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Expression of the human neuropeptide tyrosine Y1 receptor

(*in situ* hybridization/colon/kidney/placenta/cardiovascular system)

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ABSTRACT **Neuropeptide tyrosine (NPY) is the predominant peptide in the innervation of many human tissues and is considered to play a role in the regulation of blood flow, gastrointestinal secretion and motility, and renal function. Three NPY receptors have been identified (Y1, Y2, and Y3) and the cDNAs encoding the human Y1 and bovine Y3 receptors have recently been cloned. We have demonstrated the expression of the Y1 receptor subtype in several fetal and adult human tissues, including the colon, kidney, adrenal gland, heart, and placenta. A single transcript was identified (≈2.2 kb) and localized in tissue sections by *in situ* hybridization. In the colon the receptor is expressed in the mucosa and basal glands, as well as the myenteric and submucous plexuses. Y1 receptor mRNA was detected in renal collecting ducts, loop of Henle, and juxtaglomerular apparatus and in the syncytiotrophoblast layer of placental villi. Fetal aorta and adult intramyocardial, colonic, and renal blood vessels also exhibited receptor expression, localized to the intima as well as the media. The distribution of Y1 receptor expression correlates with that of NPY-immunoreactive nerves and the apparent actions of NPY in the intestine, kidney, and heart. Although the placenta is devoid of nerves, an NPY-like transcript was detected in the villous trophoblast layer. The results indicate a tissue-specific regulation of NPY Y1 receptor expression.**

Neuropeptide tyrosine (NPY) is an amidated 36-amino acid peptide found in both the central and peripheral nervous systems and is a member of a family of regulatory peptides which also includes peptide tyrosine tyrosine (PYY) (1). NPY is localized together with norepinephrine in sympathetic nerves and is widely distributed in the innervation of the human cardiovascular system, kidney, and gastrointestinal tract, as well as other organs (2, 3). In addition to its neural localization, NPY-like immunoreactivity has also been demonstrated in adrenal medullary cells (4) and the villous trophoblast layer in the placenta (5). PYY immunoreactivity exhibits a distinct distribution pattern and is localized to endocrine cells in the human colon (6). These peptides have both direct and indirect effects on blood flow, gastrointestinal secretion and motility (3), and renal function (7) which are mediated via specific receptors. Three receptor subtypes have been identified, designated Y1, Y2, and Y3 receptors according to their affinity for NPY, PYY, the analogue [Leu^{31},Pro^{34}]NPY, and C-terminal NPY fragments such as NPY-(13–36) (8). Although several studies have demonstrated NPY receptors in peripheral mammalian tissues, few have reported the localization of ligand binding sites in tissue sections (3, 5, 9–11). Furthermore, the relevance of these studies to humans is uncertain due to species variation in both receptor distribution and peptide metabolism and the apparent difficulty in demonstrating specific NPY binding sites in human tissues (12). Following the recent cloning of human and bovine NPY receptor cDNA sequences (13, 14), it is now possible to circumvent many of these problems by using specific molecular probes and *in situ* hybridization techniques to determine the localization of mRNA encoding NPY receptor subtypes. When expressed *in vitro*, the human receptor displays a rank order of agonist potency [NPY = PYY > [Leu^{31},Pro^{34}]NPY >> NPY-(13–36)] characteristic of the Y1 receptor subtype (13). We have used radiolabeled RNA probes derived from the human NPY Y1 receptor cDNA clone to investigate the localization of receptor expression in human tissues.

MATERIALS AND METHODS

Tissue Preparation. The tissues investigated included adult right atrial appendage, placenta, kidney, adrenal, and colon, and samples of fetal kidney, adrenal, and heart. Adult tissues were obtained from patients undergoing coronary artery bypass grafts, caesarean section, colectomy, and adrenalectomy. Renal tissues were obtained at autopsy, 3–4 hr after death, and fetal tissues (9–14 weeks of gestation) were collected after legal abortion by uterine evacuation. Tissues were obtained according to the ethical standards of the institutions in which the procedures were performed. Tissues for *in situ* hybridization were fixed in a solution of 4% (wt/vol) paraformaldehyde in 0.1 M phosphate buffer (pH 7.2) for 4–6 hr at 4°C and transferred to phosphate-buffered saline (PBS) containing 15% (wt/vol) sucrose; after washing, cryostat blocks were prepared. Sections (10 μm thick) were mounted on Vectabond (Vector Laboratories)-treated slides and dried overnight at 37°C before further processing. Tissues for Northern blot analysis were snap frozen and stored in liquid nitrogen until required for RNA extraction.

