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LOCALISATION OF P2X2 AND P2X3 RECEPTORS IN RAT TRIGEMINAL GANGLION NEURONS

V. Staikopoulos¹, B. J. Sessle², J.B. Furness¹, and E. A. Jennings¹

¹ Department of Anatomy and Cell Biology, University of Melbourne, Melbourne, Australia

² Faculty of Dentistry, University of Toronto, Toronto, ON M5G 1G6, Canada

Abstract

Purine receptors have been implicated in central neurotransmission from nociceptive primary afferent neurons, and adenosine 5'tri-phosphate (ATP)-mediated currents in sensory neurons have been shown to be mediated by both P2X₃ and P2X_{2/3} receptors. The aim of the present study was to quantitatively examine the distribution of P2X₂ and P2X₃ receptors in primary afferent cell bodies in the rat trigeminal ganglion, including those innervating the dura. In order to determine the classes of neurons that express these receptor subtypes, purine receptor immunoreactivity was examined for colocalisation with markers of myelinated (neurofilament 200; NF200) or mostly unmyelinated, non-peptidergic fibres (*Bandeiraea simplicifolia* isolectin B4; IB4). 40 % of P2X₂ and 64 % of P2X₃ receptor-expressing cells were IB4 positive, and 33 % of P2X₂ and 31 % of P2X₃ receptor-expressing cells were NF200 positive. Approximately 40 % of cells expressing P2X₂ receptors also expressed P2X₃ receptors and vice versa. Trigeminal ganglion neurons innervating the dura mater were retrogradely labelled and 52 % of these neurons expressed either P2X₂ or P2X₃ or both receptors. These results are consistent with electrophysiological findings that P2X receptors exist on the central terminals of trigeminal afferent neurons, and provide evidence that afferents supplying the dura express both receptors. In addition, the data suggest specific differences exist in P2X receptor expression between the spinal and trigeminal nociceptive systems.

Keywords

purine receptors; trigeminal ganglia; sensory neuron; migraine; pain

Purine receptors have been widely implicated in nociceptive processing (Burnstock, 2000; Khakh, 2001; Liu and Salter, 2005). The receptors are of two types: ionotropic (P2X) and metabotropic (P2Y), both of which are stimulated by adenosine 5'tri-phosphate (ATP). Seven P2X receptor subtypes have been cloned, and some occur as heteromultimers (e.g. P2X_{2/3}, P2X_{1/5} and P2X_{4/6} receptors). In the spinal somatosensory system, all P2X receptor subtypes with the exception of P2X₇ are expressed in the spinal dorsal horn, the dorsal root ganglion (DRG), and the central terminals of primary afferent neurons (Burnstock, 2000; Khakh, 2001; North, 2002). *In vivo* and *in vitro* electrophysiological recordings from single dorsal horn neurons have implicated P2X receptors on primary afferent terminals in spinal nociceptive transmission (Nakatsuka and Gu, 2001; Nakatsuka et al., 2002; Nakatsuka et al., 2003; Tsuzuki

Correspondence details: Dr. Ernest Jennings Dept. Anatomy & Cell Biology The University of Melbourne Parkville, Victoria, 3010, Australia Tel: +61 3 8344 5802 Fax: +61 3 9347 9619 Email: e.jennings@unimelb.edu.au.

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et al., 2003). Furthermore, behavioural studies in rodents have reported that intrathecal application of α,β -meATP (agonist at P2X₁, P2X_{2/3} and P2X₃ receptors) but not α,γ -meATP (agonist at P2X₁ receptors), elicits thermal hyperalgesia that can be prevented by intrathecal pre-treatment with the selective P2X₁, P2X₃, and P2X_{2/3} receptor antagonist 2,3-O-2,4,6-trinitrophenyl-ATP (TNP-ATP) (Tsuda et al., 1999; Stanfa et al., 2000; Tsuda et al., 2000; Honore et al., 2002). In combination, these results suggest a role for P2X₃ and P2X₂ receptors in central nociceptive mechanisms, consistent with studies in P2X₂ and P2X₃ double-knockout animals (Cockayne et al., 2005) where there is a complete loss of α,β -meATP-induced current in dissociated DRG neurons.

