Neuroscience 138 (2006) 1361–1376

THE NEUROPEPTIDE TYROSINE Y1R IS EXPRESSED IN INTERNEURONS AND PROJECTION NEURONS IN THE DORSAL HORN AND AREA X OF THE RAT SPINAL CORD

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Abstract—The localization of the neuropeptide tyrosine Y1 receptor was studied with immunohistochemistry in parasagittal and transverse, free-floating sections of the rat lumbar spinal cord. At least seven distinct Y1 receptor-positive populations could tentatively be recognized: Type 1) abundant small, fusiform Y1 receptor-positive neurons in laminae I–II, producing a profuse neuropil; Type 2) Y1 receptor-positive projection neurons in lamina I; Type 3) small Y1 receptor-positive neurons in lamina III, similar to Type 1 neurons, but less densely packed; Type 4) a number of large, multipolar Y1 receptor-positive neurons in the border area between laminae III–IV, with dendrites projecting toward laminae I–II; Type 5) a considerable number of large, multipolar Y1 receptor-positive neurons in laminae V–VI; Type 6) many large Y1 receptor-positive neurons around the central canal (area X); and Type 7) a small number of large Y1 receptor-positive neurons in the medial aspect of the ventral horns (lamina VIII). Many of the neurons present in laminae V–VI and area X produce craniocaudal processes extending for several hundred micrometers. Retrograde tracing using cholera toxin B subunit injected at the 9th thoracic spinal cord level shows that several Type 5 neurons in laminae V–VI, and at least a few Type 2 in lamina I and Type 6 in area X have projections extending to the lower segments of the thoracic spinal cord (and perhaps to supraspinal levels). The present results define distinct subpopulations of neuropeptide tyrosine-sensitive neurons, localized in superficial and deep layers of the dorsal, in the ventral horns and in area X. The lamina II neurons express somatostatin [Zhang X, Tong YG, Bao L, Hökfelt T (1999) The neuropeptide Y Y1 receptor is a somatic receptor on dorsal root ganglion neurons and a postsynaptic receptor on somatostatin dorsal horn neurons. Eur J Neurosci 11:2211–2225] and are presumably glutamatergic [Todd AJ, Hughes DI, Polgar E, Nagy GG, Mackie M, Ottersen OP, Maxwell DJ (2003) The expression of vesicular glutamate transporters VGLUT1 and VGLUT2 in neurochemically defined axonal populations in the rat spinal cord with emphasis on the dorsal horn. Eur J Neurosci 17:13–27], that is they are excitatory interneurons under a Y1 receptor-mediated inhibitory influence. The remaining Y1 receptor-positive spinal neurons need to be phenotyped, for example if the large Y1 receptor-positive laminae III–IV neurons (Type 5) are identical to the neurokinin (NK)1R-positive neurons previously shown to receive neuropeptide tyrosine positive dendritic contacts [Polgár E, Shehab SA, Watt C, Todd AJ (1999) GABAergic neurons that contain neuropeptide Y selectively target cells with the NK1 receptor in laminae III and IV of the rat spinal cord. J Neurosci 19:2637–2646]. If so, neuropeptide tyrosine could have an antinociceptive action not only via Y1 receptor-positive interneurons (Type 1) but also projection neurons. The present results show neuropeptide tyrosine-sensitive neuron populations virtually in all parts of the lumbar spinal cord, suggesting a role for neuropeptide tyrosine signaling in many spinal functions, including pain. © 2005 Published by Elsevier Ltd on behalf of IBRO.

Key words: cholera toxin, interneurons, pain, neuropeptides, projection neurons, spinal transmission.

In his now classic work on the morphology and arrangement of Nissl-stained cell bodies in transverse sections of the cat spinal cord, Rexed (1952) described a characteristic lamination of the gray matter. This work, identifying 10 regions, lamina (L) I–IX and an area around the central canal (area X), is also valid for other species including rat (Molander et al., 1984, 1989), and has since provided the cytoarchitectonic framework for the study of the anatomy, histochemistry and physiology of the spinal cord (see Grant and Koerber, 2004; Willis and Coggeshall, 2004). In fact, the rat dorsal horn harbors a rich variety of neurons of different size, shape and dendritic arborizations, some being interneurons participating in the local spinal network and others projection neurons with axons traveling for variable distances, many reaching thalamic structures (see Millan, 1999; Grant and Koerber, 2004; Morris et al., 2004; Willis and Coggeshall, 2004).

Histochemical studies have revealed that subpopulations of dorsal horn neurons in the rat spinal cord express different types of neuropeptide receptors, for example opiate receptors (Besse et al., 1990; Aicher et al., 2000a,b; Spike et al., 2002) as well as the substance P receptors NK1 (Moussaoui et al., 1992; Mantyh et al., 1995) and NK3 (Ding et al., 2002). Neuropeptide tyrosine (NPY) is a 36 amino acid peptide (Tatemoto, 1982; Tatemoto et al., 1982), and so far five different receptor subtypes have

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Abbreviations: CGRP, calcitonin gene-related peptide; CTB, cholera toxin B subunit; Cy3, cyanine 3; DRG, dorsal root ganglion; HRP, horseradish peroxidase; i.t., intrathecally; L, lamina(e); LI, -like immunoreactivity; NK, neurokinin; NPY, neuropeptide tyrosine (Y); PBS, phosphate-buffered saline; RT, room temperature; SOM, somatostatin; TMR, tetramethylrhodamine; TSA, tyramide signal amplification; VGLUT-2, vesicular glutamate transporter 2; Y1R, neuropeptide tyrosine receptor type 1; Y2R, neuropeptide tyrosine receptor type 2.

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doi:10.1016/j.neuroscience.2005.11.069

been identified (see Cabrele and Beck-Sickinger, 2000; Pheng and Regoli, 2000; Larhammar et al., 2001; Silva et al., 2002; Balasubramaniam, 2003; Berglund et al., 2003; Herzog, 2003; Dumont et al., 2004; Holliday et al., 2004). In the spinal cord a considerable number of neurons in LI–II express the Y1-type receptor (Y1R), giving rise to an intricate plexus of Y1R-positive (+) processes, in particular in inner LII (LIIi) (Zhang et al., 1994a,b, 1995a, 1999), and extending into LI and LIII (Brumovsky et al., 2002; Kopp et al., 2002). A small proportion of this $Y1R^+$ plexus may represent primary afferents, since this receptor is centrifugally transported both into the sciatic nerve and dorsal roots (Brumovsky et al., 2002). In addition, $Y1R^+$ neurons have been detected in area X at different spinal levels, as well as in the lateral horns of the thoracic spinal cord, the latter presumably representing preganglionic sympathetic neurons (Kopp et al., 2002). Nevertheless, only recently, studies on rat (Brumovsky et al., 2002; Kopp et al., 2002) and mouse (Shi et al., submitted for publication) spinal cord indicate the existence of another subpopulation of $Y1R^+$ neurons present in deep L of the dorsal horn.

$Y1R^+$ neurons are also found in dorsal root ganglia (DRGs) in normal conditions. They account for 25% of all rat DRG neurons, being mostly small- and medium-sized, and usually colocalize with calcitonin gene-related peptide (CGRP) (Jazin et al., 1993; Zhang et al., 1994a,b, 1995a,b). In the mouse the proportion of Y1R transcript is lower (~7%) (Shi et al., 1998), but as in rat (Zhang et al., 1994a,b, 1999) the Y1R-like immunoreactivity (LI) is observed in close association with the plasma membrane (Shi et al., 1998).

NPY (Tatemoto, 1982; Tatemoto et al., 1982), the preferred ligand for the Y1R, is present in a large number of interneurons and their dendritic and axonal processes in the superficial dorsal horn, but in DRG neurons the NPY levels are very low (Gibson et al., 1984). After peripheral nerve injury, a dramatic upregulation of NPY is observed, mainly in large- and medium-sized DRG neurons in rat, leading to an impressive increase in NPY levels in deep dorsal horn afferents and the gracile nucleus (Wakisaka et al., 1991, 1992; Noguchi et al., 1993; Zhang et al., 1993; Nahin et al., 1994; Ma and Bisby, 1998; Shi et al., 1999; Landry et al., 2000) and mouse (Corness et al., 1996; Shi et al., 1998). In fact, using the 'antibody-coated microprobe' technique (Duggan, 1990), it has been shown that NPY release is markedly increased in the dorsal horn after peripheral axotomy (Mark et al., 1998). Thus, NPY could not only affect the activity of the numerous $Y1R^+$ LI–II interneurons, but also potentially modulate neurons located in deeper layers of the dorsal horn expressing any of the receptors.

In the present study, using a powerful Y1R antibody (Brumovsky et al., 2002; Kopp et al., 2002) combined with a sensitive immunohistochemical approach, the tyramide signal amplification (TSA) method (Adams, 1992), we immunohistochemically 'dissect' different subpopulations of $Y1R^+$ neurons localized in various layers of the rat spinal cord, and we label them Types 1–7. We also perform colocalization studies with CGRP and attempt to elucidate, by retrograde tracing using the cholera toxin B subunit (CTB), if $Y1R^+$ neurons, particularly those located in deep layers of the dorsal horn, are interneurons and/or projection neurons.

EXPERIMENTAL PROCEDURES

Animals

Male Sprague–Dawley rats were used (b.w.t. 250–300 g; $n=14$; B&K, Stockholm, Sweden). The animals were maintained under standard conditions on a 12 h day/night cycle (light on, 7:00 a.m.), with water and food *ad libitum*.

The experiments were conducted in accordance with the Society for Neuroscience policy for the use of research animals, and were approved by the local ethical committee (Stockholms Norra Djurförsöksetiska Nämnd; # 130/02, # 396/04, # 397/04, # N2/04). All efforts were made to minimize the number of animals used and their suffering.

