Differential Expression of mRNAs for Neuropeptide Y–related Peptides in Rat Nervous Tissues: Possible Evolutionary Conservation

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Neuropeptide Y (NPY) is the only member of its peptide family that has been isolated from the mammalian CNS. We have recently found that two different NPY-related molecules are present in the CNS of a cyclostome, the river lamprey (*Lampetra fluviatilis*) (Söderberg et al., 1991). Here we show that this is also true for the rat CNS, by demonstrating expression of peptide YY (PYY) mRNA in brainstem neurons distinct from those neurons that express NPY mRNA. Dissimilar oligonucleotide DNA probes complementary to 3' untranslated regions of the rat PYY, NPY, and pancreatic polypeptide (PP) mRNA were used in *in situ* hybridization experiments on sections of rat brain and spinal cord, visceral organs, and peripheral nerve ganglia. The PYY probe hybridized with two populations of neurons in the brainstem: one dispersed along the midline in the rostral medulla and another in the lateral caudal medulla (A1 region). No additional labeling was detected in the remainder of the neuraxis. In the periphery, PYY hybridization was seen only in endocrine cells of the colon, and not in sympathetic ganglia or the adrenal gland, suggesting that previous observations of PYY immunoreactivity in these latter structures were due to antibody cross-reactivity with NPY. The NPY probe did not hybridize with cells on the midline region that contains PYY neurons, but it did label large numbers of neurons throughout the neuraxis. No expression of PP mRNA was detected in the CNS. Northern blot analysis failed to detect PYY mRNA in the CNS, further supporting the observation that PYY is only expressed by a discrete collection of CNS neurons. The anatomy of PYY- and NPY-expressing cells in the CNS and gut shows a striking similarity between rat and lamprey (Brodin et al., 1989), vertebrates that diverged evolutionarily about 450 million years ago, suggesting that both peptide systems have been conserved throughout vertebrate evolution.

The neuropeptide Y (NPY) family consists of three related peptides: pancreatic polypeptide (PP), which was discovered first (Lin and Chance, 1974; Kimmel et al., 1975), and also NPY and peptide YY (PYY). NPY and PYY show over 70% identity in amino acid sequence, and both share 50% identity with PP (Tatemoto, 1982; Tatemoto et al., 1982).

While immunocytochemical studies have proposed that all members of the NPY family of peptides are present in the brain, as yet NPY is the only family member that has been isolated from the CNS (Tatemoto et al., 1982). Due to the structural similarities, it has been difficult to distinguish between different PP-related peptides using immunological and chromatographic methods. Thus, the results from different laboratories are in conflict and no less than four different hypotheses have been presented regarding the presence of PP-related molecules in nervous tissues. Based on the cross-reactivity patterns of a range of antisera, Inui et al. (1985) have suggested that genuine PP is present in the brain. Broomé et al. (1985) and Ekman et al. (1986) have proposed that PYY occurs in the CNS, based on immunohistochemical and RIA data. The occurrence of PYY-like immunoreactivity in sympathetic neurons (Häppölä et al., 1990) and the adrenal gland (Gaumann et al., 1989) has also been reported. Tatemoto (1989) has suggested that a novel member of the NPY family may be expressed in the brain. Finally, DiMaggio et al. (1985) and de Quidt et al. (1990; de Quidt and Emson, 1986) have proposed that NPY is the only NPY family member present in the CNS. The latter investigators have presented evidence that the PP-like material that can be distinguished from NPY in chromatographic separation of brain extracts is actually an oxidized form of NPY.

Our recent results from cDNA cloning studies in a cyclostome, *Lampetra fluviatilis*, have indeed shown that more than one PP-related peptide can be expressed in the CNS of a vertebrate (Söderberg et al., 1991). To further analyze this matter in the rodent, we have used the *in situ* hybridization technique to examine the expression of NPY, PYY, and PP in the nervous system of the rat. Due to the high degree of sequence identity between the translated regions of the NPY, PYY, and PP mRNAs, DNA oligonucleotide probes were made against 3' untranslated regions of the three mRNAs. These probes showed no significant similarity to each other. In addition, Northern blot analysis of nervous and visceral tissues was done to compare the levels of PYY and NPY mRNA.

Materials and Methods

Male Sprague–Dawley rats (100–200 gm) were used in all experiments. Animals were housed and cared for in compliance with National In-
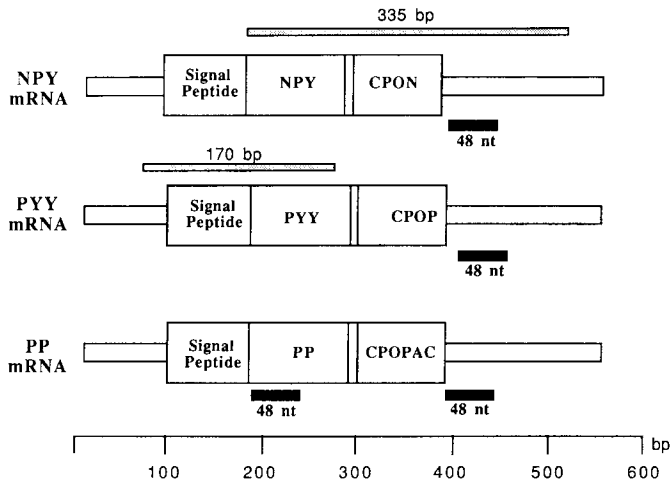
Figure 1. A schematic diagram of the NPY, PYY, and PP mRNAs detailing the locations on the mRNA where the oligonucleotide and cDNA probes were derived from for the present studies. The oligonucleotides (*solid bars* marked *48 nt* below the mRNAs) used in the *in situ* hybridization studies of NPY, PYY, and PP and Northern blot analysis of PYY and NPY were complementary to regions in the untranslated 3' ends of the corresponding mRNAs. An additional oligonucleotide DNA probe used for PP *in situ* experiments was complementary to a portion of the PP peptide-coding region of the PP mRNA. Additional Northern blot analysis was done using cDNA probes derived from the 5' end of the PYY and NPY mRNA (*open bars* above the NPY and PYY mRNAs).

stitutes of Health guidelines for the care of laboratory animals. Animals were rapidly decapitated by guillotine, and the brains, peripheral ganglia, and various visceral organs removed and rapidly frozen on dry ice.

In situ *hybridization.* Oligonucleotide probe sequences were selected for optimal GC content (approximately 60%) and minimal homology with other known RNA sequences (no eukaryotic sequences in Gene Bank had over 80% identity) and synthesized on a Pharmacia Gene Assembler. The NPY, PYY, and one of the PP probes were all 48 bases and complementary to 3' untranslated regions of the rat mRNAs, respectively (Fig. 1). The PYY probe was complementary to nucleotides 357–405. (5'-ACCAAACATGCAAGTGAAGT-CGGTGTAGTTAGCACA-TCTCACGGGAGG-3') (Leiter et al., 1987). The NPY probe was complementary to nucleotides 3146–3194 (5'-ATGAGATGTGGGGGGAAACTAGGAAAAGTCAGGAG-AGCAAGTTTCATT-3') (Larhammar et al., 1987). Two PP probes were made: one to region 2562–2610 of the rat PP gene (Yonekura et al., 1988), with the sequence 5'-CCCTGA GACCTTGT-TTCCCTTCCCCTCCTTTGGATCCCCTTTCCAGAC-3', and one to positions 676–723 the translated region of the mRNA, with the sequence 5'-CTGTTGATGCGTAGCATAGTCCCCCGGGTACATTG GCTCCAGTGGGGC-3'.

The probes were end labeled with ³⁵S-adenosine triphosphate [³⁵S-ATP; New England Nuclear (NEN); approximately 20–40 ³⁵S-ATP moles per mole of oligonucleotide DNA] by combining 80 ng of the probe with 24 U of terminal deoxynucleotidyltransferase (Amersham), ³⁵S-ATP, 2.5 μl cobalt reaction buffer (NEN), and 6 μl of water. The reaction mixture was incubated at 37°C for 2 hr, stopped with 0.1 M Tris buffer (pH 8.0), and purified on a Nensorb 20 column (NEN) as directed by the manufacturer.

