Histochem Cell Biol (2003) 120:415–426
DOI 10.1007/s00418-003-0579-3

ORIGINAL PAPER

Huai Zhen Ruan · Geoffrey Burnstock

Localisation of $P2Y_1$ and $P2Y_4$ receptors in dorsal root, nodose and trigeminal ganglia of the rat

Accepted: 16 September 2003 / Published online: 15 October 2003

Abstract The presence and distribution of P2Y (nucleotide) receptor subtypes in rat sensory neurons has been investigated. RT-PCR showed that $P2Y_1$, $P2Y_2$, $P2Y_4$ and $P2Y_6$ receptor mRNA is expressed in sensory ganglia [dorsal root ganglion (DRG), nodose ganglion (NG) and trigeminal ganglion (TG)]. The regional and cellular distribution of $P2Y_1$ and $P2Y_4$ receptor proteins in these ganglia was investigated using immunohistochemistry. $P2Y_1$ polyclonal antibodies stained over 80% of the sensory neurons, particularly the small-diameter (neurofilament-negative) neurons. The $P2Y_4$ receptor antibody stained more medium- and large- (neurofilament-positive) diameter neurons than small-diameter neurons. $P2Y_1$ and $P2Y_4$ receptor immunoreactivity ($P2Y_1$-IR and $P2Y_4$-IR) was often coexpressed with $P2X_3$ receptor immunoreactivity ($P2X_3$-IR) in subpopulations of neurons. Double immunohistochemistry showed that 73–84% of $P2X_3$ receptor-positive neurons also stained for the $P2Y_1$ receptor in DRG, TG and NG while only 25–35% also stained for the $P2Y_4$ receptor. Subpopulations of $P2Y_1$-IR neurons were coexpressed with NF200, CGRP and IB_4; most $P2Y_4$-IR neurons were coexpressed with NF200, while only a few neurons were coexpressed with CGRP (10–20%) or with IB_4 (1–2%). The results suggest that P2Y as well as P2X receptor subtypes contribute to purinergic signalling in sensory ganglia.

Keywords Purinoceptors · P2Y receptors · ATP · Sensory ganglia

H. Z. Ruan · G. Burnstock (✉)
Autonomic Neuroscience Institute,
Royal Free and University College Medical School,
Rowland Hill Street, London, NW3 2PF, UK
e-mail: g.burnstock@ucl.ac.uk
Tel.: +44-207-8302948
Fax: +44-207-8302949

H. Z. Ruan
Department of Neurobiology,
Third Military Medical University,
Chongqing 400038, China

Introduction

The diverse actions of extracellular nucleotides are mediated through the activation of specific P2 receptors in a wide variety of cell types (Ralevic and Burnstock 1998). The P2 purinoceptors can be divided into two classes on the basis of their signal transduction mechanisms and their characteristic molecular structures: the adenosine 5′-triphosphate (ATP)-gated ionotropic P2X family and the G protein-coupled (metabotropic) P2Y receptors (Burnstock and Kennedy 1985; Abbracchio and Burnstock 1994). Several P2X receptors have been localised to sensory ganglia neurons, with the $P2X_3$ receptor expressed exclusively in those small neurons that mediate responses to noxious stimuli (Chen et al. 1995; Lewis et al. 1995). Thus, P2X receptors appear to have an important role in pain sensation (Burnstock 2001). Indeed, studies of mice lacking the $P2X_3$ gene revealed a significant decrease in pain-related behaviour in several inflammatory models (Cockayne et al. 2000; Souslova et al. 2000). So far eight mammalian P2Y receptors have been cloned and shown to be activated by extracellular nucleotides, namely $P2Y_1$, $P2Y_2$, $P2Y_4$, $P2Y_6$, $P2Y_{11}$, $P2Y_{12}$ (Hollopeter et al. 2001; Zhang et al. 2001), $P2Y_{13}$ (Communi et al. 2001) and the more recent $P2Y_{14}$ receptor (Abbracchio et al. 2003). These share between 25% and 55% identity (at the amino acid level) and can be distinguished by their specific pharmacological selectivities for both purine and pyrimidine nucleotides, which overlap in some cases (King and Burnstock 2002). The P2Y receptor has a widespread tissue distribution, with its mRNA being commonly detected in gastrointestinal tract, heart, lung, liver, kidney, skeletal muscle, spleen, pancreas, spinal cord and brain. The P2Y receptor subtypes may have potentially important roles in the nervous system, but possible roles for metabotropic (P2Y) ATP receptors in nociceptive signalling have received limited attention. $P2Y_1$ has been identified in primary sensory neurons, where it has been implicated in the transduction of mechanical stimuli (Nakamura and Strittmatter 1996), and modulation of the

capsaicin receptor, VR1 (Tominaga et al. 2001). Expression of $P2Y_2$, $P2Y_4$ and $P2Y_6$ mRNA and P2Y-evoked increase in intracellular calcium has also been reported recently in dorsal root ganglion (DRG) neurons (Sanada et al. 2002), However, little is known about the distribution of the P2Y receptor protein in different sensory ganglia.

One important approach in examining the potential roles of P2Y receptor subtypes in sensory functions is to determine the subtypes expression pattern in combination with other markers of sensory neuron populations. We chose to examine the expression of the P2Y receptors in relation to the neuropeptide calcitonin gene-related peptide (CGRP), as well as binding of the isolectin B_4 from *Griffonia simplicifolia* type one (IB_4) and expression of neurofilament-200 (NF200). These markers are associated with general functional groups of sensory receptors, and could thus provide an indication of the functional types of neurons expressing and coexpressing the P2Y receptor subunits examined. IB_4 binds to cell surface carbohydrates expressed on a subpopulation of unmyelinated primary afferent neurons (Streit et al. 1985; Silverman and Kruger 1990; Wang et al. 1994). A large number of GS-IB_4-positive DRG neurons, which are primarily non-peptidergic [trkA-negative and fluoride-resistant acid phosphatase (FRAP)-positive], are believed to play a role in the processing of nociceptive information (Silverman and Kruger 1990; Molliver et al. 1995). IB_4 binding has been shown to occur in a number of small DRG neurons which display $P2X_3$ immunoreactivity (IR; Bradbury et al. 1998; Petruska et al. 2000). $P2X_3$ receptor subunits in DRG neurons have also been implicated in the coding of a wide range of sensory modalities, including nociception (Bland-Ward and Humphrey 2000; Ding et al. 2000; Hamilton and McMahon 2000). Small-diameter DRG neurons that display CGRP or lack neuropeptides, but bind IB_4, may represent two different functional groups of nociceptors (Ambalavanar and Morris 1993; Kitchener et al. 1993; Plenderleith and Snow 1993; Averill et al. 1995; Molliver et al. 1995). Lawson and co-workers (McCarthy and Lawson 1990, 1997) have claimed that most neurons expressing CGRP were involved in nociception. Neurofilament (NF) has been shown to be highly enriched in the large-diameter neurons of the sensory ganglia, while the small-diameter neurons are NF-weak, indicating that NF-IR is a reliable indicator of the myelination state of a sensory neuron (Lawson and Waddell 1991; Perry et al. 1991). In the present study, we examined the expression patterns of the P2Y receptor in relation to these population markers, in sections of sensory ganglia from naive rats.

Materials and methods

Tissue preparation

Breeding, maintenance and killing of the animals used in this study followed principles of good laboratory animal care and experimentation in compliance with UK regulations. Eight adult Sprague-Dawley rats (200–250 g) were killed by asphyxiation with CO_2. DRG, nodose ganglion (NG) and trigeminal ganglion (TG) were dissected as quickly as possible and placed in liquid nitrogen for RT-PCR. Another nine adult Sprague-Dawley rats (200–250 g) were killed by asphyxiation with CO_2 and perfused immediately through the heart (left ventricle) with 50 ml saline followed by fixative containing 4% paraformaldehyde and 0.2% saturated picric acid in 0.1 M phosphate-buffered saline (PBS; pH 7.4). DRG, NG and TG were dissected and immersed in the same fixative for 4 h at 4°C, and then transferred to 20% sucrose in PBS (overnight at 4°C). Thereafter, the tissue blocks were rapidly frozen by immersion in isopentane at −70°C for 2 min. Transverse sections through the ganglia (10 μm thickness) were cut on a cryostat and thaw-mounted on poly-L-lysine-coated slides.