CTB tracing. Four rats were deeply anesthetized using halothane (Fluothane®, AstraZeneca, Södertälje, Sweden), and the dorsal aspect of the spinal cord (corresponding to the 9th thoracic segment) was exposed by laminectomy of the 8th thoracic vertebra. CTB (List-Biological Laboratories Inc., Campbell, CA, USA) was infused into the right spinal hemisection with a 10 μl Hamilton syringe at a speed of 1 μl/min (Ultra MicroPump-II, World Precision Instruments, Sarasota, FL, USA) via a polyethylene tubing and a glass capillary (outer diameter 100 μm) mounted to a stereotaxic device. In this way, 2.5 μl CTB were injected in equal amounts at 1 mm and 1.75 mm from the surface of the spinal cord (corresponding to the dorsal corticospinal tract and the ventral funiculus, respectively). After the injections, muscles and skin were sutured in layers, and the animals received an i.p. injection of 0.03 mg/kg buprenorphine (Temgesic®, Schering-Plough, Kenilworth, NJ, USA) to alleviate surgery-induced pain during recovery from the halothane anesthesia.

Colchicine treatment. Two rats were injected intrathecally (i.t.) with the mitosis inhibitor (and axoplasmic transport-blocker) colchicine under Hypnorm/midazolam anesthesia (containing midazolam 12.5 mg/kg, fentanyl citrate 0.78 mg/kg and fluanisone 25 mg/kg i.p.; Apoteket, Stockholm, Sweden). An intrathecal catheter (PE 10, o.d. 0.61 mm; AgnThós AB, Lidingö, Sweden) was implanted between the L5 and L6 vertebrae under anesthesia (as above), with its tip at the lumbar enlargement. Colchicine was dissolved in 0.9% NaCl to a final concentration of 60 μg in 10 μl and slowly infused into the intrathecal space. Twenty-four hours later, animals were perfused and processed for immunohistochemistry.

Tissue preparation. After twenty-four hours (colchicine) or seven days (CTB) of survival, all rats, including naïve ones, were deeply anesthetized using sodium pentobarbital (60 mg/kg, i.p.; Apoteket) and perfused via the ascending aorta with 50 ml of Tyrode's buffer (37 °C), followed by 50 ml of a mixture of 4% paraformaldehyde and 0.2% picric acid dissolved in 0.16 M phosphate buffer (pH 6.9) (Pease, 1962; Zamboni and De Martino, 1967) and 300 ml of the same fixative at 4 °C, the latter for approximately 5–7 min. The lumbar (L1–L6) and thoracic spinal cords (in CTB-treated rats, at the injection site level) were dissected out and postfixed for 90 min at 4 °C in the same fixative, and finally immersed in 10% sucrose in phosphate-buffered saline (PBS) (pH 7.4) containing 0.01% sodium azide (Sigma, St. Louis, MO, USA) and 0.02% Bacitracin (Sigma) (4 °C) for 48 h. After embedding in Tissue-Tek O.C.T. compound (Sakura, Torrence, CA, USA), the spinal cords were parasagittally or transversally sectioned in a cryostat (Microm, Heidelberg, Germany) at 50–60 μm thickness. Twenty micrometer thick, transverse sections of

the thoracic spinal injection site were cut and mounted on aluminum gelatin-coated slides.

Free-floating immunohistochemistry

Single-staining (TSA plus). Cryostat sections were immediately transferred to multidish 24 wells (Nunclon, NUNC, Roskilde, Denmark), rinsed twice in PBS (20 min each) and incubated for 72 h at 4 °C with a rabbit Y1R polyclonal antiserum raised against the C-terminal 13 amino acids of the Y1R protein conjugated to keyhole limpet hemocyanin (Kopp et al., 2002) diluted in 0.01 M PBS containing 0.3% Triton X-100 and 0.5% bovine serum albumin (BSA). Five to 10 spinal cord sections were put into each well, and in all cases 300–500 μl of any solution were used. To visualize the immunoreactivity, the sections were processed using a commercial kit based on TSA (Adams, 1992; TSA Plus, NEN Life Science Products, Inc., Boston, MA, USA). Briefly, the sections were washed twice in TNT buffer (kit; 0.1 M Tris–HCl, pH 7.5; 0.15 M NaCl; 0.05% Tween 20) for 20 min, incubated with TNB buffer (kit; 0.1 M Tris–HCl, pH 7.5; 0.15 M NaCl; 0.5% blocking reagent) for 1 h at room temperature (RT) and then with a swine anti-rabbit/horseradish peroxidase (HRP) conjugate (Dako, Copenhagen, Denmark) diluted 1:400 in TNB buffer for 2 h at 37 °C. The sections were washed twice in TNT buffer (20 min each) and incubated in a biotinyl tyramide-fluorescein isothiocyanate (BT-FITC) conjugate diluted 1:1000 in amplification diluent (kit) for 1 h at RT, then washed twice in TNT (20 min each) and stored in PBS overnight at 4 °C.

Double-staining (TSA plus combined with indirect immunohistochemistry): Y1R plus CGRP or CTB. After 72 h incubation with Y1R antiserum, some sections were washed twice in PBS (15 min each) and further incubated for 24–48 h at 4 °C with guinea-pig anti-CGRP (1:1000; Peninsula Laboratories, Belmont, CA, USA) or goat anti-CTB (1:5000; List-Biological Laboratories Inc.) (tracing experiments) antibodies according to Coons (1958), followed by the TSA plus technique for visualization of the Y1R (as above). After the last wash in TNT and two washes in PBS (15 min each), the sections were further incubated using Texas Red or cyanine 3 (Cy3)-conjugated donkey anti-guinea-pig (1:100 or 1:400, respectively; Jackson Immuno-Research, West Grove, PA, USA) or Cy3-conjugated donkey anti-goat (1:400, Jackson) antibodies, diluted in PBS, for 2 h at 37 °C. Finally, all sections were washed overnight in PBS at 4 °C.

Double-staining ('double' TSA plus): Y1R plus CGRP. After 72 h incubation with Y1R antiserum, sections were processed with the TSA plus technique for its visualization (as above), washed twice in PBS (15 min each) and further incubated for 24 h at 4 °C with a guinea-pig anti-CGRP antibody (1:6000; Peninsula Laboratories). In a following step, sections were processed a second time with the TSA plus method for visualization of CGRP using a donkey anti-guinea-pig/HRP secondary antibody, and a biotinyl tyramide–tetramethylrhodamine (TMR) conjugate diluted 1:600 in amplification diluent (kit) for 1 h at RT. Finally, all sections were washed overnight in PBS at 4 °C.

Immunohistochemistry on slides

Coronal sections of the thoracic spinal cord were incubated with the goat anti-CTB antibody (1:5000; List-Biological), followed by indirect immunohistochemistry (see Coons, 1958) using Cy3-conjugated donkey anti-goat antibody (1:400; Jackson).

In situ hybridization

A probe complementary to the 546–585 nucleotides of the Y1R mRNA (Eva et al., 1990) was used, labeled with ^{35}S-γ-dATP (New England Nuclear, Boston, MA, USA). The lumbar spinal cord from four Sprague–Dawley rats was obtained after decapitation and frozen before transversal sectioning at 14 μm in a cryostat (as above) and mounted on ProbeOn slides (Fisher Scientific, Pittsburgh, PA, USA). After drying overnight, the sections were processed following the protocol by Dagerlind and colleagues (1992). Briefly, the sections were incubated with the Y1R oligoprobe (0.5 ng) diluted in hybridization cocktail. After 18–22 h hybridization in a humid chamber at 42 °C, the sections were washed, rapidly dehydrated and dried at RT. The slides were then dipped in NTB2 photoemulsion (Kodak, Rochester, NY, USA). After appropriate exposure time (4–6 weeks), the sections were developed using Kodak D19 developer and Kodak 3000 fixative and coverslipped using glycerol.

Controls

For control of all antibodies used in the immunohistochemical study, single-stained sections were also processed for comparison with double-stained sections; in a few sections the primary antibody was omitted to test for unspecific staining by the secondary antibodies. The Y1R and CGRP antisera were preadsorbed with an excess (10^{-5} and 10^{-6} M) of the respective antigen. Analysis of the possible coexpression of the Y1R$^+$ with CGRP in the spinal cord was carried out in sections processed using 'double TSA,' since this technique allows a more sensitive detection of any of the markers used in this study. In order to control for the overall efficacy of the technique and eventual cross-reactivity between the antibodies, parallel slides were processed with a combination of TSA plus and regular fluorescence technique. The specificity of the Y1R antiserum has previously been established in tests on Y1R knock-out mice (Kopp et al., 2002). For the *in situ* hybridization control, sections were incubated with a cocktail containing an excess (100×) of unlabeled probe.

Microscopy and image processing

Immunohistochemical analysis. Free-floating sections were mounted on aluminum gelatin-coated slides, coverslipped using 2.5% DABCO in glycerol (Sigma) and examined in a Bio-Rad Radiance Plus confocal scanning microscope (Bio-Rad, Hemel Hempstead, UK) installed on a Nikon Eclipse E600 fluorescence microscope, equipped with 10× (0.45 N.A.), 20× (0.75 N.A.), and 60× oil (1.40 N.A.) objectives. The fluorescein labeling was excited using the 488-nm line of the argon ion laser and detected after passing an HQ530/60 (Bio-Rad) emission filter. For the detection of Texas Red, Cy3 or TMR signals the 543-nm line of the green HeNe laser was used in combination with the HQ590/70 (Bio-Rad) emission filter.

Boundaries of the different layers in longitudinally cut sections of the spinal cord are difficult to define. Nevertheless, some generally accepted landmarks were used in our study in order to indicate the approximate location of Y1R$^+$ neurons in parasagittal spinal cord sections (Naim et al., 1997; Woodbury et al., 2000; Ribeiro-Da-Silva, 2004). Thus, all neuronal cell bodies located 150 μm or more below the dorsal white matter were considered to be in LIII–IV (Naim et al., 1997), whereas profiles present more than 400 μm below were defined as being present in LIV–V. In addition, Y1R$^+$ neurons and processes located deeper than most of the superficial CGRP-LI, the latter in primary afferents known to terminate mainly in LI–II, were considered to be in LIII and deeper (Ribeiro-Da-Silva, 2004). In transverse sections of the spinal cord, boundaries are more easily identified, and we followed the description by Paxinos and Watson (1986), to define the position of Y1R$^+$ neurons analyzed in such sections.