In the trigeminal somatosensory system, nociceptive primary afferents terminate in the brainstem, predominantly in the spinal trigeminal subnucleus caudalis which is also known as the medullary dorsal horn because of its morphological and functional similarities with the spinal dorsal horn (Dubner and Bennett, 1983; Bereiter et al., 2000; Sessle, 2000; Sessle, 2005). Central sensitisation in trigeminal brainstem nociceptive neurons can be elicited by noxious orofacial stimulation and its modulation by intrathecal application of P2X receptor agonists and antagonists indicates that P2X₃ or P2X_{2/3} receptors on the trigeminal primary afferent terminals in the medullary dorsal horn are involved (Hu et al., 2002; Chiang et al., 2005). *In vitro* studies (Sessle and Jennings, 2005) also suggest that activation of P2X_{2/3} or P2X₃ receptors on trigeminal nociceptive afferent endings in the medullary dorsal horn enhance trigeminal nociceptive transmission. These *in vivo* and *in vitro* electrophysiological findings are supported by findings of P2X receptor expression on the trigeminal ganglion cell bodies. These neurons supply tissues that are predominantly innervated by small-diameter nociceptive afferents, some of which have unique features related to pain (see Bereiter et al., 2000; Sessle, 2000; Sessle, 2005). These P2X receptor-expressing neurons include those innervating the rat and human tooth pulp (Cook and McCleskey, 1997; Alavi et al., 2001; Renton et al., 2003), rat temporomandibular joint (TMJ) (Ichikawa et al., 2004; Shinoda et al., 2005) and masseter muscle (Ambalavanar et al., 2005). Most of these studies reported P2X₃ receptor expression, and some noted increased P2X₃ receptor expression after inflammation or nerve injury (Eriksson et al., 1998; Ambalavanar et al., 2005; Shinoda et al., 2005).

Apart from neurons innervating the masseter muscle, P2X₂ receptor expression has not been quantified in trigeminal ganglion neurons (Xiang et al., 1998; Ambalavanar et al., 2005) and there is little information on the pattern of expression of P2X₂, P2X_{2/3} and P2X₃ receptors in neurons of the trigeminal ganglion. Therefore, the aims of this study were to examine and quantify the distribution of P2X₂ and P2X₃ receptors in trigeminal sensory neurons, and correlate this distribution with labelling by *Bandeiraea simplicifolia* isolectin B4 (IB4) and a high molecular weight neurofilament marker (NF200), which are neurochemical markers for mostly unmyelinated, non-peptidergic and medium to large-diameter myelinated afferent fibres, respectively. Furthermore, since there is no information published on P2X receptor involvement in afferent neurons innervating the dura mater, a structure supplied by nociceptive afferents and implicated in headaches (Moskowitz, 1984; Strassman et al., 1996; Messlinger and Ellrich, 2001; Goadsby, 2005), we examined the distribution of P2X₂ and P2X₃ receptor immunoreactivity in neurons retrogradely labelled from the dura mater. Some of this work has been presented in abstract form (Staikopoulos and Jennings, 2005).

Experimental Procedures

Animals

Experiments were conducted on 17 male Sprague-Dawley rats from the Anatomy and Cell Biology colony at the University of Melbourne. All procedures were approved by the University of Melbourne Animal Experimentation Ethics Committee.

Immunohistochemistry

All animals were terminally anaesthetised with an overdose of pentobarbitone (240 mg/ kg; Nembutal; Merial Labs, Australia) and perfused through the aorta with 250 ml of heparinised phosphate-buffered saline (PBS; pH 7.2), followed by 250ml of 4% paraformaldehyde in 0.2 M phosphate buffer (pH 7.3). Left trigeminal ganglia were removed from 13 animals, and postfixed for 4 hr. Tissue was then cryo-protected for 48 hr before being embedded in 100% optimal cutting temperature solution (OCT; Tissue Tek, Elkhart, Ind., USA). Frozen sections of 14 µm thickness were cut with a cryostat, and every 14th section (~ every 200 µm) was collected and thaw-mounted on 1 % gelatinised microscope slides and left to air dry for 1 hr at room temperature.

To characterise fibre types, localisation and cell size frequency distribution profiles of trigeminal ganglion neurons expressing P2X receptors, double labelling for P2X receptors and markers for subpopulations of C-type fibres (IB4) and A-type fibres (NF200) was performed; these markers are expressed by different neuronal populations. IB4 has been shown to predominantly label non-peptidergic unmyelinated fibres and neurons (Ambalavanar and Morris, 1993; Kitchener et al., 1993; Gerke and Plenderleith, 2004) whilst the widely used high molecular weight neurofilament marker, NF200, is known to specifically label myelinated primary afferent neurons (Lawson and Waddell, 1991).

