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AMPA and NMDA glutamate receptors are found in both peptidergic and non-peptidergic primary afferent neurons in the rat

Helen Willcockson and **Juli Valtschanoff**
Department of Cell and Developmental Biology, University of North Carolina, CB# 7090, Chapel Hill, NC 27599, USA, Phone: 919-962-6882; Fax: 919-966-1856

Helen Willcockson: hhw@med.unc.edu; Juli Valtschanoff:

Abstract

Two distinct classes of nociceptive primary afferents, peptidergic and non-peptidergic, respond similarly to acute noxious stimulation; however the peptidergic afferents are more likely to play a role in inflammatory pain, while the non-peptidergic afferents may be more characteristically involved in neuropathic pain. Using multiple immunofluorescence, we determined the proportions of neurons in the rat L4 dorsal root ganglion (DRG) that co-express AMPA or NMDA glutamate receptors and markers for the peptidergic and non-peptidergic classes of primary afferents, substance P and $P2X_3$, respectively. The fraction of DRG neurons immunostained for the NR1 subunit of the NMDA receptor (40%) was significantly higher than that of DRG neurons immunostained for the GluR2/3 (27%) or the GluR4 (34%) subunits of the AMPA receptor. Of all DRG neurons double-immunostained for glutamate receptor subunits and either marker for peptidergic and non-peptidergic afferents, a significantly larger proportion expressed GluR4 than GluR2/3 or NR1 and in a significantly larger proportion of $P2X_3$- than SP-positive DRG neurons. These observations support the idea that nociceptors, involved primarily in the mediation of neuropathic pain, may be presynaptically modulated by GluR4-containing AMPA receptors.

Keywords

Glutamate receptor; Dorsal root ganglion; Substance P; $P2X_3$; Rat

Introduction

The involvement of glutamate receptors in the mediation of nociception is documented by a large body of literature (for a review, see Bleakman et al. 2006). Besides being expressed in the membrane of spinal neurons postsynaptic to nociceptive afferents, subunits of both ionotropic and metabotropic glutamate receptors are synthesized by dorsal root ganglion (DRG) neurons and transported both peripherally and centrally (Willis and Coggeshall 2004). Peripheral terminals of primary afferents in the skin (Nunzi et al. 2004; Brumovsky et al. 2007) and palatine mucosa (Nunzi et al. 2004; Brumovsky et al. 2007) express vesicular glutamate transporters, suggesting that they release glutamate. Conversely, activation of the same peripheral terminals by glutamate or its agonists can induce aversive behavior (Carlton et al. 1995), suggesting that they also express glutamate receptors. Moreover, activation of glutamate receptors expressed in primary afferent terminals can decrease their release of

Correspondence to: Helen Willcockson, hhw@med.unc.edu.

glutamate (Lee et al. 2002; Bardoni et al. 2004) and/or facilitate their release of the neuropeptide substance P (SP, (Liu et al. 1997; Marvizón et al. 1997; Malcangio et al. 1998).

In our previous work we used immunohistochemistry to show that central terminals of DRG neurons in the superficial laminae of the dorsal horn express subunits of ionotropic and metabotropic glutamate receptors (Jia et al. 1999; Hwang et al. 2001a, 2001b; Lu et al. 2002, 2003). To provide further insight into the role that presynaptic glutamate receptors may play in pain modulation, we also identified the subunits of the kainate glutamate receptor that are expressed by nociceptive primary afferents (Lucifora et al. 2006). As a direct continuation of this study, we here aimed at determining whether α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) or *N*-methyl-D-aspartic acid (NMDA) receptors are preferentially expressed in either one or both of the two major classes of nociceptors, i.e. peptidergic (identified by immunostaining for SP) and non-peptidergic (identified by immunostaining for the $P2X_3$ subunit of the purinergic receptor).

Materials and Methods

All animals were treated according to the guidelines of the Institutional Animal Care and Use Committee (IACUC) of the University of North Carolina. Nine Sprague-Dawley rats (200–300 g, Charles River, Raleigh, NC) were deeply anesthetized with sodium pentobarbital (60 mg/kg, i.p.) and perfused through the heart with 100 ml normal saline, containing 500U heparin sodium followed by 4% paraformaldehyde (PF) in phosphate buffer (PB, 0.1M, pH 7.4) at room temperature. L4 DRG were removed and post-fixed in 4% PF for 2 hrs at 4 °C. The DRG were cryoprotected in 30% sucrose in PB overnight at 4 °C, embedded in Tissue-Tek O.C.T. compound (Sakura Finetek, Torrance, CA), and sectioned on a cryostat at 15 μm, thaw-mounted on slides, and stored at −20 °C.