For the measurement of neuronal size in neurons present in different dorsal horn L, the area of all nucleated Y1R$^+$ NPs in a naïve rat was measured using the public domain NIH program (developed at the U.S. National Institutes of Health and available on the Internet at http://rsb.info.nih.gov/nih-image/). A total of 19–21 NPs per LI–II, IV–V and area X were used.

In situ *hybridization analysis.* The sections were examined using a Nikon Eclipse E800 microscope equipped with dark- and brightfield illumination.

Resolution, brightness and contrast of the images were optimized using the Adobe Photoshop 7.0 software (Adobe Systems Inc., San Jose, CA, USA).

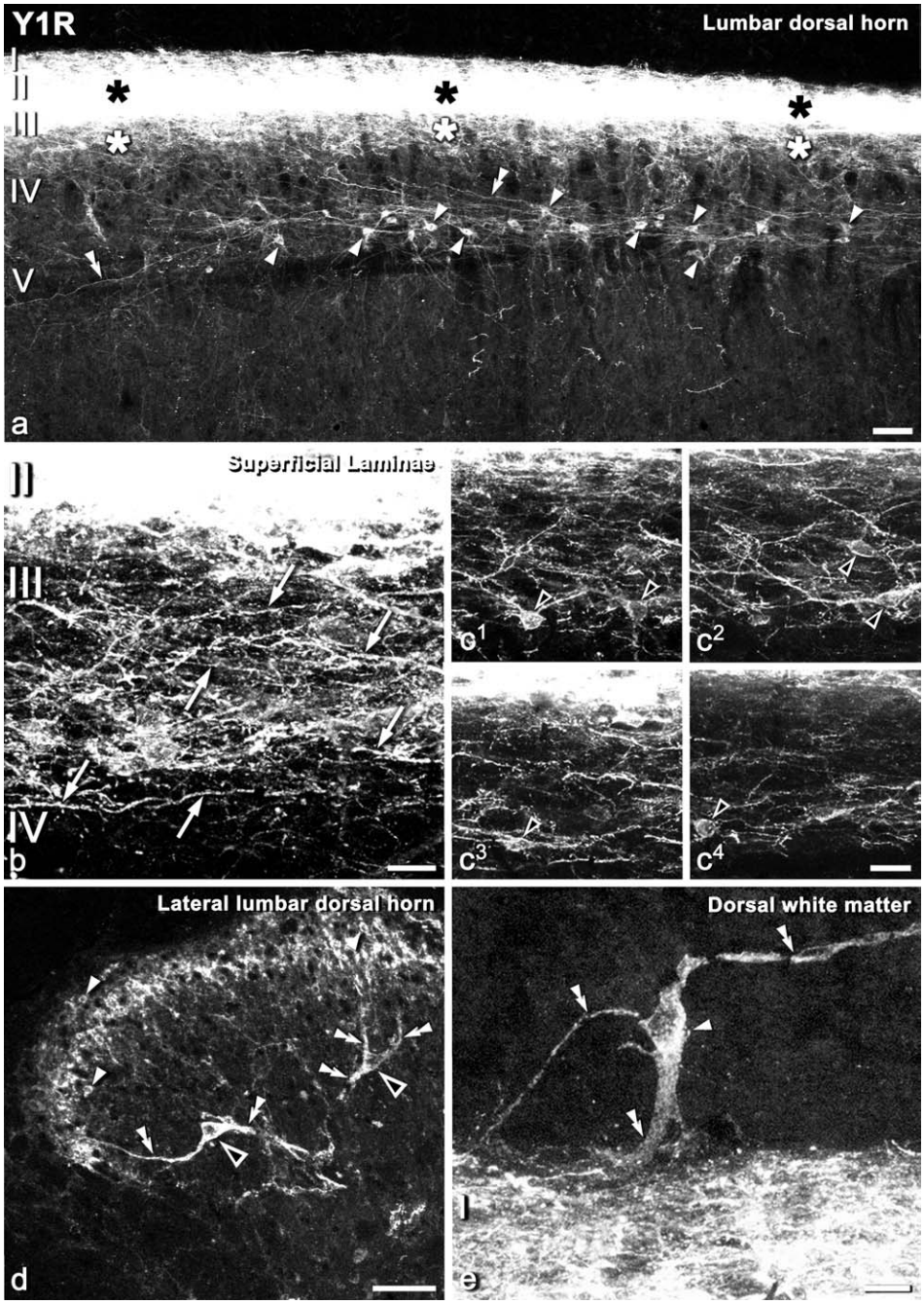

Fig. 1. Confocal immunofluorescence micrographs of parasagittal (a–c^{1-4}, e) and transversal (d) sections of the dorsal horn of the lumbar spinal cord after incubation with Y1R antiserum (a: 20 Z-sections; b: 43 Z-sections; c^{1-4}: single Z-sections; d: 13 Z-sections; e: 38 Z-sections. a–c^{1-4}, e: 1 μm Z-spacing; d: 0.5 μm Z-spacing) (roman numbers, from I to V, indicate approximate limits of dorsal horn L). (a) Note an intense Y1R-LI in LI–II (black asterisks), becoming less profuse in LIII (white asterisks). In addition, there are also Type 5 $Y1R^+$ neurons localized in deeper L (IV–V) (arrowheads), as well as abundant, and sometimes long, $Y1R^+$ processes in the rostro-caudal direction (double arrowheads). (b–c^{1-4}). Considerable Y1R-LI is observed in LIII, encompassing a profuse network of processes (arrows), as well as cell bodies (Type 3 neurons), which are more easily detected when analyzing 1 μm thick optical sections (black arrowheads in c^{1-4}). (d) In colchicine-treated rats, some large $Y1R^+$ neurons (Type 4) are easily detected in the border area between LIII–IV (black arrowheads), and these produce a number of projections, some of them directed toward LI–II (double arrowheads). Small $Y1R^+$ neurons (Type 1) are also observed in LI–II (white arrowheads). Note a considerable decrease in Y1R-LI in LI–II, probably derived from the colchicine-treatment. (e) An isolated $Y1R^+$ neuron in the Lissauer's tract is shown (arrowhead), producing several processes (double arrowheads). Scale bars=100 μm (a, e); 25 μm (b–$c^{4=c1-3}$); 50 μm (d).

RESULTS

LI–II

Intense Y1R-LI was observed in LI–II (Fig. 1a, b). This immunohistochemical pattern represented cell bodies and processes of numerous Y1R$^+$ neurons, present in the highest numbers in LII (Fig. 2a, b^{1,2}). The great majority of these neurons was tightly packed and embedded in abundant, strongly Y1R$^+$ processes, and was more easily identifiable by confocal analysis of thin optical sections (Fig. 2a, b^{1,2}). These Y1R$^+$ neurons had a fusiform shape and rapidly dividing bipolar processes (Fig. 2b^{1,2}) and a cross sectional area between 120 and 200 μm^2. The receptor was almost always in close relation to the plasma membrane (Fig. 2a, b$^{1-2}$). In addition, a few large Y1R$^+$ neurons were detected in the dorsal white matter (Fig. 1e) and in LI after colchicine treatment (data not shown).

LIII–IV

Two types of Y1R$^+$ neurons were detected in LIII–IV. First, mostly in LIII, many neurons similar to those in LI–II but more sparsely distributed, and producing a less dense Y1R$^+$ neuropil were observed (Figs. 1a, b, c$^{1-4}$; 3a, b). They exhibited similar morphological characteristics, including membrane-bound Y1Rs. Some neurons also showed a round shape (Fig. 1b, c$^{1-4}$). The second type was located in the border area between LIII and LIV (200–300 μm below the dorsal white matter). In most cases, these neurons exhibited one or more thick dendrites directed toward LI–II (Figs. 1d; 3c; 4g, h), and sometimes showed a presumable axon directed toward deeper layers of the dorsal horn (Fig. 4g, h). In colchicine-treated rats, this type of multipolar Y1R$^+$ neurons was more frequently observed (Fig. 1d). The Y1R-LI was mainly associated with the plasma membrane (Fig. 1d).