RT-PCR

Total RNA was extracted from DRG, NG and TG using the Total RNA Isolation System (Promega, Madison, USA). RT-PCR was performed using Ready-to-Go RT-PCR beads (Amersham Pharmacia Biotech, NJ, USA). Reverse transcription was performed using the Moloney murine leukaemia virus reverse transcriptase. Primer sequences for $P2Y_{1, 2, 4, 6}$ (Table 1) were used for amplification reactions. The amplification reaction, performed in the same reaction tube, was conducted under the following conditions: 95°C for 30 s, the relevant annealing temperature ranging from 55 to 60°C (Table 1) for 1 min, and 72°C for 1.5 min. PCR products were obtained after 30 cycles. Amplification products were separated by electrophoresis and visualised by ethidium bromide staining. The presence of possible contaminants was investigated in all experiments, using control RT-PCR reactions in which either mRNA was omitted or the reverse transcriptase had been inactivated by heating to 95°C.

Immunostaining

Antisera

The following primary antisera were used in the current studies: rabbit anti-$P2Y_1$ and $P2Y_4$ receptors (Alomone Laboratories,

Table 1 P2Yr primers: sequences, annealing temperatures and product lengths. (*FORW* Sense forward PCR primer, *REV* antisense reverse PCR primer)

Gene	Sequence	Annealing temperature (°C)	Product lengths
$P2Y_1$-FORW	5′ to 3′ACGTCAGATGAGTACCTGCG	58	289
$P2Y_1$-REV	5′ to 3′CCCTGTCGTTGAAATCACAC		
$P2Y_2$-FORW	5′ to 3′CTGGTCCGCTTTGCCCGAGATG	60	311
$P2Y_2$-REV	5′ to 3′TATCCTGAGTCCCTGCCAAATGAGA		
$P2Y_4$-FORW	5′ to 3′TGTTCCACCTGGCATTGTCAG	55	294
$P2Y_4$-REV	5′ to 3′AAAGATTGGGCACGAGGCAG		
$P2Y_6$-FORW	5′ to 3′TGCTTGGGTGGTATGTGGAGTC	60	339
$P2Y_6$-REV	5′ to 3′TGGAAAGGCAGGAAGCTGATAAC		

Jerusalem, Israel; 3 μg/ml). The immunogens used for production of polyclonal $P2Y_1$ and $P2Y_4$ antibody were synthetic peptides corresponding to the carboxyl terminal of the cloned rat $P2Y_1$ and $P2Y_4$ receptors, covalently linked to keyhole limpet hemocyanin. The peptide sequences of the $P2Y_1$ and $P2Y_4$ receptors are of amino acid sequence 242–258 (RALIYKDLDNSPLRRKS) and 337–350 (HEESISRWADTHQD), respectively. Rabbit anti-$P2X_3$ receptor (1:400) was provided by Roche Bioscience (Palo Alto, CA, USA). The immunogens used for production of polyclonal $P2X_3$ antibody were synthetic peptides corresponding to the carboxyl terminal of the cloned rat $P2X_3$ receptors, covalently linked to keyhole limpet hemocyanin. The peptide sequences of the $P2X_3$ receptors are of amino acid sequence 383–397 (VEKQSTDSGAYSIGH). Mouse anti-NF200 (clone N52; 1:400) and biotin-conjugated IB_4 (10 μg/ml) were from Sigma, and mouse anti-CGRP (1:2,000) was from Affiniti.

Immunostaining procedure

An indirect immunofluorescence method with three layers of antibodies was used. Antibodies to $P2Y_1$ and $P2Y_4$ receptors from rabbit were allowed to react with biotinylated donkey anti-rabbit IgG secondary antibody (Jackson Immunoresearch, PA, USA) and detected with Streptavidin-Texas Red (red fluorophore; Sigma). Briefly, the sections were incubated overnight with the primary antibodies diluted to 3 μg/ml with 10% normal horse serum (NHS) in PBS containing 0.05% Merthiolate and 0.2% Triton X-100. Subsequently, the sections were incubated with biotinylated donkey anti-rabbit IgG, diluted 1:500 in 1% NHS in PBS containing 0.05% Merthiolate for 1 h, followed by incubation in Streptavidin-Texas Red diluted 1:200 in PBS containing 0.05% Merthiolate for 1 h. All incubations were held at room temperature and separated by three 5-min washes in PBS. Slides were mounted with Citifluor (Citifluor, London, UK) and examined with fluorescence microscopy. Control experiments were performed using an excess of the appropriate homologue peptide antigen to absorb the primary antibodies and thus confirm a specific immunoreaction.

Immunofluorescence double labelling

In colocalisation studies investigating the coexpression of P2Y and $P2X_3$ receptors, P2Y receptor immunoreactivity was enhanced with tyramide amplification, which allows high sensitivity and low background specificity (Renaissance, TSA indirect, NEN, USA). The use of TSA allows immunostaining with two rabbit antisera, as described previously (Bradbury et al. 1998). Briefly, sections were incubated in 10% NHS in PBS for 30 min at room temperature, followed by incubation with the P2Y ($P2Y_1$ and $P2Y_4$) antibody (1 μg/ml) in 10% NHS and 0.2% Triton X-100 in PBS, overnight. Subsequently the sections were incubated with biotinylated donkey anti-rabbit IgG (Jackson Immunoresearch; 1:500) for 1 h, with ExtrAvidin peroxidase (1:1,500) for 1 h, biotinylated tyramide for 8 min, and then in Streptavidin-FITC (1:200) for 10 min. Polyclonal rabbit antibody against the $P2X_3$ receptor subtype (1:400) was applied as a second primary antibody and detected with Cy3-conjugated donkey anti-rabbit IgG (Jackson Immunoresearch). To check for non-crossreactivity, $P2Y_1$ and $P2Y_4$ receptor immunostaining using indirect TSA was performed alone on some sections, as was $P2X_3$ indirect immunofluorescence. The localisation of each marker appeared identical to the localisation observed with the double staining technique, with no apparent crossreactivity.

To demonstrate the colocalisation of the P2Y receptor with a medium molecular weight neurofilament marker (NF200) or CGRP, sections were incubated with the P2Y antibody overnight and detected with Cy3-conjugated donkey anti-rabbit IgG or FITC-conjugated donkey anti-rabbit IgG, and then incubated with monoclonal NF200 antibody (1:400, overnight) or monoclonal CGRP antibody (1:2,000, overnight) and FITC-conjugated mouse antibody or TRITC-conjugated mouse antibody (raised in goat; Sigma; 1:200, 1 h). For colocalisation with the IB_4, sections were immunostained for the P2Y receptor, as above, and then incubated with biotin-conjugated IB_4 (10 μg/ml, 16 h) and Streptavidin-FITC (1:200, 1 h). The sections were washed and mounted in Citifluor.

For immunostaining, a number of controls were performed on sections where either the primary or secondary antibody stage was omitted from the staining procedure.

Photomicroscopy

Images of immunofluorescence labelling were taken with the Leica DC 200 digital camera (Leica, Switzerland) attached to a Zeiss Axioplan microscope (Zeiss, Germany). Images were imported into a graphics package (Adobe Photoshop 5.0, USA). The two-channel readings for green and red fluorescence were merged by using Adobe Photoshop 5.0.

Analysis

All analyses were performed at ×20 objective magnification. P2Y receptor expression in ganglia was determined by counting all P2Y receptor-positive cell profiles in every sixth section throughout the ganglia (approximately eight sections per animal). Cell sizes were determined by the method of Rose and Rohrlich (1988). Cell diameters less than 30 μm were small-diameter neurons, cell diameters from 30 to 50 μm were medium-diameter neurons and cell diameters greater than 50 μm were large-diameter neurons.

To calculate percentages of P2Y receptor colocalisation with $P2X_3$ and other markers, four randomly selected ganglia sections were chosen for each pair of markers for each animal. For each section, counts were made of the number of profiles positive for P2Y receptor, the number of profiles positive for the $P2X_3$ receptor and the other marker (NF200, IB_4 or CGRP) and the number of profiles expressing both antigens and percentages were calculated.

Results

Expression of P2Y receptor mRNA in DRG, NG and TG

We investigated the presence of different P2Y receptor subunits in DRG, NG and TG by using RT-PCR with primers specific for various subclasses of P2Y. The mRNAs for metabotropic $P2Y_1$, $P2Y_2$, $P2Y_4$ and $P2Y_6$ receptors were detected in rat DRG, NG and TG. Amplification of mRNAs for $P2Y_1$, $P2Y_2$, $P2Y_4$ and $P2Y_6$ receptors resulted in single bands around the predicted sizes of 289, 311, 294 and 339 bp, respectively (Fig. 1).

