Distribution of purinergic P2X receptors in the equine digit, cervical spinal cord and dorsal root ganglia

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Abstract Purinergic pathways are considered important in pain transmission, and P2X receptors are a key part of this system which has received little attention in the horse. The aim of this study was to identify and characterise the distribution of P2X receptor subtypes in the equine digit and associated vasculature and nervous tissue, including peripheral nerves, dorsal root ganglia and cervical spinal cord, using PCR, Western blot analysis and immunohistochemistry. mRNA signal for most of the tested P2X receptor subunits (P2X_{1–5}, 7) was detected in all sampled equine tissues, whereas P2X₆ receptor subunit was predominantly expressed in the dorsal root ganglia and spinal cord. Western blot analysis validated the specificity of P2X_{1–3}, 7 antibodies, and these were used in immunohistochemistry studies. P2X_{1–3}, 7 receptor subunits were found in smooth muscle cells in the palmar digital artery and vein with the exception of the P2X₃ subunit that was present only in the

vein. However, endothelial cells in the palmar digital artery and vein were positive only for P2X₂ and P2X₃ receptor subunits. Neurons and nerve fibres in the peripheral and central nervous system were positive for P2X_{1–3} receptor subunits, whereas glial cells were positive for P2X₇ and P2X₁ and 2 receptor subunits. This previously unreported distribution of P2X subtypes may suggest important tissue specific roles in physiological and pathological processes.

Keywords Horse · Purinergic P2X receptors · Fore limb · Dorsal root ganglia

Introduction

P2X receptors are part of the P2 purinergic family of proteins that use extracellular adenosine-based nucleotides, such as, adenosine triphosphate (ATP), as ligands [1] and are often implicated in pain-related pathways [2]. Upon binding ATP (and related nucleotides), conformational changes result in channel opening and flux of monovalent and divalent cations including Na⁺, K⁺ and Ca²⁺ [3]. Currently seven P2X receptor subunits are recognised in vertebrates (P2X_{1–7}), each being encoded by its own gene [4]. P2X receptors consist of three individual subunits that can assemble to form homomeric or heteromeric receptors, resulting in the functional and pharmacological diversity seen with P2X receptor phenotypes [5].

The recognition of ATP as an extracellular signalling molecule was noted in the 1920s [6], but it was the late 1970s when ATP was reported to induce pain sensation [1]. Since this time, ATP activation of purinergic signalling pathways has been described in neurons and related cells in both the peripheral and central nervous systems [2, 7]. In addition to functions in the nervous tissues, the role of P2X receptor activation by ATP in non-excitable cells (such as leukocytes and epithelial cells) is being increasingly

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recognised [8]. Endogenous ATP release by endothelial and epithelial cells in response to alterations in the local tissue environment (for example, hypoxia/acidosis) can result in responses such as cell proliferation, differentiation and survival [9, 10]. Differences in P2X receptor subtype expression within and between tissue types may therefore allow for tissue- and zonal-specific response to extracellular ATP release and help to understand the potential role of P2X receptors in a number of physiological and pathological processes.

Purinergic P2X receptor distribution in the horse has received little attention [for example, 11] and is of interest with regard to pain pathways, where a number of conditions exist resulting in significant pain (for example, laminitis of the hooves). The main aim of this study was to identify and characterise the distribution of P2X receptor subtypes in tissues in the equine digit and associated nervous tissue, including the peripheral nerves, dorsal root ganglia and cervical spinal cord with a view to understanding their role in these tissues.

Materials and methods

Preparation of samples

Tissue samples were collected with owner consent immediately post-mortem from euthanised horses ($n=4$; mean age, 12 years old) in accordance with institutional ethical approval. Animals had no previous history or evidence of forelimb or neurological disease. Sampled tissues included the palmar digital artery (*a. digitalis lateralis/medialis*), palmar digital vein (*v. digitalis lateralis/medialis*), palmar digital nerve (*n. digitalis palmaris lateralis/medialis*), hoof, ipsilateral eighth dorsal root ganglion and spinal cord (forming median nerve (*n. medianus*) innervating the hoof) and ipsilateral fourth dorsal root ganglion and spinal cord (not innervating the hoof).