For immunohistochemistry, all incubations were carried out on a shaker at room temperature. Sections were permeabilized with 50% ethanol, rinsed with phosphate-buffered saline (PBS, 0.01M, pH 7.2), blocked in 10% normal donkey serum (NDS) for 30 min, and incubated overnight in a mixture containing one antibody against a glutamate receptor subunit and either an anti-$P2X_3$ or an anti-SP antibody (Table 1). Following rinses with PBS and blocking with 2% NDS, the sections were incubated in Cy3- or FITC-conjugated secondary antibodies raised in donkey (1:200 in PBS; Jackson ImmunoResearch, West Grove, PA) for 2 hrs, then rinsed and coverslipped with Vectashield (Vector Laboratories, Burlingame, CA). For optimal staining for NR1 (Table 1), we used tyramide signal amplification (TSA; Perkin Elmer, Boston, MA): Sections were pretreated with 1% H_2O_2 followed by blocking buffer (5% NDS/0.1% Triton-X100/0.1 M PBS), and then incubated overnight in a mixture of anti-NR1 and anti-$P2X_3$ or anti-SP antibodies in blocking buffer. After brief rinses with PBS and blocking buffer, sections were incubated for 2 hrs in biotinylated donkey anti-rabbit and FITC-conjugated donkey anti-guinea pig antibodies (1:200; Jackson Immunoresearch). After several rinses, the sections were incubated with streptavidin-horseradish peroxidase (1:100 in amplification diluent) for 30 min, and then with Cy3-tyramide (1:100 in amplification diluent) for 8 min.

All antibodies used in this study are well characterized and are in common use in our laboratory (Lu et al. 2002, 2003; Bae et al. 2004; Lucifora et al. 2006; Kim et al. 2008). As a matter of routine control, sections were processed according to the above protocols, except that primary or secondary antibodies were omitted, or blocking peptides were added; omission of primary or secondary antibodies or preadsorption with blocking peptides completely abolished specific staining.

Dual-color images from DRG sections were acquired with a Retiga EX cooled CCD camera (QImaging, Burnaby, Canada) attached to a Leitz DMR fluorescent microscope (Leitz,

Wetzlar, Germany) at 10X, and saved as TIFF files using OpenLab software (Improvision, Lexington, MA). Brightness and contrast were adjusted with Photoshop CS2 (Adobe Systems, San Jose, CA); all enhancements were applied to the entire image. To quantify colocalization of GluR2/3, GluR4, and NR1 with SP or $P2X_3$ in DRG neurons, NP were counted in 4–5 sections per ganglion at 90 μm intervals from each of 6 ganglia from 3 rats for each double staining by an investigator blinded to the source material. Neuronal profiles were identified by their characteristic cellular morphology and clearly visible nucleus. In every section, NP were counted in one gray-scale image for each of the two fluorescent channels. For each channel, the cut-off brightness level (labeling density threshold) was determined by averaging the integral brightness of three neuronal profiles per image that were judged to be minimally positive using Image J 1.38x software (NIH, Bethesda, MD); all profiles whose mean labeling density exceeded this threshold were counted as positive. To avoid counting the same NP more than once, we used a modified version of the physical dissector principle (Coggeshall 1992; Carlton and Hargett 2002): each positive NP was labeled with a dot, color-coded after the respective channel, as they were counted. Immuno-negative NP with clearly visible nuclei were identified using sub-threshold ("background") labeling on the green channel and labeled with white dots; in some cases, these were verified in images of the same sections taken with DIC optics. To determine colocalization, the two gray-scale images containing the colored dots were overimposed and the fraction of NP in each of the four categories [green, red, yellow (green+red, double-labeled), and white (unlabeled)] in each section were normalized to the total number of NP and expressed as a percentage of total counted NP (Marvizón et al. 2002). Data were analyzed with one-way analysis of variance (ANOVA), followed by Tukey's post hoc test, using SPSS 11.5x software (SPSS, Chicago, IL) and graphed using Microsoft Excel.