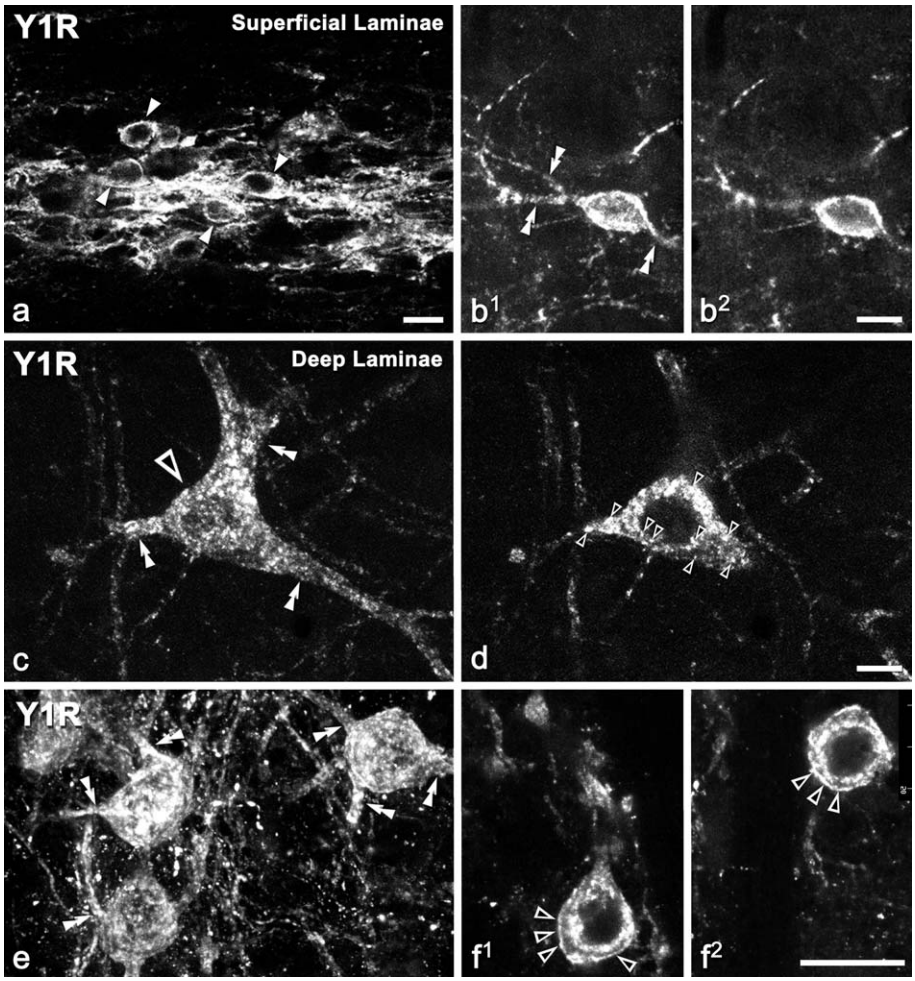

Fig. 2. Confocal immunofluorescence micrographs of parasagittal sections of the dorsal horn of the lumbar spinal cord after incubation with Y1R antiserum (a: single Z-section; b$^{1-2}$, c, d, f$^{1-2}$: see below; e: 33 Z-sections. In all cases, 1 μm Z-spacing). (a) Many Y1R$^+$ NPs are observed in LII (Type I neurons) (arrowheads). (b$^{1-2}$) Confocal analysis of a single Type 1 Y1R$^+$ neuron in LII shows the fusiform shape of these frequently bipolar neurons (double arrowheads) (b^1; 9 μm optic section). Also characteristic is the Y1R$^+$ membrane staining (b^2; 3 μm thick optic section). (c, d) A multipolar, Type 5 Y1R$^+$ neuron (black arrowhead), exhibiting several projections (double arrowheads) (c; 17 μm thick optic section). Confocal analysis of a 5 μm thick optical section of the same neuron as in (c) shows a granular Y1R$^+$ staining spread through the cytoplasm (black arrowheads in d). Note no distinct membrane staining. (e, f$^{1-2}$) Some Type 5 dorsal horn neurons, with multipolar processes (double arrowheads) also show membrane staining (black arrowheads) (f$^{1-2}$: 7 μm thick optic section). Scale bars=10 μm (a; b$^{1-2}$, d=c); 25 μm (f^2=e, f^1).

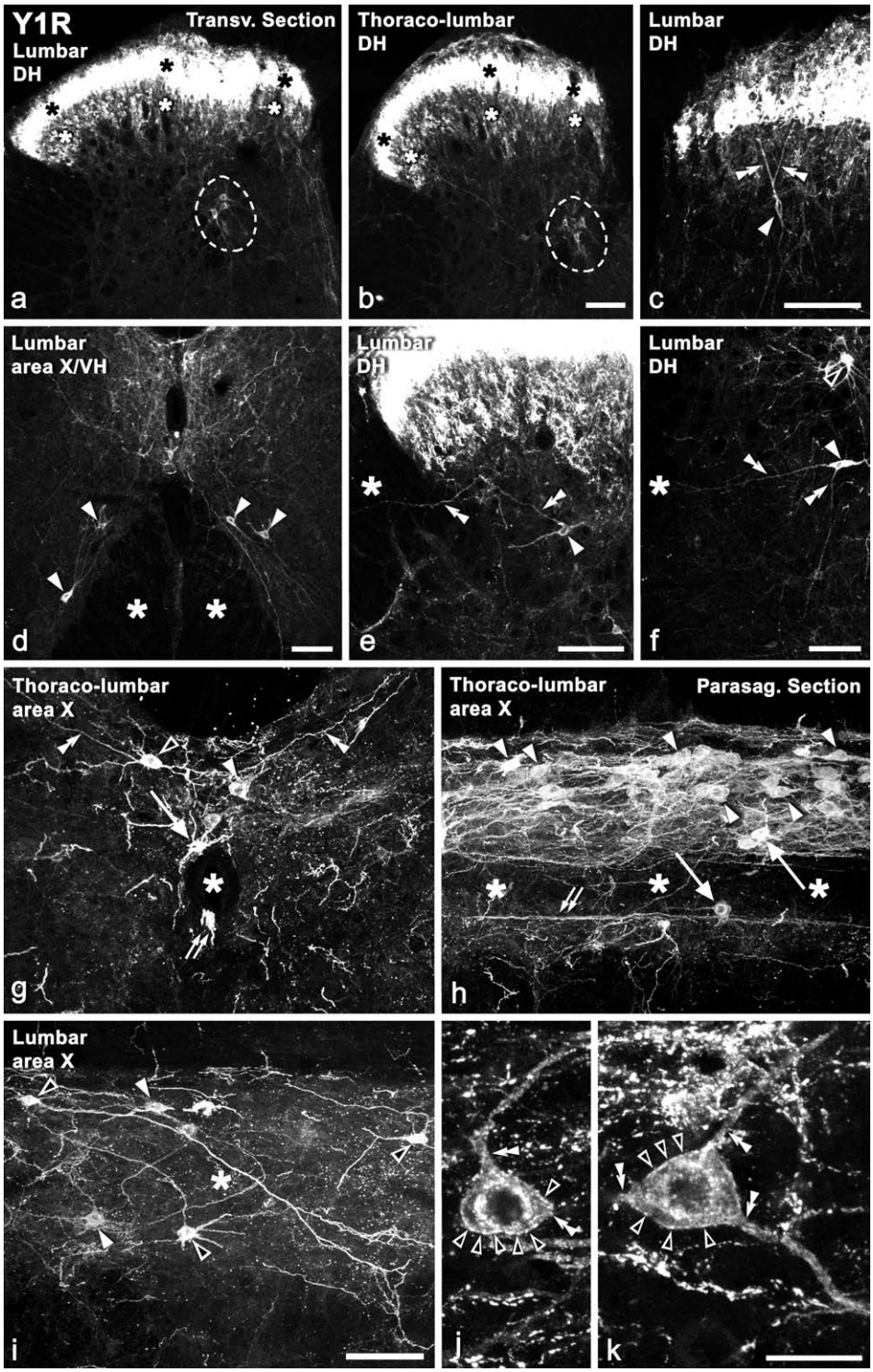

Fig. 3. Confocal immunofluorescence micrographs of transverse (a–g) and parasagittal (h–k) sections of the dorsal (a–c, e, f) and ventral (d) horns and area X (g–k) at lumbar (a, c–f, i–k) and thoraco-lumbar (b, g, h) spinal cord levels, after incubation with Y1R antiserum (a: 16 Z-sections; b: 17 Z-sections; c: 13 Z-sections; d: 27 Z-sections; e: 31 Z-sections; f: 23 Z-sections; g: 17 Z-sections; h: 18 Z-sections; I: 22 Z-sections; j, k: 5 Z-sections. a–d, f–k: 1 μm Z-spacing; e: 0.7 μm Z-spacing). (a, b) Transverse sections of the dorsal horn at lumbar (a) and thoraco-lumbar (b) levels show intense Y1R-LI in LI–II (black asterisks), becoming less profuse in LIII (white asterisks). In addition, a cluster of Type 5 $Y1R^+$ NPs is observed in deeper L (dotted circle). This group of neurons seems to be more lateral in lumbar (a) than thoraco-lumbar (b) levels. (c) A Type 4 $Y1R^+$ neuron, with its cell body located in the border area between LIII–IV (arrowhead) sends two presumably dendritic processes toward the superficial layers of the dorsal horn (double arrowheads). (d) Also in the ventral horns, in LVIII, a number of $Y1R^+$ neurons (Type 7) are observed, in most cases medially located and close to the medial aspects of the ventral funiculus (asterisks). (e, f) Two Type 5 $Y1R^+$ neurons present in LIV (e) and deeper L (f) produce long

LIV–V

Another subpopulation of Y1R$^+$ neurons was detected in deep L, 400–500 μm or more below the dorsal white matter (Figs. 1a; 3a, b). Interestingly, many of these neurons seemed to be grouped in a 'cluster,' clearly seen in longitudinal sections of the spinal cord (Fig. 1a). Analysis of transversal sections at lumbar (Fig. 3a) and thoraco-lumbar levels (Fig. 3b) confirmed this view, and it could be seen that these neurons were usually grouped in the mid/medial aspects of, most likely, LV (Fig. 3a, b). The cell bodies of many of these neurons had a round, sometimes elongated, shape (Figs. 1a; 2e; 3c; 4g, h), whereas others were characterized by a pyramidal shape (Fig. 2c, d). In most cases, these neurons were multipolar (Figs. 2c, e; 4g, h), consistently larger (areas ranging from 190 to 690 μm^2) than the small Y1R$^+$ neurons described in LI–II. In addition, some neurons also exhibited transversally oriented (axonal?) processes reaching the dorso- (Fig. 3e) and ventrolateral (Fig. 3f) funiculus of the spinal cord.

The clustered Y1R$^+$ neurons were frequently embedded in a Y1R$^+$ plexus (Figs. 1a; 2e) and exhibited a granular cytoplasmic staining, distinctly shown by analysis of 1 μm confocal optical sections (Fig. 2c, d). Other neurons had a membrane staining (Fig. 2f$^{1-2}$) similar to that observed in LI–II neurons (cf. Fig. 2b$^{1-2}$, f$^{1-2}$ with 2c, d).

Area X

A number of Y1R$^+$ neurons were observed around the central canal (area X) in transverse (Fig. 3g) and parasagittal (Fig. 3h, i) thoraco-lumbar sections. These neurons sent out and/or were surrounded by transversally (Fig. 3g, i) and longitudinally (Fig. 3h, i) oriented Y1R$^+$ processes, some extending several hundred micrometers (Fig. 3g–i). The great majority of these neurons was located dorsal to the central canal (Fig. 3g, h) and had areas ranging from 260 to 580 μm^2. In fact, some were found immediately adjacent to the basal part of ependymal cells (Fig. 3g, h). Also, a thick ventral, parasagittally running Y1R$^+$ fiber bundle was observed in this area, bordering the central canal and usually detected in sections cut through the widest part of the canal (Fig. 3g, h; 6a, b–d). Y1R$^+$ neurons were also detected in area X of the lumbar enlargement, although they seemed to be less numerous in this location (cf. Fig. 3d and 3g, h).

Finally, a number of processes passed through the layer of ependymal cells around the central canal and terminated with an expansion within the lumen of the central canal (Fig. 6e–g).

