Neuropeptide Y in the Cerebral Cortex and the Caudate-Putamen Nuclei: Ultrastructural Basis for Interactions with GABAergic and non-GABAergic Neurons

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In the cerebral cortex and caudate-putamen (CP) nuclei, neuropeptide Y (NPY) immunoreactivity is detectable within 1–2% of all neurons. The NPY-immunoreactive neurons are interneuronal and are believed to be mostly GABAergic in the cerebral cortex but not in the CP nuclei. Thus NPY and GABA may play different roles in the circuitry within these 2 regions. We tested this possibility by comparing the ultrastructure of NPY-containing neurons between (1) cortex (somatosensory and anterior cingulate areas) versus dorsolateral CP; and (2) GABAergic versus non-GABAergic NPY neurons within each area. Single coronal sections through the rat forebrain were dually labeled for GABA and NPY by combining immunautoradiography with the immunoperoxidase method. NPY-containing neurons with or without GABA occurred throughout the rostrocaudal portions of CP and all laminae of somatosensory and anterior cingulate cortex. Comparisons between the areas confirmed that somata and terminals dually labeled for GABA and NPY were more prevalent in the cortex. NPY terminals lacking detectable GABA immunoreactivity also were found within the cortex, thus suggesting additional heterogeneity in cortical NPY innervation. The ultrastructural features of NPY perikarya in both regions were morphologically similar regardless of whether the cells also contained GABA. Most synaptic inputs to NPY neurons occurred at distal dendrites. In comparison to neighboring neurons, synaptic inputs to proximal dendrites and somata of NPY neurons of cortex and CP were rare, suggesting that fewer and weaker inputs may modulate the excitability of NPY-containing neurons. In both regions, nearly all NPY- and NPY-GABA-labeled terminals formed symmetric junctions suggestive of inhibitory action. The majority of these junctions were with dendrites containing neither NPY nor GABA. NPY terminals formed few contacts on proximal dendrites and somata of GABAergic neurons (8% of 179 contacts in cortex; 12% of 73 contacts in CP) which, unlike most singly-labeled GABAergic neurons, were sparsely innervated. Thus, NPY may play a more prominent role in modulation of certain GABAergic neurons than would be predicted by the observed frequency of NPY-to-GABA contacts in the two regions. One notable regional difference was the

greater prevalence in cortex of axoaxonic associations between NPY-immunoreactive terminals and other terminals, some of which also contained NPY. These nonsynaptic associations may be involved in the modulation of (1) the release of NPY by another transmitter or (2) NPY's modulation of release of other transmitters in cortex.

Neuropeptide Y (NPY) is a 36-amino acid polypeptide with structural similarity to pancreatic polypeptide (Tatemoto et al., 1982). This peptide was first isolated from porcine brain (Tatemoto et al., 1982) and has since been shown to occur in highest concentration within nervous tissue. Consistent with its wide distribution (O'Donohue et al., 1985; Chronwall et al., 1986), a broad spectrum of responses is elicited by the central application of NPY (reviewed by Gray and Morley, 1986). At least some of these effects may be mediated by NPY's modulation of the release of various transmitters (Colmers et al., 1987; Walker et al., 1988). Alternatively, the diversity may depend on the intrinsic versus efferent connectivities of NPY-containing neurons or the species of transmitters coreleased with NPY in different brain regions. Specifically, coexistence of catecholamines with NPY within the brain-stem neurons that project to forebrain areas, and of GABA, somatostatin, and tachykinins with NPY within forebrain interneurons, have already been demonstrated (reviewed in Allen et al., 1986; Jones et al., 1988). Ultrastructural immunocytochemical studies suggest that the diversity in the action of NPY may additionally reflect the different parts of cells that NPY terminals contact (Vincent and Johansson, 1983; Hendry et al., 1984a; Pelletier et al., 1984; Vuillet et al., 1989) and the types of neurons that are pre- and postsynaptic to NPY neurons (Aoki and Pickel, 1988; Kubota et al., 1988; Massari et al., 1988; Pickel et al., 1989).

In the cerebral cortex and caudate-putamen (CP) nuclei, NPY-immunoreactive neurons consist of approximately 1–2% of all neurons (Vincent et al., 1983; Vincent and Johansson, 1983). The NPY-immunoreactive neurons are considered to be almost all interneurons (Hendry et al., 1984a; Smith and Parent, 1986). Results using light microscopic immunocytochemistry have demonstrated that most NPY neurons in the cortex are also GABAergic (Hendry et al., 1984b; Demeulemeester et al., 1988). In contrast, cytological and chemical lesion studies suggest that most NPY neurons in the CP are not GABAergic (Bolam et al., 1982; Takagi et al., 1983; Beal et al., 1986b; O'Kusky et al., 1988). Although dual immunocytochemical studies have not yet been done to examine CP, previous dual immunocytochemical studies on the ventral striatum are supportive of these findings since co-localization of GABA and NPY was not observed within terminals. However, this study did not examine GA-

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BAergic somata due to methodological limitations. To date, direct comparisons between the cortex and CP regarding the relative distribution of GABA and NPY have not been made. Thus, several important issues remain unresolved. In the cerebral cortex: (1) are there cellular bases for interaction between neurons containing both GABA and NPY versus those containing only GABA; (2) are there also axon terminals that contain NPY without GABA; and if so, (3) are there differences in termination between the 2 populations of NPY-containing axons? In the CP nuclei: (1) might there also be a smaller population of NPY neurons co-storing GABA; and if so, (2) are NPY terminals also less frequently colabeled for GABA; and (3) is there a cellular basis for interaction between the GABAergic and NPY-containing neurons? These questions are particularly pertinent to understanding the role of NPY in the intrinsic circuitry of the cerebral cortex and striatum. We have examined these issues by combining immunohistochemistry with peroxidase-immunocytochemistry for the simultaneous localization of NPY and GABA in the cerebral cortex and striatum of rat brain. Specifically, we focused our study on: the anterior cingulate area of the mesocortex (Reep, 1984), the somatosensory area of neocortex, and the dorsolateral portion of the CP nuclei receiving monosynaptic input from the somatosensory cortex (reviewed in Alexander et al., 1986). The results establish morphological similarities among the NPY-containing neurons as to whether or not they co-store GABA or reside in cortex or striatum. We also demonstrate that the NPY may have a prominent role in influencing excitability of certain GABAergic neurons in cortex.

Materials and Methods

Antibodies

Goat and rabbit antisera against the C-terminal hexapeptide of pancreatic polypeptide (Peninsula Labs, San Carlos, CA) were generous gifts from the laboratory of Dr. T. L. O'Donohue, NIH, Bethesda, MD. These antisera specifically recognize NPY in rat brain (DiMaggio et al., 1985; Chronwall et al., 1986).

Rat antisera against hemocyanin-conjugated GABA was generously supplied by Dr. Andrew C. Towle, Division of Molecular Biology, Department of Neurology and Neuroscience, Cornell University Medical College, NY. Methods for its production and characterization of its specificity for GABA in rat brain have been described previously (Lauder et al., 1986).

Secondary immunoglobulins (IgG) were obtained from the following commercial suppliers: sheep anti-goat IgG from Cappel; goat anti-rabbit IgG from Miles, Inc.; 125 I-iodinated donkey anti-rabbit IgG from Amersham; rabbit anti-rat IgG from Dako; goat anti-rat IgG from Arnel; and 125 I-iodinated sheep anti-rat IgG from Amersham.

Rabbit peroxidase-antiperoxidase (PAP) complex was generously supplied by Dr. Cory Abate, then with the Division of Molecular Biology, Department of Neurology, Cornell University Medical College, NY; goat and rat PAP were purchased from Cappel and Sternberger-Meyer, respectively.

Tissue preparation

Male adult Sprague-Dawley rats (BW 180–250 gm) were anesthetized with Nembutal (50 mg i.p. kg/BW), perfused through the ascending aorta with 10 ml of heparin-saline, followed by 50 ml of 3.75% acrolein/2% paraformaldehyde in 0.1 M phosphate buffer (pH 7.4), and then by 200 ml of 2% paraformaldehyde in the same buffer. Some of the animals received intraventricular injections of colchicine (75 μ l in 10 μ l of saline) under halothane anesthesia (1% mixed with O₂) 24 hr prior to perfusion so as to enhance immunoreactivity in somata.

