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# Serotonin-evoked cytosolic $Ca^{2+}$ release and opioid receptor expression are upregulated in articular cartilage chondrocytes from osteoarthritic joints in horses

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ABSTRACT

Osteoarthritis is a pain-associated progressive disease and pain mediators, such as opioid receptors, expressed in articular cartilage could represent novel therapeutic targets. Acute and chronic stages of OA indicate different metabolic abilities of the chondrocytes depending on inflammatory state.

This study aimed to investigate the response of healthy and osteoarthritic chondrocytes and their expression and release of pain mediators in response to acute inflammation.

Interleukin-1 beta (IL-1β) and lipopolysaccharide (LPS) were used to induce an acute inflammatory response in cultured equine chondrocytes harvested from healthy joints (HC) and osteoarthritic joints (OAC), the latter representing acute exacerbation of a chronic inflammatory state. Intracellular $Ca^{2+}$ release was determined after exposure to serotonin (5-hydroxytryptamine (5-HT), glutamate or ATP. Protein expression levels of F- and G-actin, representing actin rearrangement, and opioid receptors were investigated. Glutamate concentrations in culture media were measured. Cartilage was immunohistochemically stained for μ (MOR), κ (KOR), and δ (DOR) opioid receptors.

Upon exposure to acute inflammatory stimuli, OAC showed increased intracellular $Ca^{2+}$ release after 5-HT stimulation and increased expression of MOR and KOR. When cells were stimulated by inflammatory mediators, glutamate release was increased in both HC and OAC. Immunostaining for MOR was strong in OA cartilage, whereas KOR was less strongly expressed. DOR was not expressed by cultured HC and OAC and immunostaining of OA cartilage equivocal.

We show that chondrocytes in different inflammatory stages react differently to the neurotransmitter 5-HT with respect to intracellular $Ca^{2+}$ release and expression of peripheral pain mediators.

Our findings suggest that opioids and neurotransmitters are important in the progression of equine OA. The inflammatory stage of OA (acute *versus* chronic) should be taken into consideration when therapeutic strategies are being developed.

## Introduction

Pain and lameness due to osteoarthritis (OA) is the most common reason for failure to train and race among racehorses (Perkins, R & Morris, 2002). Local pathologic processes in osteoarthritic cartilage result in chondrocyte phenotypic changes, including the production of inflammatory mediators that might be relevant to the pain process in chronic OA (Bohm et al., 2012). Pain mediators in acute and chronic pain can differ, and so also the underlying mechanisms leading to peripheral or centrally mediated pain (Clauw et al., 2017). OA is seen as a peripheral mediated pain state where acute stages are responsive to non-steroidal anti-inflammatory drugs (NSAIDs) and opioids. However chronic stages of OA have been shown to additionally involve the central nervous system and are therefore considered as mixed pain states (Clauw et al., 2017).

1 5-Hydroxytryptamine (5-HT, serotonin) is a well-known neurotransmitter in the central nervous system (CNS), where it is a key contributor to behavioural and physiological functions (Robson, Quinlan, & Blakely, 2017). In addition, 5-HT is an immune

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system modulator in both the peripheral nervous system and the CNS and has been recently shown to be involved in autoimmune arthritis (Chabbi-Achengli et al., 2016). Pharmaceutical treatments for central pain conditions includes serotonin reuptake inhibitors (SRI) which acts as reuptake inhibitors of the neurotransmitter serotonin (5-hydroxytryptamine (5-HT)) by blocking the action of the serotonin transporter (Clauw et al., 2017).

Glutamate is an excitatory amino acid neurotransmitter in the CNS involved in nociception and pain sensitization (Dickenson, Chapman, & Green, 1997), and an important signalling molecule in various tissues, including skin, bone, and cartilage (Hinoi, Takarada, & Yoneda, 2004; Hinoi, Takarada, Ueshima, Tsuchihashi, & Yoneda, 2004). In joints, glutamate participates in inflammatory and nociceptive responses (Wen, Chang, & Jean, 2015). It induces pain in arthritic rats (Zhang et al., 2003), and increased glutamate concentration in the synovial fluid correlates with the severity of joint destruction in OA patients (Bonnet et al., 2015).

Chondrocytes in the superficial layer of the articular cartilage are coupled into networks and are equipped with intercellular and/or extracellular $Ca^{2+}$ signalling systems (Hansson et al., 2015). Changes in intracellular $Ca^{2+}$ levels influence matrix degradation and synthesis and can promote the release of signalling molecules, such as neurotransmitters and cytokines (Millan et al., 2002; Patetsos & Horjales-Araujo, 2016).

Opioid receptors are distributed in the nervous system and in peripheral non-neuronal tissues. In the presence of inflammation, the synthesis of these receptors is increased, and clinical studies have provided support for their involvement in analgesia (Sehgal, Smith, & Manchikanti, 2011). Endogenous opioids, such as β-endorphin, enkephalins, and dynorphins, induce their biological effects by binding to opioid receptors μ (MOR), κ (KOR), and δ (DOR) and opioid receptor like-1 (ORL1) (Al-Hasani et al., 2011; Elvenes et al., 2003). The presence of opioid receptors in equine articular cartilage has not been studied to date, although such receptor systems in the articular cartilage could represent a novel therapeutic target for pain control in chronic joint disorders, such as OA. Thus, investigations into their presence are warranted.

The actin cytoskeleton is critical to numerous cellular functions. In chondrocytes, the regulation of cell shape in response to mechanical compression is a fundamental property that relies on the actin cytoskeleton (Chen et al., 2015). Quantification of the monomer and filamentous forms of actin may be helpful in further understanding the direct effects of inflammation on the cytoskeleton (dos Remedios et al., 2003).

