles (Grillo and Palay, 1962; Richardson, 1962, 1964; Taxi, 1965). There is general agreement that the dense core in the *small* vesicles in all probability reflects the presence of NA. This is supported by depletion experiments using drugs such as reserpine which completely prevent the appearance of the small dense cores *in vivo* (Hökfelt, 1966; Van Orden et al., 1966, 1967; Tranzer and Thoenen, 1967; Farrell, 1968), as well as by using drugs which enhance the electron density in the vesicles (Tranzer and Thoenen, 1967, 1968). With regard to the large dense-core vesicles, the situation is more complex, and the electron microscopic appearance depends on the fixation technique. As shown in this and earlier studies, many large vesicles in $KMnO_4$-fixed tissue contain a strongly electron-dense core probably reflecting the presence of NA at the moment of fixation (Hökfelt, 1968). However, studies on glutaraldehyde/OsO_4-fixed material suggest that components other than the amine are essential for the electron density, although NA may contribute to the density of the core (Hökfelt, 1966, 1968, 1973; Bloom and Aghajanian, 1968; Pfenninger, 1973; Bloom, 1972; Thureson-Klein et al., 1974; Fried et al., 1978, 1981a, b; Fried, 1981; Pollard et al., 1982; Thureson-Klein, 1983).

In agreement with earlier studies (Bisby, 1971; Bisby and Fillenz, 1971; Fried et al., 1978), the present subcellular analysis in sucrose density gradients demonstrates the presence of NA in both a low and a high density fraction. In contrast, NPY-like immunoreactivity is contained only in a subcellular organelle with sedimentation characteristics similar to those of large noradrenergic vesicles. In gradients of the type used (0.25 to 1.2 M sucrose), such vesicles typically equilibrate at 0.8 to 1.1