All preparations except DiI preparations (see below) were pre-incubated in 10% normal horse serum or 10 % normal goat serum and 1% Triton X-100 in PBS for 30 min at room temperature to reduce non-specific binding and to permeabilise the tissue. Trigeminal ganglion sections were incubated for 48 hr at 4 °C with the following combinations of primary antibodies/ labels: P2X₂:IB4; P2X₂:NF200; P2X₃:IB4; P2X₃:NF200; or P2X₂:P2X₃.

Antibodies and labels

The following antibodies and labels were used: 1) Rabbit anti-P2X₂ (1:200, Chemicon International, Temecula, CA, USA—raised against amino acids 457-472 (SQQDSTSTDPKGLAQL) of the rat P2X₂ protein N terminal); 2) Rabbit anti-P2X₃ (1:1000, RA10109, Neuromics, Northfield, MN, USA -raised against amino acids 383-397 (VEKQSTDSGAYSIGH) of the rat P2X₃ protein); 3) FITC-conjugated isolectin B4 (5 µg/ ml) Vector Laboratories, Burlingame, CA, USA); 4) Mouse anti-NF200 (1:750; against neurofilaments of ~ 200 kDa molecular weight; Sigma, St Louis, MO, USA); and 5) anti-P2X₃ antibody raised in guinea-pig (1:1000, AB5896, Chemicon International, Temecula, CA, USA—raised against amino acids 383–397 (VEKQSTDSGAYSIGH) of the rat P2X₃ protein for the P2X₂, P2X₃ receptor double-label experiments). Following incubation in primary antibodies, sections were washed in PBS and then incubated for 1 hr at room temperature with the appropriate secondary antibody. The sections were incubated with either donkey anti-rabbit IgG coupled to Alexa 594 (1:1000; Molecular Probes Inc, Eugene, OR, USA), a mixture of donkey anti-rabbit IgG coupled to Alexa 594 (1:1000) and goat anti-mouse IgG coupled to Alexa 488 (1:500; Molecular Probes Inc, Eugene, OR, USA) or a combination of donkey anti-rabbit IgG Alexa 488 (1:500; Molecular Probes Inc, Eugene, OR, USA) and goat anti-guinea-pig IgG Alexa 594 (1:500; Molecular Probes Inc, Eugene, OR, USA). Tissue was then given 3 washes in PBS before being mounted in Dako fluorescence mounting medium (Dako Corp, CA., USA).

Controls

Both the P2X₂ (Castelucci et al., 2002) and P2X₃ (Poole et al., 2002) receptor antibodies have been previously characterised in this laboratory. In each tissue run, one sample was exposed to secondary antibody in the absence of the primary to test for non-specific staining. There was no non-specific binding in these experiments. In every section there were neurons which

labelled by only one of the primary-secondary antibody combinations, indicating lack of cross reactivity between secondary antibodies.

Retrograde tracing

In another set of experiments, animals were anaesthetised with halothane (4% for induction, maintained at 1–2 % in N₂O and O₂ in a 2:1 mix). A short parasagittal incision was made and a small burr hole (2–3 mm²) was drilled in the skull between lambda and bregma. DiI paste (Invitrogen, USA) or Fast Blue (3 µl; Sigma-Aldrich, Sydney, Australia) dissolved in 10 % dimethylsulphoxide in distilled water at 2 % w/v was applied between the skull and underlying meninges on the left side, and the hole in the skull was quickly sealed with a small drop of superglue to prevent spread of tracer to overlying tissues. The wound was closed with sutures and antibiotic cover provided (Terramycin, 200 mg/ kg i.m. Pfizer laboratories, USA). Animals were allowed to recover and 5 days later were terminally anaesthetised, perfused and tissue collected and processed for immunohistochemistry as described above.

Microscopy

Trigeminal ganglion sections were examined and analysed by confocal microscopy on a Biorad MRC1024 confocal scanning laser system installed on a Zeiss Axioplan 2 microscope. The confocal system was configured to visualise fluorescence in the green, red and far-red parts of the spectrum (images: 1024x1024 pixels). Immunoreactive cells were scanned and one optical section captured on the confocal microscope, with all images taken with a 20x objective. Images were taken of the complete mounted section to analyse regions throughout the trigeminal ganglion (usually 9-12 sections). All images were analysed and quantified using the Image J program (v1.32j, National Institute of Health, USA). For retrogradely labelled tissue, the skin overlying the burr hole was checked under a dissecting microscope (after perfusion) to confirm lack of tracer spread and thus non-specific afferent labelling.