Following vascular perfusion, the brains were removed from the skull, cut into 2 mm slabs, placed for an additional 30 min in buffered 2% paraformaldehyde solution and cut into 40 μ m-thick coronal sections using a Vibratome. The Vibratome sections were collected in phosphate buffer, and then placed for 30 min in 1% sodium borohydride/phosphate

buffer so as to terminate further cross-linking activity of the fixatives. The sections were rinsed several times in phosphate buffer, until bubbles disappeared, and then transferred to 0.1 M Tris-HCl (pH 7.6) buffer containing 0.9% NaCl (Tris-saline).

Immunocytochemical labeling

Each Vibratome section was labeled dually by combining the immunoperoxidase and immunohistochemical methods first developed by Pickel et al. (1986). In method 1, sections were labeled for GABA by immunohistochemistry, followed by the immunolabeling for NPY using a modification (Pickel, 1981) of Sternberger's PAP method (1979). In method 2, sections were labeled for NPY immunohistochemically and for GABA by the PAP method.

Method 1. Sections were preincubated in 0.1% BSA/Tris-saline for 30 min to block nonspecific binding of antibodies. The sections then were processed for immunohistochemical labeling by sequential incubations in the following solutions together with 0.1% BSA/Tris-saline at room temperature under constant agitation: (1) 1:3000–1:4000 dilution of rat anti-GABA antiserum for 12–16 hr; (2) three 15-min washes in Tris-saline; (3) 1:100 dilution of sheep 125 I-anti-rat IgG with a radioactive concentration of 1 μ Ci/ml for 2 hr; (4) at least ten 30-min washes in Tris-saline or until radioactivity in the wash solution became negligible. This series was followed by incubations for immunoperoxidase labeling: (1) 1:2000 dilution of goat anti-NPY for 12–16 hr; (2) three 15-min washes in Tris-saline; (3) 1:50 dilution of sheep anti-goat IgG for 1 hr; (4) three 15-min washes; (5) 1:100 dilution of goat PAP for 1 hr. Alternatively, sections were incubated in 1:2000 dilution of rabbit anti-NPY antiserum, followed by 1:50 dilution of goat anti-rabbit IgG, followed by rabbit PAP. Peroxidase reaction product was visualized by incubating the sections for 6 min in Tris-saline solution containing 3,3'-diaminobenzidine and hydrogen peroxide. Sections then were rinsed 3 times in Tris-saline, followed by 0.1 M phosphate buffer (pH 7.4).

Method 2 followed the same protocol as method 1 except for the following changes to achieve immunohistochemical labeling: (1) 1:2000 dilution of rabbit anti-NPY antiserum; (2) 125 I-iodinated donkey anti-rabbit IgG. This was followed by a series of incubations for immunoperoxidase labeling: (1) 1:3000 dilution of rat anti-GABA antiserum; (2) 1:50 dilution of goat anti-rat IgG; (3) rat PAP. In both series of experiments, the PAP labeling was intensified in some of the sections by repetition of the incubations with secondary IgGs and PAP complexes (Houser et al., 1983b).

Some of the sections were processed for light microscopic autoradiography. The remaining sections were postfixed for 10 min in 1% glutaraldehyde, rinsed, fixed further with 2% osmium tetroxide for 2 hr, dehydrated, and flat-embedded in Epon 812 by sandwiching between 2 sheets of Aclar plastic (Masurovsky and Bunge, 1968) and curing for 24 hr. Areas containing anatomically identified structures within flat-embedded sections were capsule-embedded and cured for another 24 hr. Ultrathin sections were collected from the Epon-tissue interface containing PAP-labeled perikarya.

Controls. Secondary IgGs and PAPs were tested for cross-reactivity by the following methods: (1) application of anti-rat IgG/rat PAP or anti-goat IgG/goat PAP to tissue incubated overnight with rabbit primary antibodies; (2) application of anti-rabbit IgG/rabbit PAP or anti-goat IgG/goat PAP to tissue incubated overnight with rat primary antibodies; (3) application of anti-rat IgG/rat PAP or anti-rabbit IgG/rabbit PAP to tissue incubated overnight with goat primary antibody. None of these combinations gave detectable levels of immunolabeling in cortex or striatum.

Autoradiography

For light microscopy, the PAP-labeled Vibratome sections were mounted on glass slides and dipped in Ilford L4 emulsion diluted 1:1 with distilled water at 50°C, and then air-dried under 70% humidity. The dipped slides were stored in light-proof boxes for 4–25 d at 4°C, then developed in Kodak D-19, fixed with Ektaflo, dehydrated, coverslipped and visualized by differential interference contrast optics. Further details have been described elsewhere (Pickel et al., 1986).

For electron microscopic autoradiography, ultrathin serial sections were picked up with loops, placed on slides coated with 2% parlodion in amyl acetate and counterstained with uranyl acetate and lead citrate. These slides were carbon-coated, dipped in Ilford L4 emulsion diluted 1:4 with distilled water at 50°C, air-dried under 70% humidity, exposed for 6–16 months in light-tight desiccated boxes at 4°C, developed in

Kodak Microdol-X, and fixed with 30% sodium thiosulfate. At the termination of the exposure, the ultrathin sections were transferred to grids, immersed in amyl acetate for 5 min to thin the parlodion layer, and then viewed with a Philips 201 electron microscope. Further details of the electron microscopic autoradiographic procedure are described elsewhere (Beaudet, 1982; Descarries and Beaudet, 1983).

Analysis of immunolabeled somata and processes

Immunautoradiographic labeling of somata, dendrites, and axons was determined by analyzing the distribution of silver grains over the cellular profiles that occurred within a 2- μ m radius from Epon-tissue interface where penetration of immunoreagents was optimal. Recurrence of silver grains over suspected source was the criterion used to differentiate and confirm specific labeling from background labeling (Salpeter et al., 1969, 1977, 1978).

Following method 1, all cells and processes exhibiting immunoperoxidase labeling with NPY antisera near the Epon-tissue interface were included in the analysis. These profiles were considered to contain NPY or NPY-like peptides and categorized as NPY-immunoreactive. Of these, the profiles exhibiting recurrence of silver grains were considered to contain both NPY (or NPY-like peptides) and GABA and categorized as "dually labeled for NPY and GABA." The NPY-immunoreactive profiles exhibiting only one silver grain in one or serially taken sections were categorized separately as "possibly dual." Other profiles exhibiting no silver grain in 3 or more sections were categorized as "possibly single." Somata and processes in the vicinity of NPY-immunoreactive profiles with recurrence of silver grains also were included in the sampling and categorized as GABA-immunoreactive/not NPY-immunoreactive.

Following method 2, NPY immunoreactivity within cellular profiles was identified by the recurrence of silver grains as described above.

Analysis of cellular relationship between NPY-containing and GABAergic profiles

Light microscopic preparations were used to determine the laminar distribution of somata that were immunoreactive for NPY alone, for GABA alone, or for both NPY and GABA. Electron microscopic preparations were used to identify the immunoreactive somata and dendrites and to determine the frequency of axosomatic and axodendritic contacts made with labeled and unlabeled axon terminals. Cellular associations made with NPY-immunoreactive axons were categorized as with: (1) somata; (2) dendrites; (3) dendritic spines; (4) axons; (5) myelin; (6) blood vessels; or (7) unidentifiable. Each cellular profile associated with NPY-immunoreactive axons was further categorized according to their immunoreactivity for GABA, for NPY, for both, or for neither (=unlabeled). For the analysis of cortical sections, the cortical depth was determined based on the light microscopic assessments of the positions before preparing trapezoids for ultrathin sectioning. Most trapezoids were made with their bases orthogonal to pial surfaces so that the ultrathin sections would span through the entire depth of the cortex. In these cases, the positions within trapezoids viewed under the electron microscope were assessed using grid bars as tick marks. For example, the thin bars of 200 mesh copper grids (Electron Microscopic Supplies, Fort Washington, PA) are placed 130 μ m apart. Thus, an area that is 4 squares away from one unparallel edge of the trapezoid could be determined to be 520 (4 $\times$ 130) μ m deep from pial surface.

Results

Light microscopic assessment of specific immunocytochemical labeling

Light microscopic dual labeling was achieved by combining GABA immunautoradiography with the immunoperoxidase method for the detection of NPY (method 1) or NPY immunautoradiography followed by the immunoperoxidase method for the detection of GABA (method 2). Regardless of the labeling sequence, 3 types of immunoreactivities, i.e., GABA only, NPY only, and dual, were detected within neuronal somata following 5–25 d of autoradiographic exposures.