P2Y receptor staining in DRG, NG and TG

The polyclonal antibodies for the $P2Y_1$ and $P2Y_4$ receptor subtypes labelled different subpopulations of neurons with differing intensity in the three sensory ganglia tested (Fig. 2). In control experiments, no signal was observed when the preimmune sera were used. In DRG, NG and TG, $P2Y_1$ polyclonal antibodies stained over 80% of the neurons. However, the small-diameter neurons (62.3%, 82.7% and 58.4% of DRG, NG and TG, respectively) were stained much more intensely than the medium- and large-diameter neurons (37.6%, 17.2% and 41.3% of DRG, NG and TG, respectively). The staining was evenly distributed

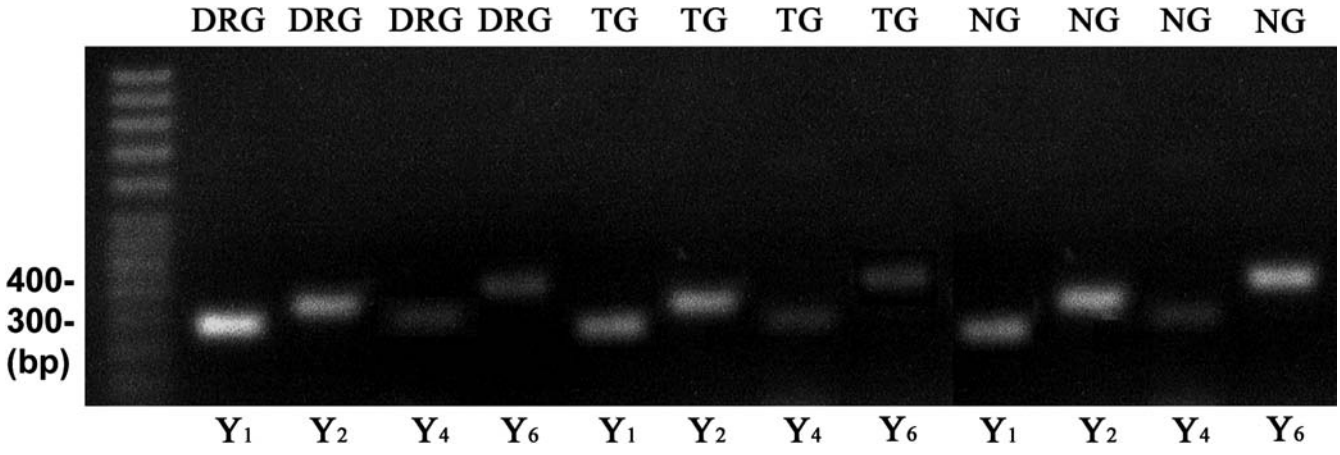


Fig. 1 Detection of mRNAs for metabotropic $P2Y_1$, $P2Y_2$, $P2Y_4$ and $P2Y_6$ receptors in rat dorsal root ganglion (*DRG*), nodose ganglion (*NG*) and trigeminal ganglion (*TG*). The expression of mRNAs encoding $P2Y_1$, $P2Y_2$, $P2Y_4$ and $P2Y_6$ receptors in rat DRG, NG and TG were detected by an RT-PCR analysis, using gene-specific primers for $P2Y_1$, $P2Y_2$, $P2Y_4$ and $P2Y_6$. PCR products were visualised with 2% agarose gel stained with ethidium bromide and viewed under UV illumination

Table 2 Size distribution of $P2Y_1$ or $P2Y_4$ receptor-immunoreactive (*IR*) cells in rat sensory neurons. (*DRG* Dorsal root ganglion, *NG* nodose ganglion, *TG* trigeminal ganglion)

Cell diameter (μm)	Percentage of $P2Y_1$-IR cells			Percentage of $P2Y_4$-IR cells		
	DRG	NG	TG	DRG	NG	TG
Small (25.5±4)	62.3±0.3	82.7±1.5	58.4±0.8	25.2±0.5	33.5±0.7	32.7±1.6
Medium (38.5±7)	34.5±1.3	17.2±0.9	39.1±0.6	66.3±1.4	60.3±0.8	58.1±1.4
Large (56±5)	3.1±0.8	0	2.2±0.9	8.4±0.4	6.1±0.3	9.1±0.6

Table 3 Colocalisation of $P2Y_1$ or $P2Y_4$ receptor immunoreactivity with $P2X_3$ receptor immunoreactivity in rat sensory neurons. (*n* Number of double-labelled cell profiles and the total number of cell profiles counted, respectively, for each combination of receptor)

	Percentage $P2X_3$ receptor profiles containing P2Y receptor			Percentage P2Y receptor profiles containing $P2X_3$ receptor		
	DRG	NG	TG	DRG	NG	TG
$P2Y_1$ (*n*)	84.1±0.3 (1,053/1,248)	78.1±1.2 (943/1,189)	73.4±0.3 (865/1,173)	62.2±0.6 (1,053/1,677)	51.7±0.6 (943/1,804)	48.7±0.6 (865/1,755)
$P2Y_4$ (*n*)	25.1±1.4 (316/1,193)	32.2±0.4 (412/1,260)	35.2±0.7 (394/1,098)	59.4±0.4 (316/528)	56.1±0.4 (412/729)	62.1±0.6 (394/628)

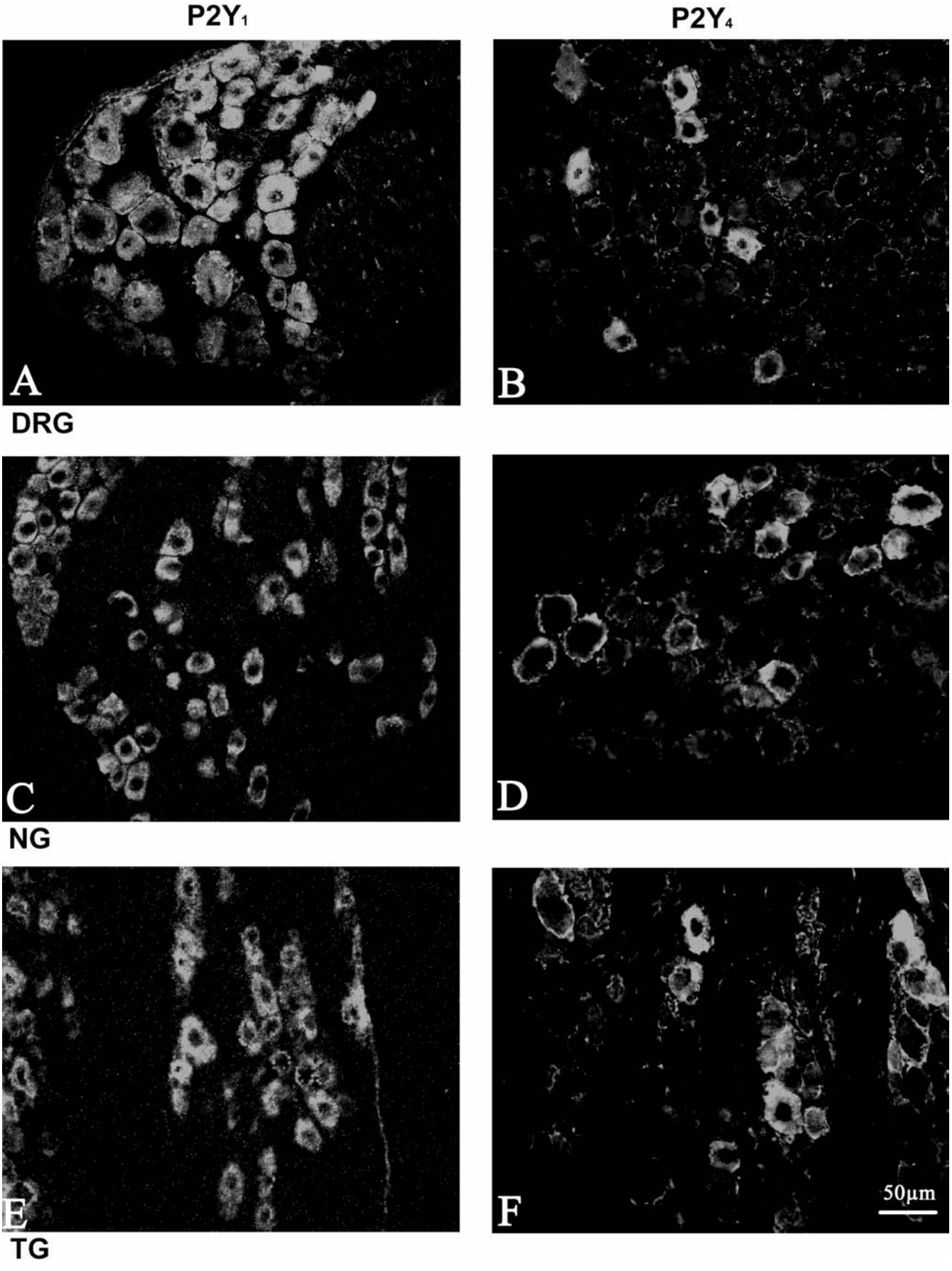
throughout the cytoplasm of these cells and positively stained cells were randomly distributed throughout the ganglia. The number of neurons staining for the $P2Y_4$ receptor was lower than that for the $P2Y_1$ receptors, and the medium- and large-diameter neurons (74.7%, 66.4% and 67.2% of DRG, NG and TG, respectively) were stained much more dominantly than the small-diameter neurons (25.2%, 33.5% and 32.7% of DRG, NG and TG, respectively; Table 2). $P2Y_2$ receptor immunostaining was less consistent, with heavy nuclear localisation and high levels of background staining that were not removed by displacement controls. It was therefore decided that due to these unsatisfactory control results, $P2Y_2$ receptor protein expression would not be further investigated. A $P2Y_6$ receptor antibody was not available.

