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Some Sensory Neurons Express Neuropeptide Y Receptors: Potential Paracrine Inhibition of Primary Afferent Nociceptors Following Peripheral Nerve Injury

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Neuropeptide Y (NPY) has been suggested to exert antinociceptive actions by inhibiting the release of neurotransmitters from trigeminal and dorsal root ganglia (DRG) neurons, but the site of direct NPY action *in vivo* and the NPY receptor subtype mediating these effects are unknown. ^{125}I-peptide YY (PYY) was used to localize and characterize NPY receptor binding sites in trigeminal ganglia, DRG, and spinal cord of the rat, rabbit, and monkey. In the rat, rabbit, and monkey, 5–20% of trigeminal ganglia and DRG neurons express NPY binding sites. Unilateral cuff-induced neuropathy or transection of the rat sciatic nerve did not significantly alter the density or number of DRG neurons expressing NPY receptors. A unimodal size distribution for L4 and L5 DRG neurons expressing NPY binding sites in the rat was determined, with a mean cross-sectional area of 947 μm^2. In the spinal cord the highest concentration of NPY receptors is found in laminae I, II, V, X, and Onuf's nucleus. Pharmacological experiments using selective Y1 and Y2 receptor antagonists suggest that Y2 is the prominent NPY receptor subtype expressed in trigeminal ganglia neurons, DRG neurons, and spinal cord. Previous studies have demonstrated that a population of large-diameter, presumably myelinated primary afferents express NPY after peripheral nerve injury. NPY released from these injured large-diameter DRG neurons may act in a paracrine fashion to block the transmission of nociceptive information from the small- and medium-diameter DRG neurons that constitutively express NPY receptors. NPY receptors are therefore uniquely positioned to inhibit primary afferent nociceptors directly, especially after peripheral nerve injury.

[Key words: dorsal root ganglion, sensory neurons, neuropeptide Y, nociceptor, neuropeptide Y receptors, peripheral nerves, trigeminal ganglion]

Neurons with cell bodies located in a trigeminal ganglion or dorsal root ganglion (DRG) convey somatic sensory information from peripheral tissues to the CNS. These pseudounipolar sensory neurons are unique in that they span the blood–brain barrier, having one process that terminates in the spinal cord and another terminating in the peripheral tissue. Modulation of sensory input into the CNS is a universal feature of mammalian sensory systems (Kandel et al., 1991). In the somatosensory system, the first level where this modulation could occur is at the sensory neuron itself, that is, presynaptic modulation of the trigeminal ganglion or DRG neuron.

Recent work has suggested that in addition to their afferent functions, DRG neurons also play an efferent role in regulating the tissues they innervate. DRG neurons have been implicated in regulating blood flow, gastric motility, inflammation, plasma extravasation, and the response to tissue injury (Lembeck and Holzer, 1979; Mantyh et al., 1988; Kruger et al., 1989; Barnes et al., 1990). One mechanism by which this efferent regulation can occur is via antidromic activation of sensory terminals with the resulting release of neurotransmitters from the peripheral terminals of DRG neurons (Lam and Ferrell, 1991; Santicioli et al., 1993). An example of this is the release of substance P from the primary afferent terminal after tissue injury. This release of substance P in the innervated tissue results in vasodilatation and plasma extravasation, both of which are components of the phenomenon known as neurogenic inflammation (Louis et al., 1989; Barnes et al., 1990; Lötvall et al., 1990; Pedersen-Bjergaard et al., 1991; Lei et al., 1992). What has also become clear is that the innervated tissue can exert effects on the primary afferent neuron by the release of ligands that bind to receptors expressed by the sensory nerve terminals. An example of this is the release of opioid peptides from immunocytes. Peripherally released opioids interact with opiate receptors on sensory neurons and might thereby inhibit the transmission of nociceptive input by the DRG neurons that normally occurs after tissue injury (Levine and Taiwo, 1989; Stein et al., 1990a,b).

Neuropeptide Y (NPY) is a 36 amino acid peptide that belongs to the pancreatic polypeptide (PP) family of structurally related peptides. The PP family consists of PP, NPY, and peptide YY (PYY), all of which share a common secondary structure containing a β fold (the "PP fold") (Fuhlendorff et al., 1990; Jorgensen et al., 1990). Immunochemical studies have shown that NPY is synthesized by neurons in the spinal cord and by postganglionic sympathetic and parasympathetic neurons, and thus is widely distributed in both the CNS and in peripheral tissues (Lacroix et al., 1990; Lundberg et al., 1990; Schalling et al., 1991). Recently it has been reported that NPY is present in

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a population of DRG neurons, but only after peripheral nerve injury (Wakisaka et al., 1991, 1992; Noguchi et al., 1993). Previous *in vitro* studies have suggested that NPY receptors are expressed by cultured DRG neurons obtained from neonatal rats (Ewald et al., 1988; Bleakman et al., 1991), and *in vivo* studies have demonstrated that NPY can potently inhibit the release of several neurotransmitters from sensory neurons (Giuliani et al., 1989; Duggan et al., 1991; Hua et al., 1991). Together these results suggest that NPY may play a role in regulating the transmission of nociceptive input by trigeminal and dorsal root ganglion neurons, particularly after peripheral nerve injury.

The present studies were designed to (1) determine whether trigeminal ganglia neurons, DRG neurons, and/or their supporting cells express NPY receptor binding sites, (2) define the percentage and diameters of the trigeminal ganglia and DRG neurons expressing NPY receptors, (3) determine the pharmacological profile of these receptors, (4) determine if there are substantial species differences in the expression of these receptors, and (5) compare expression of NPY receptors in normal rats and in rats with a painful mononeuropathy and with peripheral nerve transection.

Materials and Methods

Radioligand preparation

PYY was used instead of NPY as the radioligand because PYY binding resulted in higher specific:nonspecific binding ratios and because PYY has been demonstrated to bind to the same population of receptors as NPY (Sheikh et al., 1989). Porcine PYY (pPYY) was iodinated by mixing 5 μg of pPYY, 5 μl of $Na^{125}I$ (500 μCi), 600 ng of lactoperoxidase, and 10 μl of 0.006% hydrogen peroxide in 85 μl of 200 mM sodium phosphate buffer, pH 7.4. After incubation for 5 min at room temperature, an additional 10 μl of 0.006% hydrogen peroxide was added and the reaction was terminated after incubation for an additional 5 min by addition of 95 μl of 50% acetic acid. The reaction mixture was fractionated by reversed-phase high-performance liquid chromatography to a specific activity of approximately 2000 Ci/mmol. The second major peak of radioactivity corresponding to ^{125}I-Tyr^{36}-monoiodoPYY was used for all binding studies (Sheikh et al., 1989). After purification, ^{125}I-PYY was stored at −20°C in a solution containing 1% BSA. This radioligand stock solution was diluted to a working concentration of 100 pM shortly before incubation with either membranes or tissue sections.