The present study aimed to investigate the effects of neurotransmitters and inflammatory mediators on chondrocytes to further understand the expression of pain mediators in cellular responses of acute and chronic inflammation in horses.

## Materials and methods

In order to detect differences and similarities across physiologic and pathologic responses, chondrocytes were harvested from healthy and osteoarthritic equine joints. To mimic acute inflammation, the cells were stimulated with interleukin (IL) − 1β and lipopolysaccharide (LPS) *in vitro*. Cellular parameters, including intracellular $Ca^{2+}$ release, alterations in the actin cytoskeleton, and extracellular glutamate release were studied. The protein expression of MOR, KOR, and DOR was investigated both *in vitro* and *ex vivo*.

### *Study population and tissue sampling*

Articular cartilage samples intended for chondrocyte isolation and culture were obtained within 48 h of euthanasia from the middle carpal joints of six horses (1–8 years old). Three horses showed macroscopically intact articular cartilage, and three horses showed mild structural osteoarthritic changes in the articular cartilage of the radial facet region of the third carpal bone. The horses had been euthanized due to disease or for research purposes unrelated to this study.

In addition, spinal cord, macroscopically normal articular cartilage with adjacent subchondral bone, cartilage shavings with mild structural OA, and a small osteochondral fragment (chip fracture) were sampled and used for histology and immunohistochemistry. All but one sample was collected post-mortem. The osteochondral fragment was removed at arthroscopic surgery as part of therapeutic intervention (sampling approved by the Ethical Committee on Animal Experiments, Uppsala, Sweden, Dnr: C154/4).

All material was fixed in 10% formalin. Following fixation, osteochondral tissue was decalcified using formic acid. The material was embedded in paraffin and routinely prepared for light microscopy and immunohistochemistry.

### *Chondrocyte isolation and culture*

Following aseptic preparation and incision of the middle carpal joint, cartilage of the dorsal aspect of the radial facet of the third carpal bone was incised with a scalpel, and full-thickness cartilage samples were collected with forceps. The tissue was placed in a sterile saline (0.9% NaCl) solution with gentamicin sulphate (50 mg/l) and amphotericin B (250 μg/ml). Cartilage samples were transported chilled (~5 °C) to the laboratory. Chondrocytes from healthy joints (HC) and OA-affected joints (OAC) from each horse were expanded separately in monolayers as previously described. To maintain a chondrogenic phenotype, cells were expanded in passage 1 and seeded at 20,000 cells/$cm^2$ in chondrogenic medium (Ley et al., 2011).

Cells intended for use in $Ca^{2+}$ analyses were cultured in 96-well plates, and those intended for western blotting and F-/G-actin analyses were cultured in 6-well plates. All cells were cultured for 4 days until near confluence. On day 4, cells were stimulated with LPS (10 ng/ml, *Escherichia coli* 055:B5; List Biological Laboratories, Campbell, CA, USA) or IL-1β (5 ng/ml; R&D Systems, Abingdon, UK) or left untreated (controls) for 24 h. On day 5, intracellular $Ca^{2+}$ release was measured, cell culture medium was collected, and cells were harvested and immediately frozen and stored at –80 °C until further analyses.

### *$Ca^{2+}$ assay*

To detect intracellular $Ca^{2+}$ release in chondrocyte monolayers, cells were incubated with the $Ca^{2+}$-sensitive fluorescent indicator dye FLIPR Calcium 6 (FLIPR Calcium 6 Assay Kit, Molecular Devices, Sunnyvale, CA, USA), according to the manufacturer's instructions. After adding the masking dye, 5-HT ($10^{-5}$ M; Sigma-Aldrich, St. Louis, MO, USA), ATP ($10^{-4}$ M; Sigma-Aldrich), or glutamate ($10^{-4}$ M; Sigma-Aldrich) was added to the 96-well culture plates. The area under the curve (AUC), which reflects the amount of $Ca^{2+}$ released intracellularly, was calculated in each treatment group to measure the strength of the $Ca^{2+}$ responses (FlexStation 3 Multi-Mode Microplate Reader; Molecular Devices, Sunnyvale, CA, USA).

### *Western blotting*

Western blotting analyses were carried out according to standard protocols. Briefly, protein extracts were prepared by cell lysis in RIPA buffer (Sigma-Aldrich) supplemented with a mammalian protease inhibitor cocktail (P8340; Sigma-Aldrich). Protein concentrations were determined using the Pierce BCA Protein Assay Kit (Life Technologies, Carlsbad, CA, USA), according to the manufacturer's instructions. Equal amounts of extract (5 μg) were resolved on 4–12% Bis-Tris pre-cast gels (Life Technologies) and transferred onto nitrocellulose membranes (GE Healthcare, Danderyd, Sweden). Equal protein loading and transfer were confirmed by Ponceau staining (0.1% in acetic acid, Sigma-

Aldrich). Membranes were probed with the following primary antibodies: polyclonal rabbit anti-DOR-1 (sc-9111), polyclonal rabbit anti-KOR-1 (sc-9112), and polyclonal rabbit anti-MOR-1 (sc-15,310; all from Santa Cruz Biotechnology, Santa Cruz, CA, USA). Membranes were then probed with horseradish peroxidase-conjugated secondary antibodies (Jackson ImmunoResearch, Newmarket, UK). Protein bands were detected using Immobilon Western Chemiluminescent HRP Substrate (Millipore, Billerica, MA, USA) with a ChemiDoc XRS+ instrument (Bio-Rad, Hercules, CA, USA). The relative intensities of the protein bands in the linear exposure range were quantified using ImageLab software (Bio-Rad). The intensity of the control protein band was set to 100%.