Colocalisation of $P2Y_1$ or $P2Y_4$ with $P2X_3$ receptors

In DRG, NG and TG, $P2Y_1$ receptor immunoreactivity ($P2Y_1$-IR) was very often coexpressed with $P2X_3$ receptor immunoreactivity ($P2X_3$-IR). Relatively 84.1%, 78.1% and 73.4% $P2X_3$-IR profiles were also found to be $P2Y_1$ receptor immunoreactive in DRG, NG and TG, respectively. $P2X_3$-IR was consistently much stronger than $P2Y_1$-IR. $P2Y_1$-IR intensity was more variable among labelled neurons than $P2X_3$-IR. In some cases, $P2Y_1$-IR and $P2X_3$-IR were exclusive of each other. In general, a greater number of neurons appeared to display $P2Y_1$-IR than $P2X_3$-IR (Table 3; Fig. 3).

There were, however, some neurons in the medium to large size range that displayed $P2X_3$-IR that was weaker than that observed in small cells. These neurons coexpressed $P2Y_4$-IR (25.1%, 32.2% and 35.2% of DRG, NG and TG, respectively; Table 3; Fig. 3).

Fig. 2A–F Localisation of $P2Y_1$ and $P2Y_4$ receptor immunoreactivity in rat sensory ganglia. **A** $P2Y_1$ receptor immunoreactivity in DRG. **B** $P2Y_4$ receptor immunoreactivity in DRG. **C** $P2Y_1$ receptor immunoreactivity in NG. **D** $P2Y_4$ receptor immunoreactivity in NG. **E** $P2Y_1$ receptor immunoreactivity in TG. **F** $P2Y_4$ receptor immunoreactivity in TG


P2Y1
P2Y4
A
B
DRG
C
D
NG
E
F
50μm
TG

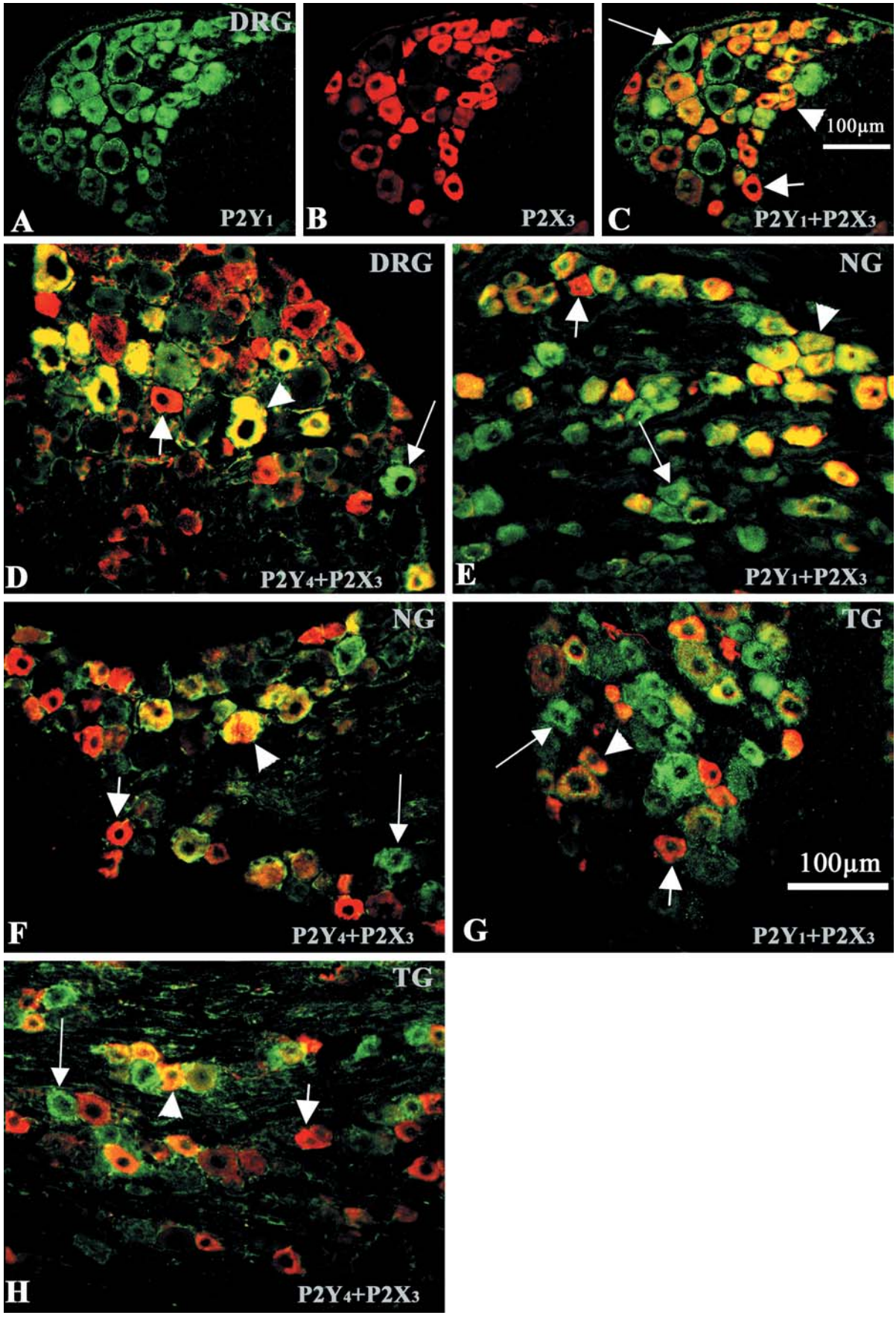

DRG
A
P2Y1
B
P2X3
C
100μm
P2Y1+P2X3
DRG
D
P2Y4+P2X3
NG
E
P2Y1+P2X3
NG
F
P2Y4+P2X3
TG
100μm
G
P2Y1+P2X3
TG
H
P2Y4+P2X3

Table 4 Colocalisation of $P2Y_1$ or $P2Y_4$ receptor immunoreactivity with NF200 in rat sensory neurons. (*NF200* Medium molecular weight neurofilament, *n* number of double-labelled cell profiles and the total number of cell profiles counted, respectively, for each combination of receptor)

	Percentage NF200 profiles containing P2Y receptor			Percentage P2Y receptor profiles containing NF200		
	DRG	NG	TG	DRG	NG	TG
$P2Y_1$ (*n*)	66.7±0.4 (561/838)	71.2±1.2 (819/1,131)	62.2±0.3 (610/976)	34.2±0.3 (561/1,628)	45.3±0.4 (819/1,794)	35.8±0.4 (610/1,688)
$P2Y_4$ (*n*)	30.6±0.4 (294/950)	46.6±1.4 (492/1,026)	31.5±0.9 (268/828)	97.4±0.2 (294/302)	73.7±1.2 (492/657)	45.2±0.8 (268/583)