Hybridization Probes. A 276-bp *Pst* I–*Eco*RV cDNA fragment (coding region 655–931) of the human NPY (Y1) receptor, isolated from an adult human hippocampus cDNA library (13), and a 591-bp *Bam*HI–*Hin*dIII insert, derived from the human pNPY 3-75 cDNA (15), were subcloned in pBluescript SK vectors (Stratagene). RNA probes were generated in both orientations, using T7 and T3 promoters to produce complementary RNA (cRNA) and mRNA transcripts, after linearization of the NPY Y1 receptor vector with *Sma* I or *Hin*dIII and the NPY vector with *Hin*dIII or *Bam*HI. Labeled transcripts were synthesized by incubating 1 μg of linearized template in a 10-μl reaction mixture containing 2 μl of transcription buffer [containing 200 mM Tris·HCl (pH 7.5), 30 mM $MgCl_2$, 10 mM spermidine, 50 mM dithiothreitol (DTT),

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Abbreviations: NPY, neuropeptide tyrosine; PYY, peptide tyrosine tyrosine; DTT, dithiothreitol.

0.5 mg of bovine serum albumin per ml, 5000 units of human placental ribonuclease inhibitor per ml, and 2.5 mM each unlabeled ATP, GTP, and UTP], 1 μl of 400 mM DTT, 5 μl of 12.5 μM [α-[^{35}S]thio]CTP (Amersham plc; specific activity, 800 Ci/mmol; 1 Ci = 37 GBq), and 20 units of RNA polymerase, for 1 hr at 40°C. The cDNA template was removed by the addition of 1 μl of RNase-free DNase for 10 min at 40°C and the RNA probe was purified by successive extractions in phenol/chloroform and chloroform/isoamyl alcohol, followed by ethanol precipitation at −20°C. The pellet was dissolved in sterile distilled water to a final concentration of 5 ng/μl.

***In Situ* Hybridization.** Tissue sections were permeabilized first with 0.2% Triton X-100 in PBS for 15 min and then with a solution of proteinase K (1 μg/ml) in 0.1 M Tris, pH 8/50 mM EDTA for 5–15 min at 37°C. The reaction was stopped by washing sections in 0.1 M glycine in PBS followed by brief postfixation with 4% paraformaldehyde in PBS. Autoradiography background was minimized by immersion in a solution containing 0.25% acetic anhydride in 0.1 M triethanolamine (pH 8) for 10 min. Five nanograms of RNA probe (≈5 × 10^5 cpm per section; 0.5 ng/μl) was diluted in buffer containing 50% (vol/vol) deionized formamide, 5× standard saline citrate (SSC), 10% (wt/vol) dextran sulfate, 5× Denhardt's solution, 2% sodium dodecyl sulfate (SDS), 100 μg of denatured salmon sperm DNA per ml, and 100 mM DTT. Preparations were covered with dimethyldichlorosilane-coated coverslips to prevent evaporation and incubated at 42°C for 16–24 hr in a humid chamber. After hybridization the coverslips were removed and the sections were subjected to high-stringency washing by immersion four times (10 min each) in 2× SSC/0.1% SDS/100 mM DTT at room temperature, twice (15 min each) in 0.1× SSC/0.1% SDS/100 mM DTT, and twice (15 min each) in 0.05× SSC/0.1% SDS/100 mM DTT at 42°C. Unhybridized, single-stranded RNA probe was removed by treatment with a solution of RNase A (10 μg/ml) in 2× SSC for 15 min at 37°C. The sections were dehydrated through graded concentrations of ethanol containing 0.3 M ammonium acetate, air dried, dipped in Ilford K-5 emulsion, and stored at 4°C for 4 days. Autoradiographs were developed in Kodak D19 developer and lightly counterstained with Harris's hematoxylin.