*F-/G-actin assay*

F- and G-actin were quantified using the F-actin/G-actin *In vivo* Assay Kit (Cytoskeleton, Denver, CO, USA). Cells were lysed in LAS2 buffer (pH 6.9), which stabilizes and maintains the F- and G-forms of cellular actin, and the wells were scraped to disrupt the cell membrane. After centrifugation to pellet the lysate, the supernatant fraction was centrifuged at 100,000 × *g* for 1 h to separate F-actin from soluble G-actin. The actin content in the supernatant fraction was visualized by immunoblotting with anti-actin antibody (Cytoskeleton).

*Determination of glutamate in culture media*

The concentration of glutamate in cell culture media was measured using ninhydrin reagent for photometric determination. All analyses were performed on duplicate samples, and results were correlated to the total protein concentration of the cell culture media. The lower limit of detection was 5 μmol/l (Moore & Stein, 1954).

*Immunohistochemistry for MOR, KOR, and DOR*

Approximately 4-μm-thick sections were mounted on microscope slides (SuperFrost® Plus; Menzel-Gläser, Braunschweig, Germany). Slides were placed at 60 °C for 40 min, then deparaffinised in xylene, rehydrated in graded alcohol, and rinsed in deionized water and phosphate-buffered saline (PBS). All spinal cord and osteochondral sections analysed with the MOR antibody were pre-treated in sodium-citrate buffer at 92 °C for 20 min, then left to cool for 20 min in the same solution. Because osteochondral tissue tended to detach from the glass slides, sections with bone and cartilage analysed with the KOR and DOR antibodies were pre-treated in sodium-citrate buffer at 60 °C for 2 h. Endogenous peroxide activity was quenched by immersion in 3% hydrogen peroxide solution for 5 min. For washing of sections and dilution of antibodies, 0.01 M PBS (pH 7.4) was used. To block non-specific antibody binding, normal goat serum (diluted 1:50; X0907; Dako, Glostrup, Denmark) was applied to sections at 25 °C and slides were incubated in a humid chamber for 20 min. Sections were then drained and incubated with polyclonal rabbit MOR antibody (diluted 1:2000; AB5511; Merck, Solna, Sweden), polyclonal rabbit KOR antibody (diluted 1:250; sc-9112; Santa Cruz Biotechnology), or polyclonal rabbit DOR antibody (diluted 1:250; sc-9111, Santa Cruz Biotechnology) overnight at 4 °C. Control sections were incubated with irrelevant antibody (X0936; Dako) at the same concentration. The following day, sections were rinsed and antibody-antigen complexes detected using the Dako REAL™ EnVision™ Detection System, Peroxidase/DAB+, Rabbit/Mouse (K500711-2; Dako), with a secondary antibody [(HRP, rabbit/mouse (ENV)] applied for 30 min at room temperature. 3, 3′-Diaminobenzidine was used to visualize target antigens. Sections were then rinsed in tap water and counterstained with Mayer's haematoxylin.

*Statistical methods*

Data were analysed using mixed models. Median values and 95% confidence intervals for each of the parameters, including $Ca^{2+}$ release (AUC), glutamate concentration (μmol/g), and F-actin, G-actin, MOR, and KOR (integrated densities of protein expression from western blots), were calculated for each combination of group (HC and OAC) and treatment. Glutamate concentrations, $Ca^{2+}$ release, and western blotting parameters were regarded as dependent variables. Groups and treatments were considered fixed factors, and subjects were regarded as random factors. Multiple measurements from the same subject were regarded as pseudoreplicates. An interaction term between groups and treatments was included in the model. *P*-values $<0.05$ were considered statistically significant. All statistical analyses were performed using SAS version 9.3 (SAS Institute Inc., Cary, NC, USA).

**Table 1**
Median values and 95% confidence intervals (in brackets) for intracytoplasmic $Ca^{2+}$ release (area under the curve, AUC), glutamate concentrations in culture media (μmol/g cell protein), cell lysate F- and G-actin content (integrated densities), and μ (MOR) and κ (KOR) expression (integrated densities) for cultured chondrocytes from healthy joints (HC) and osteoarthritic joints (OAC).

| Parameter | Treatment group | HC | OAC |
|---|---|---|---|
| **$Ca^{2+}$ release** | Ctrl | 2847 (2072–3913) | 5957 (4356–8149) |
| | IL-1β | 3450 (2510–4741) | 7351 (5374–10,055) |
| | LPS | 2859 (2081–3930) | 7637 (5584–10,446) |
| **Glutamate** | Ctrl | 1.8 (0.8–3.9) | 1.4 (0.7–2.7) |
| | IL-1β | 2.2 (1.0–5.0) | 2.1 (1.1–4.2) |
| | LPS | 2.7 (1.2- 6.0) | 2.7 (1.4- 5.2) |
| **F-actin** | Ctrl | 601 (321–1124) | 401 (210–766) |
| | IL-1β | 1042 (557–1950) | 820 (429–1564) |
| | LPS | 1155 (587–2272) | 987 (517–1883) |
| **G-actin** | Ctrl | 1268 (804–2001) | 887 (580–1357) |
| | IL-1β | 1395 (884–2201) | 1329 (869–2033) |
| | LPS | 1185 (751–1869) | 1478 (967–2261) |
| **MOR** | Ctrl | 42 (18–96) | 30 (12–74) |
| | IL-1β | 29 (13–66) | 88 (36–216) |
| | LPS | 59 (26–136) | 140 (57–343) |
| **KOR** | Ctrl | 262 (140–490) | 186 (89–390) |
| | IL-1β | 213 (8114–399) | 467 (223–976) |
| | LPS | 275 (147–514) | 360 (172–753) |

Ctrl = untreated controls; IL-1β = interleukin-1β stimulated; LPS = lipopolysaccharide stimulated.