Results

The percentages of immunostained DRG neuronal profiles (NP) and the patterns of colocalization varied according to the antibodies employed (Fig. 1, 2). Among the glutamate receptor subunits studied, the sparsest number of glutamate receptor-positive NP was immunostained with an antibody against GluR2/3 (26.7±0.9 of all NP; mean % ± S.E.M.); a larger proportion of NP were immunostained for either GluR4 (33.9±1.0) or NR1 (39.3±1.1). Of the markers for peptidergic and non-peptidergic primary afferent neurons, the proportion of NP immunostained for $P2X_3$ (22.1±0.8%) was higher than that for SP (13.1±0.4%).

The proportions of double-labeled NP for each glutamate receptor subunit and either SP or $P2X_3$ are presented in Fig. 3. Since inter-animal variability of these proportions was insignificant, results from different animals and ganglia were pooled together. More SP-expressing NP were immunopositive for GluR4 (56.1±3.3) or NR1 (48.6±2.5) than for GluR2/3 (28.7±2.4). Similarly, more $P2X_3$-expressing NP were immunopositive for GluR4 (53.0±2.8) or NR1 (41.9±3.5) than for GluR2/3 (11.7±1.3). The results also showed that: a) the proportion of glutamate receptor-expressing NP that also express either marker for peptidergic and non-peptidergic primary afferents was significantly higher for GluR4 (SP=23.6±1.4; $P2X_3$=35.2 ±2.1) than for either NR1 (SP=15.7±1.5; $P2X_3$=17.7±1.6) or GluR2/3 (SP=14.4±1.0; $P2X_3$=10.6±1.1), and b) the proportion of GluR4-positive NP that express $P2X_3$ was significantly higher than that of GluR4- positive NP that express SP (Fig. 3).

Discussion

This study shows that i) a significantly larger proportion of glutamate receptor-positive primary afferents express GluR4 than GluR2/3 or NR1, and ii) GluR4 is expressed in a significantly larger number of $P2X_3$- than SP-positive DRG neurons.

Nociceptive afferents have been classified into two largely distinct groups, peptidergic and non-peptidergic, based not only on their expression of neuropeptides, but also on their dependence on different growth factors for survival during postnatal development (Molliver et al. 1997) and topographic segregation of their termination, both centrally in different laminae of the dorsal horn, and peripherally in the skin (Hunt and Rossi 1985; Zylka et al. 2005). Functional differences include lower threshold and shorter duration of action potentials and shorter TTX-resistant currents and bigger heat currents (Stucky and Lewin 1999) and stronger tendency to sprout extensively after dorsal rhizotomy for the peptidergic nociceptors (Bennett et al. 1996). Moreover, even though both classes may respond similarly to acute noxious stimulation (Malmberg et al. 1997), the peptidergic afferents are more likely to play a role in inflammatory pain (Hunt and Mantyh 2001), while the non-peptidergic afferents may be more characteristically involved in neuropathic pain (Julius and Basbaum 2001).

The most commonly used histochemical markers for peptidergic and non-peptidergic nociceptive afferents are the expression of SP (Lawson et al. 1997; Nichols et al. 1999) and the propensity for binding the isolectin B4 of *Griffonia Simplicifolia* (IB4, (Silverman and Kruger 1990), respectively. However, since IB4 has been reported to bind more than one-half of the peptidergic afferents (Wang et al. 1994; Bergman et al. 1999; Kashiba et al. 2001), the classification of nociceptors based on the use of IB4 may be flawed (Hwang et al. 2005; Price and Flores 2007), and segregating peptidergic from non-peptidergic nociceptors remains controversial (Woolf and Ma 2007). Conversely, the colocalization of SP with $P2X_3$ is negligible (only 3% of the $P2X_3$-immunopositive DRG neurons were found to be SP-positive, (Vulchanova et al. 1998), making $P2X_3$ a marker of choice for a class of nociceptors that is distinct from those that could be identified by immunostaining for SP (Lucifora et al. 2006).

Depending on the ganglion level, species, and, perhaps more importantly, different criteria for determining immunopositivity, counts of SP-expressing neurons and of $P2X_3$- expressing neurons may vary, up to about 20% (Hokfelt et al. 1975; Battaglia and Rustioni 1988), and 35–65% (Bradbury et al. 1998; Vulchanova et al. 1998; Novakovic et al. 1999; Fukuoka et al. 2002), respectively, of the total DRG neuronal population. Counts in the present study were within the lower ranges reported previously for all antigens, including glutamate receptors (see also below).