Control Experiments. Tissue sections were hybridized with ^{35}S-labeled mRNA as well as cRNA probes. Some preparations were also treated with a solution of RNase A (100 mg/ml) at 37°C for 30 min prior to hybridization.

RNA Isolation and Northern Blot Analysis. Total RNA was extracted from samples of placenta and kidney (medulla) by acid guanidinium thiocyanate/phenol/chloroform extraction (16), and poly(A)$^+$ RNA was purified on an oligo(dT)-cellulose column. RNA species were separated by electrophoresis in a Mops/formaldehyde/1% (wt/vol) agarose gel, transferred to a Hybond-N membrane (Amersham) by capillary blotting overnight (17), and fixed by baking at 80°C for 2 hr. RNA size markers were run simultaneously and stained with ethidium bromide. The blots were probed as described (18) with the same NPY Y1 receptor and NPY cRNA probes used for *in situ* hybridization.

RESULTS

***In Situ* Hybridization.** NPY Y1 receptor mRNA was localized by *in situ* hybridization in both fetal and adult human

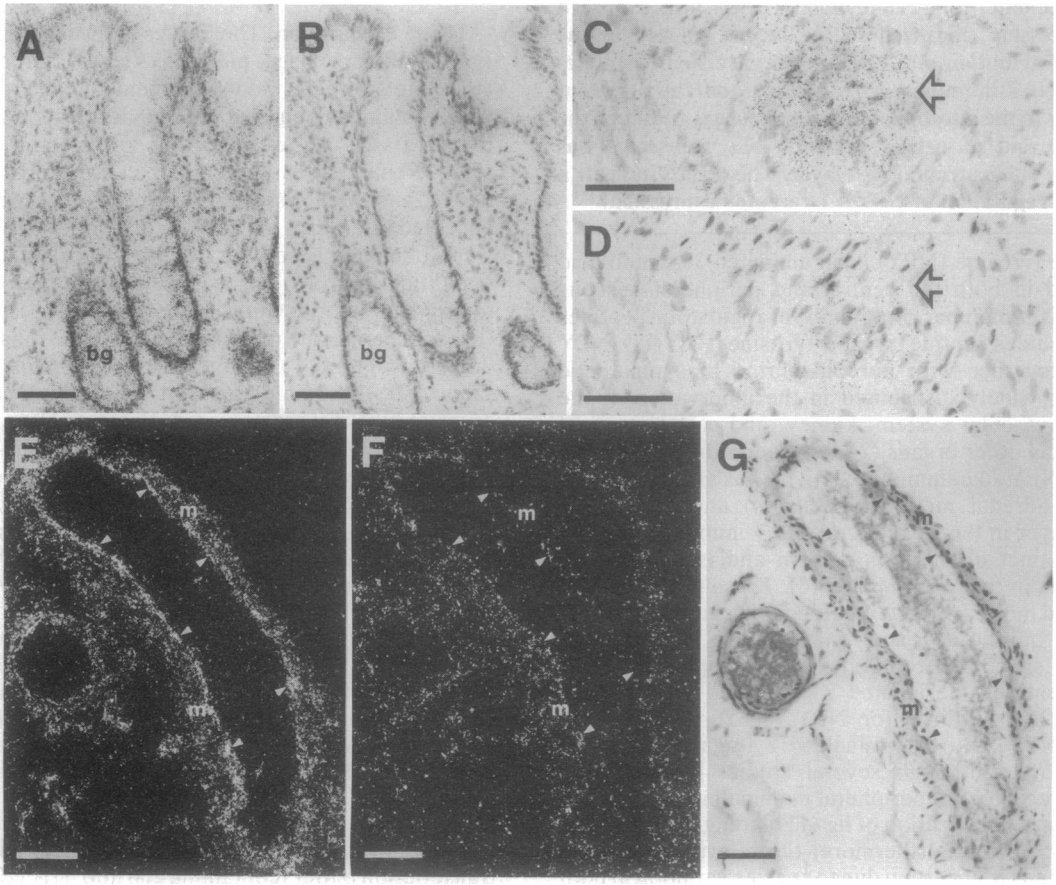

FIG. 1. Transmitted light (*A–D* and *G*) and darkfield (*E* and *F*) photomicrographs showing the localization of human NPY Y1 receptor mRNA in serial sections of human colon with an ^{35}S-labeled cRNA probe (*A*, *C*, and *E*). Only background signal was detected with a mRNA probe (*B*, *D*, and *F*). Receptor expression is localized to the basal glands (bg) and mucosa (*A* and *B*), myenteric ganglion (*C* and *D*; arrow), as well as to the intima (arrowheads) and media (m) in submucosal blood vessels. *G* shows an adjacent hematoxylin/eosin-stained section. (Bars = 50 μm.)