In situ hybridization studies confirmed presence of the Y1R in the superficial L of the dorsal horn, and occasional Y1R mRNA$^+$ cell bodies were detected in deeper layers (data not shown).

Ventral horns

A small number of Y1R$^+$ neurons with processes of variable length was seen in the medial aspect of the ventral horns, corresponding to LVIII (Fig. 3d). In most cases, these neurons were large and in close proximity to the medial aspects of the ventral funiculus.

Colocalization with CGRP

Colocalization analysis (Fig. 4a–i) showed abundant overlap of CGRP$^+$ and Y1R$^+$ staining in LI–II. However, coexistence of receptor and peptide could not be established in these layers. CGRP was much less abundant in LIII and most of the immunostaining in this layer corresponded to Y1R$^+$ NPs and processes, with virtually no overlap (Fig. 4a–f). However, CGRP$^+$ fiber bundles could be seen penetrating into deep layers (Fig. 4c, f, i), sometimes surrounding deeply localized Y1R$^+$ neurons (Fig. 4g–i). Occasional, apparently Y1R$^+$/CGRP$^+$ fibers could be detected in the dorsal roots (Fig. 5a–c) and also in LIII (Fig. 5d–f).

In area X, and parallel to the parasagittally running Y1R$^+$ bundles (Fig. 6c), CGRP$^+$ (Fig. 6d) fibers were also observed, but they were almost never Y1R$^+$ (Fig. 6a–d).

CTB tracing studies

A considerable number of the clustered LIV–V Y1R$^+$ neurons showed CTB labeling seven days after injection of the tracer into the 9th thoracic segment of the spinal cord (Fig. 7a–c; 8a, b). Double-labeling experiments showed that several CTB$^+$ neurons were Y1R-negative (Fig. 7a–c).

CTB$^+$ neurons were also observed in superficial L, sometimes even in the dorsal white matter (Fig. 8d), and occasionally some LI neurons were also Y1R$^+$ (Fig. 8c–f). In area X, a small number of Y1R$^+$ neurons showed CTB staining (Fig. 8g, h).

Analysis of coronal sections at the site of CTB injection in the left thoracic hemi-segment revealed that the two rats with the most 'effective' retrograde CTB staining at lumbar levels had a spread of tracer into the right hemisection. This included the dorsal, dorsolateral or ventrolateral funiculus, as well as the dorsal and ventral horns. In the other two rats, most of the tracer spread into the dorsal and dorsolateral funiculus, as well as the dorsal horn. In all rats, the labeling

processes (double arrowheads) projecting toward the dorso- (e) and ventrolateral (f) funiculus. Asterisks show the lateral white matter. Black arrowhead in (f) shows one Type 5 Y1R$^+$ neuron. (g) Several Type 6 Y1R$^+$ neurons (arrowheads) are detected in area X, dorsal to the central canal (asterisk), and some are seen immediately adjacent to the ependymal cell layer (arrow). Transversally running processes are abundant (double arrowheads); sometimes they can be shown to originate from Y1R$^+$ neurons (black arrowhead). Double arrows show a Y1R$^+$ bundle running ventral to the central canal (see h). (h, i) Parasagittal sections of area X show many Type 6 Y1R$^+$ neurons (white arrowheads) located dorsal to the central canal (asterisk). As in (g), occasional Y1R$^+$ neurons lie very close to the ependymal cell layer (arrows in h). These neurons are embedded in a profuse Y1R$^+$ neuropil. Many of these fibers can be seen originating from Y1R$^+$ neurons (black arrowheads in i). In addition, a Y1R$^+$ bundle runs parallel to the central canal along its ventral aspect (double arrows in g, h). (j, k) In most cases, Type 6 Y1R$^+$ neurons in area X have several processes (double arrowheads) and exhibit membrane staining (black arrowheads), in addition to a Golgi-like staining around the nucleus (j, k: 7 μm thick optic sections). Scale bars=100 μm (b=a; c; d; e; f; i=g, h); 25 μm (j, k).

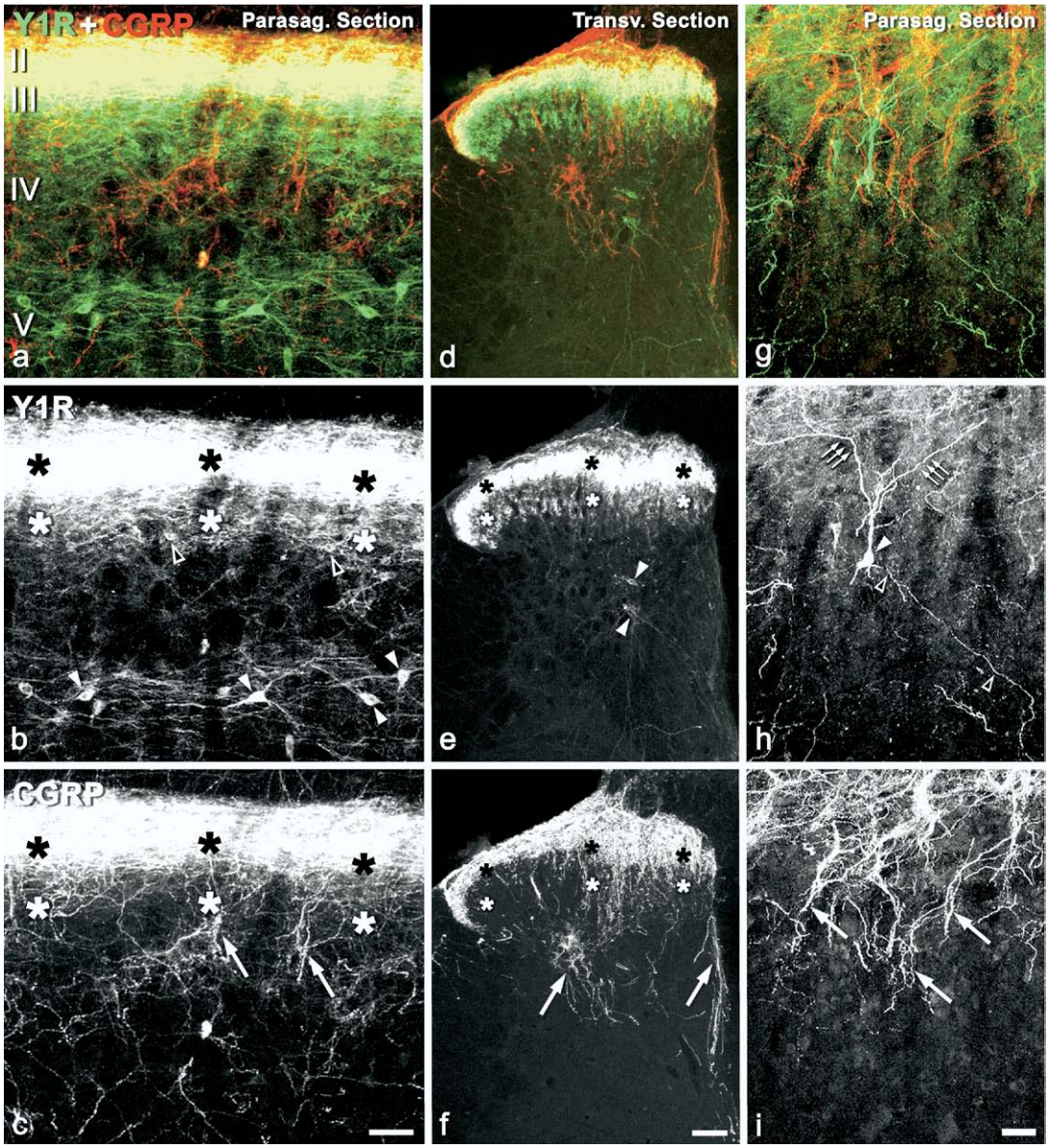

Fig. 4. Confocal immunofluorescence micrographs of parasagittal (a–c; g–i) and transverse (d–f) sections of the dorsal horn of the lumbar spinal cord after incubation with Y1R and CGRP antisera (a, d, g show merged micrographs) (a–c: 40 Z-sections; d–f: 28 Z-sections; g–i: 43 Z-sections. a–c: 0.5 μm Z-spacing; d–f: 1 μm Z-spacing; g–i: 0.5 μm Z-spacing) (roman numbers, from I to IV, indicate approximate limits of dorsal horn L). (a–f) An intense $Y1R^+$ and $CGRP^+$ neuropil is observed in LI–II (black asterisks in b, c, e, f). In LIII (white asterisks), a less profuse Y1R-LI is observed, and $Y1R^+$ neurons are frequently detected (black arrowheads). In deeper layers, a considerable number of $Y1R^+$ neurons is observed (white arrowheads in b, e) as well as $CGRP^+$ fiber bundles extending ventrally for several hundreds of micrometers (arrows in c, f). (g–i) A $Y1R^+$ neuron (arrowhead in h) with distinct dendritic arborizations in superficial L (triple arrows in h) and, possibly, an axonal process directed to deeper layers (black arrowheads in h). This neuron is occasionally reached by $CGRP^+$ fibers (arrows in i). Scale bar=100 μm (c=a, b; f=d, e); 50 μm (i=g, h).

extended rostro-caudally for about 1.5 mm in each direction from the injection site (data not shown).