## Results

*Data analysis*

Since the residuals had a skewed distribution, analyses were performed on log-transformed data. Therefore, we report the results as medians with their confidence intervals (Table 1) and, for relevant comparisons, ratios between medians with their confidence intervals, as well as *P*-values for tests of equality to one. *P*-values $<0.05$ were considered statistically significant, and the Bonferroni-Holm correction was used for multiple comparisons ($P_{BH}$). While $P_{BH}$ in the text are given only when statistically significant, both significant uncorrected *P*-values and all $P_{BH}$ are given in Table 2.

*Intracytoplasmic $Ca^{2+}$ release in chondrocytes after 5-HT stimulation*

OAC and HC were momentaneously exposed to glutamate, ATP or 5-HT, and then intracellular $Ca^{2+}$ release was measured. No changes in intracellular $Ca^{2+}$ release were observed after ATP or glutamate stimulation (data not shown); however, control OAC stimulated with 5-HT showed an increase in intracellular $Ca^{2+}$ release compared to control HC ($P < 0.005$, $P_{BH} < 0.01$), indicating a more sensitive $Ca^{2+}$ signalling system in diseased *vs.* healthy chondrocytes. LPS-stimulated OAC showed an increased intracellular $Ca^{2+}$ release compared to LPS-stimulated HC ($P < 0.0001$, $P_{BH} < 0.001$). Similarly, IL-1β-stimulated OAC showed an increased intracellular $Ca^{2+}$ release compared to IL-1β-

**Table 2**
Statistical differences in intracytoplasmic $Ca^{2+}$ release (area under the curve, AUC), glutamate concentrations in culture media (μmol/g cell protein), cell lysate F- and G-actin content (integrated densities), and μ (MOR) and κ (KOR) expressions (integrated densities) for cultured chondrocytes from healthy joints (HC) and osteoarthritic joints (OAC). Uncorrected *P*-values <0.05 were considered statistically significant. Also shown are *P*–values corrected for mass-significance using Bonferroni-Holm method ($P_{BH}$).

| Parameter | Groups and treatments | *P*-value | $P_{BH}$-value |
|---|---|---|---|
| **$Ca^{2+}$ release** | HC Ctrl *vs* HC IL-1β | 0.0350 | 0.1050 |
| | HC Ctrl *vs* OAC Ctrl | 0.0016 | 0.0096 |
| | HC IL-1β *vs* OAC IL-1β | 0.0013 | 0.0091 |
| | HC LPS *vs* OAC LPS | <0.0001 | <0.0009 |
| | OAC Ctrl *vs* OAC IL-1β | 0.0122 | 0.0488 |
| | OAC Ctrl *vs* OAC LPS | 0.0034 | 0.0170 |
| **Glutamate** | HC Ctrl *vs* HC LPS | 0.0230 | 0.0161 |
| | OAC Ctrl *vs* OAC IL-1β | 0.011 | 0.088 |
| | OAC Ctrl *vs* OAC LPS | 0.0004 | 0.0036 |
| **F-actin** | OAC Ctrl *vs* OAC IL-1β | 0.033 | 0.264 |
| | OAC Ctrl *vs* OAC LPS | 0.009 | 0.081 |
| **MOR** | HC IL-1β *vs* HC LPS | 0.0691 | 0.4837 |
| | HC IL-1β *vs* OAC IL-1β | 0.0692 | 0.4837 |
| | OAC IL-1β *vs* OAC Ctrl | 0.0237 | 0.1896 |
| | OAC LPS *vs* OAC Ctrl | 0.0029 | 0.0261 |
| **KOR** | OAC Ctrl *vs* OAC IL-1β | 0.0536 | 0.4824 |

Ctrl = untreated controls, IL-1β = interleukin-1beta stimulated, LPS = lipopolysaccharide stimulated.

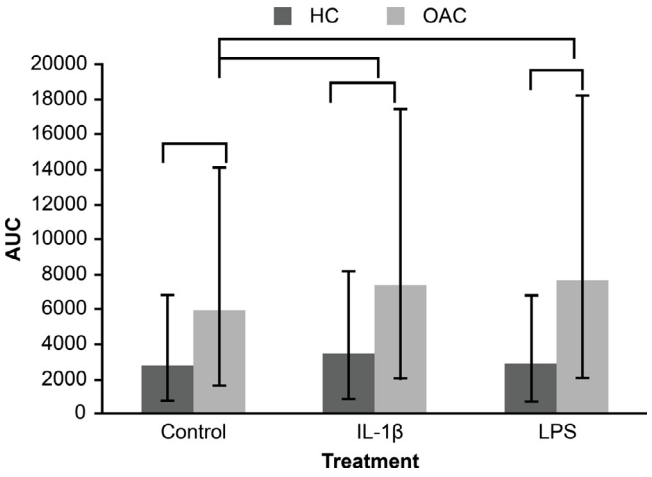


**Fig. 1. $Ca^{2+}$ release in chondrocytes in response to 5-HT** Medians and 95% confidence intervals (bars) for 5-HT-evoked ($10^{-5}$ M) $Ca^{2+}$ responses [area under the curve (AUC)] in cultured chondrocytes from healthy (HC) and osteoarthritic (OAC) joints stimulated with interleukin-1β (IL-1β, 5 ng/ml) or lipopolysaccharide (LPS, 10 ng/ml), or non-stimulated for 24 h. $n = 20$/group. *P*-values < 0.05 were considered statistically significant (indicated by brackets), and the Bonferroni-Holm correction was used for multiple comparisons.