Control experiments tested for the specificity of secondary antibodies. When secondary antibodies recognizing rat or goat

were applied following rabbit NPY or rabbit antityrosine hydroxylase, there was no detectable immunolabeling. Similarly, when secondary antibodies recognizing rabbit and rat were applied following goat NPY, or when anti-rabbit and anti-goat secondary IgG were applied following rat GABA, there was no detectable immunolabeling.

GABAergic neurons. GABA-immunoreactive somata were more easily identified by method 1 due to the intense clustering of black grains into ovoid shapes ranging from 5 to 25 μ m in diameter (Fig. 1). These somata occurred throughout the CP nuclei (Fig. 1, *D, E*) and somatosensory (Fig. 1, *A–C*) and anterior cingulate areas of cortex, but were more evident within laminae II/III (Fig. 1*A*) and V/VI (Fig. 1*C*). The GABA-immunoreactive neurons varied in density of silver grains within both areas but were, overall, more heavily labeled in the cortex than in the CP nuclei. A lower density of silver grains, probably reflecting immunoreactivity within fibers and dendrites, occurred throughout the neuropil and was markedly less over the corpus callosum (Fig. 1*C*) or fiber bundles in the CP nuclei (Fig. 1*D*). Occasionally, streaks of silver grains resembling neuronal processes also were identified in the neuropil of the cortex and the CP nuclei (Fig. 1*C*).

NPY-containing neurons. Following method 1, NPY immunoreactivity was easily differentiated from the GABA immunoreactivity by the presence of orange-brown precipitate reflecting immunoperoxidase reaction within somata and processes. Some of these processes appeared dendritic, since, by shifting the plane of focus of the light microscope, the processes could be seen to emanate from the somata with diameter > 1 μ m and gradually taper along distal portions. Other varicose processes of smaller caliber probably were axons. As was seen with GABA-immunoreactive neurons, somata with NPY-like immunoreactivity occurred throughout the laminae of cortex (Fig. 1, *A–C*) and in the CP nuclei (Fig. 1, *D, E*). NPY-immunoreactive neurons and processes were also seen in the corpus callosum (Fig. 1*G*).

Many more of the NPY-immunoreactive somata were GABA-immunoreactive in anterior and somatosensory areas of cortex (Fig. 1, *B, C*) compared to the CP nuclei. Conversely, peroxidase-positive, silver-grain-negative somata were prevalent in the CP nuclei (Fig. 1, *D, E*), but also were detectable in the 2 cortical areas (Fig. 1*C*). The labeling within these singly labeled neurons could be interpreted as containing NPY only or, alternatively, as lacking immunolabeling for GABA due to the greater limitations of immunautoradiographic detection. However, the detection of somata containing NPY immunoreactivity without detectable levels of GABA was confirmed using method 2 in which NPY was recognized autoradiographically and GABA by the PAP method (Fig. 1*F*).

In both the cortex and CP nuclei, the morphology of neurons immunolabeled singly for NPY were indistinguishable from those labeled dually for GABA and NPY. These NPY-containing neurons were fusiform or multipolar, but never pyramidal (Fig. 1, *B, D–G*). Frequently, processes resembling dendrites (Fig. 1*B*) and axons (Fig. 1*D*) were seen emanating from or near labeled somata.

Applicability of immunautoradiography for ultrastructural localizations of GABA

The location of silver grains in serial sections was analyzed using electron microscopic autoradiographs that were developed after exposures of 6, 11 and 16 months. At 6 months, 60% of the

Table 1. Frequency of synaptic contacts between NPY terminals and GABAergic dendrites in cortex

Parameters measured	Dually labeled	NPY-receptive and GABAergic	Non-NPY-receptive and GABAergic
A) <i>N</i>	20	8	22
B) Total length of dendritic plasma membrane	87.6 μm	73.3 μm	214.1 μm
C) Total number of junctions on dendrites	19	15	108
D) Intersynaptic length ^a	4.6 μm	4.8 μm	2.0 μm

^a D was calculated by dividing C into B.

800 silver grains recurred in serial sections over profiles thought to be the source of radiation. These profiles included somata, dendrites, and axons. Only 2% of the silver grains occurred within a 200-nm radius outside the cellular boundaries of the identified profile. This distance is the theoretical limit of silver grains occurring due to radiation scatter (Salpeter et al., 1969, 1977, 1978). The remaining 35% of the silver grains in surrounding neuropil were greater than 1 μm away from the suspected sources of radiation. These were attributed to background. Longer exposure periods increased the number of silver grains seen over identified profiles in adjacent sections (73% of 680 at 11 months, 72% of 830 at 16 months) relative to the nonrecurring silver grains (24% at 11 months, 26% at 16 months, respectively) while minimally affecting the frequency of scatter (3% at 11 months, 2% at 16 months).

Ultrastructural localization of GABA and NPY in cortex

Ultrastructural analyses of cellular associations formed by cortical neurons labeled for NPY and GABA using method 1 were based on observations made in serial sections representing 376,870 μm^2 of tissue area. These areas were sampled from tissues prepared in 3 separate experiments. Areas sampled included all laminae of the somatosensory and anterior cingulate cortices. The regional differences were minor. Thus, ultrastructural descriptions of the immunoreactive neurons and their cellular associations in the different areas will be described jointly.

Perikarya that contained peroxidase reaction product indicative of NPY immunoreactivity almost always exhibited silver grains reflecting immunoautoradiographic labeling for GABA.

The immunoperoxidase product was seen along various cytoplasmic membrane surfaces, such as the endoplasmic reticulum, outer membrane of mitochondria, and plasma membrane (Fig. 2). The peroxidase product was associated with large dense-core vesicles in the region of the Golgi apparatus of NPY-immunoreactive cells (Fig. 2A). This labeling was most easily recognized by comparing the dense-core vesicles in Figure 2A with similar vesicles within other neurons not labeled for NPY (e.g., dendrite in Fig. 3B).

The dually labeled somata (Fig. 2A) contained nuclei with evenly distributed chromatin, a large rim of cytoplasm, and an extensive network of endoplasmic reticulum. Almost all NPY-immunoreactive *dendrites* also were immunoreactive for GABA. These dendrites ranged from 0.26 to 1.89 μm in minimal diameter and were never found in continuity with spines. Symmetric and asymmetric junctions were apparent along small-caliber (probably distal) dendrites. However, synaptic junctions were rarely detected along perikaryal and dendritic plasma membrane (Fig. 2, B, C). Instead, the NPY-immunoreactive perikarya and dendrites were more frequently apposed to plasma membrane of glial cells (Fig. 2). Spines were not detected in continuity with NPY-immunoreactive dendrites.

The majority of the GABA-immunoreactive neurons did not contain detectable levels of NPY immunoreactivity (Fig. 3). In contrast to the dually labeled neurons, these GABAergic cells (1) received numerous synapses on their somata and proximal dendrites, except when postsynaptic to NPY terminals (see below and Table 1); and (2) rarely contained dense-core vesicles. As with the neurons dually labeled for GABA and NPY, plasma

Figure 1. Dual light microscopic localization of NPY and GABA in the somatosensory cortex and the caudate-putamen nuclei. Light micrographs in A–E show somata in the somatosensory cortex and the caudate-putamen nuclei labeled by immunoautoradiography for GABA (black grains) and by the immunoperoxidase reaction product for NPY (orange precipitate). Dendrites are present but out of focus. A, The low-magnification micrograph of the supragranular laminae (I–III) of cortex show somata that are intensely labeled for GABA (arrowheads). Somata with NPY immunoreactivity (open arrow in lamina III) are fewer. B, At higher magnification, here taken from the granular layer, GABA immunoreactivity is evident within somata with weak (open arrows) or intense (large arrow) immunoreactivity for NPY. GABA immunoreactivity within the NPY-immunoreactive process (small arrow) is difficult to discriminate from GABA immunoreactivity and background labeling in the rest of the neuropil. C, Three classes of neurons are evident in this micrograph of the infragranular laminae. The dually labeled somata exhibit weak immunoreactivity for GABA and varying intensities of immunoreactivity for NPY (open arrows versus closed arrow near corpus callosum, cc). Somata and processes labeled singly for GABA occur with varying intensities of immunoreactivity (arrowheads). The somata labeled singly for NPY in this panel appears juxtaposed to 2 GABAergic processes (small arrows pointing toward each other). Streaks of silver grains reflecting GABAergic processes also are evident elsewhere (single small arrows). D, Low-magnification micrograph of the caudate-putamen nucleus show somata with intense immunoreactivity for GABA (arrowheads) or for NPY (large arrows). A varicose process with NPY immunoreactivity (small arrow in fiber bundle, fb) can be traced back to an NPY-immunoreactive soma. E, At higher magnification, the autoradiographic labeling within NPY-immunoreactive somata (e.g., arrow) cannot be differentiated from the GABA- and background-labeling in the surrounding neuropil. These somata can be differentiated from the intensely GABA-immunoreactive somata (arrowheads) that are without detectable levels of NPY immunoreactivity. Light micrographs in F and G show somata and processes labeled by the reversed method, i.e., immunoautoradiography for NPY and immunoperoxidase reaction method for GABA. F, In the infragranular layer of anterior cingulate cortex, somata labeled singly either for NPY (arrow) or for GABA (arrowhead) are evident. Note the small NPY-immunoreactive process (small arrow) that appears juxtaposed to a weakly GABA-immunoreactive soma. G, Within the corpus callosum, NPY-immunoreactive somata (arrows) and processes (small arrows) as well as GABA-immunoreactive somata (arrowhead) are evident. Scale bars, 50 μm in A and D, 10 μm in the other panels; autoradiographic exposure period, 4 d in A–E, 25 d in F and G.