DISCUSSION

In the present study $Y1R^+$ neurons in 'superficial' (LI–II), 'intermediate' (LIII) and 'deep' L of the rat dorsal horn (IV–V), as well as in area X and the ventral horns (LVIII) are described with regard to localization and morphology, thus defining several NPY-sensitive spinal systems. These neurons exhibit different shapes, as well as subcellular distribution patterns of Y1R-LI (membrane vs. cytoplasmic staining), and extend processes with different orientations, depending on their location in the gray matter. In fact, taken together, our results tentatively suggest presence of at least seven different $Y1R^+$ neuron populations (Types 1–7) in the spinal cord. Thus, embedded into an intricate $Y1R^+$ neuropil in LI–II, abundant small, bipolar neurons were observed (Type 1). They have a fusiform-like shape, producing rapidly branching processes mainly oriented in

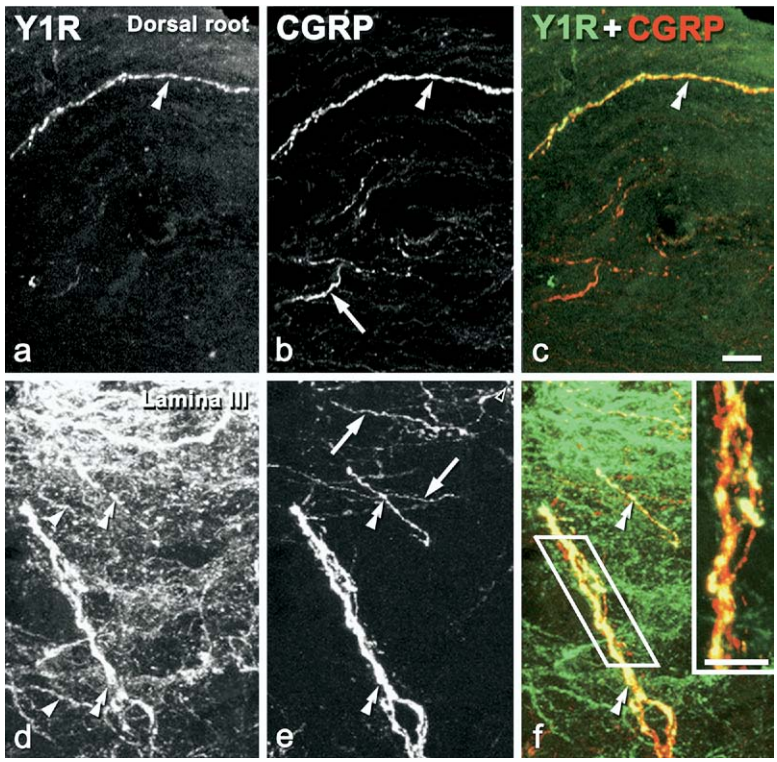

Fig. 5. Confocal immunofluorescence micrographs of parasagittal sections of the dorsal roots (a–c) and LIII (d–f) of the dorsal horn of the lumbar spinal cord after incubation with Y1R and CGRP antisera (c, f show merged micrographs) (a–c: 23 Z-sections; d–f: 56 Z-sections; inset in f: 51 Z-sections. a–f: 0.5 μm Z-spacing; inset in f: 0.2 μm Z-spacing). (a–c) Occasional CGRP$^+$/Y1R$^+$ fibers are seen in the dorsal roots (double arrowhead), close to the superficial L. In addition, CGRP$^+$ thin fibers are also seen (arrow in b). (d–f) Rare, apparently, Y1R$^+$/CGRP$^+$ double-stained fibers can also be seen in LIII (double arrowheads in d–f), along with single stained Y1R$^+$ (arrowheads in d) and CGRP$^+$ (arrows in e) fibers. Scale bars=20 μm (c=a, b; d–f); 10 μm (inset in f).

the longitudinal axis (Zhang et al., 1994a, 1999). There were also somewhat larger superficial neurons, which were retrogradely labeled with CTB and thus considered projection neurons (Type 2). The few neurons located in the dorsal white matter, and some larger Y1R$^+$ neurons seen after colchicine-treatment (unpublished data) in LI will not be 'typed' at this point. In LIII we found a considerable number of small fusiform Y1R$^+$ neurons (Type 3), similar to the Type 1 neurons in LI–II, but fewer and producing a less profuse neuropil. In the border area between LIII–IV, we detected a number of large Y1R$^+$ neurons, especially after colchicine-treatment, exhibiting a pyramidal-like shape and extending dendritic processes toward the superficial layers of the dorsal horn (Type 4). Deeper in the dorsal horn, in the border area between LIV and LV, there was a further Y1R$^+$ system. These neurons were either round- or pyramidal-like, and intermingled with Y1R$^+$ processes, mostly oriented along the longitudinal axis of the spinal cord (Type 5). A considerable number of Y1R$^+$ neurons was detected in area X producing a fiber-network oriented in the transversal and longitudinal axis (Type 6). Finally, there was a small number of neurons expressing the receptor (Type 7) in the medial aspects of the ventral horns (LVIII). A further unexpected finding was Y1R$^+$ processes passing between the ependymal cells and terminating with large 'boutons' in the spinal canal. Presence of some Y1R$^+$ fibers located in the dorsal roots and dorsal horn exhibited CGRP-LI, supporting a previous study on the presence and central transport of the Y1R in a number of primary afferents (Brumovsky et al., 2002).

The spinal cord contains many different subpopulations of Y1R$^+$ neurons

LI–II (Types 1 and 2). The presence of a subpopulation of small Y1R$^+$ neurons localized in the superficial dorsal horn has previously been well documented (Zhang et al., 1994a,b, 1995a, 1999; Brumovsky et al., 2002), and ~70% of them colocalize somatostatin (SOM) (Zhang et al., 1999). Many of these neurons, especially those found in LII, here labeled Type 1 neurons, are presumably interneurons. This is supported by studies showing that in general only few neurons present in this L project to upper levels of the neural axis, namely the caudal ventrolateral reticular formation (Lima and Coimbra, 1991). In contrast, subpopulations of neurons in LI project to several different brainstem and diencephalic areas (see Grant and Koerber, 2004). In fact, we occasionally found Y1R$^+$ neurons with a fusiform shape in LI, which exhibited retrograde labeling after CTB injection at the level of the 9th thoracic spinal cord segment. These neurons may represent a small subpopulation of superficial Y1R$^+$ projection neurons and are here termed Type 2 neurons. Fusiform cells in LI have been

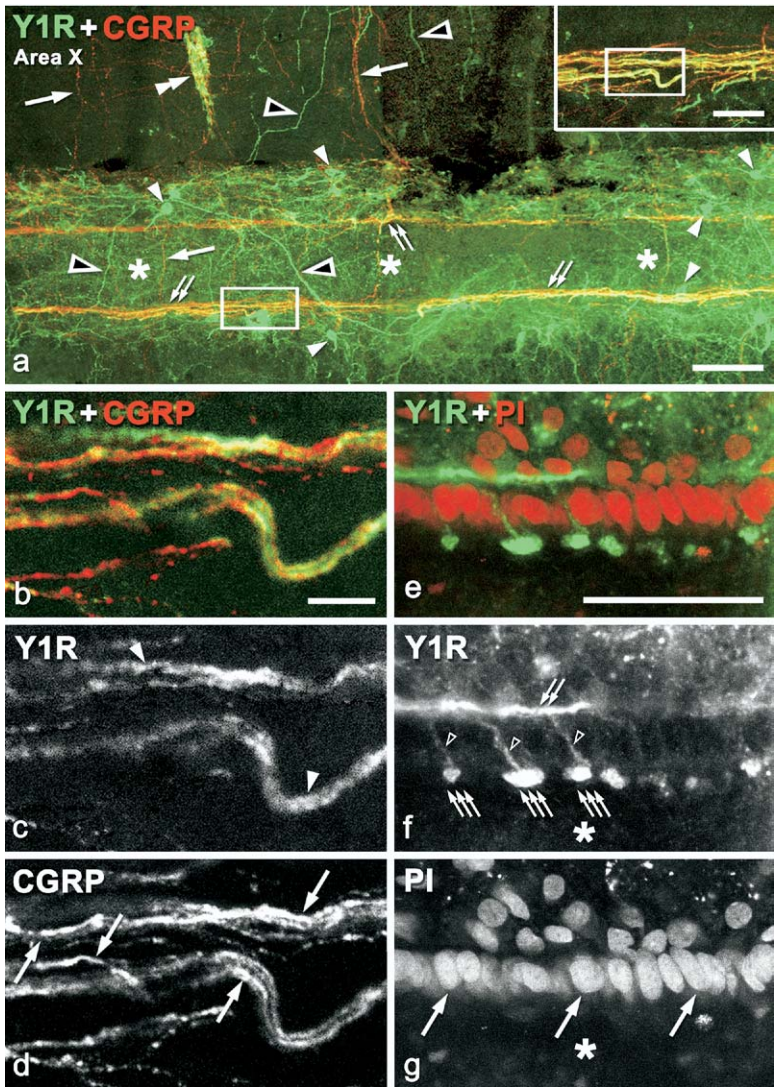

Fig. 6. Confocal immunofluorescence micrographs of parasagittal sections of area X of the lumbar spinal cord, after incubation with Y1R (a–g) or CGRP (a–d) antisera, or propidium iodide (PI) (e–g) (a, b, e show merged micrographs) (a: 51 Z-sections; b–d: five Z-sections; e–g: six Z-sections; inset in a: 51 Z-sections. a–d, inset in a: 0.2 μm Z-spacing; e–g: 0.4 μm Z-spacing). (a) Several Type 6 $Y1R^+$ neurons are detected in area X (white arrowheads), close to the central canal (asterisks). Transversally (black arrowheads) running processes are often seen, sometimes extending for several micrometers. In addition, several single-stained $CGRP^+$ fibers are detected (arrows). Also, fiber bundles in deep L (double arrowhead) and dorsal and ventral to the central canal (double arrows) appear to colocalize CGRP and the Y1R. (b–d) However, a close look at the bundles associated with the central canal shows that independent $CGRP^+$ fibers (arrows in d) are running in very close association to the $Y1R^+$ bundles (arrowheads in c). (e–g) A number of fine $Y1R^+$ processes (black arrowheads) passing through the ependymal cells layer (arrows in g) around the central canal (asterisk in f, g) are seen. These processes seem to expand into a large varicose structure within the central canal (triple arrows). Fibers probably associated with the $Y1R^+$ bundle described in 'a' are also seen (double arrows). Scale bars=100 μm (a); 50 μm (e=f, g); 33 μm (inset in a); 8 μm (b=c, d).

found to respond to nociceptive stimuli (Han et al., 1998), and it would be interesting to know, if such neurons also express the Y1R. In both Type 1 and 2 neurons the Y1R is membrane-associated.

In addition to Types 1 and 2 neurons, a few larger $Y1R^+$ neurons were observed in the dorsal white matter. The nature of these neurons is unknown. Nevertheless, previous studies have analyzed the presence of a number of neurons present in the dorsal white matter, perhaps associated with muscle sensitivity (see Light and Perl, 2003 and references therein). Further research is required to establish an association of 'our' $Y1R^+$ neurons with such an activity.