stimulated HC ($P < 0.005$, $P_{BH} < 0.01$). Both IL-1β- ($P < 0.05$, $P_{BH} < 0.05$) and LPS-stimulated ($P < 0.005$, $P_{BH} < 0.05$) OAC showed an increased intracellular $Ca^{2+}$ release (AUC) compared to non-treated control OAC. IL-1β-stimulated HC showed an increased intracellular $Ca^{2+}$ release compared to control HC, although the difference was not significant when *P* was corrected for multiple comparisons. Values presented in Fig. 1 and Table 2.

### *Effects of IL-1β and lps stimulation on MOR, KOR, and dor expression*

LPS-stimulated OAC showed statistically significant increases in MOR protein levels compared with control OAC ($P < 0.005$, $P_{BH} < 0.05$) (Fig. 2, Table 2). IL-1β-stimulated OAC showed an increase in MOR expression compared to non-stimulated OAC, although the difference was not significant when *P* was corrected for multiple comparisons. KOR expression tended to increase in IL-1β-stimulated OAC. DOR expression was not detected.

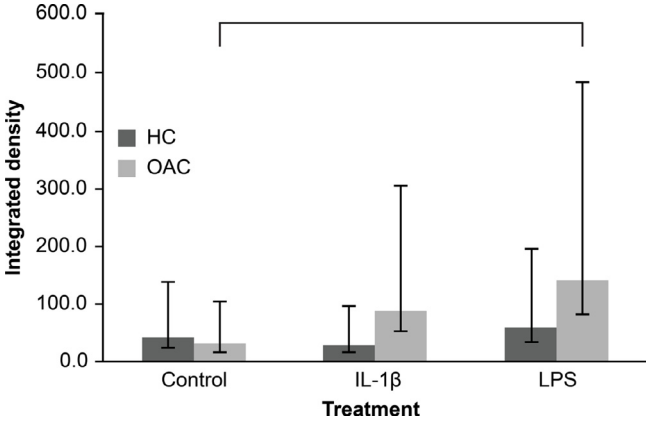


**Fig. 2. Expression of MOR in cultured chondrocytes**. Immunoblot analysis was performed on cell lysates from cultured chondrocytes from healthy (HC) and osteoarthritic (OAC) joints stimulated with interleukin-1β (IL-1β, 5 ng/ml) or lipopolysaccharide (LPS, 10 ng/ml) or non-stimulated for 24 h. The median integrated density and 95% confidence intervals (bars) for HC and OAC are shown. *P*-values < 0.05 were considered statistically significant (indicated by brackets), and the Bonferroni-Holm correction was used for multiple comparisons.

### *F- and G-actin*

Although F-actin was increased in both IL-1β- and LPS-stimulated OAC compared to control OAC, the differences were not significant when *P* was corrected for multiple comparisons (Table 2). There were no significant differences in F-actin between stimulated HC and control HC or in G-actin in stimulated HC or OAC. Further, there were no significant differences in F-actin or G-actin intensities between HC and OAC for any treatment group.

### *Glutamate release in media*

LPS-stimulated OAC showed increased glutamate release compared to non-stimulated OAC ($P < 0.0005$, $P_{BH} < 0.005$) (Fig. 3, Table 2). LPS-stimulated HC also showed an increase in glutamate release compared to non-stimulated cells ($P < 0.05$, $P_{BH} < 0.05$) (Fig. 3, Table 2). No significant difference was observed between HC and OAC for any treatment group.

### *Histologic features and immunohistochemical opioid receptor staining*

Macroscopically normal cartilage showed intact extracellular matrix and no cellular changes. In shavings from joints with mild structural OA cartilage, areas exhibited undulation of the cartilage surface, superficial fibrillation, and mild chondrocyte hypertrophy (histologically mild OA), as well as areas of deeper fibrillation, fissures, loss of matrix, and chondrocyte hypertrophy and clusters (histologically moderate OA). Cartilage of the chip fragment showed areas with chondrocyte hypocellularity, cluster formation, matrix fissures and loss, and areas of fibrocartilage (histologically severe OA).

All three opioid receptors were detected in spinal cord sections. For MOR and KOR, but not DOR, a distinct band of staining was present at the border of the grey and white matter in the region of the dorsal horn (Fig. 4a-c). Immunostaining for MOR and KOR was seen in both dorsal and ventral horn neurons and in cells of the surrounding white matter. For MOR, staining was both nuclear and cytoplasmic/membranous (Fig. 4d), whereas for KOR, only cytoplasmic/membranous staining was clearly detected (Fig. 4e). For DOR, there was distinct nuclear and rare