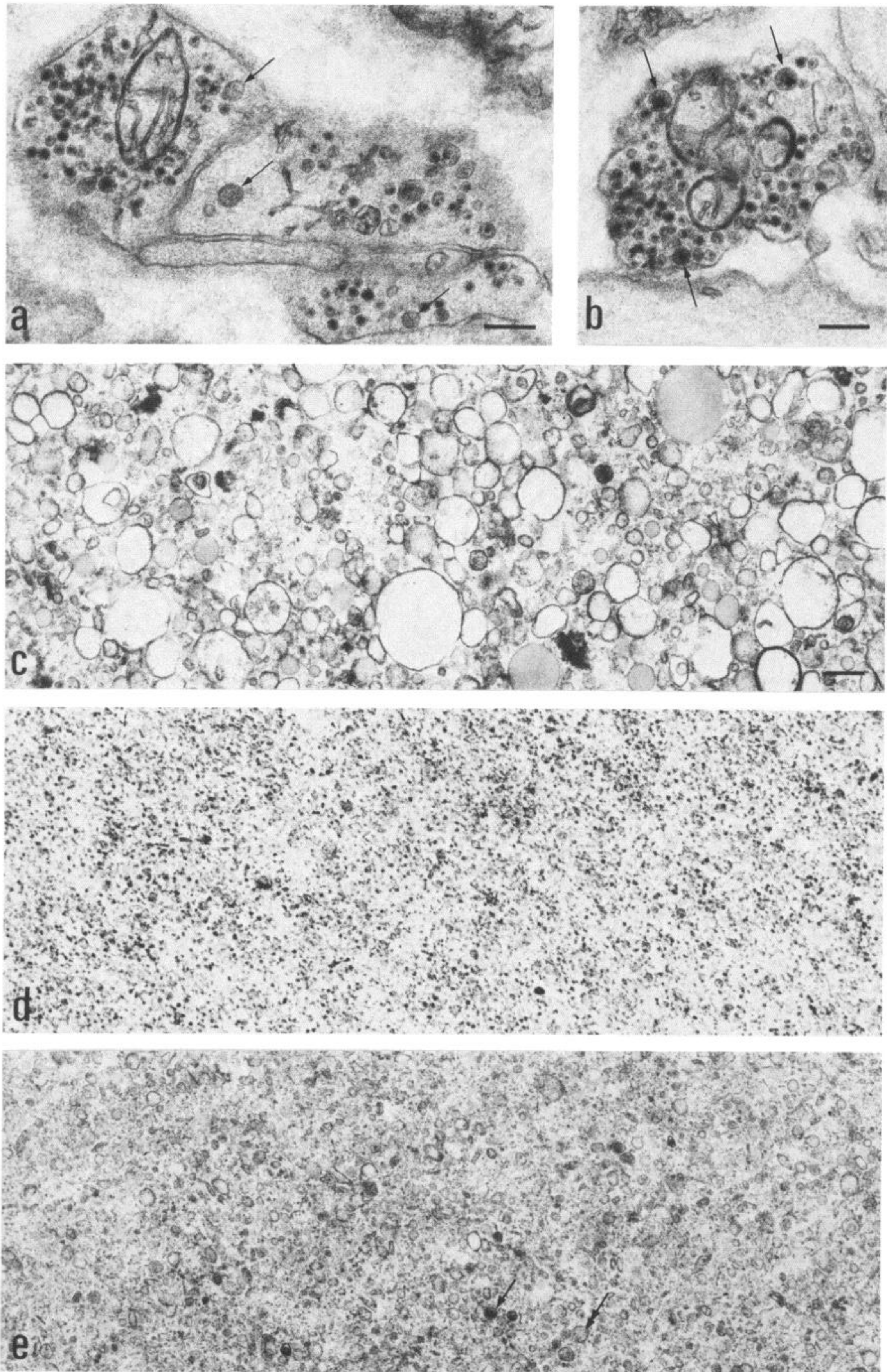
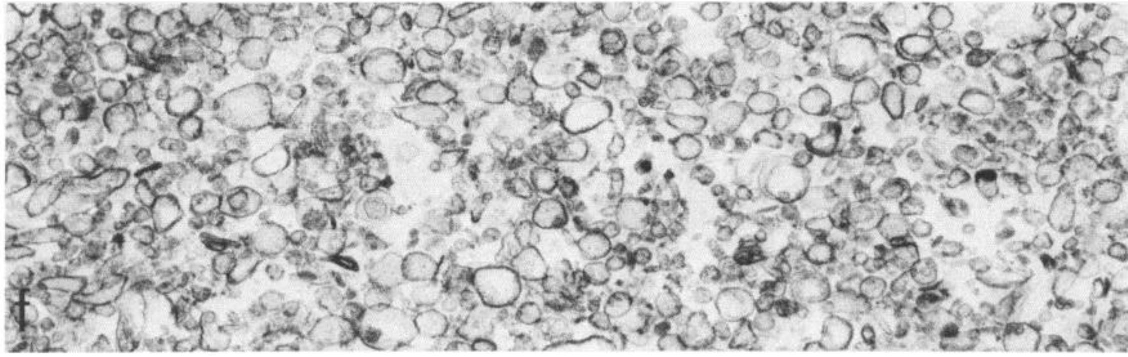
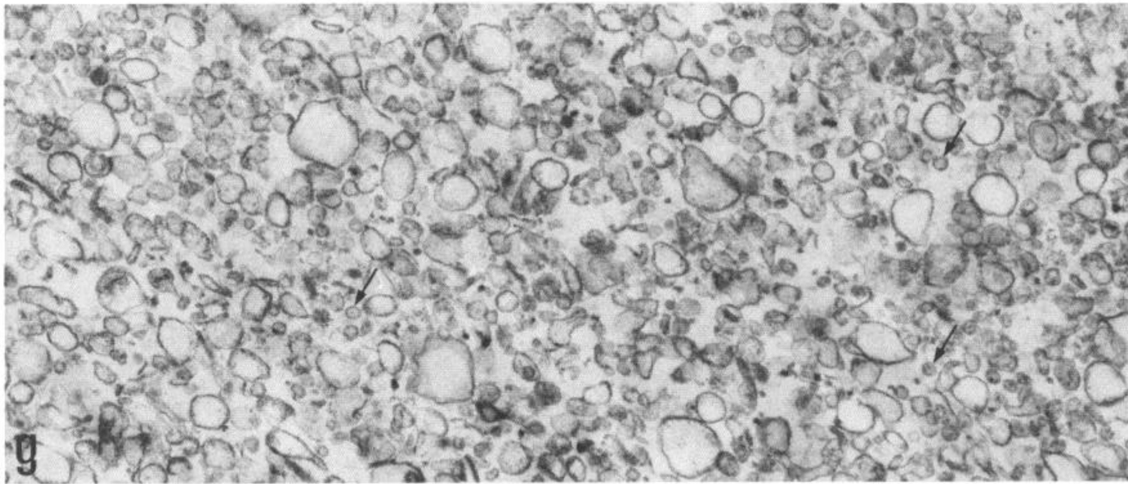