LIII–IV (Types 3 and 4). The expression of the Y1R in LIII was more discrete as compared with LII. The majority of the $Y1R^+$ neurons in LIII, here named Type 3 neurons, was similar in shape to the ones in LII, exhibited Y1R-LI associated with the plasmalemma and were embedded in a $Y1R^+$ neuropil of presumably local origin. As indicated above, a large percentage of the $Y1R^+$ neurons present in LI–II also synthesize SOM (Zhang et al., 1999). Also some SOM^+ neurons in outer LIII have been described (Proudlock et al., 1993). However, the possible colocalization between Y1R and SOM in Type 3 neurons has not been explored.

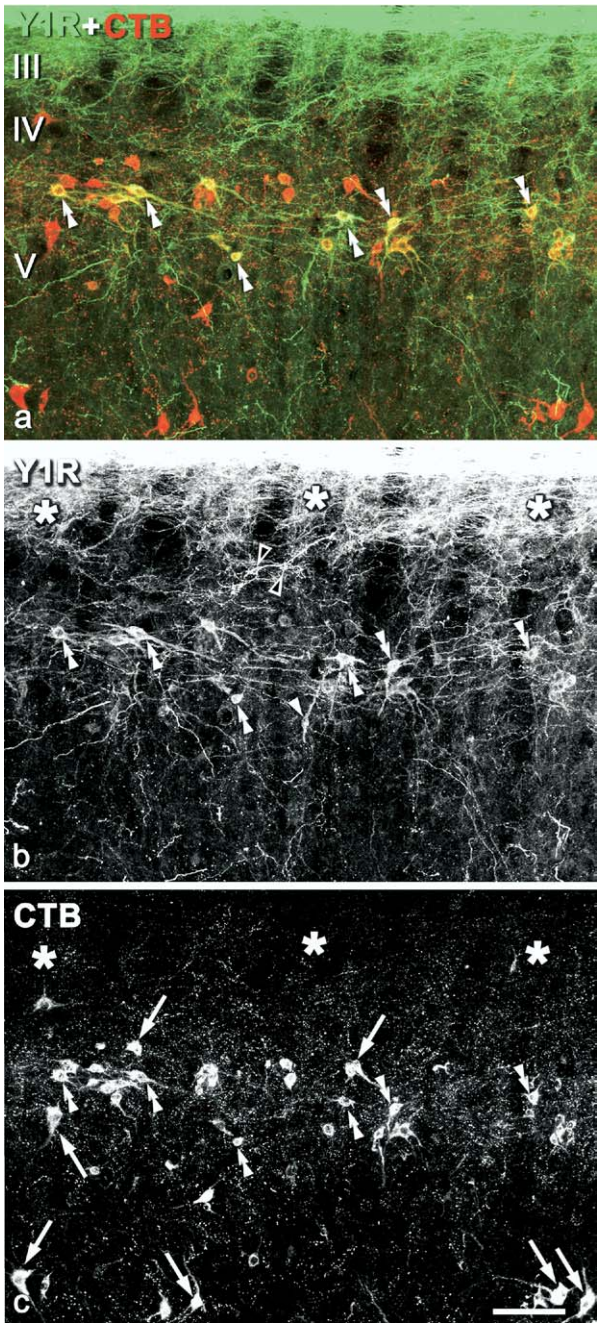

Fig. 7. Confocal immunofluorescence micrographs of parasagittal sections of the dorsal horn of the lumbar spinal cord after 7 days' infusion of CTB at Th9 (a–c) and after incubation with Y1R and CTB antisera (a shows merged micrographs) (a–c: 20 Z-sections, 1 μm Z-spacing) (roman numbers, from III to V, indicate approximate limits of dorsal horn L). Many CTB$^+$ neurons are detected, particularly abundant in the border area between LIV and LV (arrows in c). In addition, several Type 5 Y1R$^+$ neurons are seen, and many colocalize CTB (double arrowheads). In LIII (white asterisks in b, c), single-stained Type 3 Y1R$^+$ neurons are also seen (black arrowheads). Scale bars=100 μm (c=a, b).

Some neurons, located in the border area between LIII and LIV, had dendrites projecting toward superficial L and were more easily detected in colchicine-treated animals. These neurons are here grouped together as Type 4 neurons. They have large cell bodies, a distinct Y1R-decorated dendritic tree extending dorsally and were usually found ~200–300 μm below the dorsal white matter. In previous studies, a subpopulation of large neurons mostly localized in LIII–IV (~150–300 μm deep), but also detected in LVI were shown to express NK1R, the receptor binding substance P (Mantyh et al., 1995; Marshall et al., 1996; Allen et al., 1997; Naim et al., 1997; Todd et al., 2000). NK1R-expressing neurons have been shown to be fundamental for the transmission of pain information in the rat spinal cord (see Mantyh, 2002). In many cases, these NK1R$^+$ neurons exhibit a thick dendritic process, oriented toward superficial L and have a profuse branching in LI–II (Littlewood et al., 1995; Mantyh et al., 1995; Naim et al., 1997). Interestingly, it has been reported that these neurons are densely innervated by local inhibitory neurons containing GABA and NPY (Polgár et al., 1999; see Todd, 2002), suggesting their likely expression of NPY receptors. It could thus be possible that at least some of 'our' Type 4 Y1R$^+$ neurons also express NK1Rs, but this has not been analyzed in the present study. Another issue is to what extent our antibody directed against the Y1R is not sufficiently sensitive to 'decorate' the full extent of the branches of the Type 4 neurons in superficial layers, as does the NK1R antibody. In addition, it is possible that the Y1R is mostly associated with the soma and the initial dendritic segments, while the NK1R is present both in soma and the dendritic tree.

LIV–V (Type 5). Occurrence of Y1R$^+$ neurons localized in the very deep layers of the rat dorsal horn (usually 400–600 μm below the dorsal white matter) was only recently reported in a preliminary form (Brumovsky et al., 2002). Here, by employing free-floating immunohistochemistry, we show that these 'deep', large, multipolar Y1R$^+$ neurons often exhibit a 'granular' pattern of Y1R-LI in their cytoplasm, in contrast to the membrane-staining observed in Types 1–4 and 6–7 Y1R$^+$ neurons and in DRG neurons of rat and mouse (Zhang et al., 1994a,b; Shi et al., 1998; Zhang et al., 1999). Many Type 5 Y1R$^+$ neurons were clustered primarily in the mid/medial aspect of LIV–V, as seen in transverse and longitudinal sections of the spinal cord, where they extended for several hundred micrometers.

Type 5 Y1R$^+$ neurons exhibited rostrocaudally oriented dendritic processes and could represent a subpopulation of interneurons modulating the electrical activity within the same spinal segment. In fact, it has been shown in monkey and cat that cells in LIII could have such a projection pattern (Light and Kavookjian, 1988). However, neurons in this group may also belong, at least in part, to the subpopulation of projection neurons present in deep L (see Grant and Koerber, 2004), and reaching the thalamus through the spinothalamic tract (see Willis and Coggeshall, 2004). In fact, our retrograde tracing analysis shows that many of them project at least to Th9 or close to that. Further studies are needed to demonstrate, if 'our' CTB-labeled Type 5 Y1R$^+$ neurons project to supraspinal regions and what their functional role could be.