All AMPA receptor subunits may be synthesized by DRG neurons (Chambille and Rampin 2002). Microscopic evidence for central transport has been provided mainly for the GluR2/3 and GluR4 subunits (Lu et al. 2002), suggesting a selective expression of Ca^{2+}-permeable homomeric or heteromeric complexes of AMPA receptors in primary afferents to the spinal cord. Presynaptic GluR4 is mainly expressed in terminals in lamina I and II of the dorsal horn, while presynaptic GluR3 is mainly expressed in terminals of primary afferents to deeper laminae that receive low-threshold mechanoreceptor input (Lu et al. 2002). Based on this pattern of termination, it is more likely that nociceptors express GluR4-containing presynaptic glutamate receptors. However, the anatomical definition of nociceptors as primarily small DRG neurons with central terminations in the superficial laminae of the dorsal horn may need to be revised (Light and Perl, 2003).

That NR1-expressing afferents include nociceptors is suggested by their pattern of termination in the spinal cord (Lu et al. 2003). After having been reported in central terminals of C fibers (Liu et al. 1994), presynaptic NMDA receptors were assigned a tentative role in the facilitation of release of SP from these terminals (Liu et al. 1997). The present observations suggest that, if indeed activation of presynaptic NMDA receptors facilitates SP release, this may occur in a fraction of central afferents that express the NMDA receptor. In the present work, we applied more stringent criteria of what is considered immunopositive, which lead to a more conservative estimate of NR1 expression in DRG neurons (40%) than the previously reported

84–90% (Wang et al. 1999; Marvizón et al. 2002). In an earlier study, we found that virtually all DRG neurons stain for NR1 but noted that the staining displayed a gradient of immunoreactivity making it difficult to sort out “positive” from “negative” cell bodies in DRG (Lu et al. 2003). In the same study, we also suggested that NR1-expressing primary afferents are mostly non-peptidergic since we saw no colocalization with CGRP. Since all DRG neurons possess high levels of mRNA for NR1 (Sato et al. 1993), but apparently express varying levels of the protein, the accuracy of any estimates obtained with immunohistochemistry will be limited by the inherent arbitrariness in choosing the cutoff labeling density. The use of TSA and higher cutoff density for counting in the present study may have accounted for the discrepancies between the current and our previous results.

In a previous report, we demonstrated that the kainate receptor (predominantly the GluR5 subunit) is expressed by a significantly larger fraction of $P2X_3$- than SP-positive DRG neurons (Lucifora et al. 2006). In the present work, we found that a significantly larger proportion of both classes of nociceptive afferents express GluR4 than GluR2/3 or NR1. This result is in agreement with a possible selectivity of GluR4 expression in nociceptive afferents (Lu et al. 2002). Furthermore, the results also show that, like in the case of GluR5, GluR4 is expressed in a significantly larger number of $P2X_3$- than SP-positive DRG neurons. Although both classes of primary afferents considered here may contribute to the sensation of acute pain, peptidergic afferents may play a role in inflammatory pain while afferents expressing $P2X_3$ receptors are more likely to be involved in neuropathic pain (Malmberg et al. 1997; Hunt and Mantyh 2001). Thus, the results of this study support the idea that nociceptive primary afferents, particularly those involved in the mediation of neuropathic pain, may be presynaptically modulated by GluR4-containing AMPA receptors.