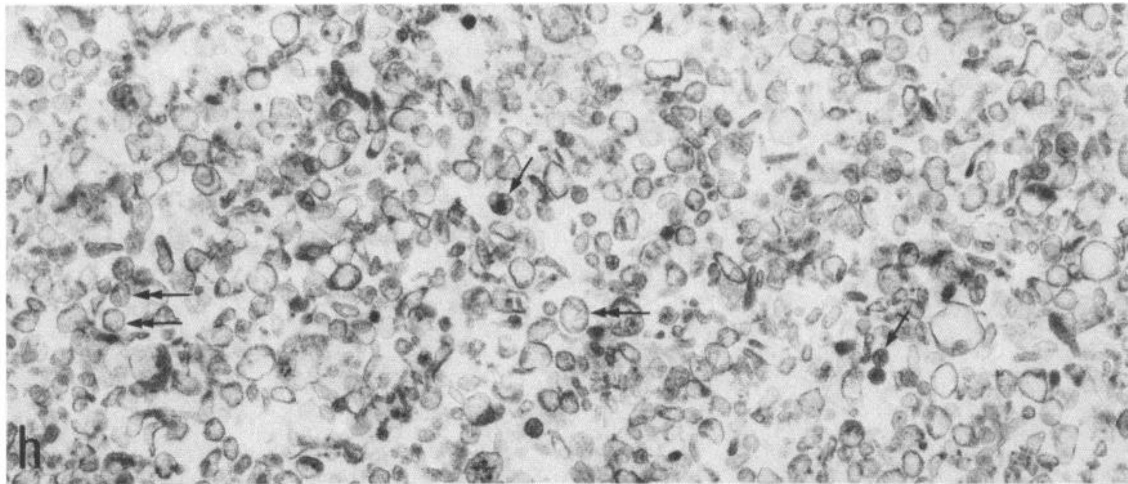
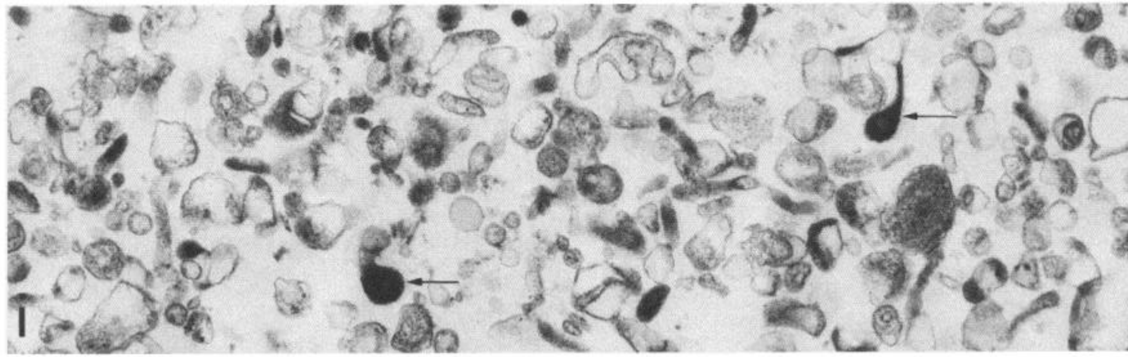