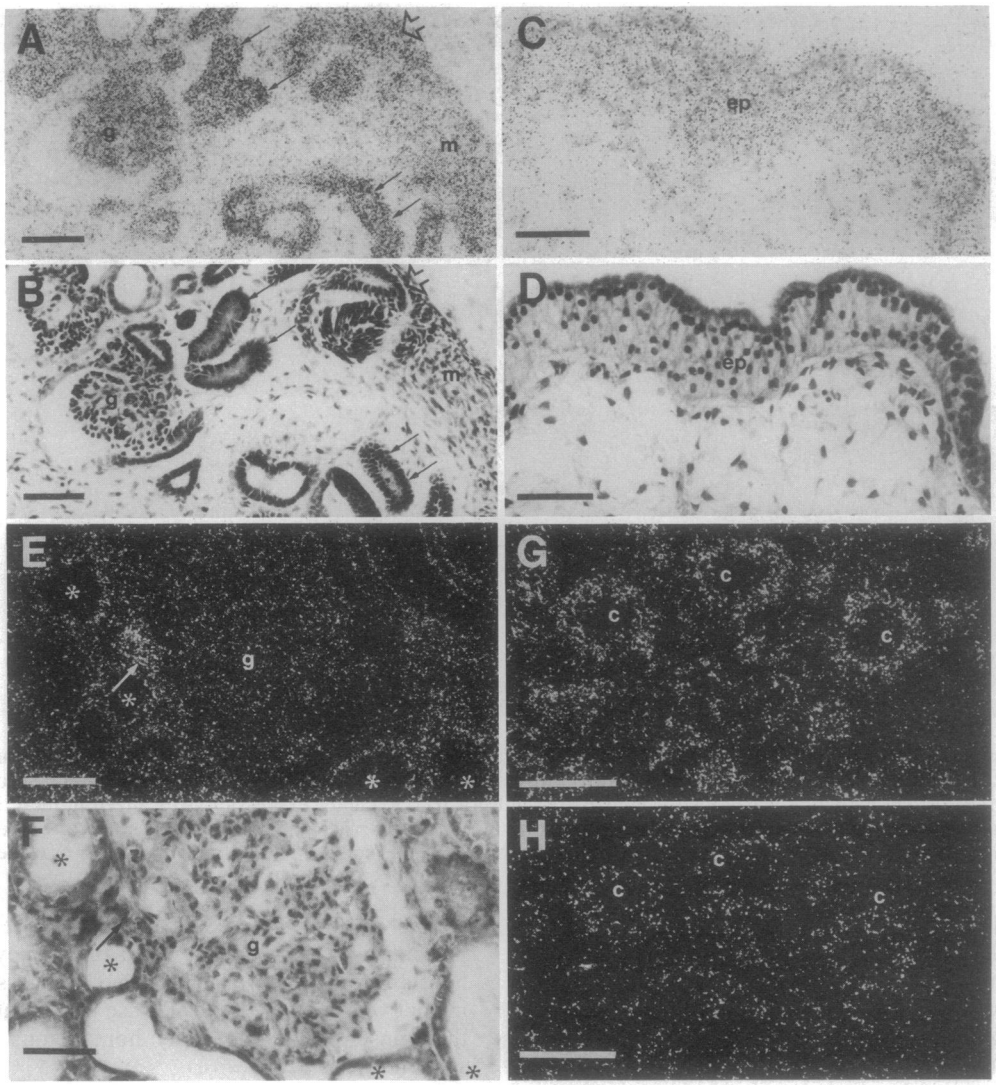

FIG. 2. Transmitted light (*A–D* and *F*) and darkfield (*E*, *G*, and *H*) photomicrographs showing the expression of NPY Y1 receptor mRNA in serial sections of fetal (*A–D*) and adult (*E–H*) human kidney hybridized with ^{35}S-labeled cRNA (*A*, *C*, *E*, and *G*) and mRNA (*H*) probes. Receptor expression is localized to metanephrogenic mesenchyme (m), terminal ampullae (open arrow), glomeruli (g), tubules (arrows) and the epithelial (ep) lining of the pelvic cavity in the fetal kidney (*A–D*). Silver grains are distributed over the juxtaglomerular apparatus (arrow), glomeruli (g), tubules (asterisks) and collecting ducts (c) in the adult kidney (*E–H*). *B*, *D*, and *F* show adjacent hematoxylin/eosin-stained sections. (Bars = 50 μm.)

organs and exhibited a tissue-specific pattern of expression. Hybridization between labeled cRNA probe and mRNA encoding the NPY Y1 receptor was demonstrated by the presence of discrete silver grains in the emulsion overlying distinct tissue structures. In contrast, no specific signal was found in the control preparations. Only background levels of silver grains were detected in sections hybridized with labeled mRNA probe, identical to the coding sequence of human NPY Y1 receptor mRNA, and specific autoradiographic labeling was abolished by RNase treatment of sections prior to *in situ* hybridization.

Relatively high receptor expression was apparent in the colon, kidney, and placenta. In the colon, silver grains were found distributed over the mucosa and basal glands (Fig. 1 *A* and *B*). Ganglia in the myenteric (Fig. 1 *C* and *D*) and submucous plexuses also exhibited