Figure 2. Ultrastructural localization of NPY and GABA in a perikaryon and dendrites in the infragranular layer of somatosensory cortex. *A*, Electron micrograph shows a dually labeled neuronal perikaryon. Immunoautoradiographic labeling for GABA (silver grains, *sg*) occurs throughout the cytoplasm. Immunoperoxidase reaction product (electron-dense precipitates) also is distributed throughout the perikaryon but is particularly dense near the plasma membrane and in association with dense-core vesicles (*dcv*). Dense-core vesicles (*dcv*) occur in the vicinity and away from the Golgi apparatus (*G*) that is relatively less labeled. The NPY-immunoreactive neurons are contacted by the plasma membrane of a glial cell (*GC* over its nucleus). However, contacts by axon terminals are not apparent. *B* and *C*, Electron micrographs show dendrites labeled for NPY (immunoperoxidase product) to be contrasted with unlabeled dendrites in the surrounding neuropil (*uD* in *C*). Silver grains do not occur over the dendrite (*D*) of *B* in this or its two adjacent sections but recur over the dendrite (*D*) in *C* in all 3 serial sections, indicating that only the latter is GABA-immunoreactive. The dendrite in *B* is almost entirely enveloped in glial processes (asterisks in cytoplasm) except where it is postsynaptic (arrowhead) to a terminal (*T*). The dendrite in panel *C* never formed junctions with the surrounding terminals (e.g., *T*) within the serial sections. *N* = nucleus of the dually labeled perikaryon; *dcv* = dense-core vesicles; *m* = mitochondria; *er* = endoplasmic reticulum. Scale bar, 1 μ m in *A*, 0.5 μ m in *B* and *C*; autoradiographic exposure period, 11 months in *A*, 16½ month in *B* and *C*.

membrane of glial cells often were found in direct contact with the perikaryal membrane of neurons containing only GABA.

Axons labeled by the peroxidase method for NPY occurred throughout the laminae of anterior cingulate and somatosensory cortices. These included a few sparsely myelinated, and many small unmyelinated axons. However, the majority of the axons contained numerous vesicles. These were considered to be axon terminals, even when no synaptic densities were seen within the plane of section. The peroxidase reaction product mainly rimmed small-clear vesicles (Fig. 4) but also was associated with mitochondria and plasma membranes. In larger terminals, clusters of vesicles were seen near appositions with both dendrites and axons (Fig. 5). Occasionally, larger vesicles (ca. 100 nm in diameter) with intense immunoreactivity within their lumina also were found within axon terminals (T2 in Fig. 4A). These were morphologically similar to the immunolabeled large dense-core vesicles seen in the neuronal perikarya. Intensity of the immunoperoxidase labeling was, at least in part, dependent on the proximity of the cellular profile to the Epon-tissue interface. This was particularly evident in terminals T1 and T3 in Figure 4. Here, the immunoperoxidase labeling was most intense in panel A, showing an electron micrograph taken from the most superficial ultrathin section, and least intense in panel C, showing a section at greater depth.

Silver grains indicative of GABA immunoreactivity occurred over the majority (147 out of 166) of NPY-immunoreactive axons. Eighty-five out of the 147 axons were confirmed to be GABA-immunoreactive by the recurrence of silver grains directly over the axonal cytoplasm in 2 or more serial sections (e.g., T2 and T3 in Fig. 4). Compared to the immunoperoxidase method, the detectability of GABA immunoreactivity by the autoradiographic method was even more dependent on the proximity of the cellular profile to the Epon-tissue interface (e.g., cf. T1 with T3 of Fig. 4; also, note the progressive decrease in silver grains over T3 in Fig. 4). Thus, often, only 2 of the 3 sections would exhibit silver grains over an axon labeled by the peroxidase method (e.g., T1 in Fig. 3).

The type and relative frequencies of cellular associations formed with NPY-immunoreactive axon terminals were determined based on analysis of electron micrographs of immunoperoxidase-labeled, vesicle-containing profiles, some of which were dually labeled for GABA. Of the 179 such NPY-immunoreactive axon terminals, 30 were without identifiable contacts, even in serial sections. The 149 remaining terminals formed a total of 164 identifiable contacts with dendrites or somata. These targets were principally with unlabeled dendrites $>0.3 \mu\text{m}$ in diameter (see Table 2 and Figs. 4, 5). However, GABA-immunoreactive (Fig. 6) and NPY-immunoreactive dendrites (Fig. 7, C, D) also were found postsynaptic to NPY-containing terminals. Not infrequently, a single NPY-containing terminal formed synaptic junctions with more than one dendrite within a single plane of sections.

Although NPY-receptive GABAergic dendrites constituted only a small fraction of targets for NPY terminals (4.3%), these were strikingly different from the GABAergic dendrites lacking apparent NPY afferents. The frequency of synaptic contacts between NPY-immunoreactive terminals and GABAergic dendrites was determined by counting the number of synaptic junctions (NPY-immunoreactive versus nonimmunoreactive) per plasma membrane length of dendrites that were GABAergic singly versus dually for GABA and NPY. The NPY-receptive GABAergic dendrites exhibited uncharacteristically few syn-

Table 2. Cellular associations with NPY-immunoreactive (ir) axon terminals in cortex

	All NPY-ir terminals (<i>N</i> = 179) (%)	NPY-ir terminals confirmed to be GABAergic (<i>N</i> = 79) (%)
Synaptic contacts (<i>N</i> = 164)		
With somata		
GABA-ir ^a	3.7	6.3
NPY-ir ^b	0	0
Unlabeled	11.0	17.1
With dendrites ($>0.3 \mu\text{m}$ d)		
GABA-ir ^a	4.3	3.2
NPY-ir ^b	0.1	0
Unlabeled	57.2	44.7
With spines ($<0.3 \mu\text{m}$ d)		
GABA-ir	0	0
NPY-ir	0	0
Unlabeled	23.7	28.8
Juxtapositions with other axons (<i>N</i> = 87)		
En passant axons		
GABA-ir ^c	2.3	1.7
NPY-ir ^c	2.3	1.7
Unlabeled	81.6	79.2
Junctional ^d	13.8	17.4

d = minimal cross-sectional diameter.

^a Singly immunolabeled.

^b Singly or dually labeled.

^c Dually labeled.

^d Associations at synaptic junctions, all with unlabeled axons.

aptic inputs, whether or not they were dually labeled (cf. first and second column in Table 1; also cf. the GABAergic dendrite in Fig. 3B with that in Fig. 6). Conversely, GABAergic dendrites exhibiting numerous synaptic contacts rarely were postsynaptic to NPY-containing terminals (cf. second and third column in Table 1). GABAergic dendrites exhibiting few synaptic contacts (<1 junction per $5 \mu\text{m}$ of dendritic plasma membrane) and without apparent NPY innervation were less frequently encountered.

Associations of NPY-immunoreactive axon terminals with distal dendrites and dendritic spines ($<0.3 \mu\text{m}$ in diameter) were less common than with the more proximal dendrites, whether unlabeled or labeled for GABA (Table 2). Most contact sites resembled symmetric junctions with no identifiable postsynaptic densities. Other unlabeled terminals often formed asymmetric junctions on the same dendritic spine.