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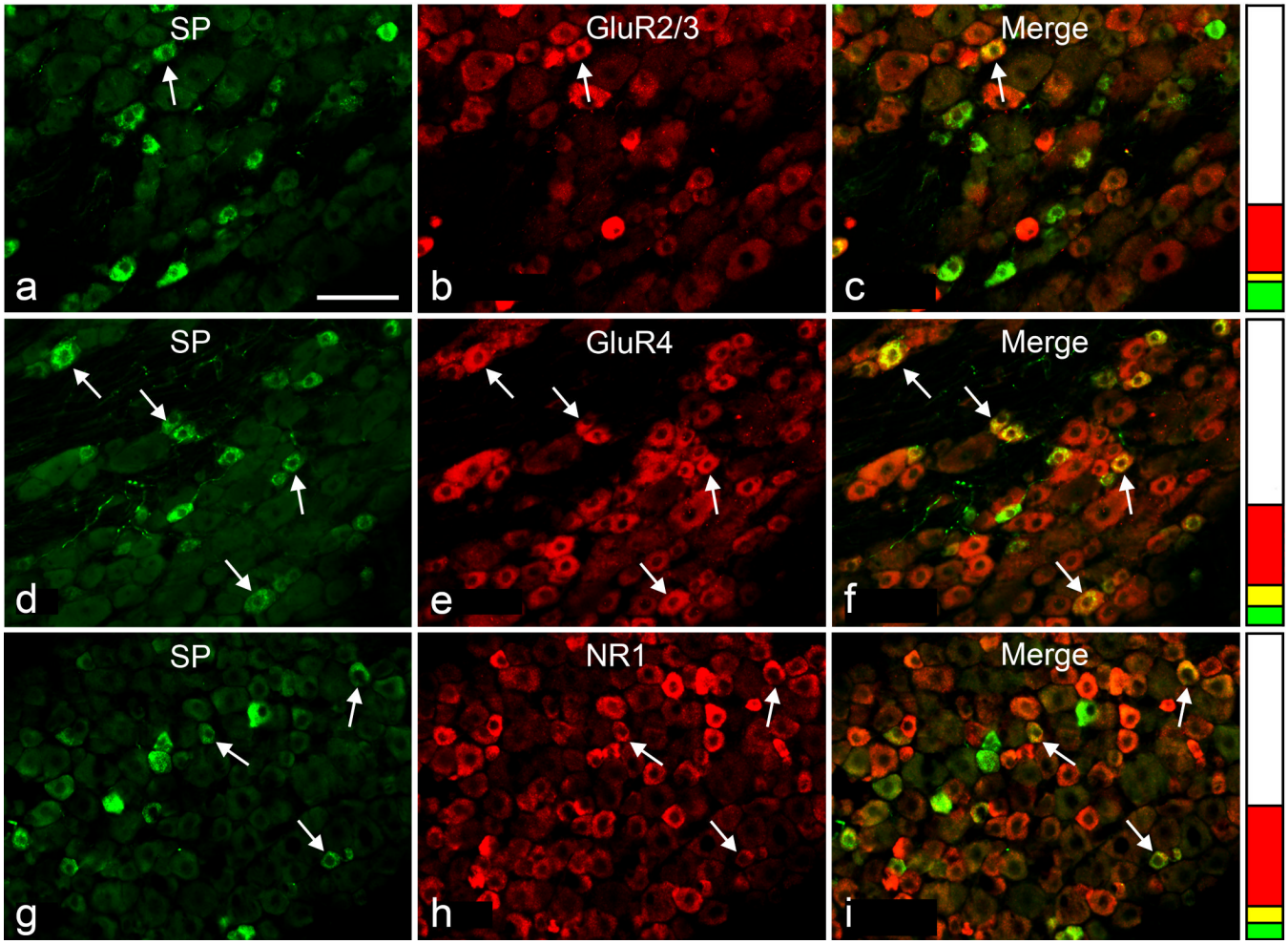

Fig. 1.

Double immunofluorescence staining for SP (a, d, g) and either GluR2/3 (b), GluR4 (e) or NR1 (h) in sections of L4 DRG; examples of double-stained neuronal profiles (arrows) appear yellow in the merged images (c, f, i). Stacked columns next to images in each row represent the neuronal profiles single-stained for SP (green), single-stained for glutamate receptors (red), double-stained (yellow), and unstained (white), as a fraction of all profiles for each double immunostaining. Scale bar, 100 μm