Quantitative Analysis

Images from each staining combination (P2X₂/NF200, P2X₂/IB4, P2X₃/NF200, P2X₃/IB4 and P2X₂/P2X₃) were taken to measure and calculate the size frequency distribution of the stained trigeminal ganglion neurons in the 13 rats used; sections from one ganglion from each of 3 rats were stained with one of the combinations above. In many cases more than one series of sections, and hence more than one series of antibodies/ labels was tested per ganglion. Only cell bodies with a nucleus in the plane of the section and a clearly defined cell perimeter were counted. The cross-sectional areas of positively stained neuron cell bodies were measured using NIH ImageJ software. In 4 experiments where retrograde labelling was performed with DiI, retrogradely labelled trigeminal ganglion neurons were counted and the fluorescence filters changed to ascertain whether they also showed P2X₂ or P2X₃ receptor staining. Size frequency histograms were plotted using GraphPad Prism (GraphPad Software, San Diego, CA, USA). Data were pooled into 150 µm² bins.

Statistics

The Kolmogorov-Smirnov test (GraphPad Prism) was first used to see if the data were normally distributed. This showed that the data sets were not normally distributed and so the data were analysed using the nonparametric Kruskal-Wallis one way analysis of variance by ranks; followed by Dunn's post-hoc test. A probability of $P < 0.05$ was considered significant and data are expressed as median and spread (25th and 75th percentile range) unless otherwise specified.

Results

Co-localisation of P2X₂ receptor with IB4 & NF200

The co-localisation of P2X₂ receptors with IB4 and NF200 was examined quantitatively in order to determine the populations of trigeminal ganglion neurons expressing P2X₂ receptors. P2X₂ receptor labelling was observed in the cytoplasm of mainly small to medium-sized neurons that had a median cross-sectional area (quartile spread) of 624 μm^2 ; (25th percentile, 472–75th percentile, 874; $n = 5174$; range 161–2448 μm^2 ; Fig. 1A, C; Fig. 3). IB4 labelling was also observed in similarly sized neurons that had a median cross-sectional area of 539; 426–673 μm^2 ($n = 8634$; range 132–2147 μm^2 ; Fig. 1A'; Fig. 3). IB4 immunoreactivity was seen as either an intense cytoplasmic labelling or labelling of the cytoplasm with even labelling of the cell membrane. NF200 labelling was observed in the cytoplasm and membrane of mainly medium to large-sized neurons with a median cross-sectional area of 957; 721–1258 μm^2 ($n = 8666$; range 184–2944 μm^2 ; Fig. 1C'; Fig. 2). The cross-sectional areas of IB4-labelled and NF200-labelled neurons were significantly different ($P < 0.05$).

IB4 labelling was found in 40 % of P2X₂ receptor-positive neurons whilst 24 % of IB4-positive neurons also expressed P2X₂ receptor immunoreactivity (Table 1). Cells with immunoreactivity for both P2X₂ receptors and IB4 had a median cross-sectional area of 570; 461 - 704 μm^2 ($n = 906$; range 229–1560 μm^2). NF200-positive neurons occurred in 33 % of P2X₂ receptor-positive neurons and 12 % of the NF200-positive neurons expressed P2X₂ receptor immunoreactivity. Cells expressing both P2X₂ receptors and NF200 had a mean cross-sectional area of 818; 571–1060 μm^2 ($n = 606$; range 271–1881 μm^2).

Co-localisation of P2X₃ receptor with IB4 & NF200

P2X₃ receptor labelling was observed in mainly small to medium-sized neurons with a median cross-sectional area of 596; 462–800 μm^2 ($n = 4928$; range 134–2460 μm^2 ; Fig. 1B, 1D; Fig. 3); staining occurred in the cytoplasm and varied in intensity. IB4 labelling occurred in 64 % of P2X₃ receptor-positive neurons; however, only about a third (32 %) of the IB4-positive neuron population expressed P2X₃ receptor immunoreactivity. Cells expressing both P2X₃ receptor and IB4 immunoreactivity had a median cross-sectional area of 525; 416–642 μm^2 ($n = 1472$; range 170–1223 μm^2). NF200-positive neurons represented 31 % of the P2X₃ receptor-expressing population and 15 % of the cell population staining for NF200 was immunoreactive for P2X₃ receptors (Fig 1D). Cells with immunoreactivity for both P2X₃ receptors and NF200 had a median cross-sectional area of 909; 676–1147 μm^2 ($n = 534$; range 292–1962 μm^2).