Homogenate receptor binding

New Zealand White rabbits (5–7 lb) were anesthetized with intramuscular pentobarbital followed by intravenous ketamine, and the spinal cord and dorsal root ganglia were rapidly removed. These were then flash frozen and stored at −70°C until used. At the time of the binding the tissue was thawed and homogenized with a polytron (Brinkmann Instruments, NY) in 10 ml of an ice-cold buffer that contained (in mM) 137 NaCl, 2.68 KCl, 2.05 $MgCl_2$, 1.8 $CaCl_2$, 0.1% mg/ml glucose, 4 mg/liter leupeptin, 2 mg/liter chymostatin, and 20 HEPES, pH 7.4 (Gimpl et al., 1990). The homogenate was then centrifuged at 14,000 × *g* at 4°C for 15 min (SS-34 rotor, Sorvall RC5C, Sorvall Instruments, Danbury, CT). The resulting pellet was washed and resuspended in a final volume of 4.5 ml of the same buffer.

For binding studies, 400–600 μg/ml of tissue membrane protein was incubated with 100 pM ^{125}I-PYY in 200 μl of buffer A containing 0.5% bovine serum albumin and 0.025% bacitracin. Membrane-bound ^{125}I-PYY was separated from free ^{125}I-PYY by centrifugation at 12,000 × *g* for 15 min (5413 Eppendorf microcentrifuge). The total amount of radioactivity added to each tube and the amount of radioactivity bound to the tissue membranes were measured on a Packard gamma counter. Nonsaturable binding was defined as the amount of membrane-associated radioactivity when 1 μM nonradioactive PYY was added to the incubation mixture. For studies of competitive inhibition of binding, increasing amounts of unlabeled peptides (10^{-11} to 10^{-6} M) were added to the incubation mixture.

Autoradiographic localization

Tissue preparation. Eight adult male New Zealand White rabbits (Birchwood Valley Farm, Red Wing, MN), 15 male adult Sprague–Dawley rats (250 gm; Holtzman, Madison, WI), and three adult *Macaca nemestrina* monkeys (Wisconsin Regional Primate Research Center, Madison, WI) were overdosed with Nembutal and perfused transcardially with phosphate-buffered saline (pH 7.4) at 4°C. The animals were placed on ice and the dorsal root ganglia, trigeminal ganglia, and spinal cords were rapidly dissected out. The tissue was then frozen on dry ice in Tissue-Tek O.C.T. Compound (Miles, Elkhart, IN), serially sectioned at 15 μm, thaw-mounted on gelatin-coated slides, and stored for up to 3 months at −70°C.

Sciatic nerve injury was produced in 22 male Sprague–Dawley rats (180 gm; Charles River, Boston, MA). Under Nembutal anesthesia (35 mg/kg, i.p.), the right sciatic nerve was exposed in midthigh proximal to the trifurcation and freed from connective tissue by sharp dissection. Sixteen animals received an experimental cuff neuropathy produced by applying four nonconstrictive 0.5 mm segments of polyethylene tubing (inner diameter = 0.030 inches; Clay Adams) to enclose the sciatic nerve. Six additional animals received complete sciatic nerve transection. Proximal stumps were tightly ligated, and a 1–2 mm segment of nerve was removed to prevent regeneration. Neuropathic and axotomized rats were killed on 7 d (N = 2 neuropathic, 1 axotomized), 14 d (N = 12, 4), or 28 d (N = 2, 1) postsurgery and both the ipsilateral and contralateral L4, L5, and L6 ganglia were dissected out on ice, frozen on dry ice in Tissue-Tek O.C.T. Compound (Miles, Elkart, IN), and processed for quantitative autoradiography.

Cuff neuropathy produced behavioral alterations similar to those reported in rat and human painful neuropathies, including hyperalgesia and allodynia (Bennett and Xie, 1988). Quantitative analysis of alterations in axon fiber spectrum revealed a progressive decrease in the number of large myelinated axons with a maximum deficit apparent at 14 d postsurgery (77% below the untreated contralateral nerve) (Mosconi and Kruger, 1992). By 28 d, many axons with extremely thin myelin sheathes are evident, presumably reflecting regeneration and/or remyelination, and coinciding with behavioral evidence of functional recovery. In contrast, operated limbs of the transected animals were flaccid and anesthetic and showed no signs of functional recovery by 28 d postsurgery.

Receptor binding protocol. The slide-mounted tissue sections were brought to room temperature and preincubated in a Krebs-Ringer buffer (118 mM NaCl, 1.2 mM $MgSO_4 \cdot 7H_2O$, 1.2 mM KH_2PO_4, 5.6 mM glucose, 4.7 mM KCl, 2.5 mM $CaCl_2$), pH 7.4, for 1 hr prior to incubation with the radioligand (Allen et al., 1993). The sections were then incubated at 22°C for 2 hr in a solution of 100 pM ^{125}I-PYY in Krebs-Ringer buffer, pH 7.4, containing 0.1% bovine serum albumin (BSA) and 0.05% bacitracin. To estimate nonsaturable binding, paired serial sections were incubated as described except that 1 μM nonradioactive PYY or NPY was present in the incubation medium. In all experiments the unlabeled peptides were obtained from Bachem (Torrance, CA). After incubation, the slides were washed four times in incubation buffer, pH 7.4 (4°C, 4 min each), rinsed once in distilled water (4°C, 5 sec), and then quickly dried in the cold room under a stream of cold air. After 3 hr of further drying at 4°C, sections were stored over desiccant at room temperature overnight before being apposed to x-ray film or dipped in nuclear emulsion.

Pharmacological characterization of the NPY binding sites. The specificity of the ^{125}I-PYY binding sites was characterized by performing the binding as described above except that nonradioactive NPY, PYY, PP, Leu^{31},Pro^{34}-NPY (a selective Y1 receptor agonist), or $NPY_{13\text{-}36}$ (a selective Y2 receptor agonist) was added to the incubation medium in various concentrations. The slides were then processed for autoradiography and the density of the Hyperfilm silver grains was analyzed by image analysis as described below.