Tissue samples were divided into three equal parts and stored in either RNAlater (Applied Biosystems) at -80°C (for RNA extraction), snap frozen in liquid nitrogen and stored at -80°C (for protein extraction) or fixed in 4 % *w/v* neutral buffered paraformaldehyde (PFA) (for immunohistochemical analysis).

RNA extraction and cDNA synthesis

Samples collected for mRNA signal detection and PCR experiments were homogenised in TRI reagent (Applied Biosystems), and total RNA was isolated using RNeasy Mini Kit (Qiagen). The RNA yield of the samples was determined in a NanoDrop 1000 Spectrophotometer (ThermoScientific). Reverse transcription was then performed to synthesize

complementary DNA (cDNA). Briefly, 1–2 μg of extracted RNA, 1 μL of Random Primers (0.5 $\mu\text{g}/\mu\text{L}$, Promega) per microgramme RNA and RNase-free water to a volume of 13 μL were heated at 70°C for 5 min (Px2 Thermal Cycler, Thermo Electron Corporation) and quickly chilled on ice. Following this, 12 μL of mastermix, containing 5 μL of M-MLV 5x Reaction Buffer (Promega), 1 μL each of dATP, dCTP, dGTP and dTTP (10 mM, Bioline), 1 μL M-MLV Reverse Transcriptase (200 U/ μL , Promega), 0.625 μL of RNasin® Plus RNase Inhibitor (40 U/ μL , Promega) and RNase-free water to 12 μL , were added to the mixture and the tube incubated at 37°C for 60 min followed by 10 min at 95°C for inactivation of the reverse transcriptase (Px2 Thermal Cycler, Thermo Electron Corporation). Finally, the DNA concentration of the obtained cDNA samples was measured with a NanoDrop 1000 Spectrophotometer (ThermoScientific).

PCR amplification

Primers for equine P2X₁, 2, 4–7 mRNA were designed with PrimerExpress v3.0 (Applied Biosystems) or manually (P2X₃) based on predicted sequences in NCBI and Ensembl databases (Table 1). To avoid amplification of potential contaminating genomic DNA at least one of the primers in each pair was designed to span an exon–exon junction. Additionally, the primers within a primer pair were also placed on different exons thereby spanning an intron. PCR reactions were performed in 50- μL final volumes containing 5 μL of 10x PCR Buffer (with MgCl_2 , Sigma-Aldrich), 1 μL of dNTP mix (10 mM, Bioline), 15 pmol each of the appropriate forward and reverse primers (Applied Biosystems), 0.5 μL Taq DNA Polymerase (5 U/ μL , Sigma) and 1 μL of cDNA sample in distilled water. Cycling conditions consisted of a step of denaturation at 94°C for 10 min, 35 cycles of denaturation at 94°C for 30 s, annealing at primer-specific temperature for 30 s and elongation at 72°C for 1 min, followed by a final elongation step at 72°C for 10 min (Px2 Thermal Cycler, Thermo Electron Corporation). In all experiments, negative controls were included which substituted distilled water for template. PCR amplified products were analysed by agarose gel electrophoresis, visualised under a UV transilluminator (GeneGenius BioImaging System) and a photograph was taken (GeneSnap). Acquired images were then processed using Adobe Photoshop CS3 extended v10.0 image processing programme.