P2X₂ and P2X₃ receptor co-localisation

In another series of experiments ($n = 3$), ganglion sections were stained with antibodies against both P2X₂ and P2X₃ receptors. Neurons expressing both P2X₂ and P2X₃ receptors in the cytoplasm were small to medium-sized and had a median cross-sectional area of 549; 425–791 μm^2 ($n = 440$; range 229–1552 μm^2 ; Fig. 3A; Fig 3E). P2X₂ receptor immunoreactivity was found in 41 % of P2X₃ receptor-expressing neurons and 46 % of the P2X₂ receptor-expressing population also expressed P2X₃ receptors (Table 1). The IB4-positive neurons that colocalised P2X₂ receptors had a median cross-sectional area significantly larger ($P < 0.05$) than those that colocalised P2X₃ receptors. A difference was also observed in neurons expressing NF200; neurons colocalised with P2X₃ receptors were significantly larger than those that colocalised P2X₂ receptors ($P < 0.05$).

P2X₂ and P2X₃ receptors on neurons innervating the dura

In four additional animals, trigeminal ganglion neurons projecting to the dura were retrogradely labelled with DiI (Fig. 3); 1022 cells were labelled. About half (52 %) were also immunoreactive for P2X₂, P2X₃ or both P2X₂ and P2X₃ receptors. 43 % of retrogradely labelled neurons expressed P2X₃ receptors, 19 % expressed P2X₂ receptors and 10 % of retrogradely labelled neurons expressed both P2X₂ and P2X₃ receptors. To confirm that the trigeminal ganglion neurons labelled with DiI were retrogradely labelled and that DiI had not spread to neighbouring neurons within the ganglion, a further three animals were retrogradely labelled with fast blue. The mean ($\pm$ SEM) number of neurons labelled with DiI was 303 ± 19 ($n=4$) was not significantly different from the number labelled with fast blue 319 ± 53 ($n=3$; $P > 0.5$; unpaired Student t -test), suggesting that there was no significant spread of DiI between neighbouring neurons in the ganglia.

DISCUSSION

P2X₂ and P2X₃ receptors are predominantly located on small to medium-sized trigeminal sensory neurons

This is the first quantitative study of P2X₂ immunoreactivity in neurons of the rat trigeminal ganglion to examine its co-localisation with IB4 and NF200 labelling. The study also quantified co-localisation with P2X₃ receptors, including P2X₂ and P2X₃ receptor localisation in afferent neurons innervating the dura. P2X₂ and P2X₃ receptors were found on both IB4-expressing neurons and NF200-expressing primary afferent neurons. Of the neurons that exhibited IB4 binding in the present study, 24 % were P2X₂ receptor positive and 32 % were P2X₃ receptor positive. Of the neurons that exhibited NF200 labelling, in the present study, 12 % were P2X₂ receptor positive and 15 % P2X₃ receptor positive. Thus, about a quarter to a third of IB4 positive neurons express P2X receptors in the trigeminal ganglia, and 12–15 % of NF200-positive fibres also express P2X receptors. It is also noteworthy that 52 % of neurons that projected to the dura were immunoreactive for one or both P2X receptors. The afferent innervation of the dura is primarily by C fibres innervating dural blood vessels that may be nociceptive and involved in the pain of migraine (Moskowitz, 1984; Strassman et al., 1996; Messlinger and Ellrich, 2001; Goadsby, 2005).