Associations of NPY-immunoreactive axon terminals with somata also were infrequently observed. These putative contact sites were without any interleaving glial processes. However, a significant portion (25%) of the somata associated with NPY terminals exhibited GABA immunoreactivity. The terminals confirmed to be dually labeled for NPY and GABA showed greater frequency for axosomatic contacts with GABAergic somata (cf. between the 2 columns in Table 2). These contact sites either appeared as symmetric junctions or were juxtaposed but lacked parallel alignments or densities at apposed plasma membranes.

Figure 3. Ultrastructure of a perikaryon and a dendrite in infragranular layer of somatosensory cortex exhibiting immunoreactivity for GABA but not for NPY. Electron micrograph in *A* shows a GABAergic perikaryon (silver grains in cytoplasm) in the vicinity of the dually labeled soma shown in Figure 1. Immunoperoxidase reaction product for NPY is not evident. The nucleus (*N*) is indented (curved arrow) and contains a prominent nucleolus (*Nu*). The Golgi apparatus (*G*) is elaborate and somatic contacts (arrowheads) are numerous. This neuron also is contacted by the plasma membrane of a glial cell (*GC*). Electron micrograph in *B* shows a cross-section of a dendrite (*D*) that is immunoreactive for GABA (silver grains) but not for NPY (peroxidase reaction product). This dendrite is postsynaptic to many unlabeled axon terminals (arrowheads) and contains mitochondria and isolated dense-core vesicle (*dcv*). Scale bar, 1 μ m in *A*, 0.5 μ m in *B*; autoradiographic exposure period, 11 months for *A*, 17 months for *B*.

Figure 5. Axon terminal dually labeled for NPY and GABA forms symmetric synapse with unlabeled dendrite and an apposition with unlabeled axon terminal in granular layer of somatosensory cortex. *A* and *B*, Electron micrographs show 2 of 4 serial sections through terminal *T1*. Light immunoperoxidase reaction product for NPY is seen in all three sections through *T1*. The product is most evident when compared to other nearby unlabeled terminals (*T2* and *uT*). Silver grains (immunoautoradiography for GABA) recur over *T1* in all 4 sections. The 2 more superficial sections, not shown here, exhibit 6 and 2 silver grains, respectively, over the terminal, but no synaptic specialization. By contrast, the section furthest away from the surface (*B*) has only one small grain (encircled) but exhibits 2 synaptic junctions. Symmetric synaptic specialization between *T1* and a dendrite (*D*) is evident in *A* (curved black arrows), to be contrasted with the asymmetric synaptic specialization formed between *T2* and *D* in *A* and *B* (open arrows). In addition, an axoaxonic contact between *T1* and *T2* is seen most clearly in *B* (pair of arrowheads). Scale bar, 0.25 μ m in *A*, *B*; asterisks, nonrecurrent silver grains; autoradiographic exposure period, 16 months.

NPY-immunoreactive axon terminals also were frequently juxtaposed to other axon terminals without intervention by glial processes (Table 2). From a total of 251 observed associations formed by NPY-immunoreactive terminals, 87 were with other axons as opposed to the remaining 164 that were with dendrites and somata (described above). The axon terminals associated with NPY-immunoreactive terminals usually were unlabeled and shared common postsynaptic targets (Fig. 5, *T1* and *T2*). More rarely, the second terminal also was labeled for NPY and GABA. In, at most, one of 2–7 serially collected ultrathin sections, the plasma membranes of the 2 axon terminals appeared similar to symmetric junctions. In these terminals, vesicles were clustered near the presumed presynaptic membranes which were oriented parallel to each other (e.g., between *T1* and *T2* in Fig. 5*B* and between *T1* and *T2* in Fig. 8). NPY-immunoreactive axon terminals occasionally were located immediately adjacent to the synaptic cleft zone of other axodendritic or axospinous junctions of the asymmetric type (“junctional” in Table 2). In these cases, the plasma membrane of NPY-immunoreactive axon terminals were aligned in parallel with both the pre- and postsynaptic elements.

Finally, NPY-immunoreactive axon terminals sometimes were closely associated with basement membranes of blood vessels

(Fig. 7, *A*, *B*). These associations were noted at the occasional interruptions of astrocytic processes surrounding the basement membrane.

NPY-immunoreactive axon terminals without detectable GABA immunoreactivity in cortex

In sections labeled by method 2, terminals containing autoradiographic labeling for NPY without detectable immunoperoxidase labeling for GABA indicated the presence of terminals singly immunoreactive for NPY. In sections labeled by method 1, the autoradiographic detection of GABA in profiles immediately adjacent to terminals showing only peroxidase reaction for NPY (Fig. 8) confirmed that the differential labeling was not attributed to methodological limitations. Most of the terminals singly labeled for NPY by method 1 contained dense immunoperoxidase reaction product (Figs. 7, 8) in comparison to those containing both NPY and GABA (Figs. 4–6). Otherwise, little difference from the dually labeled terminals was noted. Thus, the singly labeled terminals always contained many more small-clear than large dense-core vesicles and, where identifiable, formed symmetric junctions. The targets most frequently were dendrites. Synaptic junctions with somata and dendritic spines and close appositions with glia and basement membrane

Figure 6. Terminal, dually labeled for NPY and GABA, forms a symmetric synapse with a dendrite immunoreactive for GABA in the infragranular layer of somatosensory cortex. *A–D*, Electron micrographs taken from 4 serial sections show recurrence of silver grains (immunogold) over axon terminal *T1* and over the postsynaptic dendrite (*D*, curved arrow in *D* points to the symmetric synaptic junction). Immunoperoxidase labeling for NPY (seen as electron-dense product) is evident in *T1* of *A–C* but not *D*. Axon terminal *T2* also exhibits immunolabeling for NPY. However, it is unclear whether *T2* also contains GABA, since silver grains occur only in *B*. Scale bars, 0.5 μm in *A–D*; autoradiographic exposure period, 11 months.

of blood vessels also were evident. The targets were usually unlabeled, although a few somatic contacts were formed by NPY terminals on GABAergic perikarya.

Similarities and differences in the ultrastructural localization of GABA and NPY in the CP nuclei compared to cerebral cortex

The quantitative ultrastructural analysis of GABA- and NPY-immunoreactive profiles in the rostral CP nuclei (precommissural level) was based on serial electron micrographs taken from the dorsolateral quadrant representing 202,800 μm^2 of tissue area. These tissues were prepared from 2 separate experiments using method 1 where sections were dually labeled for NPY by

the immunoperoxidase method and for GABA by immunogold autoradiography.

The majority of NPY-immunoreactive somata contained only 0–5 silver grains per 10 μm^2 (equivalent to background levels of radioactivity) and did not exhibit these silver grains in serial sections. Thus, these NPY-immunoreactive profiles were not considered to be immunogold autoradiographically labeled for GABA. This was in sharp contrast to the NPY-immunoreactive neurons in the cortex which were almost always labeled for GABA. The NPY-immunoreactive somata in the CP nuclei also differed from those in the cortex in that they appeared to contain fewer dense-core vesicles.

A few NPY-immunoreactive somata in the CP nuclei con-

Figure 7. Association of NPY-immunoreactive axon terminals in somatosensory cortex with blood vessel and unlabeled as well as NPY-labeled dendrite. *A* and *B*, Electron micrographs through serial sections show an NPY-immunoreactive axon terminal (*T*) in the infragranular layer of somatosensory cortex that is juxtaposed to a glial process (asterisks in cytoplasm) and associated less extensively (open arrows) with the basement membrane (small circles) of a blood vessel. The same terminal also forms a symmetric synaptic junction (black curved arrow in *A*) with an unlabeled dendrite (*D*). *BVL* = blood vessel lumen. *C* and *D*, Electron micrographs taken from the granular layer of somatosensory cortex show localization of NPY immunoreactivity in both pre- and postsynaptic profiles. The junction (closed arrowhead) between the axon terminal, *T*, and dendrite, *D*, is partially obscured by immunoperoxidase reaction product but appears symmetric. The same dendrite is also postsynaptic to an unlabeled terminal (*uT*) forming an asymmetric synaptic junction (open arrows). Scale bars, 0.25 μ m in *A–D*; autoradiographic exposure period, 11 months for *A* and *B*, 16 months for *C* and *D*.