Autoradiography. The slide-mounted tissue sections were placed in apposition to β-max Hyperfilm (Amersham) alongside iodinated standards (Amersham). After 1–3 weeks the film was developed in D-19 developer, fixed, and washed. When a higher degree of histological resolution was required, the tissue was processed for standard emulsion-dipped autoradiography. The emulsion-dipped autoradiograms were developed, stained with Nissl or thionin, and mounted with Permount. Dark-field and bright-field photomicrographs were then taken of the silver grains and counterstained sections, respectively. This approach generated three complementary images: the Hyperfilm autoradiograms,

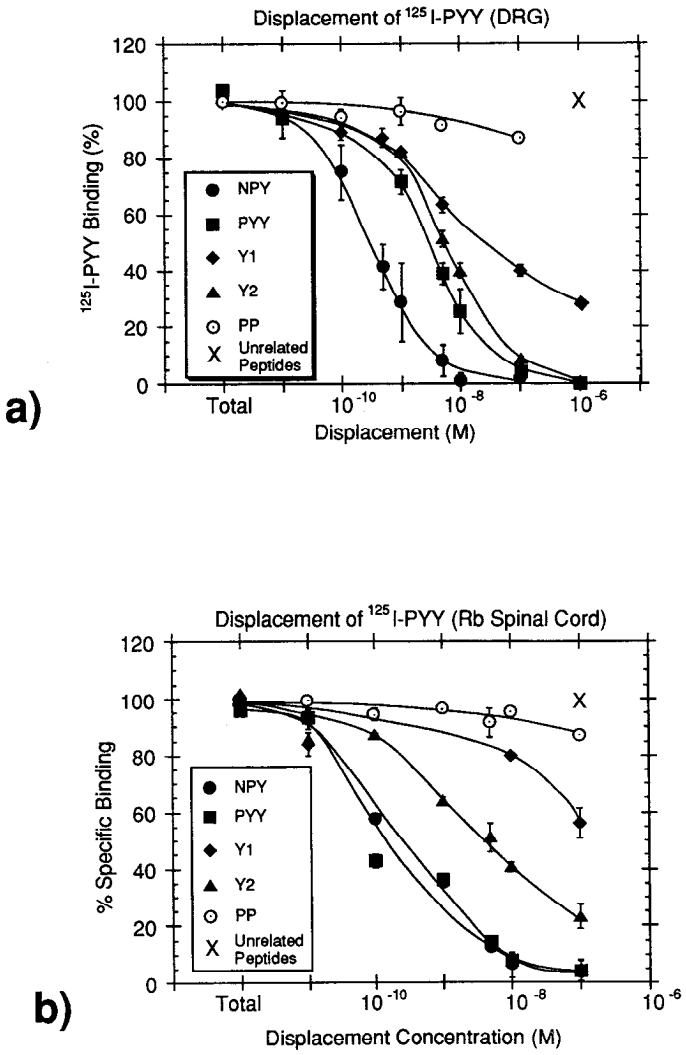


Figure 1. Pharmacological displacement of ^{125}I-PYY binding in membranes of rabbit DRG and rabbit spinal cord. Rabbit DRG (*a*) or spinal cord (*b*) from all spinal levels was pooled and homogenized as described. Note that in both the spinal cord and DRG the prominent receptor subtype appears to correspond to the Y2 subtype, as the Y2 agonist (NPY_{13-36}) appears to be significantly more potent than the Y1 agonist (Leu^{31},Pro^{34}-NPY) in displacing ^{125}I-PYY from these tissues. The unrelated peptides (*X*) that failed to show any displacement of ^{125}I-PYY included cholecystokinin, neurokinin A, neurokinin B, and substance P.

which were analyzed for quantitative densitometry; the emulsion-dipped slides, which provided detailed histological resolution of the developed silver grains; and the counterstained sections, which allowed identification of the cell type expressing the saturable binding site.

Image analysis. To estimate quantitatively the density of radiolabeled neuropeptide binding sites and to generate inhibition curves from the autoradiographic displacement experiments, microdensitometry was performed on a Macintosh-based system using the NIH IMAGE program, version 1.37.

Results

Homogenate binding analysis of ^{125}I-PYY binding to membranes of the rabbit DRG and spinal cord

Membrane preparations from rabbit DRG or spinal cord were used to characterize the ^{125}I-PYY binding sites in these tissues (Fig. 1). In rabbit DRGs the rank order of displacement of ^{125}I-PYY was NPY > PYY > NPY_{13-36} > Leu^{31},Pro^{34}-NPY > PP (Fig. 1*a*). Scatchard analysis revealed a one-site best fit with a K_d of 3.6 nM and a B_{max} of 352.4 fmol/mg protein. In rabbit spinal cord, a similar pharmacological profile was obtained with the rank order of displacement of ^{125}I-PYY being NPY > PYY > NPY_{13-36} > Leu_{31},Pro_{34}-NPY > PP (Fig. 1*b*). Scatchard analysis revealed a one-site best fit with a K_d of 1 nM and a B_{max} of 489.7 fmol/mg protein.

Autoradiographic analysis of ^{125}I-PYY binding sites in the rabbit DRG, trigeminal ganglion, and spinal cord

Several dozen rabbit DRG and trigeminal ganglia were examined for ^{125}I-PYY binding sites. In general, nearly all the specific binding sites were observed over neurons with few specific binding sites overlying the supporting cells (Fig. 2). Dorsal root ganglion neurons with a silver grain density greater than fourfold higher than background levels were considered to be labeled. The ^{125}I-PYY ligand binds to 5.2 ± 2.9% (N = 4016), 10.2 ± 3.9% (N = 2907), 6.3 ± 3.9% (N = 3060), 9.5 ± 2.6% (N = 5067), and 15.1 ± 3.3% (N = 3848) of the neurons in the cervical, thoracic, lumbar, sacral, and trigeminal ganglia, respectively. The numbers in parentheses indicate the total number of neurons examined. The specificity of the ^{125}I-PYY ligand is demonstrated by the highly localized silver grains overlying individual neurons, while only background levels of silver grains are found over the surrounding supporting cells and intercellular space (Fig. 2).

In the spinal cord, the highest density of specific ^{125}I-PYY binding sites is present in laminae I, II, V, X, and Onuf's nucleus. Low but detectable levels of specific ^{125}I-PYY binding sites were present in laminae III–IX.

Comparisons of the inhibition of ^{125}I-PYY binding by Y1 (Leu^{31},Pro^{34}-NPY) and a Y2 (NPY_{13-36}) receptor agonist in a displacement series demonstrated that the Y2 agonist was significantly more potent than the Y1 agonist in displacing ^{125}I-PYY from the DRG, trigeminal ganglia, and spinal cord tissue sections, suggesting that the receptor binding sites in these tissues are of the Y2 subtype.

Autoradiographic analysis of ^{125}I-PYY binding sites in the rat DRG, trigeminal ganglion, and spinal cord

Several hundred rat DRG and several dozen trigeminal ganglia were examined for ^{125}I-PYY binding sites. Nearly all the specific binding sites were observed over neurons with few if any specific binding sites overlying the supporting cells (Fig. 3). In the DRG and trigeminal ganglion of the rat, several thousand neurons were examined for ^{125}I-PYY labeling. In the rat, specific ^{125}I-PYY binding sites were observed overlying 12.8 ± 3.8% (N = 1607), 12.2 ± 5.4% (N = 1687), 13.1 ± 5.2% (N = 1856), 11.7 ± 7.5% (N = 1655), and 16.8 ± 4.4% (N = 2152) of the neurons in the cervical, thoracic, lumbar, sacral, and trigeminal ganglia, respectively. The frequency distribution of the cross-sectional area of L4 and L5 DRG neurons expressing NPY receptors was also calculated (Fig. 4). Neurons expressing NPY receptors were mainly small to medium sized, with a cross-sectional area of 947 ± 485 μm^2. At 7, 14, or 28 d after cuff-induced sciatic neuropathy or sciatic nerve transection there is relatively little change in either the number of DRG neurons expressing NPY receptors or the density of NPY receptors expressed per neuron (Fig. 3, Table 1).