Table 5 Colocalisation of $P2Y_1$ or $P2Y_4$ receptor immunoreactivity with CGRP immunoreactivity in the rat sensory neurons. (*CGRP* Calcitonin gene-related peptide, *n* number of double-labelled cell profiles and the total number of cell profiles counted, respectively, for each combination of receptor)

	Percentage CGRP profiles containing P2Y receptor			Percentage P2Y receptor profiles containing CGRP		
	DRG	NG	TG	DRG	NG	TG
$P2Y_1$ (*n*)	86.1±0.4 (602/696)	72.5±0.4 (551/756)	62.1±0.7 (411/656)	36.3±0.4 (602/1,642)	31.2±0.4 (551/1,742)	22.8±1.1 (411/1,722)
$P2Y_4$ (*n*)	7.5±0.2 (48/624)	22.5±1.3 (186/782)	12.3±0.5 (72/562)	9.8±0.6 (48/462)	26.5±0.5 (186/689)	11.3±0.5 (72/608)

Table 6 Colocalisation of $P2Y_1$ or $P2Y_4$ receptor immunoreactivity with IB_4 in the rat sensory neurons. (*IB_4* Isolectin B_4, *n* number of double-labelled cell profiles and the total number of cell profiles counted, respectively, for each combination of receptor)

	Percentage IB_4 profiles containing P2Y receptor			Percentage P2Y receptor profiles containing IB_4		
	DRG	NG	TG	DRG	NG	TG
$P2Y_1$ (*n*)	84.4±0.4 (1,032/1,220)	58.1±0.8 (792/1,344)	23.3±0.4 (383/1,614)	62.4±0.2 (1,032/1,648)	44.2±0.7 (792/1,763)	21.5±0.5 (383/1,744)
$P2Y_4$ (*n*)	0.6±0.3 (9/1,032)	0.6±0.3 (11/1,225)	0.4±0.2 (9/1,447)	1.6±0.3 (9/487)	1.4±0.3 (11/664)	1.3±0.2 (9/586)

Coexpression of $P2Y_1$, $P2Y_4$ receptors and other neurone markers

$P2Y_1$, $P2Y_4$/NF200

P2Y receptor immunoreactivity was examined in relation to a number of other widely used markers of subsets of sensory neurons. Costaining for P2Y and NF200 was performed in order to examine the extent to which $P2Y_1$ and $P2Y_4$ may be expressed in neurons with myelinated axons. NF200 is an anti-neurofilament antibody that has been shown to be a reliable indicator of the myelination state of sensory neurons. It stains the classically defined large-diameter neurons with myelinated axons (Lawson and Waddell 1991; Perry et al. 1991). Double immunofluorescence histochemistry showed that 34.2%, 45.3% and 35.8% of $P2Y_1$ receptor neuron profiles were also NF200 immunoreactive in DRG, NG and TG, respectively (Table 4; Fig. 4). Double immunofluorescent histochemistry showed that 97.4%, 73.7% and 45.2% of $P2Y_4$ receptor neuron profiles were also NF200 immunoreactive in DRG, TG and NG, respectively.

$P2Y_1$, $P2Y_4$/CGRP or IB_4

The classically defined small dark cells in L4/5 DRG can be subdivided into two principal groups which show only limited overlap. The first and numerically slightly larger group expressed the neuropeptide CGRP. Relatively 86.1%, 72.5% and 62.1% CGRP-immunoreactive profiles were also found to be $P2Y_1$ receptor immunoreactive in DRG, NG and TG, respectively (Table 5; Fig. 5). Relatively fewer (7.5%, 22.5% and 12.3%) CGRP-immunoreactive profiles were also found to be $P2Y_4$ receptor immunoreactive in DRG, NG and TG, respectively (Table 5; Fig. 5). The other group of small cells contains those that bind the lectin IB_4. $P2Y_1$ receptors were also highly coexpressed in many of these cells, with 84.4%, 58.1% and 23.3% of the IB_4 cells also immunoreactive for the $P2Y_1$ receptor in DRG, TG and NG, respectively (Table 6; Fig. 6); that is, 62.4%, 44.2% and 21.5% of $P2Y_1$ receptor-immunoreactive profiles contain IB_4 in DRG, TG and NG, respectively. In contrast, $P2Y_4$-IR neurons lacked IB_4 in these sensory ganglia.

Fig. 3A–H Colocalisation (*yellow/orange*) of $P2Y_1$ or $P2Y_4$ receptor immunoreactivity (*green*) with $P2X_3$ receptor immunoreactivity (*red*) in the rat sensory ganglia. In all cases *white arrowheads* show examples of double-labelled neurons, *short arrows* illustrate $P2X_3$ receptor-positive cells that are not double labelled and *long arrows* show P2Y-positive cells that are not $P2X_3$ receptor immunoreactive. **A–C** Photomicrographs of a single section double stained for $P2Y_1$-IR and $P2X_3$-IR in DRG. **D** Photomicrograph of a single section double stained for $P2Y_4$-IR and $P2X_3$-IR in DRG. **E** Photomicrograph of a single section double stained for $P2Y_1$-IR and $P2X_3$-IR in NG. **F** Photomicrograph of a single section double stained for $P2Y_4$-IR and $P2X_3$-IR in NG. **G** Photomicrograph of a single section double stained for $P2Y_1$-IR and $P2X_3$-IR in TG. **H** Photomicrograph of a single section double stained for $P2Y_4$-IR and $P2X_3$-IR in TG