Figure 8. Differential localization of NPY and GABA immunoreactivities in terminals within the infragranular layer of somatosensory cortex. *A* and *B*, Electron micrographs taken from 2 of 3 serial sections show an axon terminal with intense immunoreactivity for NPY (*T1*), but without silver grains in any of the sections. *T1* forms a symmetric junction (black arrow) with a dendrite (*uD*) and is juxtaposed (arrowhead in *A*) to an unlabeled axon terminal (*T2*). *T2* forms an asymmetric junction (open arrow) with another dendrite. A nearby dendrite (*G-D*) contacted (open curved arrow) by an axon terminal (*G-T*), both of which show recurring silver grains indicative of immunoautoradiographic localization for GABA. Scale bars, 0.5 μ m in *A* and *B*; autoradiographic exposure period, 11 months.

tained significant numbers of silver grains indicative of immunoautoradiographic labeling for GABA (Fig. 9). These shared many morphological features with other striatal neurons singly labeled for GABA or for NPY. These features included deeply indented nuclei and a well-developed endoplasmic reticular network. As was observed in the cortex, the NPY-immunoreactive somata, whether GABA-immunoreactive or not, differed from

the non-NPY-immunoreactive GABAergic neurons in that they exhibited few if any axosomatic contacts.

NPY-immunoreactive dendrites were indistinguishable from those observed in the cortex. While most NPY-immunoreactive dendrites did not contain detectable levels of GABA immunoreactivity, some dually labeled dendrites were encountered (Fig. 10). Dendritic spines were never detected on either type

Figure 9. Ultrastructure of GABAergic perikarya with and without NPY immunoreactivity in the caudate putamen. The electron micrographs in *A* and *B* show silver grains indicating GABA immunoreactivity in neuronal perikarya. The recurrence of silver grains in serial sections was established for both of these perikarya. The perikaryon in *A* also is immunoreactive for NPY (peroxidase reaction product). The peroxidase reaction product is intensely associated with endoplasmic reticulum (*er*) but is relatively sparse in the vicinity of the Golgi apparatus (*G*). The perikaryon in *B*, like that in *A*, contains a nucleus (*N*) with an invagination in nuclear envelope near the Golgi apparatus. Peroxidase labeling for NPY is not detectable in the cytoplasm of the cell in *B*. Numerous synaptic contacts (*arrowhead*) from unlabeled terminals are also evident. *N* = nucleus. Scale bars, 0.5 μ m in *A* and *B*; autoradiographic exposure period, 16 months for *A*, 6½ months for *B*.

of dendrite. These dendrites often were enveloped by glial processes. Axodendritic synaptic contacts were sparse but included junctions with GABAergic terminals.

NPY-immunoreactive axon terminals in the CP nuclei also were morphologically similar to those observed in the cortex. Striatal NPY-containing terminals had the following morphological features in common with those in cortex: (1) many more small-clear than dense-core vesicles; and (2) formation of symmetric junctions with all types of targets. However, striatal NPY-immunoreactive terminals were strikingly different from those in the cortex in that, of a total of 87 terminals that were followed in serial sections, none exhibited recurring silver grains indicative of GABA immunoreactivity and only 2 exhibited non-recurring silver grains.

The cellular associations formed with the striatal NPY-immunoreactive terminals differed slightly from the pattern seen in the cortex (cf. Table 2 and Table 3). In both regions, the terminals formed synapses most often with dendrites, some of which were GABAergic. The relative proportion of axodendritic synapses was similar. However, fewer axons formed synapses with somata and more with dendritic spines in the CP nuclei. Axospinous junctions typically occurred opposite or adjacent to an asymmetric junction formed by another unlabeled axon terminal onto the same spine. Interestingly, most of the somata associated with NPY-immunoreactive axon terminals were labeled for GABA (e.g., Fig. 11). As was observed in the cortex, most of the putative axosomatic contacts appeared as appositions uninterrupted by glial processes, but lacking clearly parallel membranes and/or synaptic densities. Cellular associations also occurred with other unlabeled axons but were more rare (14 out of 87) when compared to cortex (87 out of 251).

Discussion

We have confirmed that, in the cortex, most of the NPY-like immunoreactivity is contained within the nonpyramidal, GABAergic neurons, whereas, in the CP nuclei, most of the immunoreactivity is in neurons without GABA immunoreactivity. The quantitative ultrastructural analysis of ultrathin sections labeled dually for GABA and NPY revealed the following intracellular and intercellular features shared by NPY-containing neurons in both regions: (1) most of the GABAergic somata without NPY can be contrasted with the NPY-immunoreactive somata in that they exhibit many more axosomatic and axodendritic synapses; (2) the NPY-immunoreactive neurons exhibit extensive Golgi-endoplasmic reticular network as well as large vesicles with immunoreactive cores in somata, dendrites and terminals; (3) NPY-immunoreactive terminals contain many small-clear vesicles and form symmetric junctions irrespective of target; (4) the majority of targets for NPY-immunoreactive terminals are dendrites containing neither GABA nor NPY; (5) a small percentage of targets for NPY-immunoreactive terminals are with sparsely innervated GABA-immunoreactive somata and dendrites. The analysis revealed additional differences between these regions in that, as compared to the CP nuclei, cortical NPY-immunoreactive terminals were more frequently (1) labeled for GABA and (2) apposed to other unlabeled axons. The relevance of these observations to known functions and physiological actions of NPY in cortex and the CP nuclei will be discussed.

Specificity of the primary antisera

Radioimmunoassay performed on brain proteins and peptides separated by high-performance liquid chromatography reveal

Table 3. Cellular associations with NPY-immunoreactive (ir) axon terminals in the caudate-putamen nuclei ($N = 87$)

Synaptic contacts ($N = 73$)	
With somata	
GABA-ir	4.2%
Unlabeled	0%
With dendrites ($>0.3 \mu\text{m d}$)	
GABA-ir	4.2%
Unlabeled	51.1%
With spines ($<0.3 \mu\text{m d}$)	
GABA-ir	4.2%
Unlabeled	36.2%
Juxtapositions with other axons ($N = 14$)	
En passant axons	92.8%
Junctional	7.1%

d = minimal cross-sectional diameter.

that NPY is the overwhelmingly dominant species recognized by the antisera used in the present study (DiMaggio et al., 1985). However, the possibility that the antisera also recognize portions of proteins sharing sequences in common with NPY cannot be excluded completely. For this reason, the immunoreactivity recognized using the antisera include NPY-like peptides as well as NPY itself, even if not so specified in text. More recently, the distribution of somata immunocytochemically labeled using the same antisera was shown to closely resemble the distribution of messenger RNA transcribing NPY (Gehlert et al., 1987). This finding suggests that neurons containing NPY probably synthesize the peptide. However, labeling by immunocytochemistry and by *in situ* hybridization within single sections would be required to confirm this point.

The density of NPY-immunoreactive perikarya and extension of processes under light microscopy appeared to be less compared to those tissues processed in the presence of permeabilization agents, such as Triton X-100. The detergent dependence indicates that the differences in immunodetectability is due to the differences in the depth of penetration of immunoreagents from tissue surface. Under electron microscopy, where tissue was sampled strictly from the surface, distal and proximal portions of processes as well as perikarya were all detectable, suggesting that our sampling was not overtly biased for the proximal portions of cells.

The morphological features of neurons in cortex and striatum immunolabeled using antisera against protein-conjugated forms of GABA (Somogyi and Hogson, 1985, and unpublished observations; Pickel et al., 1988) are indistinguishable from the characteristics previously described for neurons labeled for glutamate decarboxylase (GAD), the major GABA-synthesizing enzyme (Ribak, 1978; Hendrickson et al., 1981; Hendry et al., 1983; Houser et al., 1983a; Somogyi et al., 1983; Bolam et al., 1985) or by the uptake of GABA (Bolam et al., 1983). Thus, most neurons exhibiting GABA immunoreactivity probably synthesize GABA. However, possibilities remain that some exhibit immunoreactivity to the anti-GABA antiserum due to uptake from glia and terminals in the vicinity. Ultrastructural identification of many GABA-immunoreactive somata and dendrites without apparent contacts from GABA-immunoreactive terminals make this possibility less likely.

Figure 11. An NPY-immunoreactive axon terminal forms a contact with an axon emerging from a GABA-immunoreactive soma. Electron micrographs show a GABA-immunoreactive soma (silver grains over cytoplasm and nucleus, *N*) that contains a large, unindented nucleus. An NPY-immunoreactive (peroxidase reaction product) terminal is in direct contact with an axon emerging from the GABA-labeled soma (*boxed area* and *curved arrow* in insert). Silver grains were never detected over the peroxidase-labeled axon terminal. Scale bars, 1 μ m in larger electron micrograph; 0.5 μ m in insert; autoradiographic exposure period, 6½ months.