Our observations that P2X₃ receptor immunoreactivity was predominantly in medium as well as small-sized trigeminal ganglion neurons is consistent with the few earlier reports in the trigeminal ganglion (Eriksson et al., 1998; Llewellyn-Smith and Burnstock, 1998; Jiang and Gu, 2002; Ruan and Burnstock, 2003; Ichikawa and Sugimoto, 2004; Ruan et al., 2004; Ambalavanar et al., 2005; Shinoda et al., 2005). In addition, we found that 64 % of P2X₃ receptor-expressing trigeminal ganglion neurons also express IB4, suggesting that they are mainly non-peptidergic, unmyelinated afferents, since IB4 labelling is almost entirely limited to non-peptide unmyelinated afferents in the rat (Ambalavanar and Morris, 1993; Kitchener et al., 1993; Kitchener et al., 1994; Stucky and Lewin, 1999; Gerke and Plenderleith, 2001). This proportion is in agreement with previous reports in the rat (73 %, Ambalavanar et al., 2005) and mouse (70 %, Ruan et al., 2004) trigeminal ganglia, but contrasts with studies in the DRG where almost all P2X₃-expressing neurons are IB4-positive in the mouse (99 %, Ruan et al., 2004), rat (>94 %, Bradbury et al., 1998; Vulchanova et al., 1998; Novakovic et al., 1999) and cat (88 %, Ruan et al., 2005). In rat DRG, P2X₃ is expressed by few (1–3 %) peptide containing neurons (Bradbury et al., 1998; Vulchanova et al., 1998). Another difference with the DRG was our finding that 31 % of P2X₃ receptor-expressing neurons also expressed NF200 and that P2X₃ receptor expression occurred in 15 % of NF200-labelled trigeminal ganglion neurons, presumably reflecting expression mainly in A δ afferents since the high molecular weight neurofilament marker NF200 exclusively labels myelinated neurons (Lawson and Waddell, 1991). The size-frequency plot in the present study (Fig. 2) shows that the distribution of the

two markers only partially overlaps. While our results are similar to those in the mouse trigeminal ganglion where 15 % of NF200 neurons were reported to express P2X₃ receptors (Ruan et al., 2004), P2X₃ receptors are however, rare in NF200-expressing cells in DRG, e.g. only 0.5% in the mouse (Ruan et al., 2004), less than 1% in the rat (Bradbury et al., 1998) and 1.7% in the cat (Ruan et al., 2005). Interactions may also occur between trigeminal ganglion neurons and satellite glial cells that involve P2 receptors including P2X and P2Y receptors (Ruan and Burnstock, 2003; Weick et al., 2003).

This is the first study to quantitatively examine P2X₂ receptor expression in the rat trigeminal ganglion, and examine colocalisation with NF200 and IB4. P2X₂ receptors have previously been reported in trigeminal ganglia (Xiang et al., 1998) and specifically on trigeminal sensory neurons innervating the masseter muscle (Ambalavanar et al., 2005). In DRG, immunohistochemical studies have revealed that P2X₂ receptor-expressing neurons also express IB4, with little co-expression with NF200 (Vulchanova et al., 1997; Petruska et al., 2000), and an *in situ* hybridisation study reports that P2X₂ receptor mRNA is predominantly expressed in small, NF200-negative DRG neurons, with 14 % colocalisation (Kobayashi et al., 2005). In contrast, our study has revealed that a considerable proportion of P2X₂ receptor-expressing neurons also express NF200. Together with the P2X₃ receptor expression that we noted above, these findings emphasise the differences between trigeminal ganglia and DRG in P2X receptor expression.

P2X₂ receptor expression and its colocalisation with P2X₃ receptor expression

The present study has shown that about 40 % of neurons expressing P2X₃ receptors in the trigeminal ganglia also express P2X₂ receptors, and vice-versa. Previous studies in the rat and monkey DRG and trigeminal ganglion have reported that many cells with P2X₃ receptors also express P2X₂ (Vulchanova et al., 1997; Xiang et al., 1998; Petruska et al., 2000). This has been quantified for DRG cells where 12 % express both P2X₂ and P2X₃ mRNA, and in a subgroup of trigeminal ganglion neurons where P2X₂ receptors were expressed on 75 % of P2X₃ receptor positive neurons innervating the masseter muscle (Ambalavanar et al., 2005).

Previously, expression of both P2X₂ and P2X₃ receptors in the same neurons has been taken as an indication that there may be heteromeric P2X_{2/3} receptors in these cells (Lewis et al., 1995; Petruska et al., 2000). This has been shown pharmacologically by examining the kinetics of ATP-induced currents, which are different for P2X₃ and P2X_{2/3} receptor activation (Lewis et al., 1995; Cook and McCleskey, 1997). Recent studies in P2X₂ and P2X₃ double-knockout animals have shown that these ATP-induced currents in sensory neurons are entirely mediated by P2X₃ and P2X_{2/3} receptors, as the ATP agonist at P2X₁, P2X_{2/3} and P2X₃ receptors, α,β -meATP induces no current in neurons from double-knockout animals (Cockayne et al., 2005).