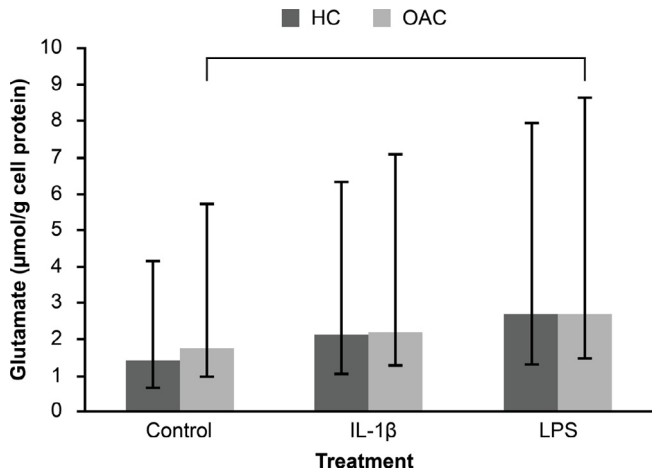


**Fig. 3. Concentrations of glutamate in media** from cultured chondrocytes from healthy (HC) and osteoarthritic (OAC) joints. Cells were stimulated with interleukin-1β (IL-1β, 5 ng/ml) or lipopolysaccharide (LPS, 10 ng/ml) or non-stimulated for 24 h. The concentration is relative to total protein in cell lysate. The median intensities and 95% confidence intervals (bars) for HC and OAC are shown. *P*-values < 0.05 were considered statistically significant (indicated by brackets), and the Bonferroni-Holm correction was used for multiple comparisons.

cytoplasmic staining in dorsal and ventral horn neurons (Fig. 4f) and weaker nuclear staining in the white matter. Staining of cells of vessel walls in the sections was seen with all antibodies.

MOR was detected in both healthy (Fig. 5a) and diseased cartilage, but with increased staining in the chondrocytes of shavings of OA cartilage (Fig. 5b-c) and of the chip fragment (not shown). Typical cytoplasmic/membranous immunostaining for KOR was not detected in chondrocytes of healthy cartilage (Fig. 5d); however, shavings of OA cartilage showed a small number of cells with weak immunostaining (Fig. 5e), and in the chip fragment, many chondrocytes were distinctly immunostained (Fig. 5f). Immunostaining for DOR was not detected in healthy cartilage. In chondrocytes of shavings of OA cartilage, weak and equivocal nuclear staining was detected in few cells, and in rare chondrocytes of the chip fragment, weak cytoplasmic immunostaining was additionally present (data not shown).

## Discussion

This study aimed to investigate the role of inflammation in healthy and diseased equine chondrocytes and the expression of pain mediators in response to inflammatory stimuli and neurotransmitters.

The results clearly showed that OAC responded differently to 5-HT stimulation compared to HC with respect to intracellular $Ca^{2+}$ release. We have previously shown that healthy chondrocytes alter $Ca^{2+}$-evoked signalling from single peaks to oscillating waves when exposed to inflammatory mediators (IL-1β and LPS) (Skiöldebrand et al., 2018). In the current study, OAC exhibited stronger intracellular $Ca^{2+}$ release than HC in response to 5-HT and inflammation. The increased concentration of cytosolic $Ca^{2+}$ in OAC may be an important pathophysiologic event in the OA pain process as it can promote the release of additional neurotransmitters and cytokines (Millan et al., 2002; Patetsos & Horjales-Araujo, 2016). Alterations in the 5-HT system have been reported in patients with chronic back pain and chronic headache, and selective 5-HT reuptake inhibitors have been used in the treatment of chronic pain (Atkinson et al., 1999; Bendtsen, Jensen, & Olesen, 1996). The roles of both centralized and peripheral pain in patients with chronic OA have been discussed (Fishbain et al., 2000), and compounds that alter neurotransmitters such as 5-HT are efficacious in pain mediation in OA patients (Clauw et al., 2017).

Our results demonstrated that MOR protein expression was up-regulated in OAC in response to LPS stimulation, whereas IL-1β stimulation tended to promote MOR and KOR expression in OAC. However, in HC these proteins were not up-regulated by IL-1β or LPS. It has been shown that under normal conditions, only a few opioid