PCR amplified product identity was confirmed by size comparison with the predicted sequences and by subsequent DNA sequencing. PCR products were purified for DNA sequencing with QIAquick PCR purification kit or gel extraction kit (Qiagen). Purified PCR products were sent along with the corresponding primer pair for sequencing at Source BioScience LifeSciences (Nottingham). The sequencing

Table 1 Primer sequences (forward/reverse) for equine P2X_{1–7} cDNAs

Primer pair		Sequence	Amplicon length	Annealing temperature	NCBI or Ensembl accession number
eqP2X ₁	Forward	TGAGTACGACACGCCTCGAA	569 bp	59 °C	XM_001504730.1 (NCBI)
	Reverse	TGCGCCTTTTGACCTTGAA			ENSECAT00000010642 (Ensembl)
eqP2X ₂	Forward	AATTCAGTTCTCTAAGGGCAACA	186 bp	59 °C	ENSECAT00000017460 (Ensembl)
	Reverse	CCCAGTTGATAATGACCCCAAT			
eqP2X ₃	Forward	CAGTGGAAATGCCTGTGCATGA	281 bp	59 °C	XM_001504914.1 (NCBI)
	Reverse	GGCCTTGTCCTCAAGTCGCA			ENSECAT00000018031 (Ensembl)
eqP2X ₄	Forward	CGGCTACAACCTTCAGGTTTGC	272 bp	59 °C	XM_001492153.2 (NCBI)
	Reverse	CCTGATCGTAATCTGCCACATATT			ENSECAT00000025636 (Ensembl)
eqP2X ₅	Forward	ACCCAGGGAGAGAACGTCTT	527 bp	60 °C	XM_001918102.1 (NCBI)
	Reverse	TGCATTGGAGGGAGCTTTAT			ENSECAT00000026624 (Ensembl)
eqP2X ₆	Forward	CAACTTCAGGACAGCCACTCACT	216 bp	59 °C	ENSECAT00000026645 (Ensembl)
	Reverse	GCTTCTCCATCCACGTACAACA			XM_001488204.2 (NCBI)
eqP2X ₇	Forward	TTCCTACGTTATCTTTGCCTTGGT	169 bp	59 °C	ENSECAT00000009106 (Ensembl)
	Reverse	GGTGTAGTCTGCGGTGTGCGAA			XM_001495572.1 (NCBI)
					ENSECAT00000010815 (Ensembl)

results were analysed and sequenced and predicted nucleotide sequences were compared with an aligner tool (www.justbio.com).

Protein extraction and Western blot analysis

Western blots were carried out to validate P2X_{1–7} antibodies used for equine tissue. Tissue samples were snap frozen in liquid nitrogen and homogenized in RIPA buffer (Sigma) supplemented with cOmplete Mini protease inhibitors cocktail (Roche). The Pierce BCA assay (ThermoScientific) was used to determine protein concentrations. Protein samples (25 µg) were mixed with lane marker non-reducing sample buffer (ThermoScientific) and DTT (0.1 M) and denatured at 70 °C for 10 min. Samples used in Western blots for P2X_{1, 3–7} proteins were denatured, whereas samples for P2X₂ protein did not undergo denaturation since P2X₂ antibody showed better results with non-denatured samples. Samples and molecular weight markers were loaded and separated on NuPage 4–12 % Bis–Tris gel (Invitrogen) and then transferred onto nitrocellulose membrane (Whatman). Membranes were blocked in 5 % skimmed dry milk in phosphate-buffered saline for 1 h at room temperature and incubated overnight at 4 °C with the primary antibody in 0.1 % Tween blocking solution, (1) anti-P2X₁ rabbit polyclonal antibody (Alomone) 1:200, (2) anti-P2X₂ rabbit polyclonal antibody (Abcam) 0.6 µg/mL, (3) anti-P2X₃ rabbit polyclonal antibody (Neuromics) 1:1,000, (4) anti-P2X₄ whole serum rabbit antibody (Abcam) 1:300, (5) anti-P2X₅ rabbit polyclonal antibody (Alomone) 1:200, (6) anti-P2X₆ rabbit polyclonal antibody (Abcam)