Functional roles of P2X receptors in nociceptive signalling

Our findings that both P2X₂ and P2X₃ receptor expression occurs in trigeminal ganglion neurons, including those innervating the dura, are relevant to recent studies implicating P2X receptors in nociceptive transmission. A number of pharmacological and behavioural studies have provided data suggesting that P2X_{2/3} and P2X₃ receptors in particular are involved in transmission of nociceptive pain signals (Burnstock, 2000; Khakh, 2001; North, 2002). The most likely site of involvement is at synapses from primary afferent neurons to second-order neurons in the medullary and spinal dorsal horns. Activation of P2X receptors on the presynaptic terminals is believed to increase the release of the excitatory transmitter glutamate (Nakatsuka and Gu, 2006). As the present and other studies have shown, the primary afferent neurons express both P2X₂ and P2X₃ receptors. *In vitro* studies in the spinal dorsal horn have shown that the P2X receptor agonist α,β -meATP causes an increase in glutamatergic

neurotransmission at synapses in the superficial dorsal horn via a P2X₃ receptor-dependent pathway and at synapses in the deep dorsal horn via slowly desensitising P2X receptors (Nakatsuka and Gu, 2001; Nakatsuka et al., 2002; Nakatsuka et al., 2003; Tsuzuki et al., 2003). We have reported analogous results in the medullary dorsal horn (Jennings et al., 2006). These *in vitro* data imply that P2X_{2/3} receptors may be located presynaptically on the central terminals of primary afferent neurons. In the case of trigeminal primary afferents with their central terminals in the medullary dorsal horn, this would include P2X receptor-expressing nerve terminals innervating the rat and human tooth pulp (Cook et al., 1997; Alavi et al., 2001; Renton et al., 2003) rat TMJ (Ichikawa et al., 2004; Shinoda et al., 2005) masseter muscle (Ambalavanar et al., 2005) and dura (present study).

Other pharmacological studies indicate that P2X_{2/3} and P2X₃ receptors play important roles. Nociceptive behaviour evoked by either peripheral inflammation in the hind-limb or intrathecal application of α,β -meATP can be inhibited by the P2X receptor antagonist TNP-ATP (Tsuda et al., 1999) or the highly selective P2X_{2/3}/P2X₃ receptor antagonist A-317491 (McGaraughty et al., 2003), and P2X₃ receptor gene ablation or antisense oligonucleotides targeting the P2X₃ receptor gene result in significant antinociception (Honore et al., 2002; Cockayne et al., 2005). While the above findings support P2X_{2/3} and/or P2X₃ receptor involvement in nociceptive processing, one study in the spinal dorsal horn has implicated P2X₁ receptors since β,γ -meATP (but not α,β -meATP) was reported to increase both evoked responses and after-discharges in deep dorsal horn neurons following C-fibre stimulation (Stanfa et al., 2000). In the trigeminal system, application of an inflammatory agent or α,β -meATP to the TMJ results in nociceptive behaviours (Oliveira et al., 2005; Shinoda et al., 2005) and our *in vivo* recordings from brainstem nociceptive neurons have shown that α,β -meATP (but not β,γ -meATP) applied to the medullary dorsal horn can mimic central sensitisation induced by mustard oil to the tooth pulp, and that this central sensitisation can be blocked by TNP-ATP (Hu et al., 2002; Chiang et al., 2005).

Our retrograde tracing studies show that there is moderate innervation of the dura and cerebrovasculature by trigeminal afferents. The tracers were applied to the dura near the superior sagittal sinus and in the territory of the middle meningeal artery in order to maximise the peripheral receptive field labelled. The numbers of neurons labelled are similar to those reported previously (Mayberg et al., 1984). Retrograde tracing studies have shown that varying proportions of neurons innervating orofacial structures express P2X₃ receptors. P2X₃ receptors are reported to be expressed in 32 % (Ichikawa and Sugimoto, 2004) or 16 % (Ambalavanar et al., 2005) of trigeminal neurons innervating the skin, 31 % innervating the tooth pulp (Ichikawa and Sugimoto, 2004), 25 % innervating the masseter muscle (Ambalavanar et al., 2005) and 49 % (Shinoda et al., 2005) or 52 % (Ichikawa et al., 2004) of neurons innervating the TMJ. In the present study we found that 43 % of trigeminal neurons innervating the dura express P2X₃ receptors, 19 % express P2X₂ receptors, and 10 % of neurons express both P2X₂ and P2X₃ receptors. Since many of the afferents supplying these tissues are nociceptive (e.g. Sessle, 2000; Sessle, 2005) our findings suggest that P2X₂ and P2X₃ receptors have a critical role in the modulation of craniofacial pain. Indeed, the present study has shown that a high proportion (52%) of afferent neurons that innervate the dura express P2X receptors and it is likely that these neurons include a high proportion of the nociceptors that convey vascular nociceptive-related information from the dura (Moskowitz, 1984; Strassman et al., 1996; Messlinger and Ellrich, 2001; Goadsby, 2005). As inflammatory nociceptive behaviour can be ameliorated by P2X receptor antagonism, it is likely that P2X receptor antagonists could have a therapeutic use in vascular pain conditions such as migraine headache.