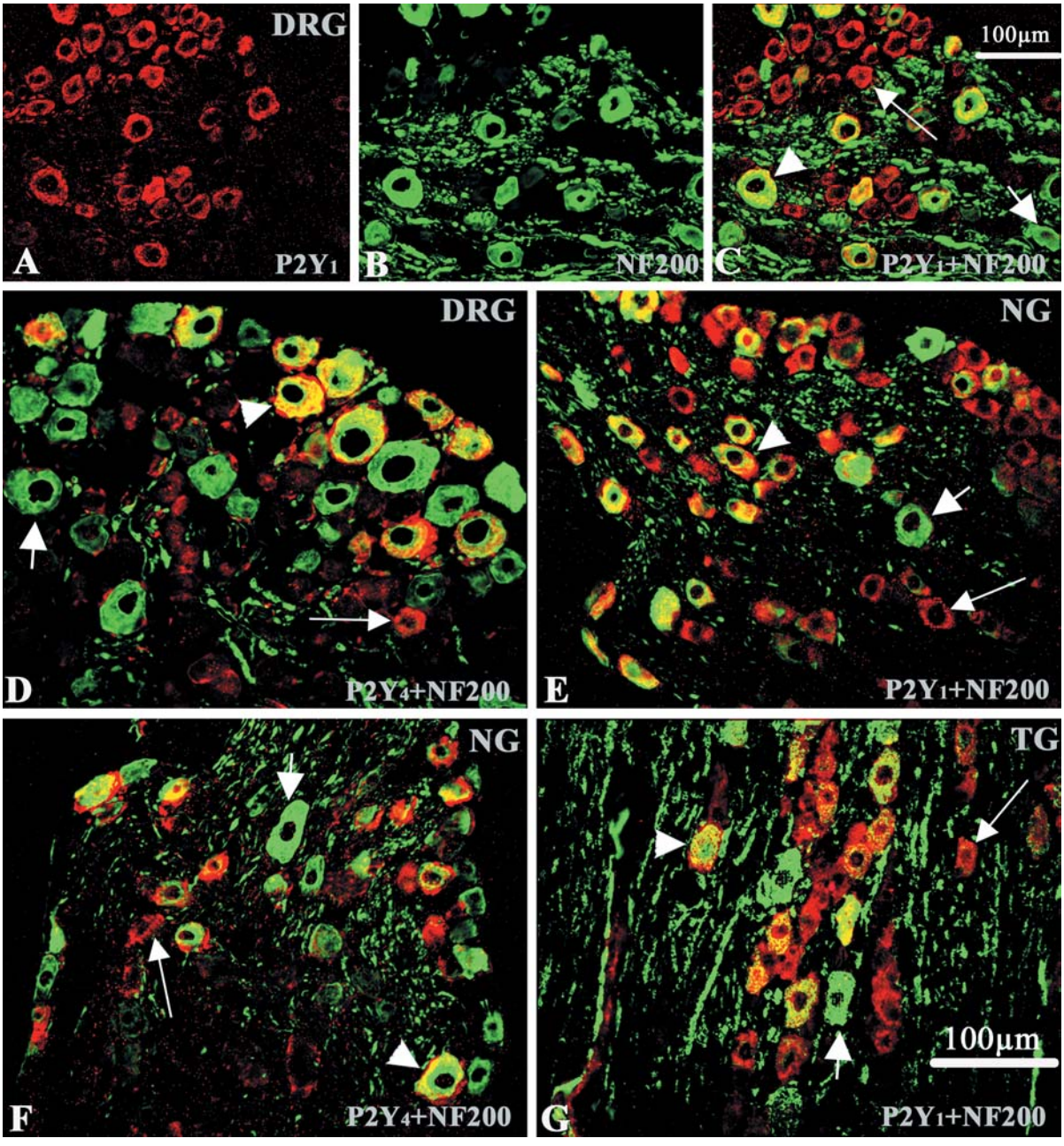


Fig. 4A–G Colocalisation (*yellow/orange*) of $P2Y_1$ or $P2Y_4$ receptor immunoreactivity (*red*) with neurofilament-200 (*NF200*) immunoreactivity (*green*) in the rat sensory ganglia. In all cases *white arrowheads* show examples of double-labelled neurons, *short arrows* illustrate NF200-positive cells that are not double labelled and *long arrows* show P2Y-positive cells that are not NF200 immunoreactive. **A–C** Photomicrographs of a single section double stained for $P2Y_1$-IR and NF200-IR in DRG. **D** Photomicrograph of a single section double stained for $P2Y_4$-IR and NF200-IR in DRG. **E** Photomicrograph of a single section double stained for $P2Y_1$-IR and NF200-IR in NG. **F** Photomicrograph of a single section double stained for $P2Y_4$-IR and NF200-IR in NG. **G** Photomicrograph of a single section double stained for $P2Y_1$-IR and NF200-IR in TG

Discussion

In this study, we have used RT-PCR and immunohistochemistry to study the expression of P2Y receptors in sensory ganglia (DRG, NG and TG). We found that mRNA for $P2Y_1$, $P2Y_2$, $P2Y_4$ and $P2Y_6$ was present in DRG, NG and TG. The $P2Y_1$ antibody stained small-diameter neurons (60–80%) and medium- and large-diameter neurons (approximately 20–40%). The expression of $P2Y_4$ receptor subtypes was mainly seen in medium-diameter neurons (approximately 60–70%).

One of the goals of the present work was to identify the particular classes of sensory neuron which express P2Y receptors. This was addressed by the use of double-immunofluorescent immunohistochemistry. The large cells within the sensory ganglia were rich in neurofila-

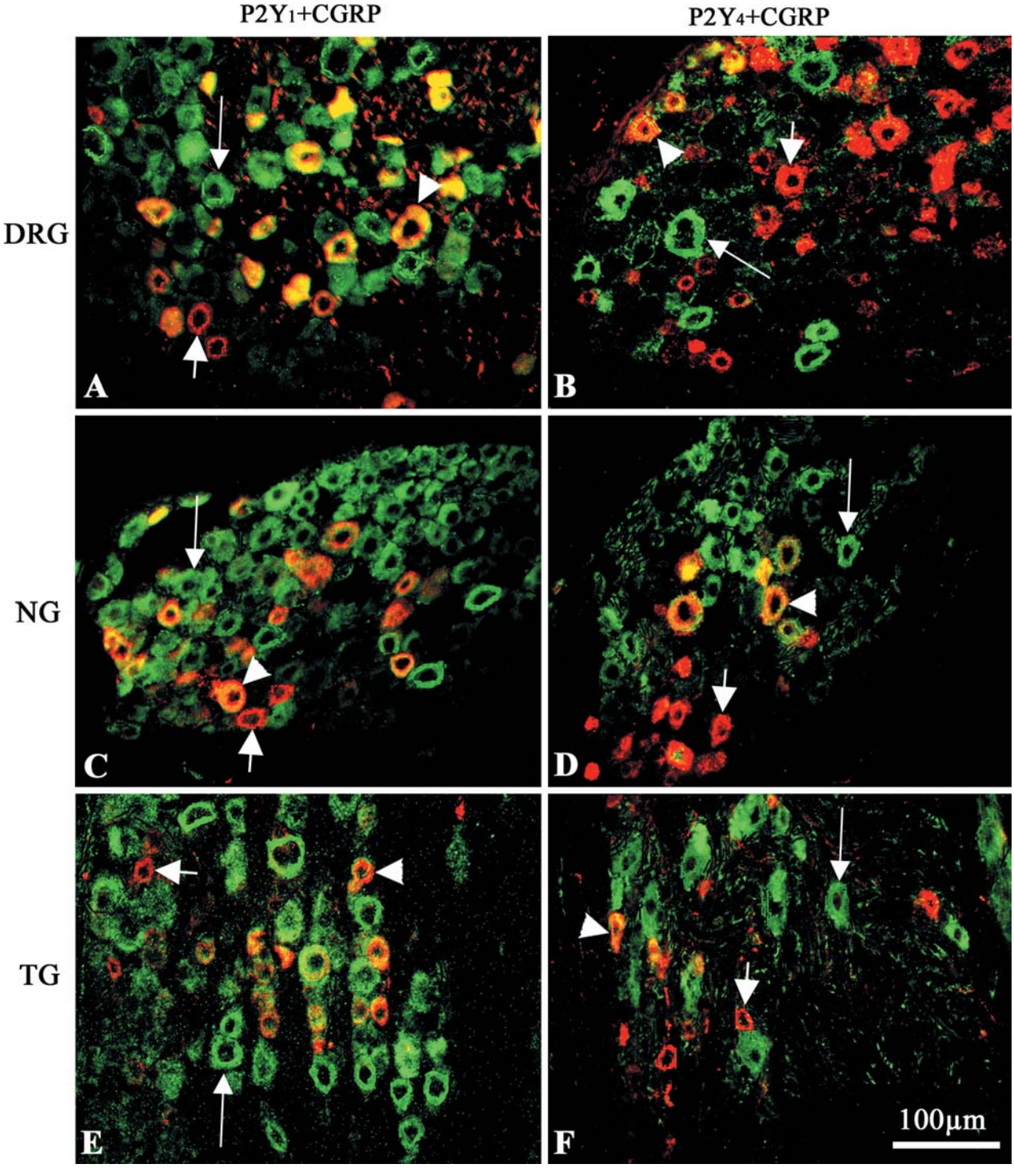


Fig. 5A–F Colocalisation (*yellow/orange*) of $P2Y_1$ or $P2Y_4$ receptor immunoreactivity (*green*) with calcitonin gene-related peptide (*CGRP*) immunoreactivity (*red*) in the rat sensory ganglia. In all cases *white arrowheads* show examples of double-labelled neurons, *short arrows* illustrate CGRP-positive cells that are not double labelled and *long arrows* show P2Y-positive cells that are not CGRP immunoreactive. **A** Photomicrograph of a single section double stained for $P2Y_1$-IR and CGRP-IR in DRG. **B** Photomicrograph of a single section double stained for $P2Y_4$-IR and CGRP-IR in DRG. **C** Photomicrograph of a single section double stained for $P2Y_1$-IR and CGRP-IR in NG. **D** Photomicrograph of a single section double stained for $P2Y_4$-IR and CGRP-IR in NG. **E** Photomicrograph of a single section double stained for $P2Y_1$-IR and CGRP-IR in TG. **F** Photomicrograph of a single section double stained for $P2Y_4$-IR and CGRP-IR in TG