In the rat spinal cord at the cervical, thoracic, lumbar, and sacral levels, the highest densities of saturable ^{125}I-PYY binding sites were found in laminae I, II, V, X, and Onuf's nucleus. A lower but still detectable level of saturable PYY binding sites

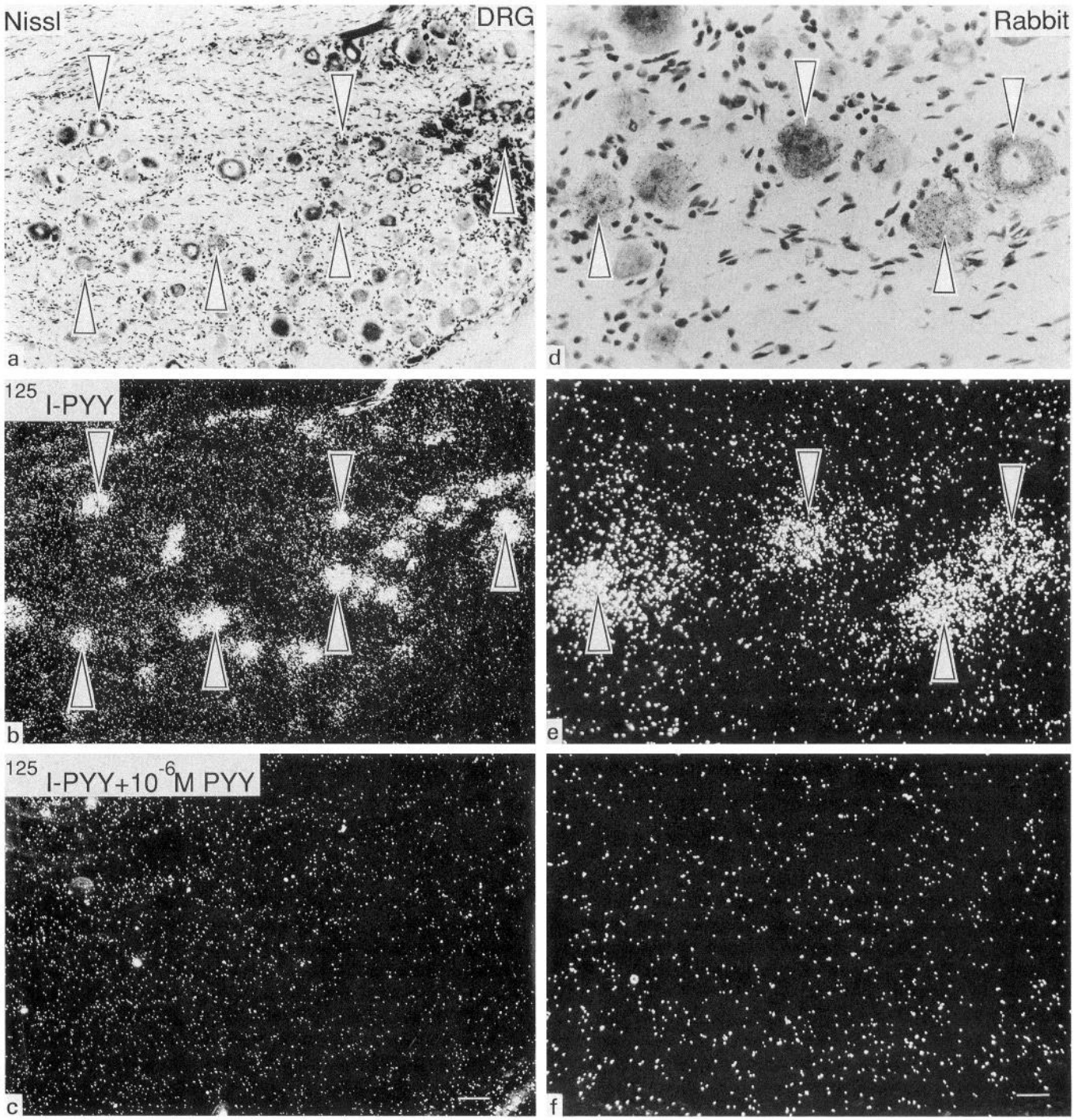


Figure 2. Autoradiographic localization of ^{125}I-PYY receptor binding sites in rabbit DRG neurons. *a* and *d*, Light-field photomicrographs of rabbit DRG (C5) neurons that have been Nissl stained to visualize the individual neurons within the ganglion. *b* and *e*, Dark-field photomicrograph of the same section shown in *a* and *d*, respectively, which has been dipped in nuclear emulsion to show individual neurons expressing the ^{125}I-PYY binding sites, which appear as *white silver grains*. *c* and *f*, Dark-field photomicrograph of sections serially adjacent to *b* and *e*, respectively, showing the nonspecific binding. The *arrowheads* point to individual DRG neurons that contain PYY receptors. Note that the silver grains are localized primarily over the neurons, with few specific binding sites present over the intercellular space or glial cells. Scale bars: 60 μm for *a–c*, 23 μm for *d–f*.

was observed in laminae III–IX. Characterization of the ^{125}I-PYY receptor binding sites in sections of the rat DRG, trigeminal ganglia, and spinal cord suggests that the predominant NPY receptor subtype expressed by these cells is the Y2 subtype. For the NPY receptor expressed by DRG and trigeminal ganglia neurons, as well as in the spinal cord, the rank order of potency for displacing ^{125}I-PYY from the tissue sections was PYY = NPY > PP. In another displacement series, NPY_{13-36} (Y2 agonist) appeared to be significantly more potent than Leu^{31},Pro^{34}-NPY (Y1 agonist) at displacing ^{125}I-PYY from the DRG, trigeminal ganglia, and spinal cord, suggesting that the receptor binding sites on these neurons are of the Y2 subtype. Thus, at