Fourteen micron serial cryostat sections of various tissues were melted onto Probe-on slides (Fisher). Alternate sections were processed for either PYY, NPY, or PP *in situ* hybridization (Schalling et al., 1986; Dagerlind et al., in press). Unfixed sections were incubated overnight at 42°C in a solution containing (per ml) 10 million cpm of the oligonucleotide probe, 31 mg of dithiothreitol, and 400 μg of heat-sheared salmon sperm DNA in a hybridization cocktail containing (per 200 ml) 40 ml of formamide, 40 ml of 20× SSC (1 × SSC is 3 M NaCl and 150 mM sodium citrate), 2 gm of sarcosyl, 20 gm of dextran sulfate, 20 ml of sodium phosphate buffer (0.1 M, pH 7.0), and 2 ml of Denhardt's solution (0.02 M Ficoll, 0.02 M bovine serum albumin, and 0.02 M polyvinylpyrrolidone). Following the incubation, sections were rinsed

for 4× 15 min in 1 × SSC buffer at 55°C and then allowed to cool for 1 hr, dehydrated in 60% then 95% ethanol, and air dried. Sections were dipped in Kodak NTB-2 photographic emulsion and allowed to expose in the dark for between 1 and 4 weeks at −20°C. Slides were developed in Kodak D-19 developer (2 min), rinsed in water, and fixed in Kodak fixer for 10 min. Sections were then rinsed in water for 20 min, dipped in PBS, and coverslipped in a glycerol/PBS solution. Some sections were counterstained with cresyl violet. Sections were observed and photographed on a Nikon Microphoto-FX microscope using dark- and bright-field illumination. Photomicrographs were made on Kodak Panatomic X film. Image analysis of autoradiographic grain densities was performed on an Apple Macintosh IIx equipped with a Dage 72 CCD camera and an Optilab framegrabber card. Software (IMAGE 3.05) was provided by Dr. Wayne Rasband of NIH.

Northern blot methodology. Male rats (*n* = 26) were rapidly decapitated, and the brain and distal gut were removed. The cerebral cortex (frontal lobes), the cerebellum, and the medial medulla oblongata were dissected out and rapidly frozen in liquid nitrogen. Total RNA was extracted from each tissue using the RNAgents (Promega). PolyA⁺ mRNA was selected from the total RNA using a column containing oligodT attached to an insoluble matrix (Pharmacia, Stockholm, Sweden). Five micrograms of polyA⁺ RNA were added to each lane of the gel along with appropriate RNA standards. The RNA was then blotted onto nitrocellulose filters, which were hybridized with either an NPY or a PYY oligonucleotide probe. Probes were end labeled as above except with ³²P-ATP. Filters were prehybridized overnight in a solution containing the following: 5× SSC, 5× Denhardt's solution, 250 μg/ml salmon sperm DNA, 50 mM sodium phosphate buffer (pH 6.5), and 0.5% sodium dodecyl sulfate. Following the prehybridization, the probes (2 × 10⁶ cpm/ml) were added and the filters hybridized overnight. Both the prehybridization and the hybridization were done at 42°C. Following hybridization, the filters were washed 2 × 5 min in 2× SSC at room temperature, and then 2 × 30 min in 0.2× SSC plus 0.2% SDS at 65°C. Filters were then apposed to autoradiographic film (Amersham β Max). The following combinations of blot experiments were done (each on all four types of tissue); polyA⁺ RNA with PYY (*n* = 2) and NPY (*n* = 1) oligonucleotide probes, and total RNA with PYY (*n* = 1) and NPY (*n* = 1) cDNA probes (Fig. 1).

Results

In situ *hybridization using the PYY probe*

Cryostat sections at virtually every level of the brain and spinal cord were examined with *in situ* hybridization. Specific binding of the PYY probe was found only over neurons in the medulla. Sections of the colon processed on the same slides as the brain sections always showed hybridization and were used as a positive control for the hybridization of the PYY probe (see Fig. 3). When emulsion-dipped sections were allowed to expose for a short time (i.e., 1 week), specific cellular hybridization was seen only over one collection of neurons at the pontomedullary junction in the brainstem (Figs. 2; 3*A,B*; 4). These neurons were found dispersed about the midline directly dorsal to the raphe magnus within and dorsal to the gigantocellular region of the reticular formation. These neurons had a very limited rostrocaudal distribution, being present over approximately 1 mm along the longitudinal axis approximately from interaural level −2.5 to −1.5 (Paxinos and Watson, 1986). The neurons were always located above the pyramidal tracts and did not extend laterally into the paragigantocellularis pars dorsalis region, and they were generally restricted to the ventral two-thirds of the medulla. In Nissl-counterstained emulsion-dipped sections an accumulation of silver grains was seen over medium-to-large (20–30 μm in long axis) neurons (Fig. 3*B*). Although double staining was not done, the distribution of these neurons did not appear to correspond with the location of the 5-HT neurons that are found in this region. When dipped sections were allowed to expose for a longer period of time, silver grains could be seen over a small collection of neurons clustered near the nucleus
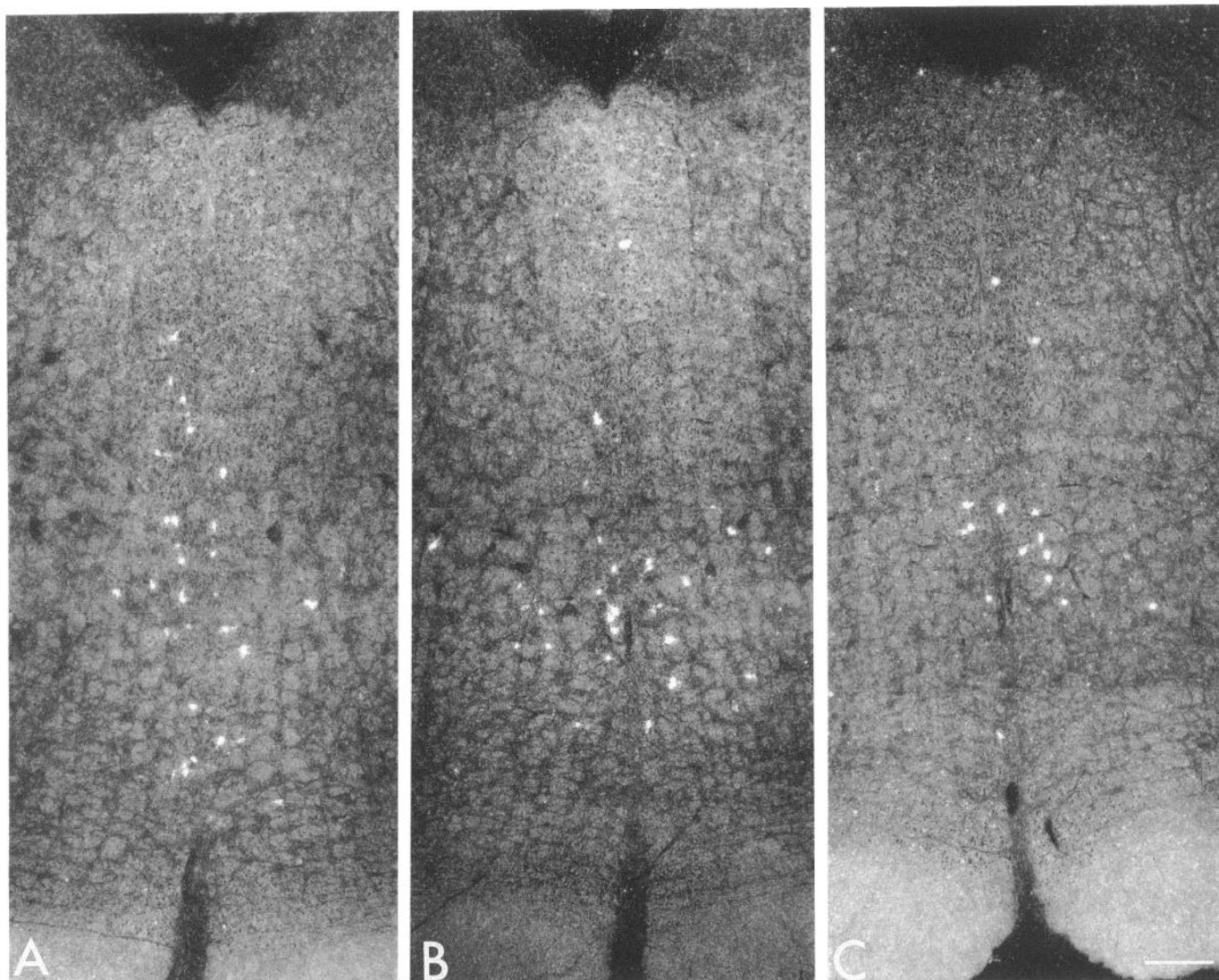
Figure 2. PYY *in situ* hybridization. *A–C,* Dark-field photomontages of silver grain collections over neurons on the midline in the rostral medulla on tissue sections hybridized with the PYY oligonucleotide probe. Note that very few cells in the field exhibit specific hybridization. *A* is caudal and *C* is rostral. Scale bar, 100 μm.