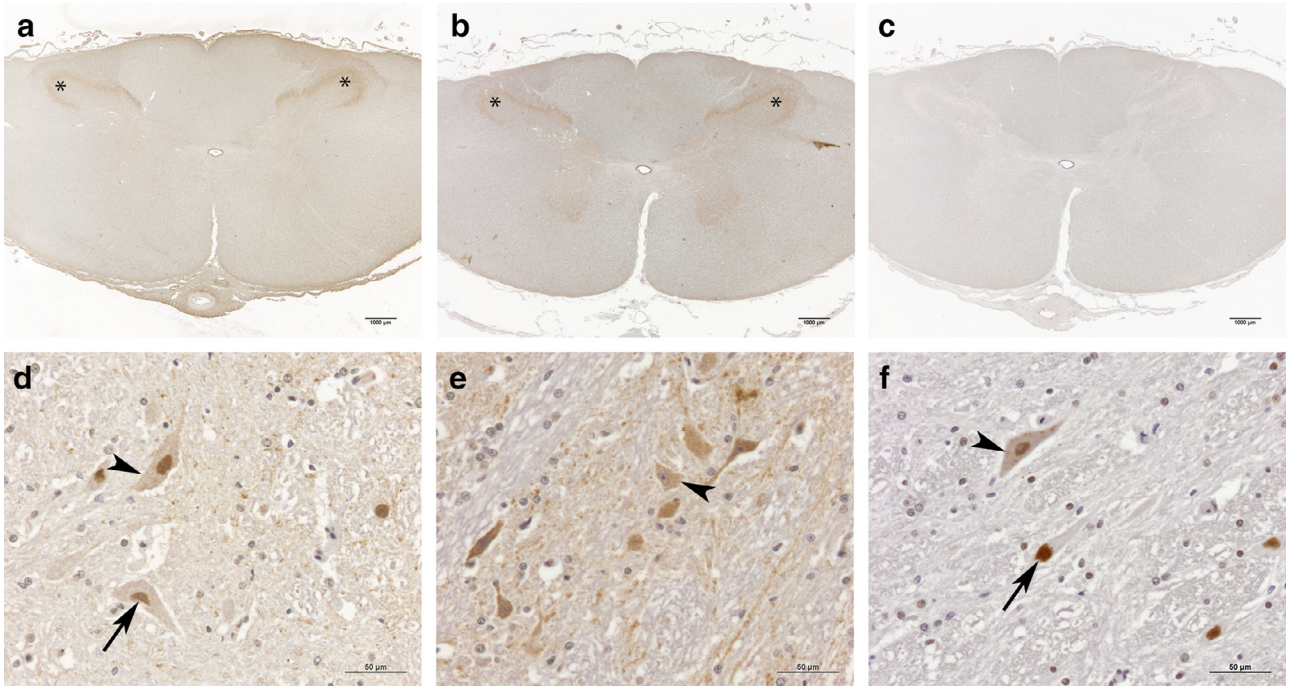


**Fig. 4. Photomicrographs of equine spinal cord immunostained for opioid receptors.** (a) Immunostaining for MOR was strong at the border between grey and white matter in the dorsal horn region (*), and (d) both nuclear (arrows) and cytoplasmic (arrowhead) immunostaining was detected in neurons. (b) Immunostaining for KOR showed a similar distribution in the dorsal horn region (*); however, (e) neuronal staining was restricted to the cytoplasm (arrowhead). (c) Immunostaining for DOR did not show a border distinct distribution in the dorsal horn region, and (f) neuronal immunostaining was dominantly nuclear (arrow), with only few cells showing cytoplasmic staining (arrowhead).

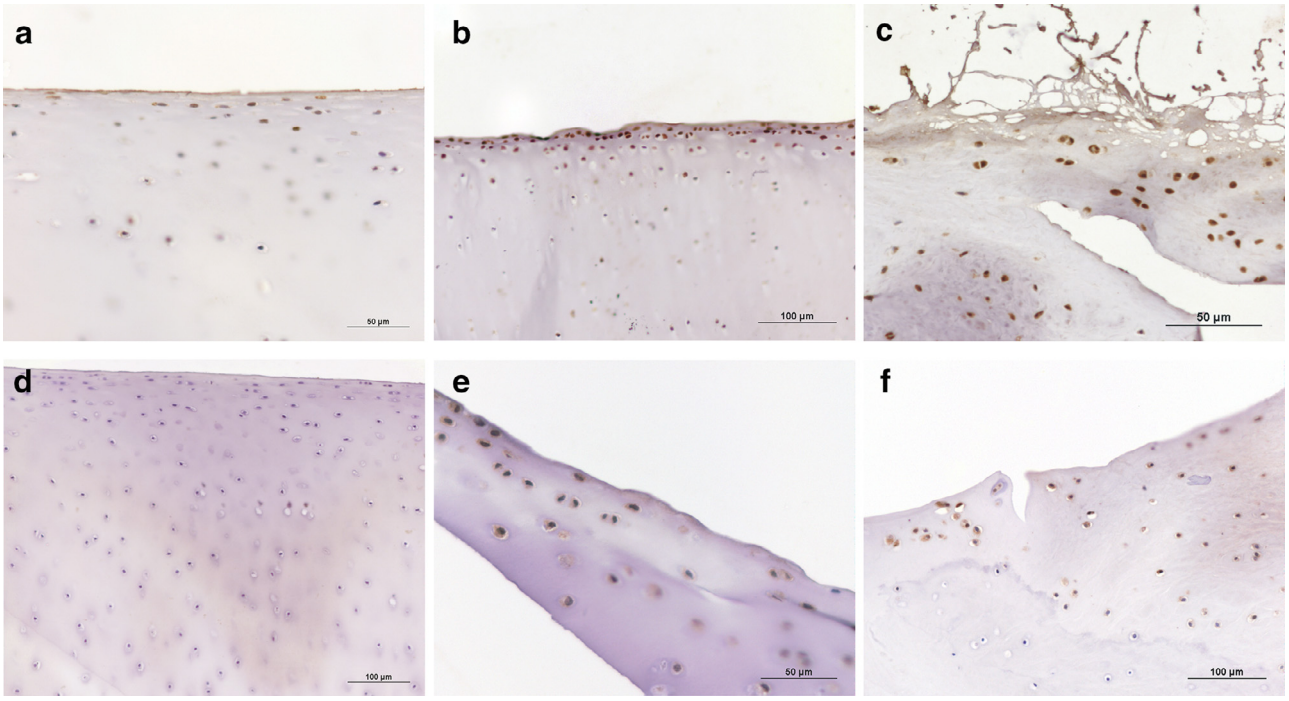


**Fig. 5. Photomicrographs of cartilage immunostained for opioid receptors MOR (a–c) and KOR (d–f).** Immunostaining for MOR was present in chondrocytes of both healthy (a) and osteoarthritic cartilage of different lesion severity (b, c), but with stronger intensity in chondrocytes of the osteoarthritic cartilage. Immunostaining for KOR was not detected in healthy cartilage (d), but was present in few chondrocytes of osteoarthritic cartilage of mild to moderate severity (e) and in chondrocytes of chip fracture fragments (f).

receptors are present in synovial tissue (Stein, Hassan, Lehrberger, Giefing & Yassouridis, 1993), which is in accordance with the reduced immunostaining for MOR in healthy *vs*. OA-affected cartilage. MOR has been previously observed in equine synovial membranes (Sheehy et al., 2001; van Loon, de Grauw, Brunott, Weerts & van Weeren, 2013) and chondrocytes from humans undergoing total knee arthroplasty (Elvenes et al., 2003). Cytoplasmic/membranous KOR was not detected in healthy cartilage, but was present in OA-affected cartilage. This is in accordance with the western blotting results, which revealed a trend of increased MOR and KOR expression in OAC after IL-1β stimulation. In our study only OAC, and not HC, responded to acute inflammatory stimuli by increasing MOR expression.