Figure 3. Electron micrographs of nerve endings in intact, potassium permanganate-fixed vas deferens (*a* and *b*) and of various subcellular fractions (*c* to *i*, fraction S2, fraction 1, fraction 2, fraction 3, fraction 5, fraction 7, and fraction 8, respectively). *c* to *i* show glutaraldehyde/osmium tetroxide-fixed pellets. *a* and *b*, The nerve endings contain many small dense-core vesicles and fewer large vesicles, some of which have an interior with a low electron density (*arrows* in *a*), whereas others (*arrows* in *b*) have a strongly electron-dense core of varying size. *c* to *i*, The S2 fraction contains a heterogeneous population of structures including small vesicles, membrane fragments forming round sacs, and lipid droplets. Numerous ribosomes are seen in fraction 2 (*d*), and fraction 3 (*e*) contains increasing numbers of vesicles with (*arrow*) and without









(*double-headed arrow*) electron-dense interior. Fractions 3 (*f*) and 5 (*g*) contain numerous vesicles, many of which have a diameter of about 500 nm (*arrows* in *g*), as well as many membranes forming rounded sac-like structures. Fraction 7 (*h*) appears fairly homogeneous with many large vesicles with (*arrow*) or without (*double-headed arrows*) dense cores as well as many smaller vesicles with dense cores (*crossed arrow*). Fraction 8 (*i*) is heterogeneous with club-shaped, strongly electron-dense structures (*arrows*), elongated sacs, and vesicles of varying size. *Bars* indicate 200 nm. *c* to *i* have the same magnification.

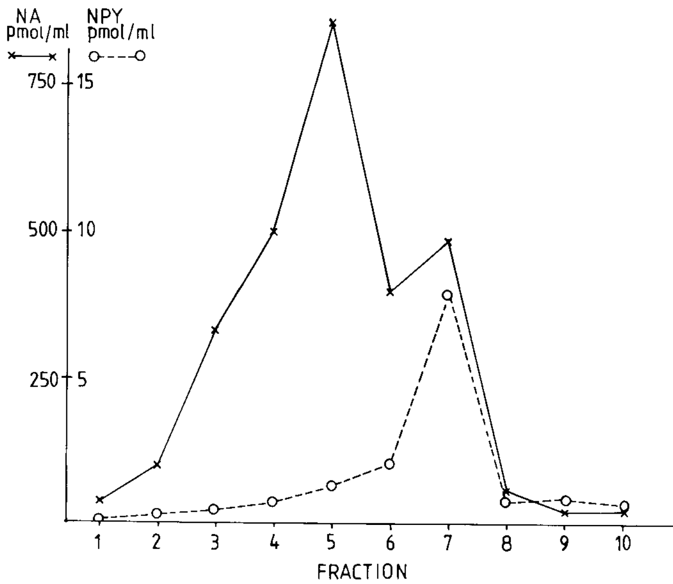


Figure 4. Subcellular distribution of NA (*x*) and NPY (O) in a density gradient of rat vas deferens. On the *abscissa*, totally recovered sedimentable substance is given as picomoles per milliliter after centrifugation at 145,000 × g_{max} for 45 min. On the *ordinate*, density gradient fractions 1 to 10 are given, corresponding to the following sucrose molarities: *1* (0.26 M), *2* (0.32 M), *3* (0.47 M), *4* (0.56 M), *5* (0.69 M), *6* (0.74 M), *7* (0.84 M), *8* (0.91 M), *9* (0.98 M), *10* (1.2 M). Recoveries of NA = 70%, of NPY = 65%, and of protein = 87%. One representative gradient out of three similar ones is shown.

M sucrose, depending on the time and G force of centrifugation (Bisby, 1971; Bisby and Fillenz, 1971).