10 µg/mL or (7) anti-P2X₇ rabbit polyclonal antibody (Alomone) 1:200. P2X_{1, 3, 5} antibodies were raised versus rat, whereas P2X_{2, 4, 6} antibodies were raised against human and P2X₇ antibody versus mouse antigens. Finally, the membranes were incubated in horseradish peroxidase-conjugated anti-rabbit IgG goat IgG 1:1,000 (Sigma) in 0.1 % Tween blocking solution for 1 h at room temperature before detection with an enhanced chemiluminescence detection kit (Western Lighting Plus) in a luminescence cabinet (UVP ChemiDoc-it Imaging System). Images were created using VisionWorksLS image acquisition and analysis software package and processed using Adobe Photoshop CS3 extended v10.0 image processing programme. Protein identification was based on size of detected band. Control experiments were carried out with the omission of the primary antibody showing absence of bands in these preparations (data not shown). In addition, the amino acid sequences of the immunogen peptides used to generate the antibodies in our study were aligned against the equivalent equine amino acid sequences (predicted) giving high homology (83–100 %) (Table S1).

Subsequent to this analysis of P2X antibody binding specificity, the antibody sera for P2X_{1–3} and P2X₇ were used for immunohistochemical analysis.

Immunohistochemistry

Tissue samples for immunohistochemical analysis of P2X_{1–3, 7} were fixed overnight in 4 % buffered PFA, paraffin embedded then cut in 6-µm sections. Sections were deparaffinised and rehydrated before being subjected to antigen retrieval

(bacterial proteinase solution for P2X₁ and P2X₂ antibodies, ethylenediaminetetraacetic acid buffer pH9 solution for P2X₃ antibody and citrate buffer pH6 solution for P2X₇ antibody) and endogenous peroxidase block (3 %w/v H₂O₂). Sections were then pre-incubated in blocking solution (10 % goat serum in Tris-buffered saline) for 1 h before incubation with primary antibody in blocking solution (1 h, 25 °C), (1) anti-P2X₁ antibody 1:200, (2) anti-P2X₂ antibody 1:125, (3) anti-P2X₃ antibody 1:1,000 or (4) anti-P2X₇ antibody 1:200. Sections were washed in Tris-buffered saline three times before being incubated in Ztyochem Plus HRP Polymer anti-rabbit (Source Bioscience) for 30 min (25 °C). Sections were washed again in Tris-buffered saline three times and immunostaining was detected with the chromogenic substrate DAB (SigmaFast 3,3-diaminobenzidine, Sigma) and sections counterstained with Delafield's haematoxylin (Fluka). Control experiments were carried out with omission of the primary antibody and substitution with non-immune rabbit IgG (Abcam). No staining was observed in the control experiments (Figs. S1, S2). Immunostained sections were visualised using Nikon eclipse 80i microscope and pictures were acquired with Nikon DS-L2 stand-alone control unit and analysed using Adobe Photoshop CS3 extended v10.0 image processing programme.

Results

P2X receptor subunit mRNA and protein expression in equine tissue

All designed primers produced PCR products and the bands were of the predicted sizes in sampled equine tissues (Fig. 1). P2X receptor subunits were expressed in most tissues, specifically P2X_{1–5, 7} mRNA was found in all tissues, whereas P2X₆ was well expressed in the dorsal root ganglia and spinal cord but only faintly in other tissues (data not shown). The identification of the PCR amplified products as P2X_{1–7} amplicons was based on their size and on subsequent DNA sequencing and genome-wide similarity searches (Blast, NCBI). A comparison of the sequenced nucleotide sequences to the predicted ones gave a perfect match for all, but the P2X₅ transcript which was found to combine parts of sequences matching the NCBI predicted transcript (XM_001918102.1), the Ensembl predicted transcripts (ENSECAT00000026624, ENSECAT00000026645) or neither the NCBI/Ensembl predicted transcripts (Tables S2 and S3). Figure 1 shows that P2X₅ amplification gave slightly smaller products raising the possibility of the existence of splice variants. However, further investigation into this possibility was not within the remit of the current study. Tissue-related differences in mRNA nucleotide sequences were not detected.