relatively high levels of receptor expression. Moderate to low expression was observed in submucosal blood vessels, localized to the intima as well as the media (Fig. 1 *E* and *G*), and an equivocal signal was detected in the longitudinal and circular muscle layers. Significant NPY Y1 receptor expression was apparent in renal tissue. In the fetal kidney, a high density of silver grains occurred over the metanephrogenic mesenchyme, glomeruli, collecting ducts and tubules, and epithelium lining the pelvic cavity (Fig. 2 *A–D*). In adult renal tissues, hybridization was localized to collecting ducts and loop of Henle in the outer medulla and to the juxtaglomerular apparatus (Fig. 2 *E–H*). Low specific hybridization signal was found associated with interlobar and arcuate arteries, tubules, and glomeruli in the renal cortex. In the adrenal gland, expression was greater in the subcapsular zone than in other regions, which displayed a diffuse signal.

NPY Y1 mRNA appeared to be present throughout the fetal myocardium, with higher expression in the atria (Fig. 3 *A* and *B*) than in the ventricles. Myocardium and intramyocardial blood vessels in the adult right atrial appendage also displayed specific hybridization. Whilst the coronary vasculature showed moderate to low expression in the fetal heart, a high density of silver grains was observed overlying the intimal layer of the ascending fetal aorta and the endocardial lining of aortic valve cusps (Fig. 3 *C* and *D*). Receptor expression was relatively high in the placenta and selectively localized to the syncytiotrophoblast in placental villi (Fig. 3 *E* and *F*). Other regions of the placenta exhibited low or