ambiguus in the ventrolateral medullary area (near the A1 noradrenergic cell group; Figs. 3*C,* 4). These neurons were present at a more caudal level than the midline PYY neurons and consistently showed much less intense hybridization. The labeled neurons were medium to large in size (10–20 μm in diameter). A schematic diagram depicting these two labeled cell groups is shown in Figure 4.

Sections of the intestines (small and large) incubated with the PYY probe revealed numerous intensely labeled cells scattered in the lumen (mucosal layer; Figs. 3*D,E;* 5*C*). No cells were seen in the submucosal or surrounding muscular layers of the intestines. Greater numbers of cells were seen in the distal colon (Fig. 3*D*) than in the rostral small intestines (Fig. 5*C*), in which fewer luminal cells exhibited hybridization. In addition, hybridization was seen over cells in the pancreas (data not shown). Although it has been previously reported (Gaumann et al., 1989) that PYY-immunoreactive material is present in the adrenal medulla, no specific PYY hybridization was detected anywhere in the adrenal gland (Fig. 5*E*). Other tissues including lung,

trachea, kidney, and liver were examined, but no specific hybridization was found in any of these tissues even following extended exposure times.

Cryostat sections of sympathetic (superior cervical) (Fig. 5*A*), parasympathetic (nodose), and primary sensory (lumbar dorsal root) ganglia were also incubated with the PYY probe. While a previous report indicated that neurons in sympathetic ganglia exhibited PYY immunoreactivity (Häppölä et al., 1990), no specific hybridization (Fig. 5*A*) could be detected in any of the above ganglia following extended exposure times (4 weeks). Sections of dorsal root ganglia that had the distal afferent processes cut either 7 or 28 d prior to removal (see below) were also examined for PYY expression. No specific PYY expression was detected in any of these tissues.

The grain density over labeled neurons in sections of the medulla and colon was compared using an image analysis system. After 21 d exposure of emulsion-dipped sections, midline medullary PYY neurons had 40% [102.1 ± 28.1 mean pixel value (out of 256 levels) over cell body of 50 measures ± SD]
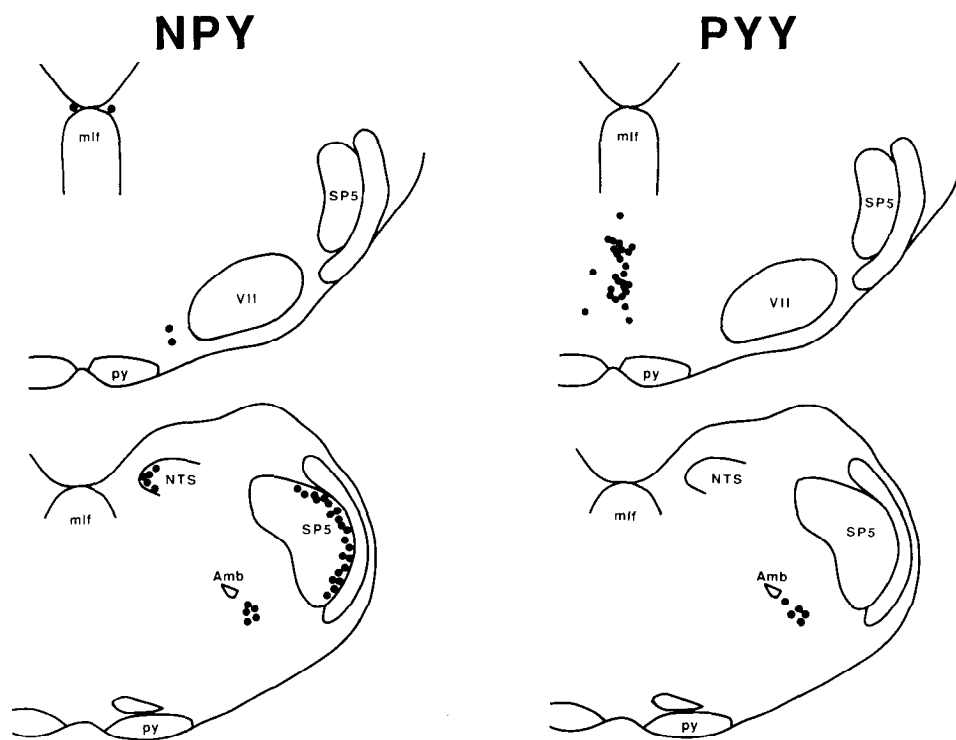
E   M