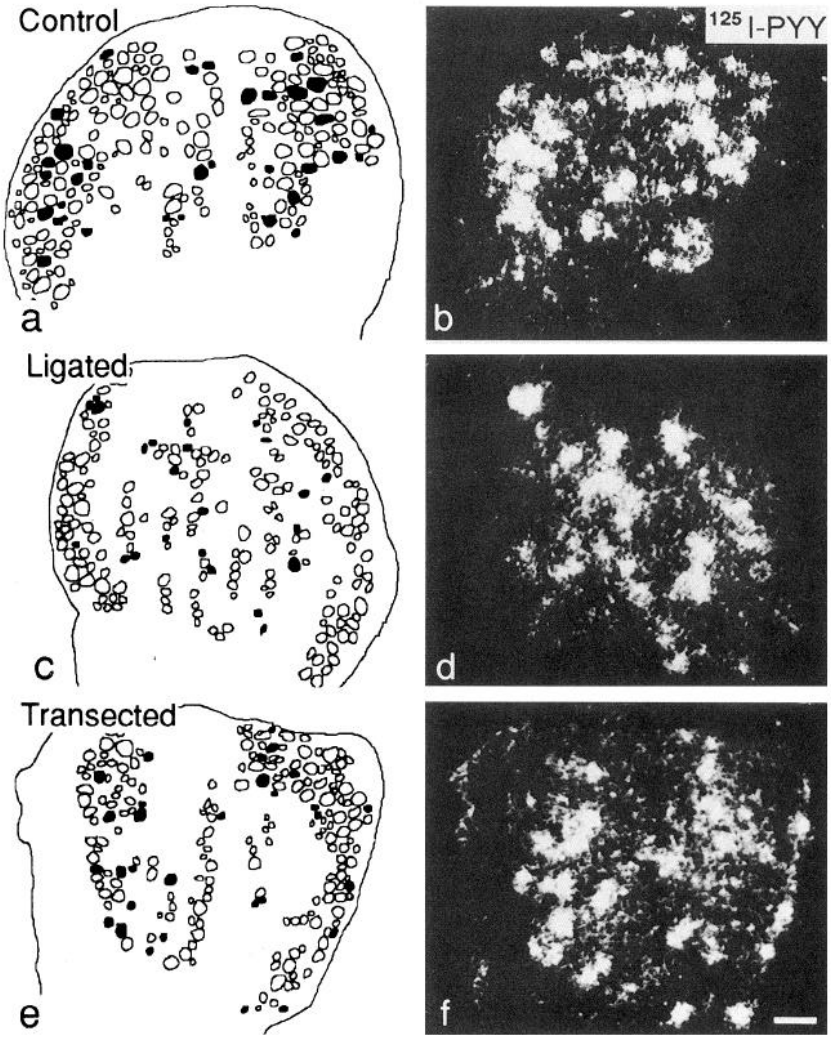


Figure 3. Autoradiographic localization of ^{125}I-PYY receptor binding sites in the normal, ligated, and transected rat DRG. Dark-field photomicrographs show the localization of ^{125}I-PYY binding sites in the normal (*b*), ligated (7 d; *d*), and transected (7 d; *e*, *f*) rat L4 DRG. These same DRG are schematically depicted in camera lucida tracings: *a*, normal; *c*, ligated (7 d); and *e*, transected (7 d). In the camera lucida drawings the neurons expressing ^{125}I-PYY receptors are denoted by *solid profiles*, whereas the nonexpressing neurons are denoted in *open profiles*. Note that although the neurons that are labeled express very high levels of ^{125}I-PYY binding sites, neither the density of binding nor the size or percentage of DRG neurons expressing the ^{125}I-PYY binding sites appears to be significantly altered after either ligation or transection of the sciatic nerve. Scale bar, 150 μm.

a 10^{-8} M concentration of the Y2 agonist NPY_{13-36}, nearly all ^{125}I-PYY binding to rat DRG neurons was displaced, whereas Leu^{31},Pro^{34}-NPY at the same concentration showed little displacement of the binding to rat DRG neurons.

Autoradiographic analysis of ^{125}I-PYY binding sites in the monkey DRG and spinal cord

Several dozen monkey DRG were examined for ^{125}I-PYY binding sites. As was found in the rat and rabbit, nearly all the saturable ^{125}I-PPY binding sites were observed over neurons with very low levels of ^{125}I-PYY saturable binding present over the surrounding supporting cells (Fig. 5*f*). Several thousand monkey DRG neurons were examined for ^{125}I-PYY labeling at all spinal cord levels. The ^{125}I-PYY ligand bound saturably to 17.6 ± 4.7% (N = 2093), 9.5 ± 3.2% (N = 1607), 9.0 ± 5.9% (N = 2205), and 12.1 ± 5.0% (N = 1632) of the neurons in the cervical, thoracic, lumbar, and sacral ganglia, respectively.

Although a full displacement curve was not performed on

Table 1. Percentages of DRG neurons expressing ^{125}I-PYY receptor binding sites

	7 day	14 day	28 day
Ligation			
Contra	11.43 ± 1.10 (746/85)	17.40 ± 9.68 (1341/220)	15.08 ± 1.31 (444/67)
Experimental	11.76 ± 3.30 (774/90)	17.88 ± 8.26 (1497/271)	10.32 ± 1.47 (690/73)
Transection			
Contra	10.05 ± 1.35 (429/43)	17.22 ± 7.07 (306/50)	17.54 ± 3.06 (1051/181)
Experimental	15.85 ± 5.56 (333/54)	15.09 ± 4.70 (606/91)	20.79 ± 4.71 (748/153)

The numbers in each column indicate the percentage of DRG neurons expressing ^{125}I-PYY binding sites. The animals had either a unilateral ligation or transection of the sciatic nerve and were killed at various time points after the peripheral nerve injury. The numbers in parentheses indicate the total number of DRG neurons counted/number of DRG expressing ^{125}I-PYY binding sites.

monkey spinal cord or DRG, the Y2 agonist NPY_{13-36} at a concentration of 1 μM displaced nearly all ^{125}I-PYY binding to monkey DRG and spinal cord (Fig. 6), whereas Leu^{31},Pro^{34}-NPY at the same concentration caused little displacement of ^{125}I-PYY binding to these same tissues.

In the monkey spinal cord, results similar to the rat and rabbit were obtained, with high concentrations of binding sites being observed in laminae I, II, V, and X at all spinal levels, whereas low but detectable concentrations of specific ^{125}I-PYY binding sites were observed in laminae III–IX (Figs. 5, 6).

Discussion

NPY actions on sensory nerve terminals in the spinal cord

Previous studies have shown that there are high concentrations of NPY immunoreactivity in the superficial spinal cord (Gibson et al., 1981; Suburo et al., 1992), where high concentrations of NPY receptors are also expressed and where small-diameter nociceptive sensory neurons are known to terminate (Willis and Coggeshall, 1991). Intrathecal infusion of NPY in the spinal cord has been shown to inhibit the release of both substance P and calcitonin gene–related peptide (CGRP) from sensory neurons (Giuliani et al., 1989; Duggan et al., 1991). Since substance P has been reported to be involved in conveying nociceptive information in the spinal cord (De Koninck and Henry, 1991; Salter and Henry, 1991), these results suggest that NPY exerts its reported antinociceptive actions (Hua et al., 1991) by directly inhibiting the release of neurotransmitters from trigeminal and DRG neurons.