Fig. 1 Example of agarose gel electrophoresis of eqP2X_{1–7} cDNA PCR amplification products in sampled equine tissues (P2X₁–P2X₄ and P2X₇ C8 DRG, P2X₅ and P2X₆ C4 DRG) showing products of the expected size. base pairs (bp)

Figure 2 shows representative Western blots for P2X_{1–3, 7} proteins and Table 2 summarises their tissue expression and predicted (NCBI/Ensembl) and detected band sizes.

Immunohistochemical distribution of P2X_{1–3, 7} receptors in equine tissue

Palmar digital artery and vein

Figure 3 shows the distribution of P2X_{1–3, 7} receptor subunits in the palmar digital artery and vein. P2X_{1, 2, 7} receptor subunits were demonstrated in the smooth muscle cells in the *tunica media* of the palmar digital artery and vein, whereas P2X₃ receptor subunit positive staining was identified in the smooth muscle cells of the artery but not the vein. P2X₇ receptor subunit staining in the smooth muscle cells exhibited characteristic patterning, being localised in a thin line adjacent to the nucleus. The palmar digital artery and vein endothelial cells were immunopositive for the P2X₂ receptor subunit, whereas only the palmar digital vein endothelial cells were immunopositive for the P2X₃ receptor subunit.

Fig. 2 Western blot analysis for P2X_{1–3, 7} proteins showing validation of rabbit antisera against equine homologues. Shown are representative bands of Western blots for P2X_{1–3, 7} proteins in equine tissue samples (P2X₁ palmar digital artery lysate, P2X₂ and P2X₃ C8 DRG lysate, P2X₇ hoof lysate). The bands observed at 55, 42, 50 and 79 kDa represent P2X_{1–3, 7} proteins, respectively, whereas the bands observed at higher molecular weights correspond to the protein's glycosylated form or multimer

Table 2 Summary of Western blot analysis plus predicted sizes for P2X_{1–3, 7} proteins

P2X receptor	Palmar digital artery	Palmar digital vein	Palmar digital nerve	Hoof	DRG (C4)	DRG (C8)	Spinal cord (C4)	Spinal cord (C8)	Molecular weight (kDa)	Predicted molecular weight (kDa) ^a
P2X ₁	+	+	+	+	+	+	+	+	55	50
P2X ₂	+/-	+/-	+/-	+	+	+	+	+	42	39
P2X ₃	+	+	+	+	+	+	+	+	50	44
P2X ₇	+	+	+	+	+	+	+	+	79	69

Present (+), not always present (+/-) in samples

DRG dorsal root ganglia

^aBased on NCBI/Ensembl sequences or manufacturers datasheet

Palmar digital nerve

P2X₁ and P2X₂ receptor subunit staining was strong in the nerve fibres but faint in the Schwann cells (Fig. 4). P2X₃ receptor subunit staining was present only in the nerve fibres, and P2X₇ receptor subunit staining was seen exclusively in the Schwann cells.

Hoof

Figure 5 shows P2X receptor subunit distribution in the equine hoof. P2X_{1–3} receptor subunit staining predominately occurred in the epidermal basal cells. P2X₇ receptor subunit staining was present in the epidermal basal cells and in the corium (dermal) fibroblasts.

Dorsal root ganglia and spinal cord

P2X₁ receptor subunit staining was strong in the dorsal root ganglia neuronal cells but faint in the satellite glial cells in the fourth and eighth cervical ganglia (Fig. 6). P2X₂ receptor subunit staining, in comparison, was present in both the dorsal root ganglia neurons and satellite glial cells. P2X₃ receptor subunit staining was present in the dorsal root ganglia neurons only, whereas P2X₇ receptor subunit staining was strong in the dorsal root ganglia Schwann cells.

P2X_{1–3, 7} receptor subunit staining was present in the dorsal horn neurons and ependymal cells, although differences in staining intensity were present between these receptor subtypes, with P2X₃ receptor subunit staining strongest in the neuronal cell body and projections (Fig. 7). P2X_{1, 2, 7} receptor subunit staining was also present in the dorsal horn glial cells.