SM

NPY

PYY
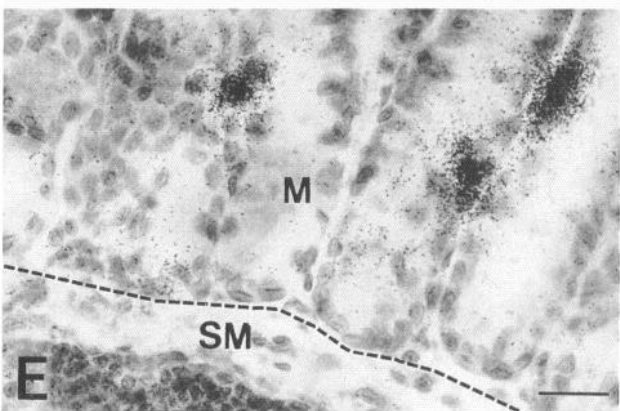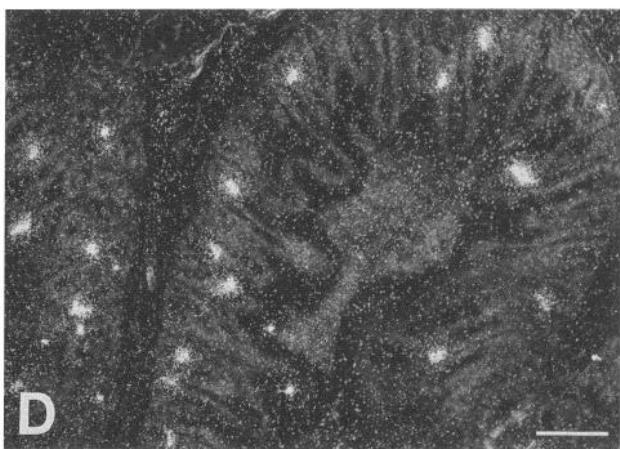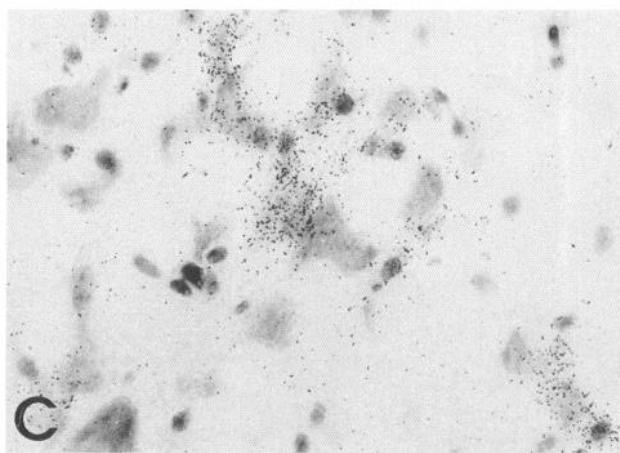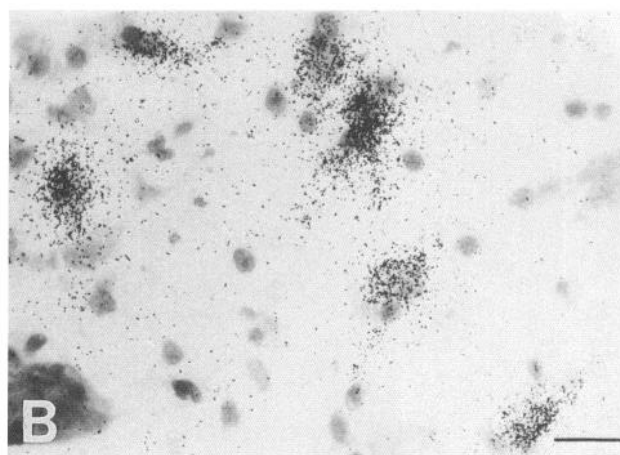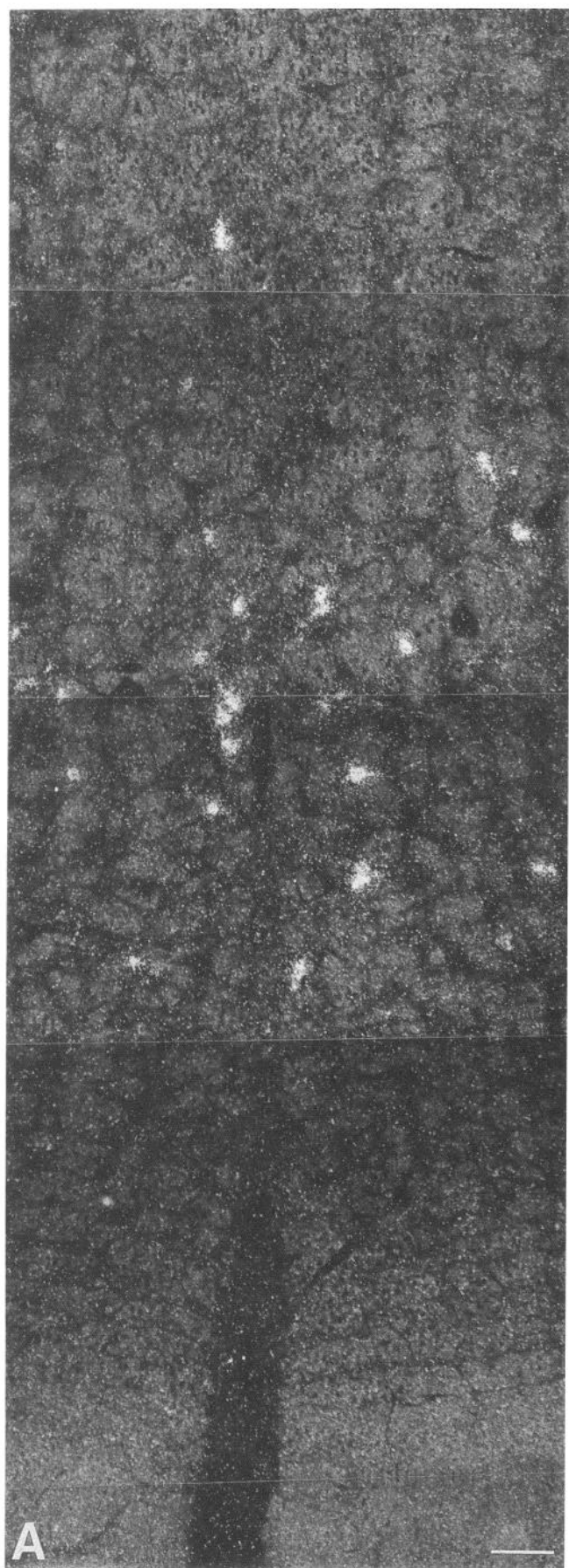
Figure 4. A schematic diagram comparing the distribution of PYY- and NPY-positive neurons in the medulla. The *left* diagrams show the location of NPY-positive neurons in rostral (*upper*) and caudal (*lower*) medulla, while the right depicts the location of PYY-positive neurons at these two levels. In the rostral medulla, NPY-positive neurons are found only occasionally in the dorsal midline region (C3 adrenaline neurons) and medial to the facial nucleus (*VII*; C1 adrenergic neurons). Caudally, NPY-positive neurons are found in the superficial layer of the spinal trigeminal nucleus (*SP5*), in the medial nucleus of the solitary tract (*NTS*), and in A1 noradrenergic cells ventral to the ambiguus nucleus (*Amb*). On the other hand, PYY-positive neurons are only found in rostral medulla on the midline and caudally in the area of noradrenergic A1 neurons. It is not known if the caudally located PYY-positive neurons are A1 adrenergic neurons; if so, they represent only a subpopulation since their numbers are much fewer than those cells found to be noradrenergic. *mlf*, medial longitudinal fasciculus; *py*, pyramidal tract.

of their somatic area covered by grains, while the endocrine cells of the colon had 70% (195.8 ± 48.8) of their cell area covered by grains. Background staining was less than 1%.

In situ *hybridization using the NPY probe*

In all of the above PYY experiments, alternate sections of each tissue were processed using the NPY probe. The distribution of labeled neurons in the CNS correlated to previous *in situ* hybridization studies showing great numbers of neurons in numerous areas of the CNS including the spinal cord, medulla (Fig. 6), hypothalamus, thalamus, amygdala, hippocampus (Fig. 7), and the cerebral cortex. However, no NPY hybridization could be detected in the midline region of the medulla that contained PYY-positive neurons in alternate sections, although NPY-positive neurons were seen in other regions of the medulla including all of the caudal catecholamine cell groups including the A1, A2, A3, C1, C2, and C3 cell groups (Dahlström and Fuxe, 1964; Hökfelt et al., 1974) (Fig. 6), the spinal trigeminal nucleus, and the inferior olivary complex.

Examination of the other peripheral organs (listed above) revealed specific hybridization with the NPY probe only in the intestine (Lundberg et al., 1984) and the adrenal gland (Fig. 5F). There was no apparent hybridization in cells of the mucosal layers of the intestine, but labeled cells (presumably neurons) were always seen in the submucosal layer, mainly in the rostral small intestine (Fig. 7D). Large cells within the adrenal medulla also stained positive with the NPY probe. If sections of the

adrenal medulla were allowed to expose for a long period of time (4 weeks), slight labeling was detected over larger numbers of cells in the adrenal medulla.

Examination of ganglia revealed very strong NPY hybridization in the sympathetic ganglia (superior cervical) (Fig. 5B), but no specific hybridization was seen in either dorsal root (C7) or nodose ganglia. However, numerous strongly labeled cells were seen in dorsal root ganglia whose afferent axons had been previously transected 7 or 28 d prior to removal.

In situ *hybridization using the PP probe*

No specific hybridization was detected with either of the PP probes in any part of the CNS or in any peripheral ganglia (normal or cut) even following extended exposure times. Strong hybridization was observed in control sections of pancreas processed on the same slide.

Northern blot analysis

PolyA⁺ RNA extracted from the medulla, frontal cortex, cerebellum, and distal colon was hybridized with the PYY and NPY oligonucleotide probes (Fig. 8). PYY probe revealed a single strong band of the appropriate size (Leiter et al., 1987) in the lane containing colon mRNA, but no specific hybridization could be detected in any of the lanes containing brain RNA. Hybridization with the NPY-specific probe revealed a heavy band in the lanes containing medulla and cortex RNA and no corresponding signal in the lanes containing cerebellar or colon RNA.