The electron microscopic analysis of the different fractions revealed that the highest proportion of small vesicles was found in fraction 5 (0.5 to 0.65 M), whereas the highest proportion of large vesicles was observed in fraction 7 (0.8 to 0.9 M). Only a rather small number of vesicles in these fractions had an electron-dense core. This is in all probability due to the fact that the amine as well as other components of the vesicular matrix have been lost during the homogenization and centrifugation procedures. In contrast to some previous studies (Fried et al., 1978, 1981b), no attempts were made to restore the electron-dense core by incubation with NA-Mg^{2+}-ATP.

The co-distribution of the heavy NA peak and the NPY peak may indicate that NPY is stored together with NA in large vesicles, or that they are stored in different organelles with similar size and density (Fig. 6). The latter hypothesis receives apparent support by electron micrographs of $KMnO_4$-fixed nerve endings demonstrating some large vesicles with low and others with high electron density, as shown in this paper. However, administration of exogenous amines *in vivo* and *in vitro* enhances electron density of all large vesicles (Tranzer and Thoenen, 1967; Hökfelt, 1968), suggesting that large vesicles in noradrenergic neurons in general have NA-storing capacity. Thus, it seems most likely that NPY is stored together with NA in large vesicles.

The distribution of NPY in the differential centrifugation was somewhat different from that of NA. In the S2 supernatant, 69.4% of the NPY was still present, as compared to 78.4% of the NA. After centrifugation of S2 for 140,000 × g_{max} for 45 min, 61% of the NPY was sedimentable as compared to 39% of the NA. Since there probably are two storage compartments for NA (large and small vesicles), but apparently only one for NPY, the distribution would be expected to be different. A higher proportion of sedimentable NPY as compared to NA is also compatible with the hypothesis of storage of NPY only in large (heavy) vesicles.

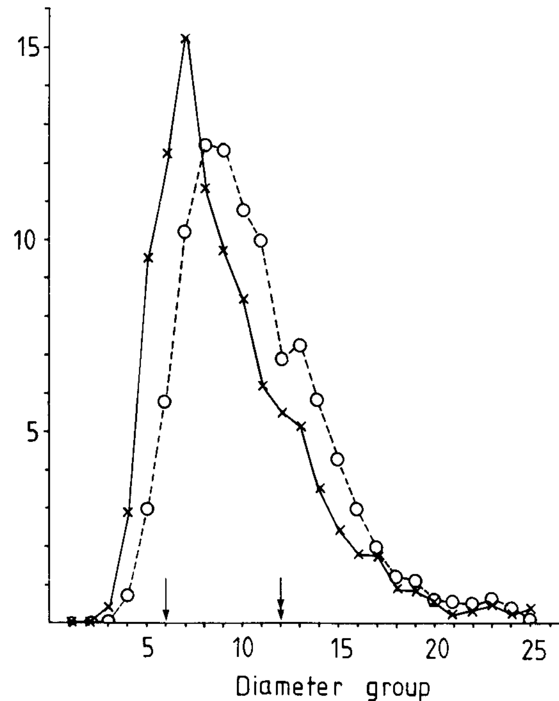


Figure 5. Distribution (percentage) of the diameters of vesicle profiles in subcellular fractions of vas deferens of castrated male rats. Fraction 5 (0.69 M sucrose) (*x*) and fraction 7 (0.84 M sucrose) (O) are shown. In fraction 5, 2756 vesicular profiles were counted (mean, 644/picture), and in fraction 7, 1929 vesicular profiles were counted (mean, 482/picture). Values shown are the mean of four pictures per group (SEM was less than 5%). The diameters of the vesicular profiles have been grouped in the particle size analyzer, and the means of the groups are as follows (in nanometers): group 1, 63; group 2, 125; group 3, 250; group 4, 375; group 5, 438; group 6, 500; group 7, 563; group 8, 625; group 9, 750; group 10, 813; group 11, 875; group 12, 1000; group 13, 1063; group 14, 1125; group 15, 1188; group 16, 1250; group 17, 1313; group 18, 1375; group 19, 1438; group 20, 1500; group 21, 1563; group 22, 1625; group 23, 1688; group 24, 1750; group 25, 1875. The *arrow* indicates 500 nm and the *double-headed arrow* indicates 1000 nm.