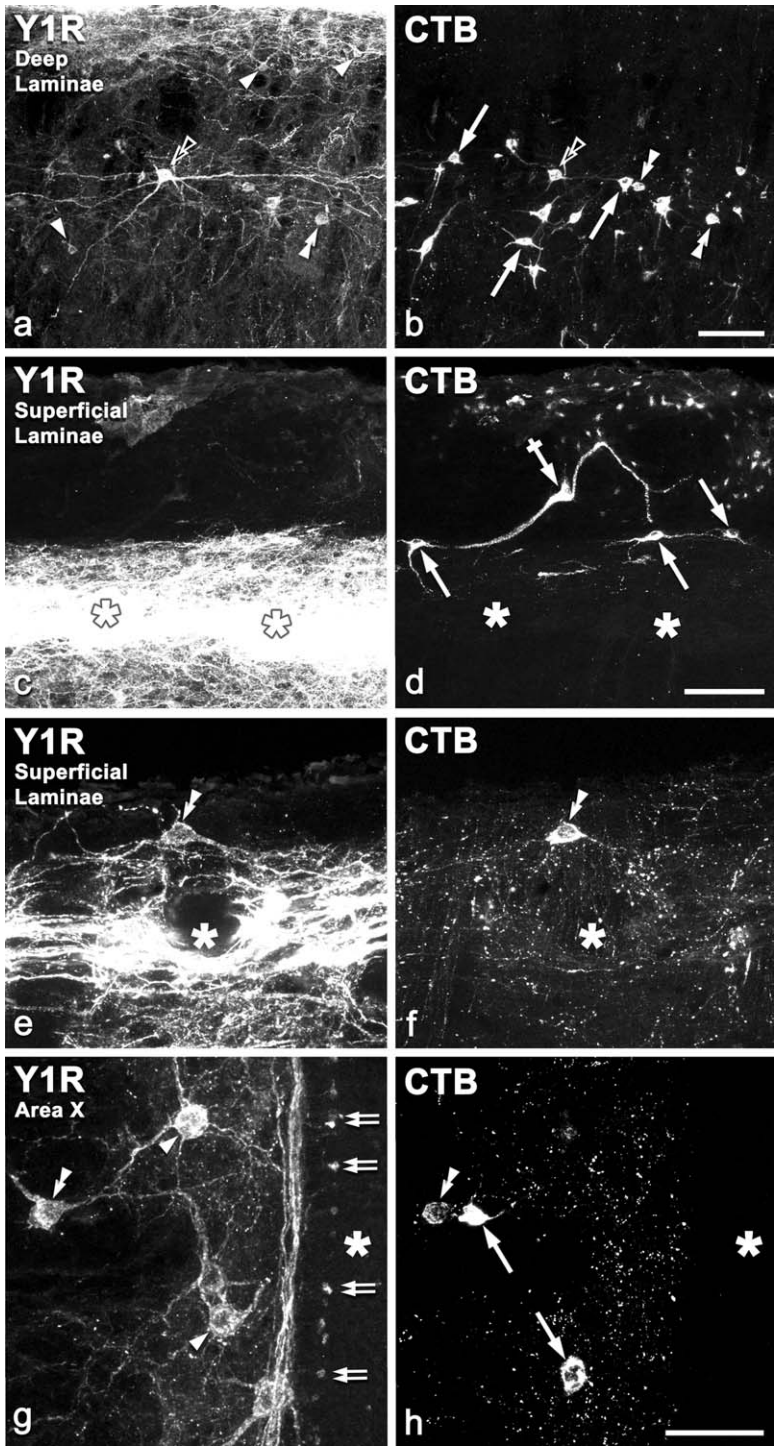

Fig. 8. Confocal immunofluorescence micrographs of parasagittal sections of the deep (a, b) and superficial (c–f) layers of the dorsal horn and area X (g, h) of the lumbar spinal cord 7 days after infusion of CTB at Th9 and after incubation with Y1R and CTB antisera (a, b: 27 Z-sections; c, d: 30 Z-sections; e, f: 13 Z-sections; g, h: 18 Z-sections. a–d: 0.8 μm Z-spacing; e, f: 1 μm Z-spacing; g, h: 1.5 μm Z-spacing). (a, b) A multipolar Type 5 $Y1R^+$ neuron produces several long parasagittal processes in deep layers and shows CTB staining (black double arrowhead in a, b). Other $Y1R^+$/CTB^+ neurons are also detected (white double arrowheads in a, b), as well as a number of single-stained $Y1R^+$ (arrowheads in a) or CTB^+ (arrows in b) neurons. (c, d) Intense Y1R-LI is observed in LI–II (asterisks). CTB^+ neurons are detected in LI (arrows in d), and occasionally in the dorsal white matter (crossed arrow in d). (e, f) A single Type 2 $Y1R^+$ neuron in LI–II (asterisks) shows CTB staining (double arrowhead in e, f). (g, h) In area X, some Type 6 $Y1R^+$ neurons close to the central canal (asterisk) exhibit CTB-LI (double arrowhead in g, h), but some single-labeled $Y1R^+$ (arrowheads in g) and CTB^+ (arrows in h) neurons are seen. $Y1R^+$ processes terminating in an expansion within the central canal are seen (double arrows in g). (g) And (h) have been rotated 90°, and dorsal is to the right. Scale bars=100 μm (b=a; d=c), 50 μm (h=g, e, f).

The Type 5 Y1R$^+$ neurons described in the present study seem both topographically, and morphologically different from the NK1R$^+$ neurons, as discussed above.

Area X (Type 6). Here we observed an abundant number of Y1R$^+$ multipolar neurons establishing a profuse network around the central canal. Some of these neurons were also retrogradely labeled, suggesting them to be projection neurons. Neurons in this area coexpress CCK and galanin and project contralaterally to the ventral posterior thalamus (Ju et al., 1987), and they also contain enkephalin (Nicholas et al., 1999). They may be important in pain processing, particularly for the transmission of visceral pain (Wang et al., 1999). More recently, these CCK/galanin/enkephalin neurons have been shown to express the NK1R (Truitt and Coolen, 2002). Deletion of these neurons by use of a saporin-substance P conjugate has provided evidence for their involvement in the ejaculation process (Truitt and Coolen, 2002).

An interesting finding was the presence of large Y1R$^+$ 'boutons' (diameter up to 11 μm) in the central canal lying on top of the ependymal cells, apparently connected to processes running just on the basal side of these cells. These receptors could thus 'sense' NPY levels in the spinal fluid. In fact, presence of NPY was early on shown in human lumbar cerebrospinal fluid (Berrettini et al., 1986) with a possible relation to mental disease (Widerlöv et al., 1988), as well as in animals (McDonald et al., 1988).

Ventral horn (Type 7). It has been previously shown in rats that a considerable number of neurons present in the medial intermediate gray matter and the ventral horn at the cervical and lumbar enlargements contributes to the production of the spinothalamic tract (Granum, 1986; Burstein et al., 1990; see Willis and Coggeshall, 2004). In the present study, we show a small number of Y1R$^+$ neurons present in this lumbar region, corresponding to LVIII. The nature of these neurons is unknown, but they may project to the thalamus and have a role in the transmission of pain and temperature sensations (Edinger, 1889, 1890; see Willis and Coggeshall, 2004). In fact, previous studies have shown that some cells in this L project to the brain stem reticular formation (Chaouch et al., 1983; Shokunbi et al., 1985) or the thalamus (Burstein et al., 1990). Nevertheless, it is also possible that these 'ventral' Y1R$^+$ neurons could be interneurons and modulate the activity of motoneurons located in their proximity, providing a role for NPY in the control of motor functions.

Functional significance of different subpopulations of Y1R$^+$ neurons present in the dorsal horn

The role of NPY in pain processing at the spinal level is complex. Pronociceptive peripheral (Tracey et al., 1995) and spinal (White, 1997), as well as antinociceptive (Xu et al., 1994, 1999; Naveilhan et al., 2001) and biphasic (Xu et al., 1994, 1999) actions have been shown for this peptide in various pain models. Deletion of the Y1R (in mouse) results in a strongly decreased pain threshold (Naveilhan et al., 2001). In fact, it has been proposed that activation of Y1Rs is primarily responsible for the depressive effect of exogenous NPY on the flexor reflex in rats (Xu et al., 1999). This diversity of actions of NPY may not be surprising, since other messengers, e.g. galanin, also have been shown to modulate pain by complex, including biphasic actions, probably dependent on the type of receptor activated (see Xu et al., 2000; Wiesenfeld-Hallin and Xu, 2001; Liu and Hökfelt, 2002; Hua et al., 2005), as well as the site of action, that is primary afferent neurons (cell body, nerve terminals) or dorsal horn neurons (local, projection).

Here we confirm the presence of the Y1R in a few rat primary afferents (Brumovsky et al., 2002). In addition, the NPY receptor type 2 (Y2R) has been recently shown to be expressed in a population of mouse DRG neurons also projecting to the dorsal horn (Brumovsky et al., 2005). NPY could then modulate neurotransmitter release from primary afferents by acting through different presynaptic receptors (Walker et al., 1988; Giuliani et al., 1989; Duggan et al., 1991; Gibbs et al., 2004). Suppression of local spinal inhibitory transmission after i.t. application of NPY or a Y1R agonist has been described, suggesting a postsynaptic role for NPY acting on Y1Rs (Moran et al., 2004; Miyakawa et al., 2005), and an antinociceptive effect for NPY acting through spinal Y1R$^+$ neurons has been described in rat models of inflammatory hyperalgesia (Taiwo and Taylor, 2002). Several Y1R$^+$ neurons in LI–II colocalize SOM (Zhang et al., 1999), and 97.5% of the axonal endings originating from local SOM-IR neurons in LI–II coexpress the vesicular glutamate transporter 2 (VGLUT-2) (Todd et al., 2003), suggesting that the great majority of these neurons should have glutamate as its principal neurotransmitter. Thus, it seems likely that NPY via the Y1R inhibits excitatory Y1R$^+$/SOM$^+$/VGLUT-2$^+$ neurons, resulting in an attenuation of the spinal excitatory tone.

Under 'normal circumstances' the main source of NPY is local dorsal horn neurons (Gibson et al., 1984), and it is important to establish under which circumstances these neurons are activated and NPY is released. One situation is presumably inflammation which increases NPY mRNA levels in the dorsal horn (Ji et al., 1994). However, also descending systems, e.g. noradrenergic neurons in the locus coeruleus coexpressing NPY, may be a source (Everitt et al., 1984; Holets et al., 1988). The locus coeruleus neurons are activated e.g. by stress (Valentino et al., 1993; Sved et al., 2002) and could then release NPY from their nerve endings in the dorsal horn and cause attenuation of dorsal horn transmission. Perhaps more importantly, after peripheral nerve lesions a new major source of NPY emerges, since many large and some small DRG neurons upregulate NPY which then is transported into the dorsal horn (Wakisaka et al., 1991, 1992). Thus, NPY released from primary afferents and/or interneurons under different conditions could have an antinociceptive effect, both by inhibiting excitatory interneurons via postsynaptic Y1Rs and the release of excitatory transmitters from primary afferents, primarily via presynaptic Y2Rs (cf. Brumovsky et al., 2005).

The role of the here-described Y1R$^+$ neurons in pain transmission, including those in LX, remains to be studied. If the large Y1R$^+$ neurons at different depths of the gray matter project to supraspinal levels and are excitatory,

then NPY released in deeper dorsal horn L, especially after peripheral nerve injury, could directly inhibit pain transmission to higher centers.

Concluding remarks

The present study introduces several further 'players' in NPY neurotransmission at the spinal cord level, by demonstrating that $Y1R^+$ neurons basically are present in all layers of the dorsal horn originally described by Rexed (1952).

The present results also provide additional information on the anatomical substrate for sensory processing at the spinal level with focus on NPY and the Y1R. Many studies on NPY's role in pain transmission are based on i.t. administration of the peptide, and it will be important to know what type of neuron is being 'reached' in such a paradigm, and in which L they are located. Several studies support the idea of volume transmission in different brain areas (see Fuxe and Agnati, 1991), that is the ligand is released at some distance from the localization of the appropriate receptor(s). Thus, diffusion over several hundred micrometers is a distinct possibility. This may be particularly relevant for peptidergic transmission, since these messenger molecules are assumed to be released outside the synapse and act on extrasynaptic receptors (Zhu et al., 1986). So it remains to be established how important the exact knowledge of the various circuitries in fact is.

Acknowledgments—This study was supported by the Swedish Research Council (04X-2887), the Marianne and Marcus Wallenberg Foundation, the Knut and Alice Wallenberg Foundation, the Wallenberg Consortium North, the antibody/RIA Core grant DK 41301 (CURE, University of California, Los Angeles), a Carrillo Oñativia Grant and the Austral University.

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APPENDIX

Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi: 10.1016/j.neuroscience.2005.11.069.

(Accepted 30 November 2005)
(Available online 31 January 2006)