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Figure 3. PYY *in situ* hybridization. *A,* A dark-field photomontage of the rostral medial medulla showing the intense hybridization seen in neurons on the midline of the medulla. These are the same neurons shown in Figure 2. *B,* A high-power bright-field photomicrograph of a Nissl-counterstained section of the rostral medulla showing silver grain accumulation over cell bodies. *C,* A high-power photomicrograph of neurons in the caudal lateral medulla (A1 region) that exhibited weak hybridization with the PYY probe. *D,* A dark-field photomicrograph of the colon demonstrating intense hybridization over endocrine cell. *E,* A high-power photomicrograph of a Nissl-counterstained section of the colon showing hybridization over luminal (endocrine) cells. *SM,* Submucosal layer; *M,* mucosal layer. Scale bars: *A* and *D,* 100 μm; *B, C,* and *E,* 50 μm.

PYY

NPY
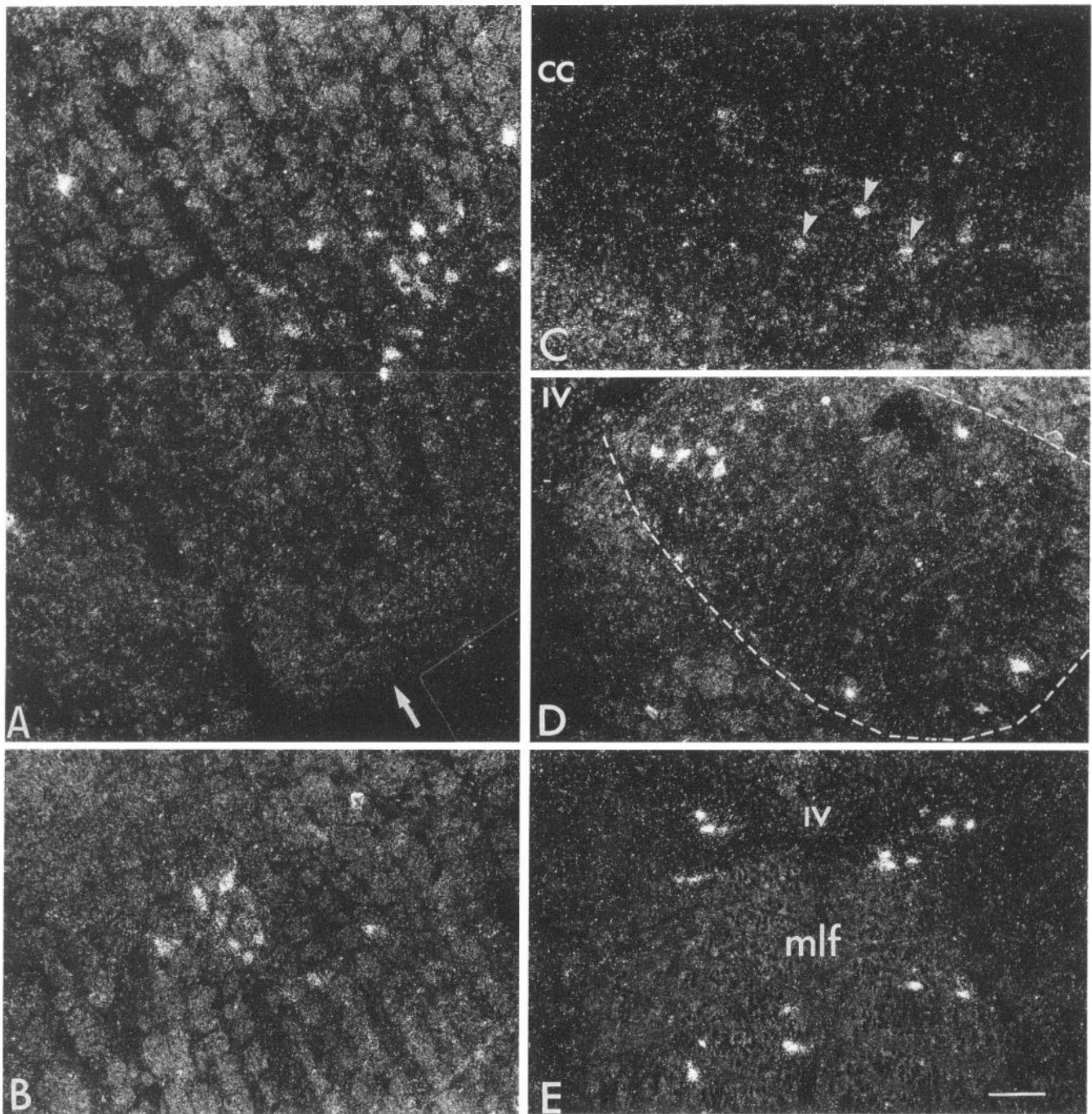
A

B

SM   M

C

SM   M

D

E

F
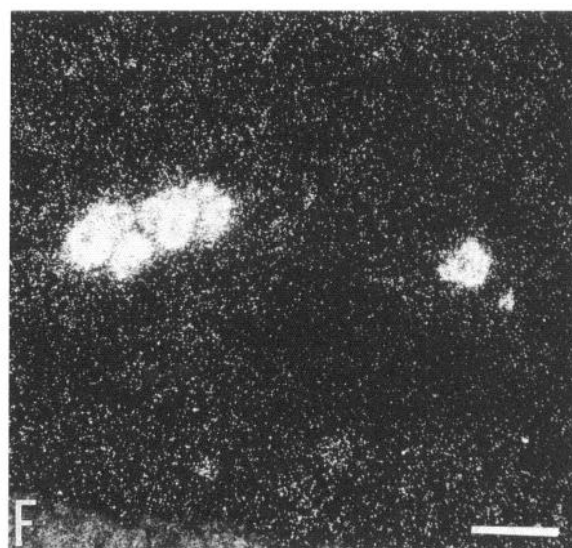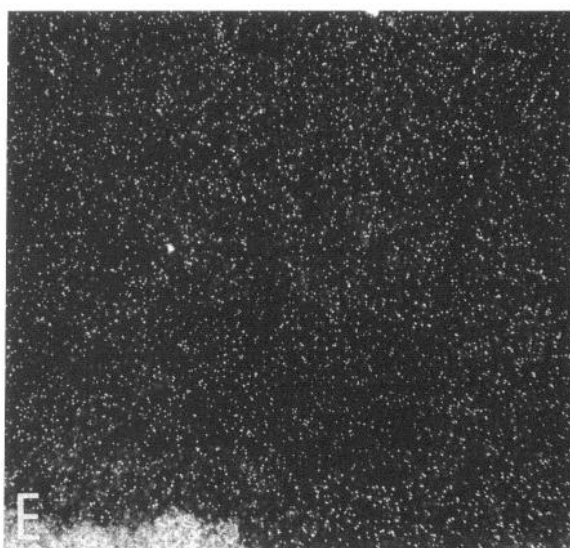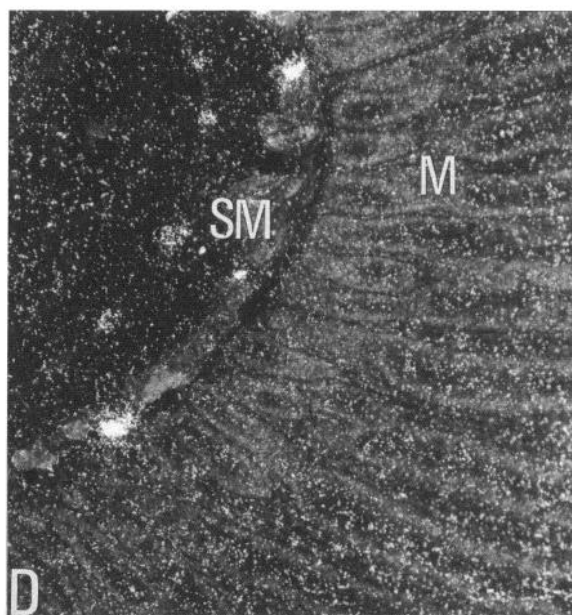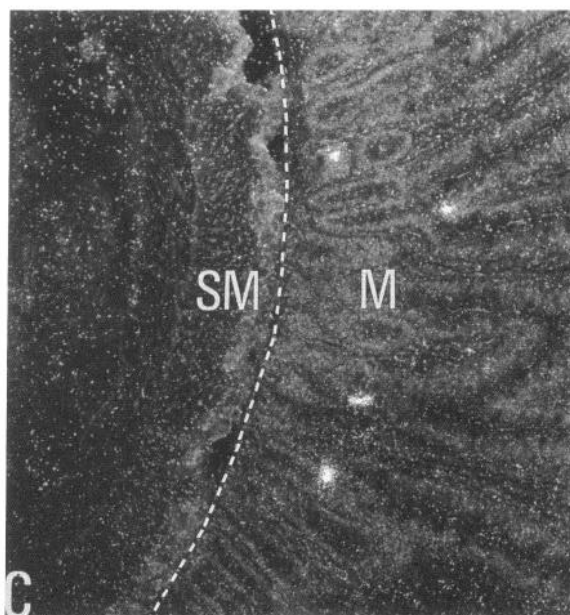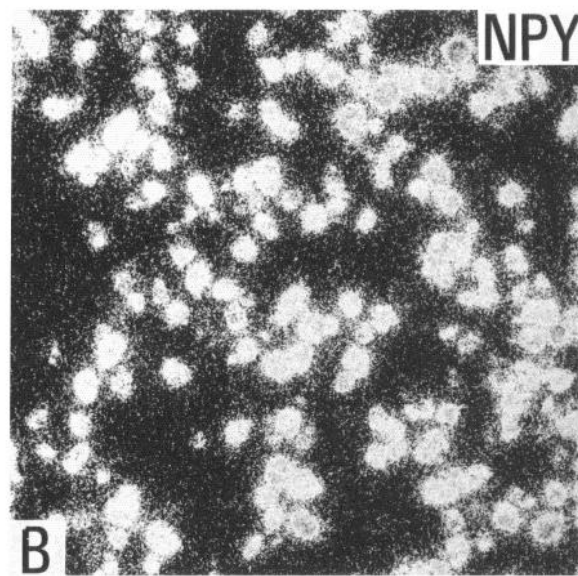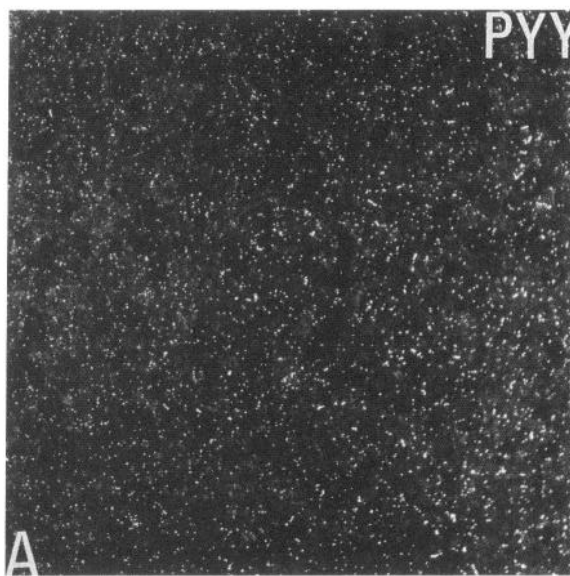
Figure 6. NPY hybridization in the medulla. *A,* A dark-field photomontage of NPY-positive neurons seen in the ventrolateral rostral medulla (C1 region; *arrow* indicates the ventral surface of the medulla). NPY-positive neurons were also seen in the caudal