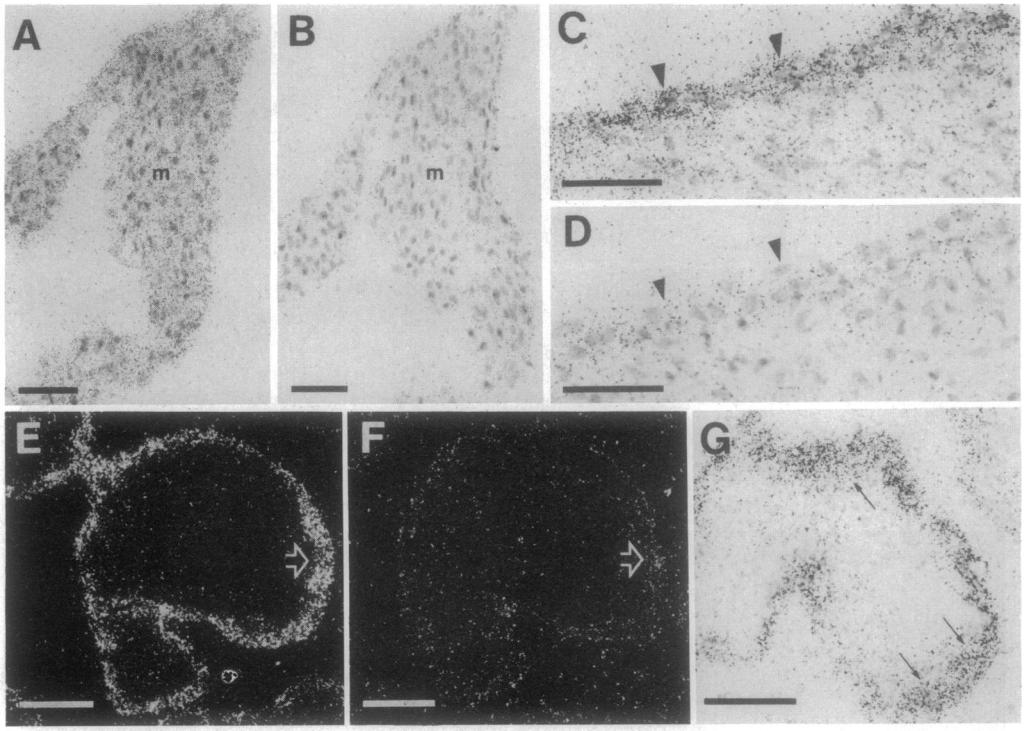

FIG. 3. Transmitted light (*A–D* and *G*) and darkfield (*E* and *F*) photomicrographs of serial sections of fetal right atrium (*A* and *B*), fetal ascending aorta (*C* and *D*), and placenta (*E–G*) hybridized with ^{35}S-labeled cRNA (*A*, *C*, and *E*) and mRNA (*B*, *D*, and *F*) probes for the human NPY Y1 receptor mRNA and with a cRNA probe for human NPY mRNA (*G*). Receptor expression is localized in fetal tissues to the atrial myocardium (*A* and *B*; m) and intimal layer (arrowheads) of the aorta (*C* and *D*). In placental villi, NPY Y1 receptor expression (*E* and *F*) is demonstrated in the syncytiotrophoblast (open arrow), and NPY-like mRNA expression (*G*) in the trophoblast layer of placental villi (arrows). (Bars = 50 μm.)

nonspecific labeling, similar results being obtained with both cRNA and mRNA probes.

The renal medulla and placenta were selected for further *in situ* hybridization studies and Northern blot analysis using RNA probes specific for human NPY mRNA. Hybridization of kidney sections with an ^{35}S-labeled cRNA probe produced equivocal results, whereas a high density of silver grains was localized to the villous trophoblast layer in placental sections (Fig. 3*G*). In contrast, sections hybridized with an mRNA probe showed no specific labeling.

Northern Blot Analysis. The placenta and renal medulla displayed relatively high levels of NPY Y1 receptor expression, and a single mRNA transcript of ≈2.2 kb was detected by Northern blot analysis, using the same RNA probe as for *in situ* hybridization (Fig. 4*A*). High-stringency washing and RNase treatment of the blot had no apparent effect on the labeled hybrid. When the same poly(A)$^+$ RNA extracts were blotted and hybridized with the human NPY cRNA probe, a distinct ≈3.3-kb band was found to be expressed in the placenta and at a lower level in the kidney (Fig. 4*B*). The hybridization signal was unaffected by high-stringency washing but was abolished after RNase treatment, indicating that the transcript was not fully homologous with the probe sequence.

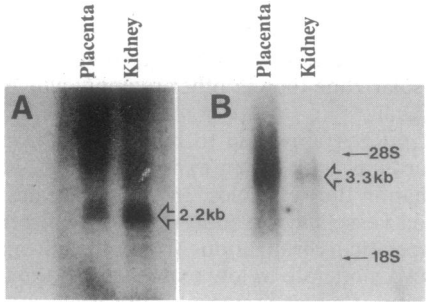

FIG. 4. Northern blot analysis of poly(A)$^+$ RNA (10–20 μg per lane) from human placenta and kidney (medulla) after hybridization with ^{32}P-labeled cRNA probes specific for human NPY Y1 receptor mRNA (*A*) and human NPY mRNA (*B*). Positions of 28S and 18S rRNAs are indicated.