Discussion

Purinergic P2X receptors have been identified in both excitable and non-excitable cells and have important roles in neuronal transmission and regulation as well as cell growth,

differentiation and survival [2, 8, 12]. In this study, we identify the presence of P2X_{1–7} receptor subunits in equine tissue, specifically the hoof and its innervation. We also show the location and distribution of four key receptor subunits (P2X_{1–3, 7}) in these tissues, showing important differences in cellular location and suggesting that heterogeneity in receptor expression may be important in normal cellular and tissue function. Further work is required to elucidate the physiological relevance of these differences.

PCR experiments identified gene expression (mRNA) for all seven P2X subunits in the equine tissues. P2X receptors are considered conserved receptors and have been found in other species in a wide variety of tissue types, supporting our findings [4]. The presence of transcripts, however, does not equate to protein expression, and using Western blot analysis, we validated the expression of P2X_{1–3, 7} proteins in our sampled tissues. In most horses, protein expression was consistent, although in occasional samples, expression of some subunits (notably P2X₂) was not always present. Although identifiable at the predicted protein size (based on NCBI/Ensembl-predicted sequences or manufacturers datasheets), Western blots for some subtypes revealed multiple bands of molecular weight greater than expected (Fig. 2). Expression of higher molecular weight bands is recognised in P2X receptor expression analysis where dimer and trimer formation occurs. Glycosylation of protein subunits or other post-translational modification also occurs in P2X proteins [13] and may explain some discrepancy between actual and predicted molecular weights for bands present (for example, 55 kDa versus predicted 50 kDa for P2X₁ protein and 79 kDa versus predicted 69 kDa for P2X₇ protein). Where results showed bands of higher molecular weight (P2X₁ and P2X₇), we subsequently performed pre-incubation experiments with the immunogen peptide, demonstrating reduction in band intensities (Fig. S3).

In our immunohistochemical analysis, palmar digital artery and vein smooth muscle cells showed staining for P2X₁, P2X₂ and P2X₇ receptor subunits, whereas P2X₃ receptor subunits were present in smooth muscle cells in the artery but not in the vein. P2X₁ receptor is the

Fig. 3 Immunostaining for P2X₁ (a, e), P2X₂ (b, f), P2X₃ (c, g) and P2X₇ (d, h) receptor subunits in equine palmar digital artery (a–d) and vein (e–h). P2X₁ receptor subunit was present in the smooth muscle cells in the *tunica media* of the palmar digital artery (a) and vein (e). *Insert* shows representative image of positive smooth muscle cells. P2X₂ receptor subunit was present in the smooth muscle cells in the *tunica media* and the endothelial cells of the palmar digital artery (b) and vein (f). *Insert* shows representative image of positive endothelial and smooth muscle cells. P2X₃ receptor subunit was not found in the palmar digital artery (c) but was present in the smooth muscle cells in the *tunica media* and the endothelial cells of the palmar digital vein (g). *Insert* shows representative image of endothelial and smooth muscle cells. P2X₇ receptor subunit was present in the smooth muscle cells in the *tunica media* of the palmar digital artery (d) and vein (h). *Insert* shows representative image of P2X₇ staining in smooth muscle cells, exhibiting characteristic patterning being localised in a thin line adjacent to the nucleus

predominant receptor type in vascular smooth muscle cells but other receptor types have also been documented in these cells [14, 15], supporting our findings. P2X receptors on vascular smooth muscle cells have been firmly linked to vasoconstrictor function [16]. The P2X₇ receptor in particular has an important role in local regulation of blood flow in diverse tissues including large coronary and cerebral arteries, hepatic mesentery, and umbilical and placental vessels [17–19]. Vascular endothelial cells were also positive for P2X₂ and P2X₃ receptor subunits. P2X₂ receptors have been described in vascular endothelial cells in the rat brain

[20], and other receptor subtypes (for example P2X₄) are recognised to regulate vasoactive substance release in primary human vascular endothelial cells following activation by extracellular ATP after local haemodynamic changes, such as shear stress [10, 21].