ventrolateral medulla (in A1 noradrenergic neurons; *B*), the commissural nucleus of the solitary tract (C2/A2 region, *C*), the rostral nucleus of the solitary tract (enclosed in *dashed line, D*), and in the dorsomedial rostral medulla (the C3 region, *E*). *CC,* central canal; *IV,* fourth ventricle; *mlf,* medial longitudinal fasciculus. (*Arrowheads* indicate labeled cells.) Scale bar, 100 μm.

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Figure 5. Comparison of PYY (*A, C, E*) and NPY (*B, D, F*) hybridization in the superior cervical (sympathetic) ganglia (*A* and *B*), the small intestine (*C* and *D*), and the adrenal medulla (*E* and *F*). The PYY probe failed to show any specific hybridization in sympathetic ganglia, while the NPY probe revealed numerous heavily labeled neurons. In the small intestine, the PYY probe labeled only a few cells in the mucosal layer (*M*), while the NPY probe labeled presumable enteric neurons in the submucosal (*SM*) and muscular (not shown) layers. No specific labeling of the adrenal medulla was seen with the PYY probe (*E*), while the NPY probe labeled scattered large cells in the medulla (*F*). Scale bar, 100 μm.
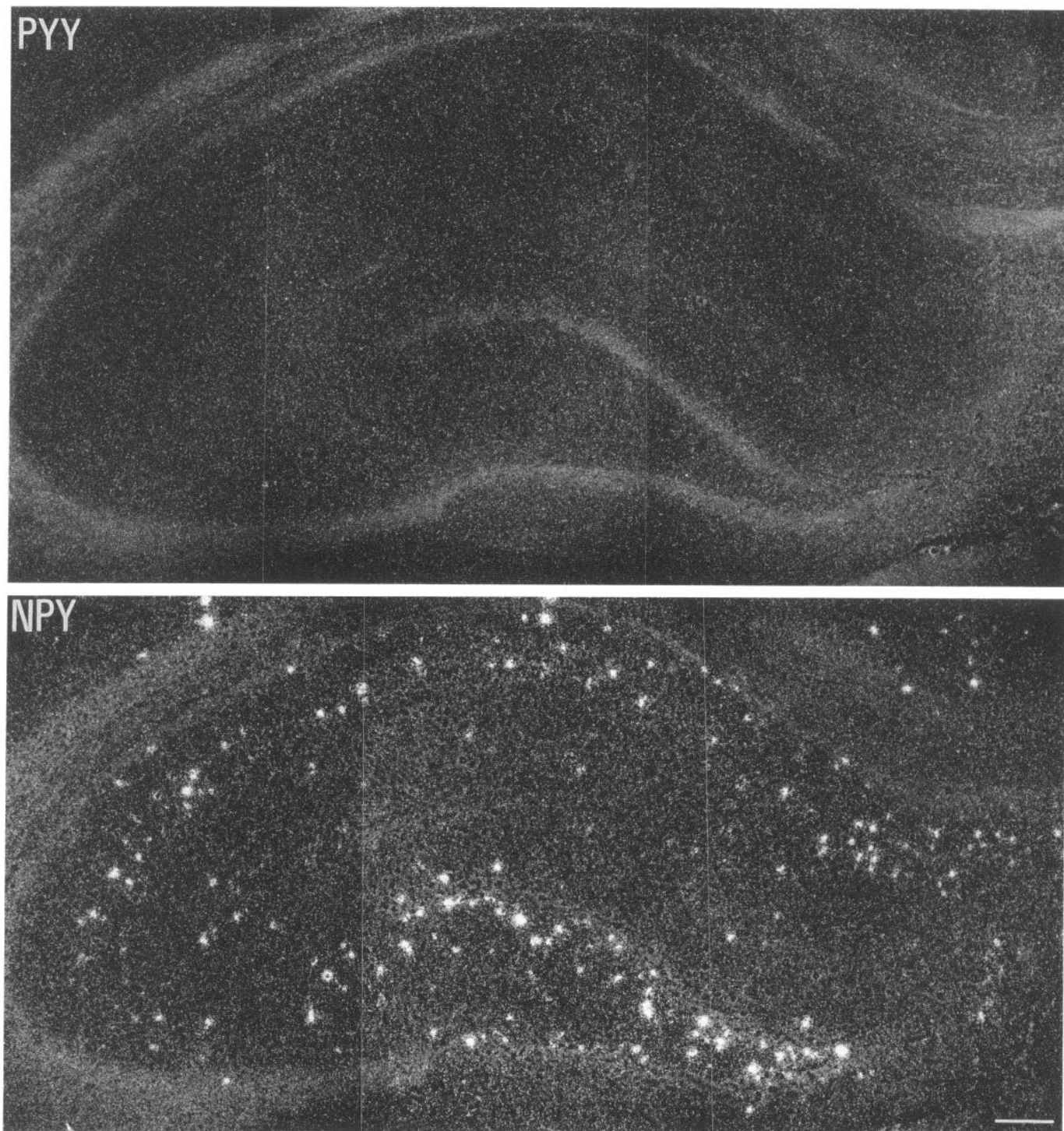
Figure 7. Dark-field photomontages comparing hybridization of PYY and NPY in adjacent sections of the dorsal hippocampus. No hybridization is apparent with the PYY probe, but intense labeling can be seen with the NPY probe, particularly in the dentate gyrus. Scale bar, 100 μm.