DOR was not detected in HC or OAC in western blot analyses, nor was it clearly detected in cartilage samples. MOR and DOR signalling act through endogenous endorphins and enkephalins, with differential expression in primary sensory neurons. In nociception, MOR agonists reduce heat pain, and DOR agonists regulate mechanical pain; thus, the two proteins exhibit different physiologic functions, which may explain their differential expression in healthy and diseased cartilage (Scherrer et al., 2009).

In an acute LPS- induced synovitis model in healthy horses, intra-articular and epidural injection of morphine showed analgesic and anti-inflammatory effects (Lindegaard et al., 2010, a, 2010, b; van Loon et al., 2010, 2012). Intra-articular administration of morphine decreased numbers of white blood cells and concentrations of amyloid A in synovial fluid (Lindegaard et al., 2010 b). In the present study, we detected MOR in chondrocytes of both healthy and osteoarthritic cartilage.

Our finding of increased expression of KOR in OA-affected *vs*. normal cartilage was consistent with the results in chondrocytes cultured with inflammatory mediators. KOR has shown to be involved in nociceptive, emotional and cognitive manifestation of joint pain in mice (Negrete, García Gutiérrez, Manzanares, & Maldonado, 2017). Normal fibroblast-like synoviocytes have also been shown to express DOR and KOR, and the opioid receptors were down-regulated in synoviocytes from patients with OA and RA when compared with healthy controls (Shen et al., 2005). Activation of KOR signalling have been shown to increase the expression of anabolic enzymes and inhibit cartilage degradation in response to pro-inflammatory cytokines. Additionally, selective KOR agonists increased joint lubrication, suggesting a protective role of KOR signalling against cartilage destruction (Wu et al., 2017). DOR was not found to be expressed in healthy articular cartilage and the expression in OA cartilage appeared very limited. This may suggests that DOR could be less involved in OA progression compared to MOR and KOR.

Involvement of glutamate in nociceptive signalling has been shown in animal models of acute arthritis (Carlton et al., 1999) and induced inflammation cause the release of glutamate and the nitric oxide metabolites, arginine and citrulline in the synovial fluid (Lawand, McNearney & Westlund, 2000). In a metabolomics study performed on synovial fluid from horses with OA or sepsis, metabolites such as acetate, alanine, citrate, creatine phosphate, creatinine, glucose, glutamate, glutamine, glycine, phenylalanine, pyruvate, and valine were elevated in horses with OA compared to levels in horses with sepsis (Anderson, Phelan, Clegg, Peffers & Rubio-Martinez, 2018). We have shown that cultured chondrocytes from osteoarthritic equine joints exhibit enhanced release of glutamate within 24 h of stimulation with IL-1β and LPS. Additionally, cultured chondrocytes from healthy equine joints release glutamate when stimulated with LPS (Skiöldebrand et al., 2018). Glutamate release corresponded with the stimulation of chondrocytes (both HC and OAC) with LPS and IL-1β, which could indicate that glutamate release is an early cellular response involved in acute pain mediation, in both healthy inflamed and chronically diseased cartilage.

The cytoskeleton of healthy chondrocytes reacts to the dynamic compression with contractile responses, such as deformation and contraction (Szafranski et al., 2004). OAC stimulated with IL-1β and LPS showed a trend towards increased F-actin expression compared to control OAC. It has been previously shown that the F-actin content in chondrocytes increases in response to IL-1β (Pritchard et al., 2006) and

to tumour necrosis factor (TNF)-α stimulation (Chen et al., 2015). It has also been shown that the actin polymerization status regulates chondrocyte dedifferentiation by decreasing cartilage matrix proteins (type II collagen and aggrecan concentration) and increasing fibroblast-like matrix and type I collagen (COL1) gene expression (Parreno et al., 2017). In the current study, we observed no differences in F-actin and G-actin expression between non-stimulated HC and non-stimulated OAC, indicating that the chondrogenic phenotype of the cells was similar.

## Conclusions

Chondrocytes showed different metabolic properties according to the joint status of the original tissue (healthy *vs* OA). The results indicate that chondrocytes from OA joints are predisposed to show an exaggerated response to inflammatory stimuli, such as increased expression of pain mediators, which is absent in chondrocytes of healthy joints.

Our findings suggest that the opioid receptors MOR and KOR and neurotransmitters are important in the progression of equine OA. The inflammatory stage of OA (acute *vs* chronic) and the expression of opioid receptors could be important to take into consideration when therapeutic strategies are being developed.

## Declaration of Competing Interest

None of the authors has any financial or personal relationship that could inappropriately influence or bias the content of the paper.

The authors have no conflict of interest

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## Ethical statement

Sampling of tissue at arthrocopy was approved by the Ethical Committee on Animal Experiments, Uppsala, Sweden, Dnr: C154/4).

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