Hoof epidermal basal cells were positive for P2X₁, P2X₂, P2X₃ and P2X₇ receptor subunits. To the authors' knowledge, P2X receptor expression and distribution in the equine hoof has not been previously described. The equine hoof shares a common basic structure with skin, but hoof epidermal cells have evolved as a highly modified epidermal layer

Fig. 4 Immunostaining for P2X₁ (a), P2X₂ (b), P2X₃ (c) and P2X₇ (d) receptor subunits in equine palmar digital nerve. **a** P2X₁ receptor subunit staining was strong in the nerve fibres but faint in the Schwann cells. *Insert* shows representative image of positive Schwann cells and nerve fibres. **b** P2X₂ receptor subunit staining was strong in nerve fibres but faint in the Schwann cells. *Insert* shows representative image

of positive Schwann cells and nerve fibres. **c** P2X₃ receptor subunit staining was present only in nerve fibres. *Insert* shows representative image of a positive nerve fibre. **d** P2X₇ receptor subunit staining was seen exclusively in Schwann cells. *Insert* shows representative image of longitudinal section of positive Schwann cell myelin sheath

to enable it to function in its roles within the suspensory apparatus of the distal phalanx in equids [22]. Previous studies in human and rat skin keratinocytes have reported the presence of P2X₂, P2X₃, P2X₅ and P2X₇ receptor

subunits [23, 24]. P2X receptors found in the skin are suggested to have an important physiological role as an initial sensor for external stimuli, and P2X₇ receptors, known to be involved in ATP-induced apoptosis, are likely

Fig. 5 Immunostaining for P2X₁ (a), P2X₂ (b), P2X₃ (c) and P2X₇ (d) receptor subunits in equine hoof. P2X_{1–3, 7} receptor subunit staining predominately occurred in the epidermal basal cells. *Inserts* show representative images of positive epidermal basal cells

Fig. 6 Immunostaining for P2X₁ (a, e), P2X₂ (b, f), P2X₃ (c, g) and P2X₇ (d, h) receptor subunits in equine C4 (a–d) and C8 (e–h) dorsal root ganglia (DRG). P2X₁ receptor subunit staining was strong in DRG neuronal cells but faint in satellite glial cells in C4 (a) and C8 (e) DRG. *Insert* shows representative image of positive neurons and satellite glial cells. P2X₂ receptor subunit staining was present in both DRG neurons and satellite glial cells in C4 (b) and C8 (f) DRG. *Insert* shows representative image of positive neurons and satellite glial cells. P2X₃ receptor subunit staining was present only in DRG neurons in C4 (c) and C8 (g) DRG. *Insert* shows representative image of positive neurons. P2X₇ receptor subunit staining was strong in the Schwann cells in C4 (d) and C8 (h) DRG. *Insert* shows representative image of positive Schwann cell myelin sheaths

to participate in the process of the end-stage terminal differentiation of keratinocytes [23]. The presence of P2X receptor expression in epidermal basal cells suggests a potential role for P2X receptors in relation to cell growth and differentiation in the equine hoof. This finding may have important implications in clinical conditions of the hooves, such as, laminitis, where destruction of the epidermal/dermal interface occurs, resulting in catastrophic mechanical failure of the hoof and extreme pain [25].

P2X receptor expression and function in the peripheral nerve has been extensively studied [for review see 26]. In

other species, neuronal responses to purines have important roles in signal transmission in afferent neurones in the peripheral nervous system including those from the dorsal root ganglia. Efferent autonomic neurones have been described in the palmar digital nerve of the horse involved in local vascular responses [27]. In our study, staining for P2X₁ and P2X₂ receptor subunits was present in nerve fibres and faintly in Schwann cells, whereas P2X₃ receptor subunit staining was present in the nerve fibres only and P2X₇ receptor subunit staining was strong in the Schwann cells only. Agonist studies show that C-fibre nociceptors respond