The PYY and NPY mRNA bands were of similar size (figures are aligned at the origins), in agreement with previous work (Larhammar et al., 1987; Leiter et al., 1987). In an additional set of experiments (not illustrated), blots of total RNA from the same tissues as above were probed with cDNA probes to PYY and NPY (see Fig. 1). These blots were identical to the ones presented here although a faint band was seen in the cerebellar lane using the NPY probe. This is likely due to a cerebellar dissection ($n = 20$) that contained a portion of the medulla.

Discussion

We show here by *in situ* hybridization that under normal conditions (without colchicine) some neurons in the rat CNS express PYY mRNA. *In situ* hybridization experiments with the NPY probe revealed large numbers of NPY-containing neurons in the CNS, the sympathetic ganglia as well as in neurons of the upper digestive tract. Northern blot experiments also support the wide spread distribution of NPY in the rat CNS (see also
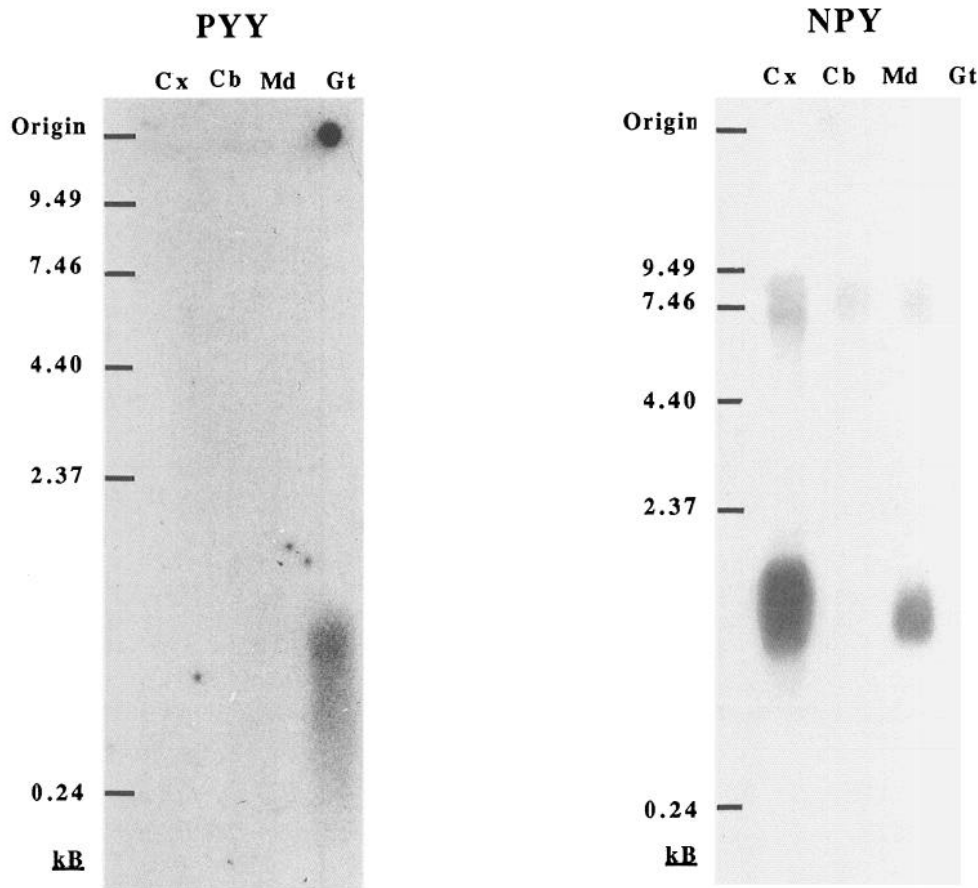
Figure 8. Film autoradiograms of Northern-blotted polyA⁺ RNA from the cerebral cortex (*Cx*), cerebellum (*Cb*), medulla (*Md*), and the distal large intestine (*Gt*) hybridized with the NPY and PYY oligonucleotide probes. The *numbers to the left of each blot* correspond to the location of size marker RNAs (in kilobases). Hybridization of filters with the PYY probe produced a single band of the appropriate size in the lane containing gut RNA, while no hybridization could be detected in RNA from other tissues. In filters hybridized with the NPY oligonucleotide probe, a strong band was present at the expected size for NPY mRNA in the lanes containing cerebral cortex and medulla RNA, while no corresponding band was found in the lanes containing cerebellar or gut RNA. The weak large-sized bands of atypical shape are probably due to contamination of the polyA⁺ RNA.

Larhammar et al., 1987). These results demonstrate that at least two members of the NPY family are synthesized by neurons in the rat brain. In contrast, no PP mRNA could be detected.

While no NPY, PYY, or PP was found in dorsal root ganglia of normal rats, a recent report has shown that peripheral nerve cuts induce NPY immunoreactivity in primary sensory neurons of the dorsal root ganglia (Wakisaka et al., 1991), and this is confirmed here showing expression of NPY mRNA. It appears, however, that peripheral nerve cuts do not enhance (or induce) significant expression of PYY or PP mRNA in dorsal root ganglia.

PP-related peptides cannot be reliably distinguished by immunological methods

When comparing the present with previous studies based on immunological methods, it seems likely that in many of the latter studies cross-reactivity may be responsible for false positive results. Two previous immunocytochemical studies agree with the present results, since in both cases a collection of PYY-immunoreactive neurons were detected in the midline of the rostral medulla (Broomé et al., 1985; Ekman et al., 1986). In contrast, reports indicating that this same area contains NPY- (Chronwall et al., 1985; de Quidt et al., 1990) and PP- (Olschowka et al., 1981) immunoreactive cells may be inaccurate.

The observations that the sympathetic ganglia contained PYY- (Häppölä et al., 1990) or PP- (Olschowka and Jacobowitz, 1983) like immunoreactivity also appear to be a result of cross-reactivity. It seems likely that the antiserum used in these studies cross-reacted with NPY, which is present in large quantities in

sympathetic ganglia (Lundberg et al., 1984). This may also be true with regard to the adrenal medulla (Gaumann et al., 1989).

Taken together, the above discrepancies between the immunocytochemical studies and the *in situ* hybridization experiments demonstrate the usefulness of comparing the *in situ* hybridization technique with immunocytochemistry in discriminating the cellular expression of closely related products. It should, however, be noted that our conclusions from *in situ* hybridization partially rest on negative results, a situation that should always be interpreted with caution. Finally, the present results were obtained without the use of colchicine, as is required in immunocytochemical studies examining the distribution of neuronal somata containing NPY family of peptides. Since colchicine, like axotomy (see above), may influence peptide synthesis (Cortés et al., 1990), our results show that PYY is synthesized in the normal rat brain.