It has been suggested recently that after injury (loose ligation, crush, or extraction of a tooth) or axotomy, large-diameter DRG neurons, which probably correspond to low-threshold myelinated mechanoreceptors (Willis and Coggeshall, 1991), express NPY immunoreactivity (Wakisaka et al., 1991, 1992; Itotagawa et al., 1993) and mRNA coding for NPY (Wakisaka et al., 1992). These results suggest that, at least in an injured state, a population of large-diameter sensory neurons may provide the ligand (in a paracrine fashion) to occupy the NPY receptors expressed by a population of the medium- and small-diameter sensory neurons. This possibility would also fit with the observation that peripheral nerve injury triggers the sprouting of myelinated afferents (which may correspond to the large-diameter NPY-containing DRG neurons) into laminae I and II of the spinal cord, which is the spinal laminae where the small thinly or unmyelinated neurons (which may correspond to the small-diameter NPY receptor–expressing DRG neurons) terminate (Woolf et al., 1992). However, unlike for the ligand NPY, DRG neurons do not appear to alter significantly their expression of NPY receptors after peripheral nerve cuff–induced neuropathy or transection. This paracrine release of NPY may inhibit the release of sensory peptides, such as substance P and CGRP, which are thought to modulate nociception in the spinal cord (De Koninck and Henry, 1991; Radhakrishnan and Henry, 1991). While the clinical relevance of this paracrine NPY interaction among DRG neurons has not yet been explored, it does suggest both a physiological pathway and a mechanism that may be pharmacologically manipulated to control the pain that often results from peripheral nerve injury or disease (Parry and Withrington, 1984).

NPY actions on sensory nerves outside the spinal cord

Previous data on other receptors expressed by DRG neurons such as opiate or cholecystokinin receptors have shown that these receptors are transported within the axon to both the central nerve terminals in the spinal cord and to peripheral nerve terminals in the innervated peripheral tissues (Zarbin et al., 1981, 1990). NPY receptors may be similarly transported to both the central and peripheral terminals. In support of this hypothesis, several reports have demonstrated that peripherally released NPY can modulate neuropeptide release from the peripheral nerve terminals of DRG neurons. In tissues as diverse as the atria, bronchi, ileum, and mesenteric arteries, NPY has been shown to inhibit potently the motor response and the release of substance P and CGRP from the sensory neurons innervating these tissues (Giuliani et al., 1989; Kawasaki et al., 1991; Takaki and Nakayama, 1991). Since substance P release from peripheral terminals of DRG neurons is known to mediate neurogenic inflammation and plasma extravasation (Lam and Ferrell, 1991; Santicioli et al., 1993), peripherally released NPY may prove to be an effective agent in blocking the neurogenic inflammation that follows tissue injury in a peripheral tissue.

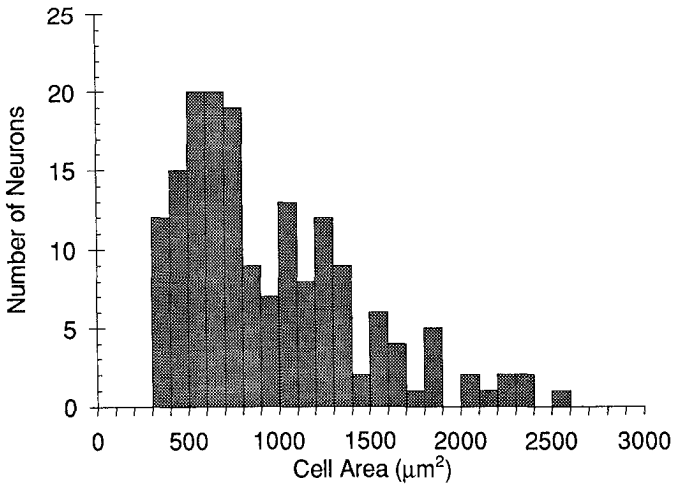


Figure 4. Histogram of the cell body size of rat (L4 and L5) DRG neurons that express NPY receptor binding sites. Note that the majority of DRG neurons that express NY binding sites are small to medium in size.

The NPY that might interact with the NPY receptors of trigeminal and DRG neurons could arise from several sources. A population of both sympathetic and parasympathetic neurons has been shown to synthesize and release NPY in peripheral tissues (Lundberg et al., 1982, 1990; Franco-Cereceda et al., 1985; Lacroix et al., 1990; Schalling et al., 1991). This interaction of sympathetically and parasympathetically released NPY with sensory nerve terminals may be one of the modes by which sympathetic nerves normally regulate the activities of sensory neurons. Since loss of sympathetic control of sensory neurons is thought to be a contributing factor in the chronic pain state of reflex sympathetic dystrophy (McLachlan et al., 1993), and NPY in sympathetic neurons can inhibit the release of excitatory neurotransmitters by sensory neurons (Giuliani et al., 1989; Duggan et al., 1991; Kawasaki et al., 1991; Takaki and Nakayama, 1991), NPY agonists may prove useful in the treatment of this chronic pain state.

In most cases, sympathetic control of DRG neurons is thought to take place at the peripheral terminals of DRG neurons (Mantyh et al., 1992). In a recent article, however, it was reported that after peripheral nerve injury, sympathetic fibers could be found surrounding the cell bodies of sensory neurons within the