Conclusions

This study has quantified the extent of the distribution of P2X₂ and P2X₃ receptor subtypes in rat trigeminal primary afferent neurons and documented that P2X₂ and P2X₃ receptor expression occurs predominantly on small to medium-sized trigeminal afferent neurons. The present findings that the majority of neurons innervating the dura express P2X₂ and/or P2X₃ receptors suggest that purines may be involved in nociceptive processing in pain conditions such as migraine headache. While these trigeminal data are consistent with several aspects of earlier findings in trigeminal and DRG neurons, they do highlight some differences between spinal and trigeminal systems in P2X receptor expression.

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Abbreviations

ATP	adenosine 5' tri-phosphate
α,β -meATP	α, β - methylene ATP
β,γ -meATP	β, γ - methylene ATP
DRG	dorsal root ganglion
IB4	<i>Bandeiraea simplicifolia</i> isolectin B4
NF200	high molecular weight neurofilament marker
PPADS	Pyridoxalphosphate-6-azophenyl-2',4'-disulfonic acid tetrasodium salt
TMJ	temporomandibular joint
TNP-ATP	2,3 -O-2,4,6-trinitrophenyl-ATP

Fig 1. P2X₂ or P2X₃ receptor expression in rat trigeminal ganglion neurons

A) P2X₂ receptor and IB4 (A') with colocalisation shown in yellow (A''). IB4 immunoreactivity labelled predominantly small-sized neurons, a sub-population of which were P2X₂ receptor immunoreactive. B) P2X₃ receptor and IB4 (B') showing colocalisation in yellow (B''). C) Localisation of P2X₂ receptors and NF200 (C') with neurons expressing both markers seen in yellow (C''). D) Labelling of P2X₃-receptor (D) and NF200 (D') with colocalisation seen in yellow (D''). P2X-receptor immunoreactivity occurred in the cytoplasm of neurons and was not detected in the nucleus. In all rows, an example of double labelling is indicated with an arrow. Scale-bar 50 μ m

Fig 2. Size frequency histograms of P2X₂, P2X₃, IB4 and NF200 labelling in trigeminal ganglion neurons

Data showing the mean number of immunoreactive cell profiles counted per ganglion. A) Histograms showing size-frequency distribution of neurons expressing P2X₂ (black), IB4 (diagonal lines) and both (white). B) Histograms of the size-frequency distribution of neurons expressing P2X₂ (black), NF200 (diagonal lines) and both (white). C) Size-frequency distribution of neurons immunostained for P2X₃ (black), IB4 (diagonal lines) and double labelled (white). D) Size-frequency distribution of neurons expressing P2X₃ (black), NF200 (diagonal lines) and both (white).

Fig 3. Distribution of P2X₂ and P2X₃ in the same trigeminal neurons

A) Size-frequency histogram of neurons expressing P2X₂ (black), P2X₃ (diagonal lines) and both receptors (white). Triple labelling for P2X₃-receptor (C) and P2X₂-receptor (D) with retrograde labelled cell seen in red (B) which co-localises with staining for both P2X₃ and P2X₂ receptors (E). Scale-bar 50 μm.

Table 1Co-localisation of P2X₂ and P2X₃ with IB4, NF200 and with each other in trigeminal ganglion neurons

Percentage		Percentage		Percentage	
P2X ₂ of IB4	23.5	P2X ₃ of IB4	32.3	P2X ₂ of P2X ₃	41.4
IB4 of P2X ₂	40.1	IB4 of P2X ₃	63.9	P2X ₃ of P2X ₂	45.6
P2X ₂ of NF200	12.0	P2X ₃ of NF200	15.1		
NF200 of P2X ₂	33.2	NF200 of P2X ₃	30.9		