The ratio of NA to NPY on a molar basis was about 50:1 in fraction 8, containing large vesicles. This is remarkably similar to the ratios of classical transmitter to neuropeptide found in other coexistence systems investigated by the technique of subcellular fractionation. In bovine splenic nerve, the ratio of NA to enkephalin in a large dense-core vesicle fraction was 60:1 (Wilson et al., 1980; Fried et al., 1981a). In cat submandibular gland, the ratio of acetylcholine to VIP was 70:1 in a fraction enriched in VIP, acetylcholine, and large dense-core vesicles (Lundberg et al., 1981).

The specific activity of NA was markedly increased in the small dense-core vesicle fraction, about 30-fold higher than that in the homogenate. The specific activity of NPY was maximally increased in the heavy fraction (0.86 M) but only 2-fold higher than that in the homogenate. However, the increase in specific activity of NA in this fraction was also about 2-fold, i.e., comparable to that of NPY. This indicates a similar degree of enrichment of NPY and NA stored in large vesicles. In addition, the observed 30-fold enrichment of NA in the small vesicle fraction as compared to a 2-fold enrichment in the large vesicle fraction fits rather well with the calculated ratio of small (95%) to large (5%) vesicles in the sympathetic terminals of rat vas deferens (see Thureson-Klein, 1983).

The results provide further evidence of compartmentalization of coexisting neurotransmitter/neuropeptides at the sub-

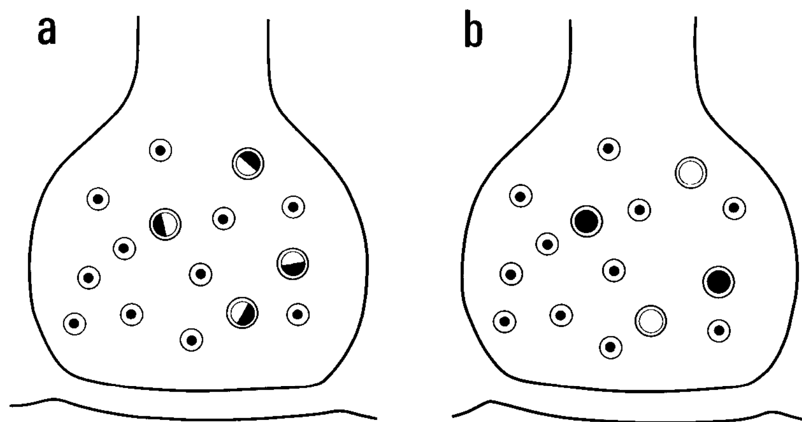


Figure 6. Schematic illustration of the storage sites of NA and NPY in a nerve ending of rat vas deferens based on the present experiments. The small vesicles store only NA, whereas the large vesicles contain both NA and NPY (*a*). However, a second alternative (*b*) cannot be excluded; NA and NPY are stored in separate large vesicles.

cellular level (Wilson et al., 1980; Fried et al., 1981a, 1984; Lundberg et al., 1981; Klein et al., 1982; Lundberg and Hökfelt, 1983). The occurrence of both large and small NA storage organelles has long been regarded as an enigmatic feature of adrenergic neurons. It seems possible that one important function of the large vesicles is storage of neuropeptide(s), as suggested also from recent immunoelectron microscopic studies on peripheral peptide neurons (Probert et al., 1981; Tapia et al., 1983), whereas the small vesicles primarily store the classical transmitter (Fried, 1982; Fried et al., 1984). This may provide a morphological basis for separate control mechanisms for differential release. Evidence that NA and NPY may cooperate to produce the biological response to sympathetic nerve stimulation has been obtained by *in vivo* experiments in the cat, where infusion of NA and NPY together, but not NA or NPY alone, mimicked the vascular effects of sympathetic nerve stimulation (Lundberg and Tatemoto, 1982).

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