The PYY system is one of the smallest peptide systems in the CNS

Although we examined numerous sections from all levels of the CNS at long exposure times, we failed to detect any PYY mRNA expression outside the two cell groups in the medulla oblongata, suggesting that this collection of PYY neurons is one of the most discrete neurochemically identified groups of neurons in the rat brain. This conclusion is further supported by the negative Northern blot data. The amount of PYY mRNA in the pooled medial medulla oblongata, as well as in the other brain regions, was below the detectable level of our Northern blot analyses contrasting with the very high levels of NPY mRNA. In addi-

Lamprey **Rat**

PYY **PYY**

Lamprey **Rat**
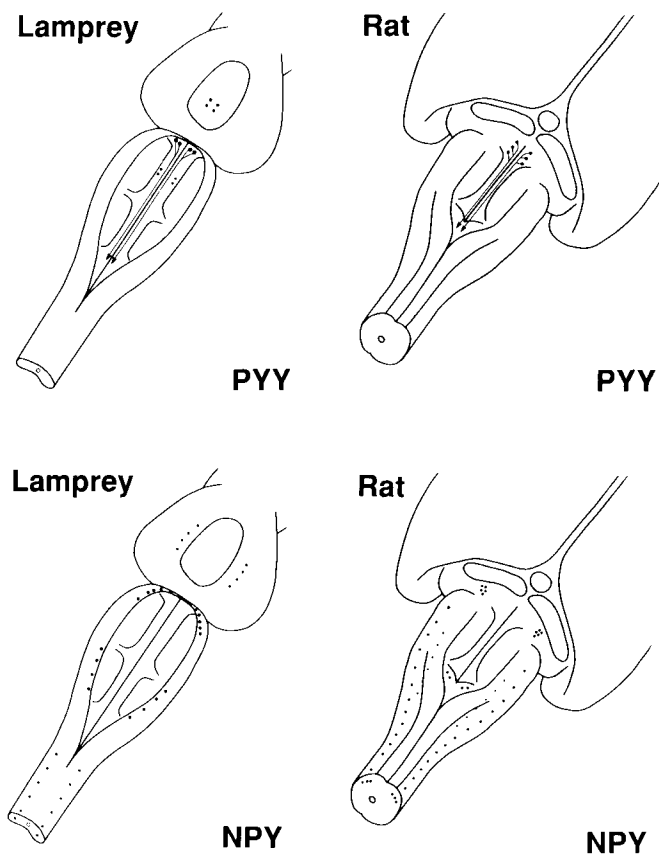
NPY **NPY**

Figure 9. Comparison of NPY-related peptides in lamprey and rat: a schematic diagram comparing the brainstem and spinal cord distribution of NPY and PYY in the rat and their presumed homologs in the lamprey. In the diagrams of rat brain, the cerebellum has been removed. In the rat, there is a collection of large bulbospinal PYY neurons on the midline in the rostral brainstem. A similar collection of large bulbospinal neurons that are PYY immunoreactive are also found around the midline in the rostral brainstem of the lamprey (Brodin et al., 1989). The NPY system in the rat and lamprey have a wider distribution, present mainly lateral to the midline, throughout the brainstem and spinal cord. NPY cells found in the dorsal medulla (C3 adrenergic cell group) have been omitted from the diagram.

tion, previous investigators have failed to detect significant levels of PYY in brain extracts (Miyachi et al., 1986). It is interesting to note that the few central PYY neurons in the rat medulla appear to synthesize a relatively large quantity of PYY mRNA (greater amounts of mRNA per cell than NPY neurons). This conclusion is based on two observations: when alternate sections of the brainstem were processed for NPY and PYY and exposed together with probes of the same length, GC ratio, and radioactive end labeling, only PYY hybridization was visible. The NPY signal generally required a much longer exposure time to be visible, indicating that the NPY neurons probably synthesize fewer mRNA molecules per cell. In addition, when the total grain density over neurons in the medulla was compared to that seen over the endocrine cells of the colon, which are known to produce large quantities of peptide, it was found that the neurons had comparably high, although fewer, numbers of grains.

The PYY and NPY systems may have been conserved throughout vertebrate evolution

Retrograde tracing experiments performed in our laboratory have shown that the medial group of PYY neurons has a spinal

projection (V. Holets and T. Hökfelt, unpublished observation). The fact that the rat brain contains a reticulospinal PYY system may appear to be of limited interest in view of the vast number of reticulospinal peptidergic systems so far identified. However, our comparative studies of a cyclostome (*Lampetra fluviatilis*), which diverged from the main vertebrate line about 450 million years ago, suggest that the reticulospinal PYY system may have persisted throughout most of the vertebrate phylogeny. We have recently isolated clones from a brain/spinal cord cDNA library of *L. fluviatilis*, which encodes two different PP-related peptides (Söderberg et al., 1991). Their deduced amino acid sequences indicate that they may correspond to NPY and PYY (cf. Larhammar et al., in press). This supports our previous immunohistochemical evidence for two distinct PP-containing neuron systems in the lamprey brainstem (Brodin et al., 1989). One rather widespread system was found to react with NPY and PP antisera, and a more restricted set of neurons including reticulospinal neurons reacted with PYY and PP antisera. Preliminary *in situ* hybridization experiments in lamprey have shown that these two patterns of immunoreactivity correspond to the cloned putative lamprey forms of NPY and PYY, respectively (V. A. Pieribone and L. Brodin, unpublished observation). It is striking to note that the location and projection of the PYY cells in the rat brainstem are virtually identical to that of the presumed PYY-containing cells in the lamprey (Fig. 9). In both animals, NPY-immunoreactive neurons and fibers are abundant in the brainstem and spinal cord (dorsal horn; cf. also amphibians, Danger et al., 1985), whereas these regions contain only a sparse PYY innervation derived from a small cell cluster in the rostromedial medulla. Thus, at least two PP-related peptides are expressed in the brains of both a cyclostome and a mammal, and it seems likely that their functions have been partly retained. This is as yet the most striking resemblance between peptidergic neuron systems to be observed in a mammal and a cyclostome (for review, see Brodin and Grillner, 1990).

Are there PPY-selective receptors in the CNS?

Two types of NPY/PYY receptors have been identified in mammalian tissues using autoradiography as well as biochemical and physiological assays (Wahlestedt et al., 1985; Inui et al., 1988; Leslie et al., 1988; Walker et al., 1988; Schwartz et al., 1989; Michel et al., 1990). Studies of the structure–function relationship of NPY and PYY suggest that only the N-terminal and C-terminal regions (residues 1–6 + 18–36 and 25–36, respectively) are important for receptor binding, whereas the 7–17 fragment can be deleted without apparent effects on the binding properties (Krstenansky et al., 1989; Michel et al., 1990). This may explain why the known receptors cannot distinguish well between PYY and NPY, which have similar amino acid sequences in their N- and C-terminals, whereas they differ most markedly in their midportion (residues 13–18). However, it seems rather unlikely that two distinct peptide systems with related peptides would have evolved if there would be no difference between their target receptors. Therefore, we speculate that additional receptor subtypes selective for either NPY or PYY exist. This is supported by recent intracellular recording experiments in rat locus coeruleus cells, which are hyperpolarized by NPY application, but were unresponsive to PYY (V. A. Pieribone, unpublished observation). If PYY-selective receptors are present in the CNS, they would be expected to occur only in restricted areas of the lower brainstem (not including the locus coeruleus) and spinal cord (see above). Recent comparative

studies of different lower vertebrate NPYs (Larhammar et al., in press) have shown that the midportion 13–18 region of NPY is highly conserved between species, indicating biological significance. It therefore seems reasonable to postulate that the binding to putative NPY- and PYY-selective receptors would involve the midportion regions of these peptides.

It will be difficult to assess the exact role of the PYY system in the rat, but the presumed homology with the lamprey system provides a unique opportunity to utilize precise physiologic methods, such as paired intracellular recordings, to characterize in the lamprey the function of a conserved peptide system (cf. Brodin and Grillner, 1990).

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