The density of GABAergic neurons in striatum was, in some cases, sparse compared to what has been reported previously using antibodies against glutamate decarboxylase (Penny et al., 1986). We suspect some of the differences to be attributable to

the antigens recognized in the 2 immunocytochemical procedures, since smaller molecules such as GABA may have a greater tendency to leach out of the tissue during the 2–3 d of incubation and rinsing procedures.

Figure 10. An axon labeled for GABA forms a synaptic junction with a dendrite dually labeled for GABA and NPY in the caudate-putamen nuclei. *A–C*, Electron micrographs taken from 3 of 6 semiadjacent sections show the recurrence of silver grains, indicative of GABA immunoreactivity, over a terminal (*T* in *A* and *B*) and a dendrite (*D* in *B* and *C*). The dendrite also is immunolabeled for NPY (peroxidase reaction product). A symmetric junction between the terminal and the dendrite is apparent in *A* (*curved arrow*). The terminal and dendrite both are enveloped by glial processes (*asterisks* in cytoplasm; *arrowhead* = gap junction formed between 2 astrocytic processes). Scale bars, 0.5 μ m in *A–C*; autoradiographic exposure period, 6½ months.

The validity, usefulness and limitations of the dual immunocytochemical method for light microscopic analyses

The simultaneous localization of GABA and NPY immunoreactivities within somata was made possible by the combined application of immunautoradiographic and immunoperoxidase labeling procedure (Pickel et al., 1986). Care was taken to minimize the potential cross-reactions between the 2 labeling procedures by choosing primary antibodies made in different species and avoiding the use of secondary antibodies made in the species of the primary antibody. The absence of cross-reactivity is suggested by both light and electron microscopic observations, since many of the somata and processes are labeled only by immunautoradiography or by the immunoperoxidase reaction product (however, see below). The striking regional difference in the prevalence of dually versus singly labeled somata between cortex and striatum also is indicative of the specificity of methods.

The identification of dually labeled somata in light microscopic preparations is greatly facilitated by the use of differential interference contrast (DIC) optics. DIC optics allows for optical sectioning (Curcio et al., 1987) of the emulsion containing silver grains from the tissue layer containing somata filled with the peroxidase reaction product. However, quantification of the relative frequency of dually versus singly labeled somata was not feasible, due to the greater difficulty in identifying somata without autoradiographic labeling as nonimmunoreactive. This was because autoradiography does not favor detection of immunoreactivity within cells away from the surface adjacent to the emulsion layer. Some of the limitations would be eliminated in thin 5–10 μm frozen sections. Use of this type of section is not advantageous for electron microscopy. Thus, we instead compared results following the reversed labeling method to further establish the extent of single versus dual labeling for NPY and GABA. In these sections, silver grain positive/peroxidase-negative somata reflecting NPY-positive/GABA-negative still were identified in the CP nuclei and less frequently, but also, in cortex. The detection of all 3 types of neurons, namely GABA-positive, NPY-positive, and dually positive, by electron microscopy further indicates that the light microscopic assessments are valid.

Validity of the dual method for semi-quantitative ultrastructural analyses

Analysis of ultrathin sections serially collected starting from the Epon-tissue interface confirmed earlier reports (Pickel et al., 1986, 1989; Aoki and Pickel, 1988) that immunolabeling is restricted to the first few microns within tissue. The immunautoradiographic labeling is relatively more restricted than the peroxidase labeling, since recurring silver grains are not seen within portions of sections still containing peroxidase reaction product. Thus relative frequencies of different types of cellular associations can be ascertained only from portions of sections exhibiting a neuropil containing both immunocytochemical labels.

The analysis of silver grains in superficial sections confirms previous findings (Pickel et al., 1986; Aoki and Pickel, 1988; Milner et al., 1988) that autoradiography is a reliable method for identifying even small caliber (<0.3 μm) profiles such as axons. Specifically, analysis based on the electron micrographs obtained from the present study indicated that only 2% of the silver grains reflected scatter [outside but within a 200-nm radius from the profile (Salpeter et al., 1969, 1977, 1978)]. Further,

since nearly all of the nonrecurring silver grains (reflecting non-specific labeling) within the surrounding neuropil were located >1 μm away from the suspected profile, these were easily differentiated from the grains reflecting specific labeling or scatter. The ratio of specific to nonspecific silver grains did not change appreciably between 11 and 16 months, indicating that the shorter periods are sufficient for electron microscopic detection of ^{125}I and that longer periods have minimal increases in background scatter.

Ultrastructural characteristics of NPY-immunoreactive neurons in cortex and CP nuclei

Similarities among perikarya in the 2 regions. The morphological characteristics of NPY-immunoreactive somata in the anterior cingulate and somatosensory areas of cortex and the CP nuclei closely resemble each other. In both regions, abundant Golgi apparatuses and extensive network of endoplasmic reticulum suggest active turnover of proteins and lipids by NPY-containing neurons (Farquar, 1985). Moreover, the dense-core vesicles near the Golgi apparatus and within proximal dendrites may reflect sites of storage of newly synthesized NPY as seen in other brain regions (Pickel et al., 1989). However, even though immunoreactivity appeared to accumulate specifically over dense-core vesicles, possibilities remain that these vesicles are sites for storage of other known or yet unknown peptides. One likely candidate for storage with NPY is somatostatin, a peptide known to co-localize with NPY in somata of both cortex and striatum (Vincent et al., 1982; Demeulemeester et al., 1988). On the other hand, although cortical tachykinin-containing neurons also contain numerous dense-core vesicles in the soma, it is less likely that tachykinins occur within cortical NPY neurons of the presently studied population, since they were reported not to co-store with GABA (Jones et al., 1988).

The numerous similarities between NPY-containing neurons in cortex and the CP nuclei were not necessarily expected, since the NPY-immunoreactive neurons in cortex are mostly GABAergic (Demeulemeester et al., 1988, and unpublished observations), a chemical characteristic not shared by all NPY-immunoreactive neurons in the striatum. On the other hand, the similarities are concordant with the above-mentioned chemical characteristic shared by the NPY neurons in the 2 regions (co-localization with somatostatin) as well as certain anatomical features shared by the NPY neurons in the 2 regions, i.e., that they are (1) nonpyramidal and intrinsic (Jones, 1975; Houser et al., 1984; Christie et al., 1986; Smith and Parent, 1986); and (2) aspiny (DiFiglia and Aronin, 1982; reviewed by Houser et al., 1984).

Similarities in the morphology of NPY-immunoreactive axons in the two regions. The NPY-immunoreactive axon terminals in the cortex and the CP nuclei mostly contact dendrites and are morphologically similar, whether or not they also contain GABA. In both regions, synaptic specializations formed by NPY-labeled terminals are overwhelmingly of the symmetric type, irrespective of the target. This pattern also was observed previously in striatum (Vincent and Johansson, 1983; Aoki and Pickel, 1988; Massari et al., 1988; Vuillet et al., 1989), cortex (Hendry et al., 1984a) and solitary tract nuclei (Pickel et al., 1989) and suggests that NPY may be predominantly inhibitory in its action (Gray, 1959) throughout the brain regions. Further, the detection within single planes of section of synaptic associations between one NPY-containing terminal and more than

one dendrite suggests that NPY may be able to modulate the excitability of a group of postsynaptic neurons synchronously.

In contrast to our results, Hendry et al. reported (1984a) on the occurrence of NPY terminals in area 4 (motor) of monkey cortex containing numerous small, nonimmunoreactive clear vesicles together with fewer, larger vesicles with immunoreactive cores. These terminals formed asymmetric junctions with dendrites. Terminals with these morphological features were never labeled for NPY in any lamina of somatosensory or anterior cingulate areas of cortex or the CP nuclei in rat brain. However, catecholaminergic terminals containing large vesicles with NPY-immunoreactive cores have been observed in the accumbens nuclei (Aoki and Pickel, 1988). These terminals may be noradrenergic, since one distinguishing feature of the accumbens nuclei compared to the CP nuclei is the presence of a higher concentration of noradrenergic terminals (Moore and Card, 1984). On the other hand, since cortex receives a significant number of noradrenergic afferents, the absence of NPY-immunoreactive terminals with asymmetric junctions in the somatosensory and anterior cingulate areas of rat cortex may reflect areal or species differences. Also, since overlying emulsion in dually labeled ultrathin sections diminish contrast for immunoperoxidase labeling, the discrete labeling of dense-core vesicles may not always have been detected.