DISCUSSION

Membrane binding and affinity crosslinking studies have shown that there are several putative NPY receptors in mammalian tissues, but they cannot be identified unequivocally by comparing agonist binding. We have adopted an alternative approach, employing *in situ* hybridization techniques to demonstrate the tissue-specific localization of NPY Y1 receptor gene expression in human tissues. Northern blot analysis also revealed the presence of a single ≈2.2-kb transcript, which is distinct from the Y3 receptor mRNA recently identified in bovine tissues (14).

NPY evokes a potent vasoconstrictor response in several vascular beds, but mammalian blood vessels exhibit little specific ^{125}I-NPY binding (10, 19) and we observed a relatively weak hybridization signal in the media of human blood vessels. This suggests that the number of vascular NPY receptors is low. The relative abundance of receptor transcripts need not reflect the number of receptors, however, as receptor density is also influenced by translation rates and receptor turnover. In addition, the vasoconstrictor action of NPY may depend not only on receptor number but also on the relative proportion of postjunctional receptor subtypes (19, 20) and the availability of secondary messenger systems coupled to NPY receptors (13). The significance of Y1 receptor expression in the intima of human blood vessels and the role of the endothelium in the vascular actions of NPY are uncertain (21). Nonetheless, it has been suggested that NPY

may induce the release of endothelium-derived relaxing factor as well as exert a direct vasoconstrictor effect in human coronary arteries (22).

NPY Y1 receptors expressed in the colon may mediate the actions of both NPY and PYY, which are released locally from nerve terminals and mucosal endocrine cells, respectively. The relatively high level of hybridization found in the colonic mucosa is consistent with a direct antisecretory effect of these peptides on mucosal cells, whereas the localization of Y1 receptor expression to the myenteric and submucous plexuses provides further evidence indicating that NPY and PYY modulate intestinal motility indirectly via the enteric nervous system (3).

Renal NPY receptors have largely been studied in the rabbit kidney, where they were localized to the proximal convoluted tubule (10) and characterized as NPY Y2 receptors (23). The apparent inability to detect similar binding sites in other mammals (10) may reflect species differences in peptide metabolism (12). However, the finding that the localization of human Y1 receptor mRNA does not correspond with ^{125}I-NPY binding sites in the rabbit suggests a possible differential distribution of receptor subtypes in the kidney. The association of NPY-containing nerves with the juxtaglomerular apparatus and renal blood vessels in the human kidney (7) is consistent with the localization of Y1 receptor mRNA in the cortex. NPY-immunoreactive nerves have not been reported to occur around collecting ducts in the renal medulla, and it is not known whether Y1 receptors are located at the luminal or basolateral membrane, but it is possible that filtered or circulating peptides represent the endogenous ligands for these receptors. Although the action of NPY on renin release and sodium excretion appears to be species-dependent and may be secondary to its hemodynamic effects (24), the localization of Y1 receptor expression indicates that NPY may directly affect human renal function, acting, for example, on the juxtaglomerular apparatus to modulate renin release.

The significance of Y1 receptor expression in fetal tissues is uncertain but occurs concurrently with the development of an NPY-immunoreactive nerve supply in the human cardiovascular system (2). Y1 receptor expression in the myocardium of the adult atrial appendage also coincides with the distribution of NPY-immunoreactive nerves (25), although studies *in vitro* have indicated that NPY has no direct effect on human myocardial contractility (26, 27).

It is generally agreed that the placenta has no autonomic innervation (28), but the localization of NPY Y1 receptor expression is very similar to that reported for both NPY-like immunoreactivity and ^{125}I-NPY binding sites in the human placenta (5), suggesting a local autocrine or paracrine function. The placental NPY-like material has not been isolated and sequenced. Northern blot analysis of poly(A)$^+$ RNA indicates, however, that the mRNA species detected in the human placenta is distinct from the ≈0.8-kb mRNA species found in NPY-producing human pheochromocytoma and brain tissues (15, 29, 30). This may suggest either alternative processing of NPY transcripts or tissue-specific expression of a distinct gene in the placenta.

The results indicate potential sites of NPY action in human tissues. With the cloning of other NPY receptors it should be possible to determine the differential distribution of receptor subtypes and hence obtain further insight into the possible significance of NPY and related peptides.

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