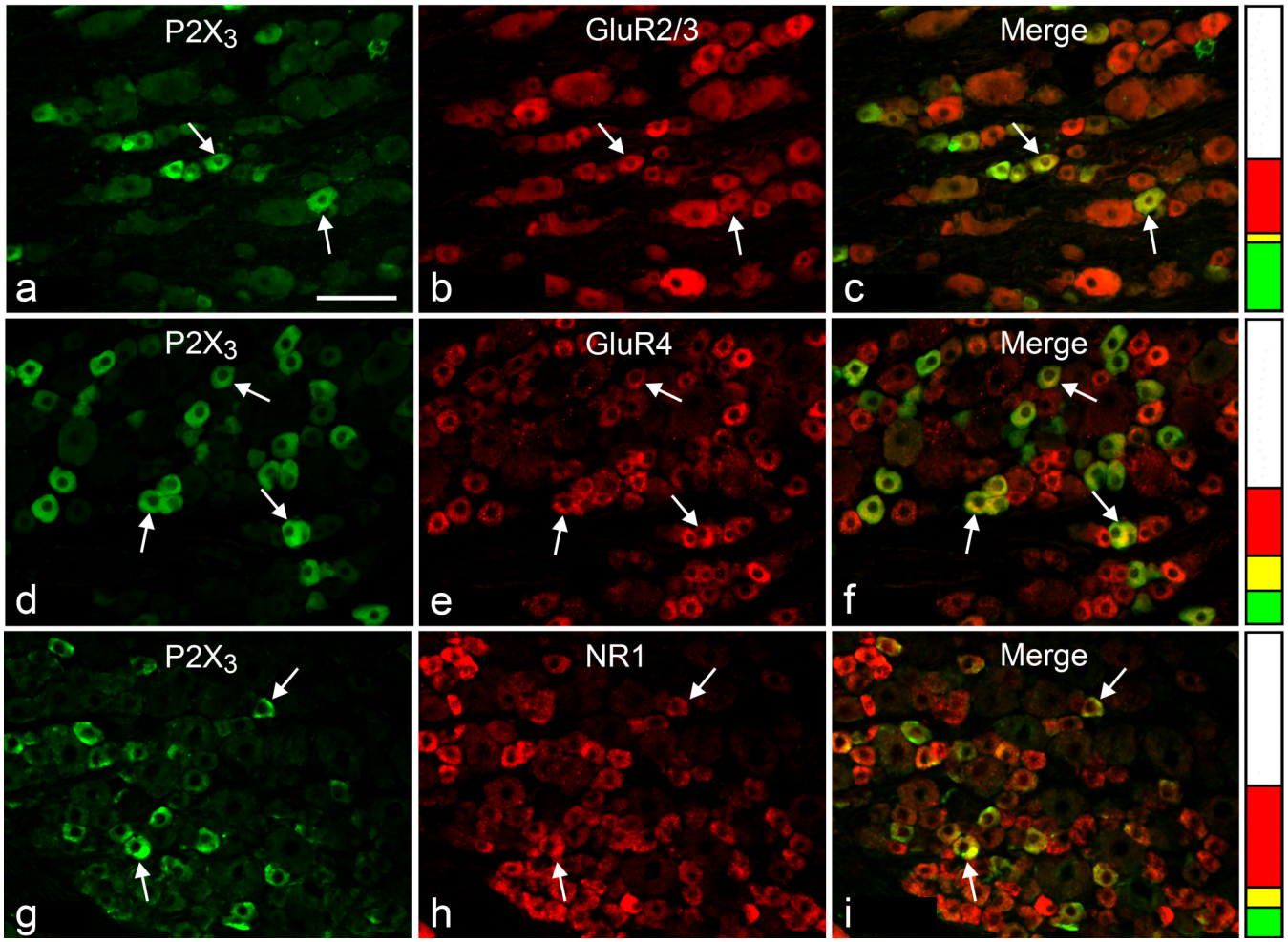

Fig. 2.

Double immunofluorescence staining for $P2X_3$ (a, d, g) and either GluR2/3 (b), GluR4 (e) or NR1 (h) in sections of L4 DRG; examples of double-stained neuronal profiles (arrows) appear yellow in the merged images (c, f, i). Stacked columns next to images in each row represent the neuronal profiles single-stained for $P2X_3$ (green), single-stained for the respective glutamate receptor (red), double-stained (yellow), and unstained (white), as a fraction of all profiles for each double immunostaining. Scale bar, 100 μm