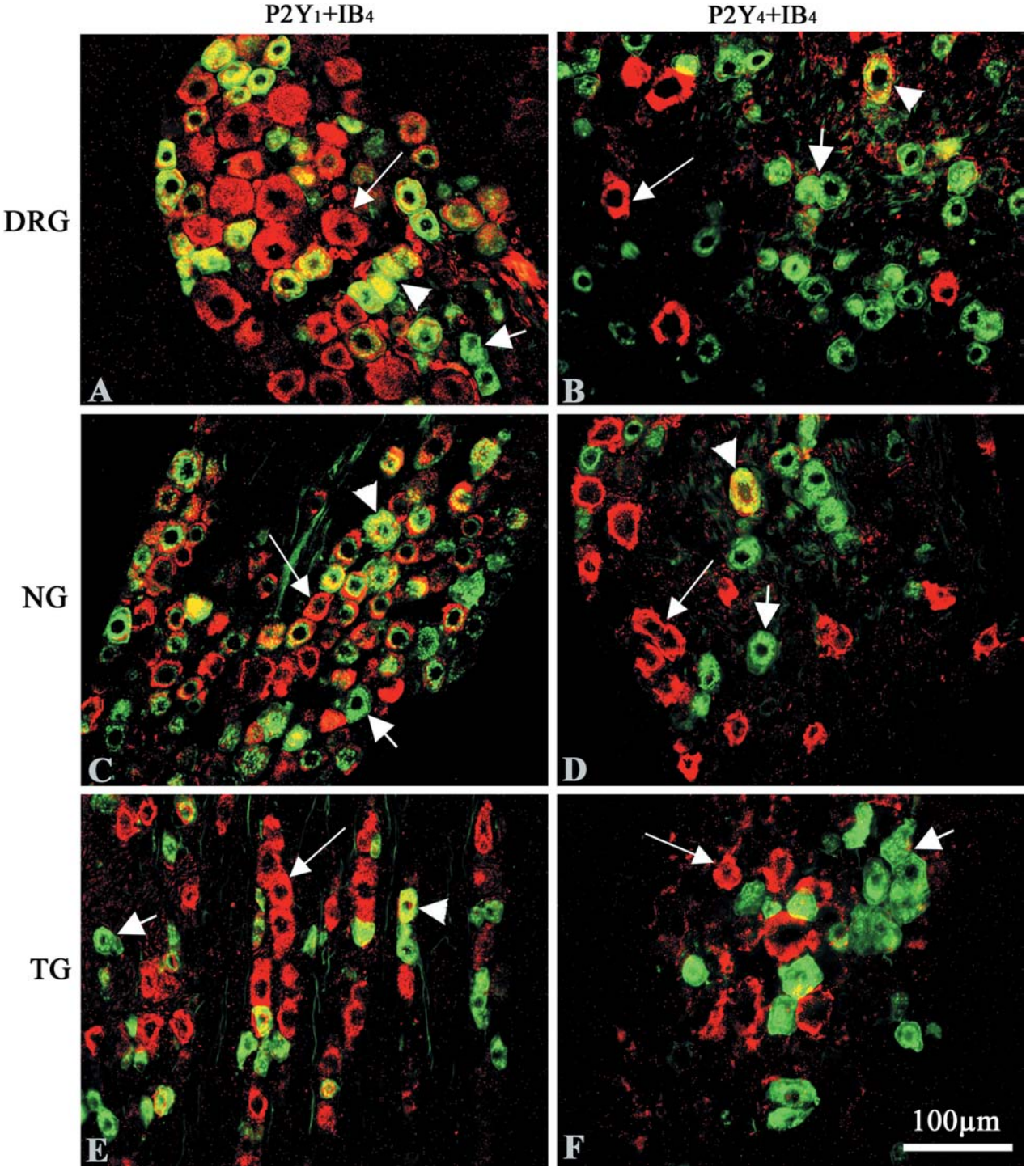


Fig. 6A–F Colocalisation (*yellow/orange*) of $P2Y_1$ or $P2Y_4$ receptor immunoreactivity (*red*) with isolectin B_4 from *Griffonia simplicifolia* type one (*IB_4*) immunostaining (*green*) in the rat sensory ganglia. In all cases *white arrowheads* show examples of double-labelled neurons, *short arrows* illustrate IB_4-positive cells that are not double labelled and *long arrows* show P2Y-positive cells that are not IB_4 immunoreactive. **A** Photomicrograph of a single section double stained for $P2Y_1$-IR and IB_4 in DRG. **B** Photomicrograph of a single section double stained for $P2Y_4$-IR and IB_4 in DRG. **C** Photomicrograph of a single section double stained for $P2Y_1$-IR and IB_4 in NG. **D** Photomicrograph of a single section double stained for $P2Y_4$-IR and IB_4 in NG. **E** Photomicrograph of a single section double stained for $P2Y_1$-IR and IB_4 in TG. **F** Photomicrograph of a single section double stained for $P2Y_4$-IR and IB_4 in TG

ment. These cells were identified with the antibody NF200 and found that many of these neurons coexpressed $P2Y_1$ (62–71%) and $P2Y_4$ (30–46%) receptor. On the other hand, only 34–45% of neurons positive for the $P2Y_1$ receptor stained for NF200 and a variable number of neurons positive for the $P2Y_4$ receptor stained for NF200 (97.4%, 73.7% and 45.2% in DRG, NG and TG, respectively). NF200 is a marker of A-fibre sensory neurons. Aδ-fibres play an important role in nociception. The normal pain threshold in humans is the threshold of Aδ-fibre nociceptors. The large-diameter neurons which are neurofilament-positive are known to possess myelinated axons and to be predominantly responsive to mechanical stimuli (Lawson and Waddell 1991; Perry et al. 1991). Therefore, our results suggest that the $P2Y_4$ receptors and perhaps some $P2Y_1$ receptors may participate in the conversion of mechanical stimuli into nerve impulses.

The remaining cells in the sensory ganglia form the classically defined 'small dark cell' population. These cells predominantly have unmyelinated C-fibre axons. Electrophysiological studies have shown that most of these cells (approximately 90%) in mouse, rat, monkey and human are nociceptive in function (Snider and McMahon 1998). These small cells can be further subdivided into two minimally overlapping groups on anatomical and functional bases: about one-half express the NGF receptors trkA and p75. Virtually all of this group contain the neuropeptide CGRP, and other neuropeptides, such as substance P, are also found in this population. We found that about 30% of $P2Y_1$ receptor-positive neurons coexpressed CGRP, while fewer $P2Y_4$-IR neurons were coexpressed with CGRP (10–20%).

The other half of the C-fibre population does not express NGF receptors (McMahon et al. 1994; Averill et al. 1995), but can be identified by a number of markers: they bind the lectin IB_4, express the enzyme TMP and stain with the antibody LA4 and $P2X_3$ receptor (Bradbury et al. 1998; Petruska et al. 2000). We have found in the current work that nearly 50% of $P2Y_1$ receptor-expressing neurons fall into this group. Conversely, the large majority of IB_4-binding neurons (more than 80%, DRG) expressed the $P2Y_1$ receptor. However, $P2Y_4$-IR neurons lacked IB_4 in these sensory ganglia. At the same time, we have found half of $P2Y_1$ receptor was coexpressed with $P2X_3$ receptor. Hence, it means that most $P2X_3$ and $P2Y_1$ receptors are located at the same class of neurons. The result is coincident with Borvendeg's work (Borvendeg et al. 2003), although in contrast to a previous report (Nakamura and Strittmatter 1996). $P2X_3$ receptor activation may lead to increased firing of DRG cells and subsequently to increased release of the sensory transmitter from their central processes. An opposite effect at the same cells is also conceivable; $P2Y_1$ receptor activation may decrease the release of the sensory transmitter and may thereby partly counterbalance the algogenic effect of ATP. P2Y purinoceptors are heptahelical, G protein-coupled receptors, mediating signal transduction via the induction of inositol triphosphate (IP3), leading to intracellular Ca^{2+} release (Strobaek et al. 1996). Thus, P2Y receptors appear to have a modulatory role, rather than directly mediating purinergic transmission.

In the present study, the $P2Y_1$ and $P2Y_4$ receptors were not colocalised directly. We have, however, colocalised these receptors with various other markers of subpopulations of primary afferent neurons. About 34–45% of $P2Y_1$ and 45–90% $P2Y_4$ receptor-positive neurons stained for NF200; about 30% of $P2Y_1$ and 10–20% $P2Y_4$ receptor-positive neurons coexpress CGRP; the percentage of $P2Y_1$ and $P2Y_4$ receptor-positive neurons coexpressing $P2X_3$ is almost the same. Therefore, there may be some overlap between expression of the $P2Y_1$ and $P2Y_4$ receptors.

In conclusion, our results suggest that sensory neurons could respond to ATP, not only through the ionotropic P2X receptors but also through metabotropic P2Y receptors. It will be of great interest to clarify the relative physiological and pathophysiological roles of these two receptor family subtypes.