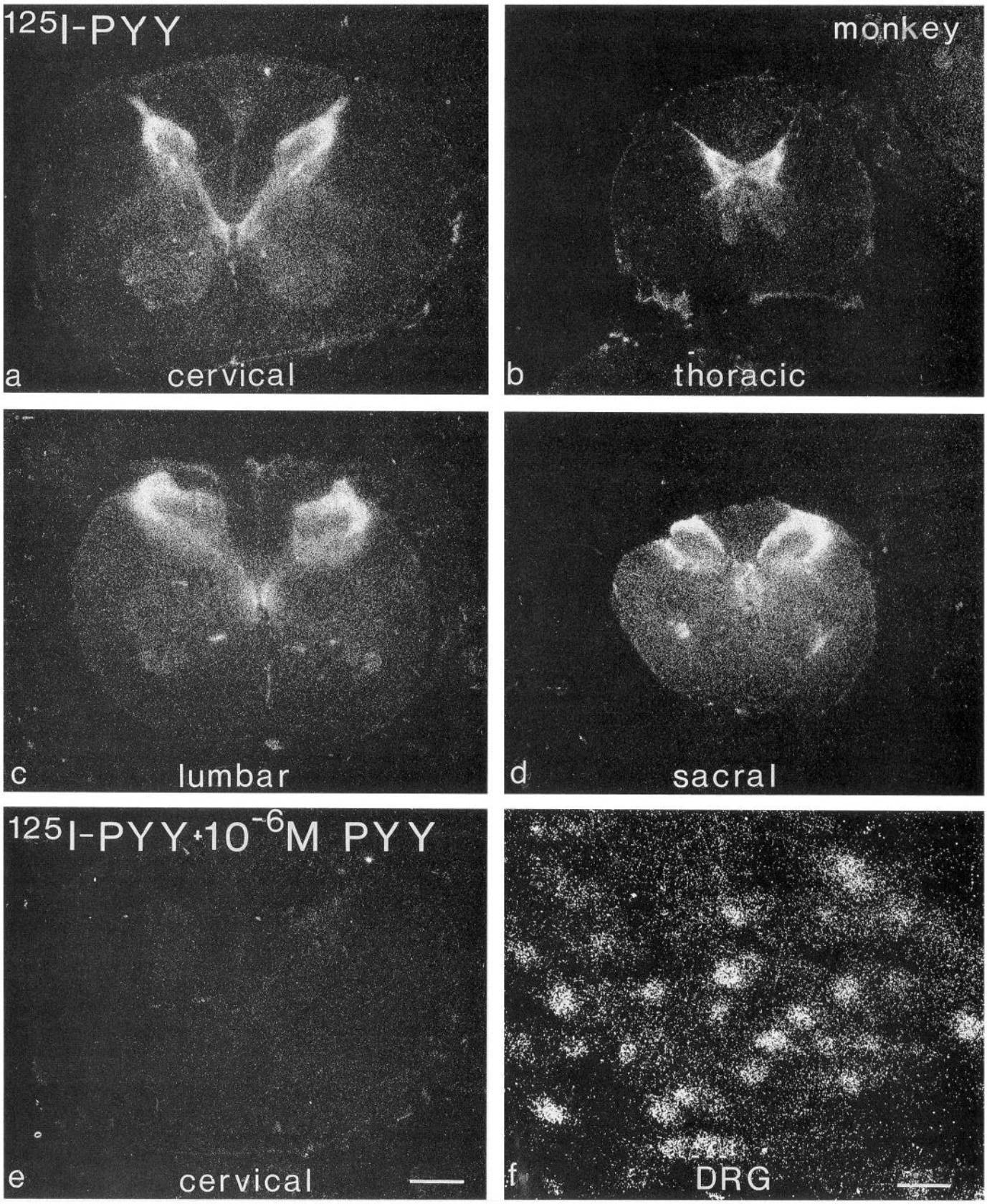
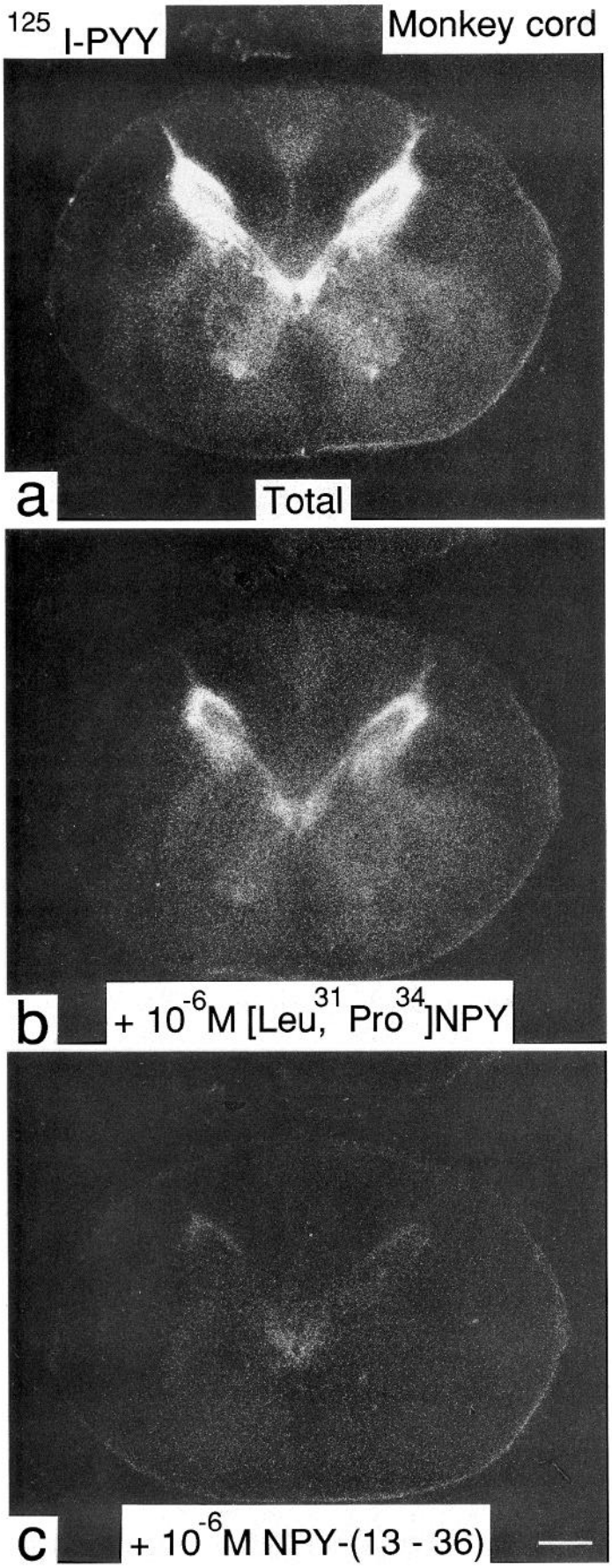


Figure 5. Autoradiographic distribution of ^{125}I-PYY receptor binding sites in monkey spinal cord and DRG neurons. Dark-field photomicrographs are autoradiograms of monkey cervical (*a*), thoracic (*b*), lumbar (*c*), and sacral (*d*) spinal cord. Note that in the spinal cord the highest density of binding sites occurs in laminae I, II, V, X, and Onuf's nucleus. The control section shown in *e* is a section serially adjacent to *a*. Note also the binding to individual monkey cervical DRG neurons in *f* is similar to that observed in rabbit (Fig. 2). Scale bars: 1.2 mm for *a–e*, 100 μm for *f*.



DRG itself (McLachlan et al., 1993). The majority of sympathetic nerves expressing noradrenaline also express NPY (Lundberg et al., 1990). These results suggest that after peripheral nerve injury, sources of NPY that might occupy NPY receptors present on the cell bodies of small- and medium-diameter DRG neurons include not only large-diameter DRG neurons but also sympathetic nerve terminals that are present next to the cell bodies of the sensory neurons within the DRG itself. Although it is generally thought that little or no synaptic activity occurs within the trigeminal ganglia or the DRG itself (Willis and Coggeshall, 1991), the demonstrations that DRG neuron cell bodies express NPY receptors, that after injury DRG neurons can express NPY, and that after injury sympathetic nerves sprout within dorsal root ganglia (McLachlan et al., 1993) suggest that after peripheral nerve injury NPY may act directly on DRG cell bodies expressing NPY receptors.