Fig. 7 Immunostaining for P2X₁ (a, e), P2X₂ (b, f), P2X₃ (c, g) and P2X₇ (d, h) receptor subunits in equine C4 (a–d) and C8 (e–h) spinal cord segments. P2X₁ receptor subunit was present in the dorsal horn neurons (*a*₁ and *e*₁) and glial cells (*inserts a*₂ and *e*₂) in C4 and C8 spinal cord segments. P2X₂ receptor subunit was present in the dorsal horn neurons (*b*₁ and *f*₁) and glial cells (*inserts b*₂ and *f*₂) in C4 and C8 spinal cord segments. P2X₃ receptor subtype staining was strong in the dorsal horn neuron cell body and projections (*c*₁ and *g*₁) but absent in the glial cells (*inserts c*₂ and *g*₂) in C4 and C8 spinal cord segments. P2X₇ receptor subunit was present in the dorsal horn neurons (*d*₁ and *h*₁) and glial cells (*inserts d*₂ and *h*₂) in C4 and C8 spinal cord segments

the most to extracellular nucleotide activation, suggesting a predominately nociceptive role for P2X receptors [28]. P2X₃ receptor, in particular, has been found to be selectively expressed on small-diameter capsaicin-positive nociceptive neurons [29], and homomeric P2X₃ and heteromeric P2X_{2/3} receptors have roles in acute and longer lasting sensitivity to nociception, respectively [30].

Particular interest has been shown in the putative role for P2X₇ in Schwann cells. Schwann cell electrophysiological investigations show ATP-evoked currents due to Ca²⁺-dependent K⁺ and Cl[−] conductance via P2X₇ receptor

activation and a P2X₇ non-selective cationic conductance [31]. P2X₇ receptor may be involved in local immunomodulation of axonal function by Schwann cells including regulation of cytokine release; this may be of particular relevance during peripheral nerve injury or neuropathies.

In the dorsal root ganglia, P2X₇ receptor subunit expression was similarly localised to supporting cells, such as, Schwann cells, and this finding is supported by published data in other species [32, 33]. Neuronal expression of P2X_{1–3} receptor subunits in the dorsal root ganglia and spinal cord seen in our study also supports the role of these receptor

subtypes in signal transmission and spinal processing [34–36]. Release of ATP can modulate neurotransmitter release, such as, glutamate through P2X receptors in central afferent terminals or second-order neurons of the spinal cord [34] leading to activation of other receptors, such as neurokinin 1 (NK1R) [36]. Additionally, P2X receptor activation is implicated in inhibitory mechanisms involving glycine and GABA in the dorsal horn cells of the spinal cord. Subpopulations of the dorsal horn cells exist with different P2X subtype expressions. P2X₃ and P2X_{2/3} receptors have been found at the central terminals of afferent neurons in the inner part of lamina II of the dorsal horn, the corresponding region of the trigeminal complex and in the *nucleus tractus solitarius* where they affect dorsal horn neurons of the spinal cord [7, 37]. Additionally, P2X₂ and P2X_{2/3} receptor subtypes are distributed in the brain where they are responsible for supraspinal P2X receptor-mediated antinociception [35]. Furthermore, P2X receptors have been found in spinal glial cells where they have a key role in the development and maintenance of pathological pain states [38, 39]. Thus, P2X receptors have a complex role in spinal transmission and are implicated in neurogenic and inflammatory pain pathways. The similar localisation of P2X receptor subtypes in the equine tissue in this study, particularly in the distribution in the nervous tissue, suggests this previously unreported area could be utilised in the understanding of (and dealing with) inflammatory and nociceptive conditions of horses.

In conclusion, this study details the expression and distribution of key members of the P2X receptor family in equine tissue, with a particular emphasis on the forelimb and associated nerve supply. Functional studies on these P2X receptor subtypes may provide further understanding of their role not only in pain-related pathways but also in the regulation of normal structures and functions in equids.

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