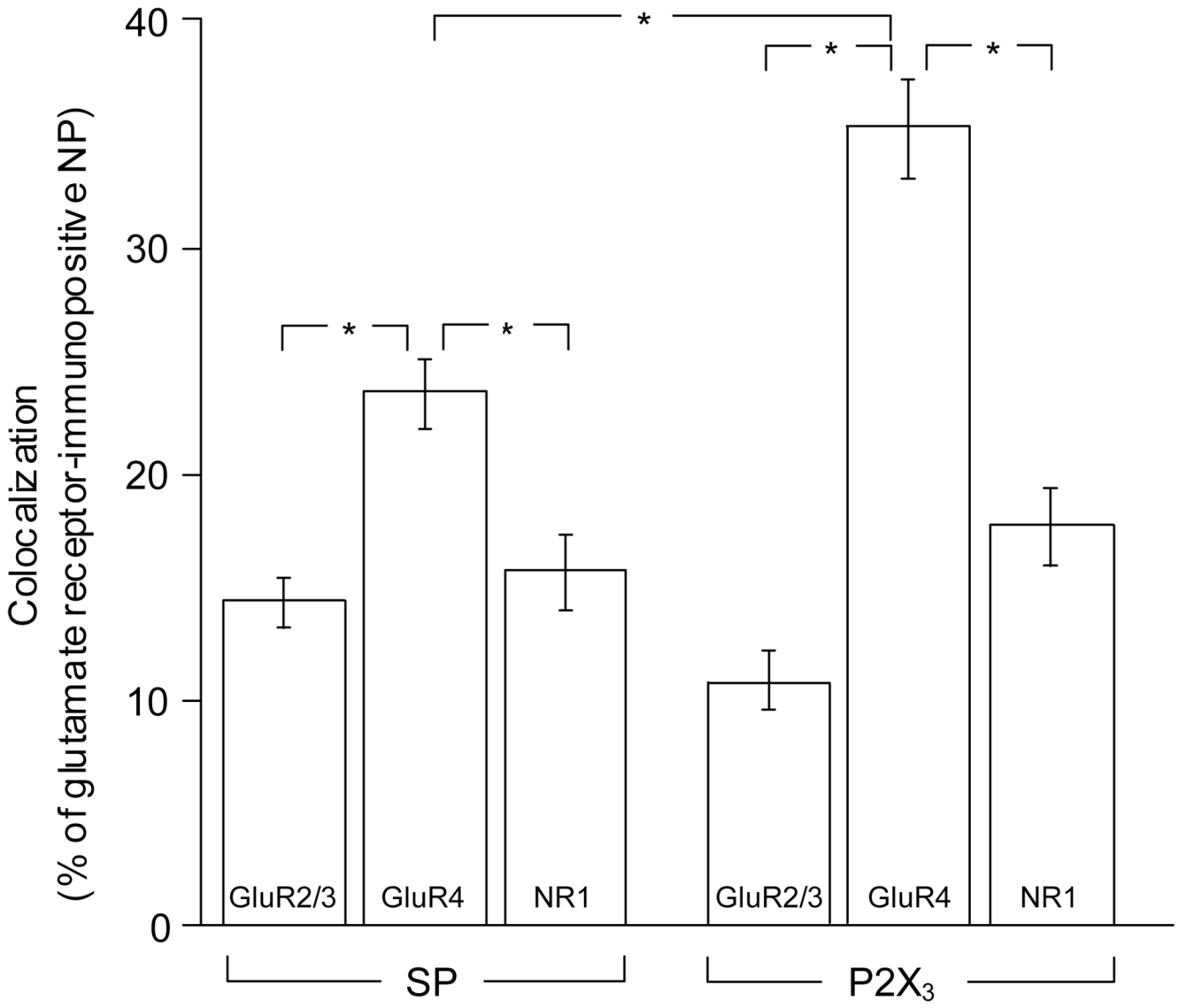

Fig. 3.

Graphs show percent (mean ± S.E.M.) of SP-positive (indicated at the bottom) or $P2X_3$-positive DRG neuronal profiles that immunostain for glutamate receptor subunits (indicated within the columns), as a fraction of all glutamate receptor-expressing neuronal profiles (NP) for each double immunostaining. *$p<0.001$, one-way ANOVA followed by Tukey's post hoc test

Table 1

Primary antibodies used in the study

Antibody	Epitope (AA)	Host	Source	Cat. #	Lot	Dilution
GluR2/3	871–883	Rabbit	Chemicon	AB1506	0507005717	1:100
GluR4	889–902	Rabbit	Chemicon	AB1508	21081551	1:250
NR1	909–938	Rabbit	Millipore	AB9864	0704057185	1:10,000
$P2X_3$	383–397	Guinea pig	Neuromics	GP10108	400457	1:250
SP	1–11	Guinea pig	Neuromics	GP14103	20046	1:250