Percentage of trigeminal ganglia and DRG neurons expressing NPY receptors

In the present study we observed that a substantial number of trigeminal and DRG neurons express NPY receptors in the rat, rabbit, and monkey. However, we never observed saturable binding sites over more than 20.7% of the neurons in any trigeminal ganglia or the DRG. In a previous report, however, using electrophysiological recordings from 2-week-old cultures of neonatal rats, NPY inhibited the Ca^{2+} current in every cell tested (Walker et al., 1988). There are several possible explanations for these apparently discrepant findings.

In the previous study the cells examined were cultured DRG neurons obtained from neonates; in the present experiments we are examining noncultured adult DRG neurons. Previous reports have noted that developing neurons are often more promiscuous in the number of neurotransmitters and receptors they express, compared to older, more differentiated neurons. For example, NPY-like immunoreactivity is transiently expressed in embryonic and fetal human DRG neurons (Suburo et al., 1992) and damaged rodent (Wakisaka et al., 1991) DRG neurons, whereas NPY has not been detected in normal adult DRG neurons (Wakisaka et al., 1991).

A second explanation may be that after 2 weeks in culture, DRG neurons that normally do not express NPY receptors may begin to express the receptors. A similar *in vivo* and *in vitro* induction of peptide receptors has been noted for substance P receptors. Thus, whereas substance P receptors cannot be detected on astrocytes in the normal brain, these same astrocytes express very high levels of substance P receptors after nearby neuronal injury (Mantyh et al., 1989). Similarly, whereas astrocytes express very low levels of substance P receptors immediately after being placed in culture, after 10 d in culture these same astrocytes dramatically upregulate their expression of substance P receptors (Torrens et al., 1989; Rogers et al., 1993).

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Figure 6. Localization and characterization of ^{125}I-PYY receptor binding sites in the monkey spinal cord. *a,* Dark-field photomicrograph of LKB tritium-sensitive film that has been apposed to a section of the monkey cord that had been incubated with the ^{125}I-PYY ligand. *b* and *c,* ^{125}I-PYY binding in a section serially adjacent to *a* that has been treated identically as *a* except that in *b* 10^{-6} M Leu^{31},Pro^{34}-NPY (a Y1 receptor agonist) and in *c* 10^{-6} M $NPY_{(13\text{-}36)}$ (a Y2 receptor agonist) has been added to the incubation medium. Note that the majority of binding sites are displaced by the Y2 agonist and remain in the presence of a similar concentration of the Y1 agonist. Scale bar, 0.6 mm.

Third, the electrophysiological techniques employed in the previous study may simply be more sensitive than the autoradiographic methods used to detect the expression of NPY receptors in the present study. We show here that there is substantial variation in the concentration of NPY receptor binding sites over individual trigeminal ganglia and DRG neurons. Thus, whereas some DRG neurons express very high levels of NPY receptors, other DRG neurons have one-fifth of the concentration (Fig. 1*a*) and in others the expression of ^{125}I-PYY binding sites is undetectable. Since we have defined labeled neurons as those neurons having a receptor level greater than fourfold higher than background, DRG neurons that express a lower density than we can detect, but are detectable by electrophysiological techniques, may give rise to the apparent discrepancy in the percent of neurons expressing NPY receptors observed in the two studies.

Lastly, in the previous report the authors did not control for the possibility that NPY perfusion of the cultured DRG neurons might itself promote the release of factors that would effect neighboring cells. Thus, the percentage of cells responding to NPY might have been spurious due to the indirect actions of NPY, rather than a direct NPY receptor–mediated action in every DRG neuron tested.

Response of sensory neurons to NPY receptor activation

NPY appears to modulate DRG neurons by affecting calcium channels, which in turn inhibit the release of neurotransmitters from sensory neurons. In cultures of neonatal rat DRG, NPY has been shown to inhibit calcium currents via a pertussis toxin–sensitive pathway and to inhibit the depolarization-induced release of substance P (Walker et al., 1988). NPY also selectively inhibits the responses produced by the ω-conotoxin–sensitive Ca^{2+} channel (Thayer and Miller, 1990), suggesting that the inhibition of voltage-sensitive calcium channels is the likely mechanism by which NPY inhibits sensory neurons.

Sensory neurons appear to express receptors for several neurotransmitters in addition to NPY, including adenosine (Taiwo and Levine, 1990), noradrenaline (Holz et al., 1988), bradykinin (Steranka et al., 1988), cholecystokinin (Ghilardi et al., 1992, 1994), GABA (Holz et al., 1988), prostaglandin E_2 (Taiwo et al., 1987), opioids (Ninkovic and Hunt, 1985), and 5-HT (Taiwo and Levine, 1992). While there is little evidence that these ligands interact at the same receptor, there are substantial data suggesting that different receptors on the same neuron ultimately integrate their actions via G-protein effects on second messenger levels, which in turn effect Ca^{2+} channels (Thayer and Miller, 1990; Levine et al., 1993). One example of this is the interaction of bradykinin and NPY on DRG neurons. Bradykinin promotes the release of substance P from sensory nerve terminals by activation of a pertussis toxin–insensitive G-protein, which in turn stimulates arachidonic acid release and thus activates a voltage-sensitive calcium channel (Thayer et al., 1988; Bleakman et al., 1990). NPY, on the other hand, inhibits the release of substance P from DRG neurons by activation of a pertussis toxin–sensitive G-protein, which in turn inhibits the same voltage-sensitive calcium channels (Ewald et al., 1988; Bleakman et al., 1991). These results together with the present study suggest that a key to understanding the sensitization and facilitation of primary afferents is to understand the repertoire of receptors and their associated second messenger systems expressed and activated in DRG neurons in normal and pathological states. This understanding might provide a rational approach for targeting analgesic agents that act specifically on the primary afferent neuron itself and thereby avoid many of the deleterious CNS side effects frequently observed with potent analgesic agents.

Summary

We have demonstrated that a significant population of small- and medium-diameter trigeminal ganglion and DRG neurons express NPY receptors in the rat, rabbit, and monkey. Previous data on other receptors expressed by DRG neurons suggest that NPY receptors synthesized in the cell bodies of trigeminal ganglion and DRG neurons are transported both centrally to the spinal cord and peripherally to the innervated tissue and constitute a target for released NPY at both sites. After peripheral nerve injury, large-diameter DRG neurons express NPY and sympathetic nerve terminals, many of which previously have been shown to contain NPY, appear near the cell bodies of DRG neurons. These data suggest that NPY is uniquely positioned to modulate DRG neurons not only in their central and peripheral terminals, but perhaps also on the DRG neuron cell body.

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