Similarities in inputs to NPY neurons. The paucity of synaptic contacts on NPY-immunoreactive somata and proximal dendrites is a feature shared by the NPY-immunoreactive neurons in the cerebral cortex and striatum as well as those in brain stem (Pickel et al., 1989). This characteristic also was noted among the NPY-immunoreactive neurons in the striatum by others (Vincent and Johansson, 1983) as well as among the tachykinin-immunoreactive neurons in the cortex, some of which may contain NPY (Jones et al., 1988). In contrast, many GABAergic neurons in cortex and striatum without NPY immunoreactivity are studded with somatic, dendritic and spinous contacts from unlabeled axon terminals. Assuming conventional electrotonic spread of synaptic potentials within dendrites and soma, the influence of synaptic inputs on a neuron's excitability would be expected to weaken with increasing distance from the impulse-initiation site (reviewed by Rall, 1964). Thus, the paucity of inputs to NPY-immunoreactive neurons, particularly on proximal dendrites and somata, suggests that the firing rate of NPY-containing neurons may be determined by fewer, weaker inputs when compared to most other GABA-immunoreactive neurons.

Similarities in cellular associations formed with NPY-immunoreactive terminals. In both regions, synaptic contacts formed by NPY-immunoreactive terminals onto GABAergic somata and dendrites were few in number. The observed frequency is lower than would be expected based on the prevalence of GABAergic neurons in the 2 regions, estimated to be about 25% of all neurons in cortex (reviewed by Jones et al., 1987) and at least 50% in the striatum (Penny et al., 1986). The lower observed incidence may at least partially reflect nonoptimal detection of one or both neurotransmitters. Thus, the dendrites may lack detectable levels of immunoreactivity due to methodological limitations or, alternatively, may comprise a mixture of many types of non-GABAergic neurons. They also may consist of a single type of cell, such as the glutamatergic pyramidal neurons in the case of the cerebral cortex (Streit, 1984) or the large, sparsely-spiny neurons in striatum that are cholinergic (Lehmann and Langer, 1983). In future studies, it would be interesting to examine whether NPY-immunoreactive terminals

in cortex form synaptic contacts more frequently with pyramidal cells labeled by immunoreactivities to glutaminase (Kaneko et al., 1987) or glutamate (Conti et al., 1987).

In spite of the rarity, direct synaptic contacts between NPY-immunoreactive terminals and GABA-immunoreactive neurons occur relatively selectively at sites expected to have strong influences on neuronal excitability or metabolism. These are the soma and proximal dendrites that are close to the impulse-initiation zone (Rall, 1964) and regions where proteins are being synthesized (reviewed by Barondes, 1972). In addition, all of the NPY-receptive GABAergic dendrites that were encountered contrast sharply from the apparently non-NPY-receptive GABAergic dendrites in that the former were associated with fewer axon terminals. Due to the relative scarcity of other competing inputs, the NPY innervation of these GABAergic dendrites would be expected to exert particularly strong influences. These sparsely innervated NPY-receptive GABAergic dendrites may belong to the same class of cortical neurons containing both tachykinins and GABA since their cytological characteristics are similar (Jones et al., 1988).

Little is known about the actions of NPY on somata in any region. In the hippocampus, Colmers et al. (1988) could not detect any electrophysiological action elicited by NPY at the soma. Since NPY alters calcium currents (Colmers et al., 1988; Walker et al., 1988) and binds to sites that are coupled to G-proteins (Walker et al., 1988), NPY's action at somata may involve slow biochemical events not easily detected using electrophysiological methods. The rarity of unambiguous synaptic specializations at these axosomatic junctions corroborate our speculations. Such biochemical events may involve alterations in the activity of intracellular enzyme(s) dependent on concentrations of second messengers like Ca^{2+} or cyclic nucleotides (reviewed by Rasmussen, 1981).

In both the CP nuclei and somatosensory and anterior cingulate areas of cortex, a few of the NPY-immunoreactive terminals were directly apposed to glia and endothelial cells around small parenchymal blood vessels. These associations may reflect another mode by which NPY modulates local cerebral blood flow and/or metabolism in the cortical and striatal regions. NPY's central mode of action may involve contraction of vascular muscles, as has been suggested for NPY peripherally (Edvinsson et al., 1983, 1987). However, many more of the blood vessels are devoid of any association with NPY-immunoreactive terminals. Further, the NPY-containing terminals often are separated from the vascular basement membrane by intervening fine glial processes. The intervening glial processes may possess receptors for NPY, in which case, NPY's influence upon blood vessels may be mediated indirectly by glia. Overall, it appears that association between NPY terminals and vasculature is much more prevalent with larger blood vessels, such as those coursing through pia mater (Edvinsson et al., 1983, 1987), than with the smaller parenchymal blood vessels.

Differences in chemical features of NPY-immunoreactive neurons in cortex versus the CP nuclei. The most striking difference between the NPY-immunoreactive neurons in the two regions is chemical rather than morphological. As already noted, most NPY-immunoreactive somata and terminals in cortex contain GABA (Hendry et al., 1984b, and unpublished observations; Demeulemeester et al., 1988), while only a subpopulation of the NPY-containing somata and terminals in the CP nuclei contain this inhibitory amino acid. Previous histochemical studies have shown that cortical NPY neurons also differ from those in the

striatum because some cortical NPY neurons have undetectable levels of NADPH-diaphorase while all NPY neurons in the CP nuclei exhibit high levels of NADPH-diaphorase activity (Vincent et al., 1983). In addition, a more recent dual immunocytochemical study indicates that the few non-GABAergic, NPY-containing neurons in cortex may be co-stored with tachykinins as well as somatostatin (Jones et al., 1988). Whether or not co-storage of the 2 peptides occurs in the non-GABAergic NPY neurons of the CP nuclei has not yet been determined. A possibility remains that some of the non-GABAergic NPY-containing terminals originate extrinsically, such as from the brainstem catecholaminergic neurons (Everitt et al., 1984). Future anatomical investigations that combine tract-tracing methods with electron microscopic immunocytochemistry would be required to address this question.

Differences in the axonal associations between the 2 regions. Appositions between NPY-immunoreactive terminals and dendritic spines resembling symmetric synaptic specializations were more frequently observed in the CP nuclei than in the cerebral cortex. Interestingly, these spine heads often were simultaneously postsynaptic to another axon terminal, forming an asymmetric [probably excitatory (Gray, 1959)] junction. This relationship between 2 axons and a spine suggest that NPY may exert potent influences on neurons by modulating excitatory inputs at spines, especially in the CP nuclei. Substance P and glutamate are likely candidates for transmitters contained within terminals presynaptic to asymmetric junctions since they both are excitatory and occur within the 2 forebrain regions (Penrow, 1983; Fonnum, 1984). The interaction between NPY and the non-NPY neurotransmitter may occur via convergence of the second-messenger systems intracellularly within the spine, since these transmitters bind to receptors that are coupled to second-messenger systems (Penrow, 1983; Nicoletti et al., 1986; Walker et al., 1988). Alternatively, NPY may alter the membrane potential of the spine by modulation of calcium channels (Colmers et al., 1988; Walker et al., 1988).

Juxtapositions of NPY-immunoreactive terminals with other unlabeled axons occur more frequently in cortex than in the CP nuclei. These axoaxonic associations were first described by Hendry et al. (1984a). In some cases, we observed that the plasma membranes of the 2 terminals form brief parallel alignments and are associated with clustering of vesicles along the aligned region. This arrangement closely resembles symmetric junctions seen at other parts of cells. The possibility that these may be the ultrastructural correlates of NPY's presynaptic modulation of transmitter release is intriguing. Review of previous studies on the action of NPY suggests that NPY can interact with many kinds of axon terminals. For example, modulations of the release of norepinephrine in areas such as the nucleus of the solitary tract, anterior hypothalamus (Ciarleglio et al., 1986), and peripheral nerves (Allen et al., 1982; Lundberg et al., 1982), or of substance P in spinal cord (Walker et al., 1988), glutamate in hippocampus (Colmers et al., 1987), luteinizing and growth hormones (LH, GH) from pituitary (McDonald et al., 1985; Crowley et al., 1987) and LH-releasing hormone from the median eminence (Sabatino and McDonald, 1987) have so far been demonstrated. In the striatum, previous reports have suggested that NPY neurons form contacts with catecholaminergic terminals since central applications of NPY can alter the turnover of striatal catecholamines (Beal et al., 1986a). This action may be mediated in part by the direct axoaxonic contacts which we detected between NPY and catecholaminergic terminals, some

of which converged onto common dendrites (Aoki and Pickel, 1988). In cortex, the prevalence of axoaxonic associations between NPY and catecholaminergic terminals remains to be determined.

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