References

Abbracchio MP, Burnstock G (1994) Purinoceptors: are there families of P2X and P2Y purinoceptors? Pharmacol Ther 64:445–475

Abbracchio MP, Boeynaems JM, Barnard EA, Boyer JL, Kennedy C, Miras-Portugal MT, King BF, Gachet C, Jacobson KA, Weisman GA, Burnstock G (2003) Characterization of the UDP-glucose receptor (re-named here the $P2Y_{14}$ receptor) adds diversity to the P2Y receptor family. Trends Pharmacol Sci 24:52–55

Ambalavanar R, Morris R (1993) An ultrastructural study of the binding of an alpha-D-galactose specific lectin from *Griffonia simplicifolia* to trigeminal ganglion neurons and the trigeminal nucleus caudalis in the rat. Neuroscience 52:699–709

Averill S, McMahon SB, Clary DO, Reichardt LF, Priestley JV (1995) Immunocytochemical localization of trkA receptors in chemically identified subgroups of adult rat sensory neurons. Eur J Neurosci 7:1484–1494

Bland-Ward PA, Humphrey PP (2000) P2X receptors mediate ATP-induced primary nociceptive neurone activation. J Auton Nerv Syst 81:146–151

Borvendeg SJ, Gerevich Z, Gillen C, Illes P (2003) P2Y receptor-mediated inhibition of voltage-dependent Ca^{2+} channels in rat dorsal root ganglion neurons. Synapse 47:159–161

Bradbury EJ, Burnstock G, McMahon SB (1998) The expression of P2X3 purinoreceptors in sensory neurons: effects of axotomy and glial-derived neurotrophic factor. Mol Cell Neurosci 12:256–268

Burnstock G (2001) Purine-mediated signaling in pain and visceral perception. Trends Pharmacol Sci 22:182–188

Burnstock G, Kennedy C (1985) Is there a basis for distinguishing 2 types of P2-purinoceptor? Gen Pharmacol 16:433–440

Chen CC, Akopian AN, Sivilotti L, Colquhoun D, Burnstock G, Wood JN (1995) A P2X purinoceptor expressed by a subset of sensory neurons. Nature 377:428–431

Cockayne DA, Hamilton SG, Zhu Q-M, Dunn PM, Zhong Y, Novakovic S, Malmberg AB, Cain G, Berson A, Kassotakis L, Hedley L, Lachnit WG, Burnstock G, McMahon SB, Ford AP (2000) Urinary bladder hyporeflexia and reduced pain-related behavior in $P2X_3$-deficient mice. Nature 407:1011–1015

Communi D, Gonzalez NS, Detheux M, Brezillon S, Lannoy V, Parmentier M, Boeynaems JM (2001) Identification of a novel

human ADP receptor coupled to G_i. J Biol Chem 276:41479–41485
Ding Y, Cesare P, Drew L, Nikitaki D, Wood JN (2000) ATP, P2X receptors and pain pathways. J Auton Nerv Syst 81:289–294
Hamilton SG, McMahon SB (2000) ATP as a peripheral mediator of pain. J Auton Nerv Syst 81:187–194
Hollopeter G, Jantzen HM, Vincent D, Li G, England L, Ramakrishnan V, Yang RB, Nurden A, Julius D, Conley PB (2001) Identification of the platelet ADP receptor targeted by antithrombotic drugs. Nature 409:202–207
King BF, Burnstock G (2002) Purinergic receptors. In: Pangalos M, Davies C (eds) Understanding G protein-coupled receptors and their role in the CNS. Oxford University Press, Oxford, pp 422–438
Kitchener PD, Wilson P, Snow PJ (1993) Selective labelling of primary sensory afferent terminals in lamina II of the dorsal horn by injection of Bandeiraea *Simplicifolia isolectin* B4 into peripheral nerves. Neuroscience 54:545–551
Lawson SN, Waddell PJ (1991) Soma neurofilament immunoreactivity is related to cell size and fibre conduction velocity in rat primary sensory neurons. J Physiol 435:41–63
Lewis C, Neidhart S, Holy C, North RA, Buell G, Surprenant A (1995) Coexpression of $P2X_2$ and $P2X_3$ receptor subunits can account for ATP-gated currents in sensory neurons. Nature 377:432–435
McCarthy PW, Lawson SN (1990) Cell type and conduction velocity of rat primary sensory neurons with calcitonin gene-related peptide-like immunoreactivity. Neuroscience 34:623–632
McCarthy PW, Lawson SN (1997) Differing action potential shapes in rat dorsal root ganglion neurones related to their substance P and calcitonin gene-related peptide immunoreactivity. J Comp Neurol 388:541–549
McMahon SB, Armanini MP, Ling LH, Phillips HS (1994) Expression and coexpression of Trk receptors in subpopulations of adult primary sensory neurons projecting to identified peripheral targets. Neuron 12:1161–1171
Molliver DC, Radeke MJ, Feinstein SC, Snider WD (1995) Presence or absence of TrkA protein distinguishes subsets of small sensory neurons with unique cytochemical characteristics and dorsal horn projections. J Comp Neurol 361:404–416
Nakamura F, Strittmatter SM (1996) P2Y1 purinergic receptors in sensory neurons: contribution to touch-induced impulse generation. Proc Natl Acad Sci U S A 93:10465–10470
Perry MJ, Lawson SN, Robertson J (1991) Neurofilament immunoreactivity in populations of rat primary afferent neurons: a quantitative study of phosphorylated and non-phosphorylated subunits. J Neurocytol 20:746–758
Petruska JC, Cooper BY, Gu JG, Rau KK, Johnson RD (2000) Distribution of P2X1, P2X2, and P2X3 receptor subunits in rat primary afferents: relation to population markers and specific cell types. J Chem Neuroanat 20:141–162
Plenderleith MB, Snow PJ (1993) The plant lectin *Bandeiraea simplicifolia* I-B4 identifies a subpopulation of small diameter primary sensory neurones which innervate the skin in the rat. Neurosci Lett 159:17–20
Ralevic V, Burnstock G (1998) Receptors for purines and pyrimidines. Pharmacol Rev 50:413–492
Rose RD, Rohrlich D (1988) Counting sectioned cells via mathematical reconstruction. J Comp Neurol 272:365–386
Sanada M, Yasuda H, Omatsu-Kanbe M, Sango K, Isono T, Matsuura H, Kikkawa R (2002) Increase in intracellular Ca^{2+} and calcitonin gene-related peptide release through metabotropic P2Y receptors in rat dorsal root ganglion neurons. Neuroscience 111:413–422
Silverman JD, Kruger L (1990) Selective neuronal glycoconjugate expression in sensory and autonomic ganglia: relation of lectin reactivity to peptide and enzyme markers. J Neurocytol 19:789–801
Snider WD, McMahon SB (1998) Tackling pain at the source: new insights into nociceptors. Neuron 20:629–632
Souslova V, Cesare P, Ding Y, Akopian AN, Stanfa L, Suzuki R, Carpenter K, Dickenson A, Boyce S, Hill R, Nebenuis-Oosthuizen D, Smith AJ, Kidd EJ, Wood JN (2000) Warm-coding deficits and aberrant inflammatory pain in mice lacking $P2X_3$ receptors. Nature 407:1015–1017
Streit WJ, Schulte BA, Balentine DJ, Spicer SS (1985) Histochemical localization of galactose-containing glycoconjugates in sensory neurons and their processes in the central and peripheral nervous system of the rat. J Histochem Cytochem 33:1042–1052
Strobaek D, Olesen SP, Christophersen P, Dissing S (1996) P2-purinoceptor-mediated formation of inositol phosphates and intracellular Ca2+ transients in human coronary artery smooth muscle cells. Br J Pharmacol 118:1645–1652
Tominaga M, Wada M, Masu M (2001) Potentiation of capsaicin receptor activity by metabotropic ATP receptors as a possible mechanism for ATP-evoked pain and hyperalgesia. Proc Natl Acad Sci U S A 98:6951–6956
Wang H, Rivero-Melian C, Robertson B, Grant G (1994) Transganglionic transport and binding of the isolectin B4 from *Griffonia simplicifolia* I in rat primary sensory neurons. Neuroscience 62:539–551
Zhang FL, Luo L, Gustafson E, Lachowicz J, Smith M, Qiao X, Liu YH, Chen G, Pramanik B, Laz TM, Palmer K, Bayne M, Monsma FJ (2001) ADP is the cognate ligand for the orphan G protein-coupled receptor SP1999. J Biol Chem 276:8608–8615