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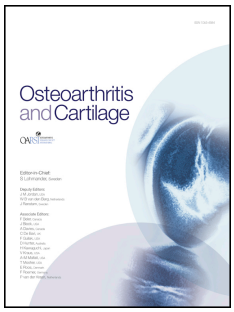

Contribution of nerves within osteochondral channels to osteoarthritis knee pain in humans and rats

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Abstract

Objectives

Subchondral bone may contribute to knee osteoarthritis (OA) pain. Nerve growth factor (NGF) can stimulate nerve growth through TrkA. We aimed to identify how sensory nerve growth at the osteochondral junction in human and rat knees associates with OA pain.

Methods

Eleven symptomatic chondropathy cases were selected from people undergoing total knee replacement for OA. Twelve asymptomatic chondropathy cases who had not presented with knee pain were selected post-mortem. OA was induced in rat knees by meniscal transection (MNX) and sham-operated rats were used as controls. Twice-daily oral doses (30 mg/kg) of TrkA inhibitor (AR786) or vehicle were administered from before and up to 28 days after OA induction. Joints were analysed for macroscopic appearances of articular surfaces, OA histopathology and calcitonin gene-related peptide-immunoreactive (CGRP-IR) sensory nerves in medial tibial plateaux, and rats were assessed for pain behaviors.

Results

The percentage of osteochondral channels containing CGRP-IR nerves in symptomatic chondropathy was higher than in asymptomatic chondropathy (difference: 2.5% [95% CI: 1.1-3.7]), and in MNX- than in sham-operated rat knees (difference: 7.8% [95%CI: 1.7-15.0]). Osteochondral CGRP-IR innervation was significantly associated with pain behavior in rats. Treatment with AR786 prevented the increase in CGRP-IR nerves in osteochondral channels and reduced pain behavior in MNX-operated rats. Structural OA was not significantly affected by AR786 treatment.

Conclusions

CGRP-IR sensory nerves within osteochondral channels are associated with pain in human and rat knee OA. Reduced pathological innervation of the osteochondral junction might contribute to analgesic effects of reduced NGF activity achieved by blocking TrkA.

Introduction

Knee osteoarthritis (OA) is a common cause of pain and disability. Pain is the most common reason sufferers seek medical help. Recent human studies showed that subchondral bone marrow lesions (BMLs) detected on magnetic resonance imaging (MRI) in knee OA are associated with pain[1-3]. Microarray analysis of subchondral BMLs in OA demonstrated upregulation of genes implicated in neurogenesis, osteochondral turnover and inflammation that might contribute to OA pain[4].

Nerve growth factor (NGF) is localized in subchondral bone of the human tibial plateau[5], cartilage[5] and synovium[6] in OA and rheumatoid arthritis and NGF plays a key role in the generation of knee OA pain through actions on its high affinity receptor tropomyosin receptor kinase A (TrkA). The NGF/TrkA pathway has emerged as an important therapeutic target for human OA pain. Antibodies that block NGF reduce pain in human and rodent knee OA[7], and selective, allosteric inhibitors of TrkA such as AR786 can inhibit pain in rat OA models[8], and in human OA[9], although a randomized controlled trial did not suggest analgesic effects of TrkA inhibition in knee OA[10].

NGF/TrkA pathway inhibitors reduce pain through direct actions on peripheral sensory nerves. TrkA is expressed by peptidergic nerves which contain the neuropeptide calcitonin gene-related peptide (CGRP)[11]. CGRP-immunoreactive (IR) sensory nerves contribute to OA pain[12,13]. NGF increases pain by sensitizing nerves[14]. NGF can also stimulate sensory nerve growth[15,16]. Sensory nerve densities have been associated with pain in nonhealed bone fractures[17], aging bone[18] and breast pain[19]. However it is unclear whether sensory nerve growth contributes to OA pain and whether NGF/TrkA pathway inhibitors are effective against pathological sensory innervation in OA. In people with OA, CGRP-IR

sensory nerves are colocalized with NGF within osteochondral channels[5], and increased NGF expression in osteochondral channels was associated with symptomatic human knee OA[20]. In this study, CGRP-like immunoreactivity was used as a well-established marker of unmyelinated sensory nerves to confirm innervation at the osteochondral junction.

The first objective of this study was to determine if CGRP-IR sensory nerves at the osteochondral junction are associated with OA pain in humans by comparing cases with similar OA structural change but with or without symptoms. One group had sought help for knee pain and undergone total knee replacement (TKR) surgery (symptomatic chondropathy), while the other group had not sought help for knee pain but had died from an unrelated illness (asymptomatic chondropathy). Our second objective was to identify the effects of blocking NGF activity by inhibiting TrkA on any OA-associated increase of CGRP-IR sensory nerves and pain behavior in rats with surgically-induced OA. We hypothesise that lower numbers of CGRP-IR sensory nerves within osteochondral channels, due either to pathological phenotype or TrkA inhibition, is associated with less OA pain.

Material and Methods

Human tissues

Eleven symptomatic chondropathy cases were selected from people who had presented with severe knee pain and had undergone TKR for OA. Twelve asymptomatic chondropathy cases who had not presented with knee pain and 11 non-arthritic control cases who had macroscopically normal articular cartilage or only mild chondropathy were selected post-mortem (PM). One knee joint from each donor was included. All asymptomatic chondropathy cases had not sought medical attention for knee pain during the last year and are highly likely to have experienced less pain than the symptomatic

chondropathy cases. Human tissues were selected according to predefined criteria from a human Joint Tissue Repository held by the University of Nottingham containing donations from >2,500 cases at arthroplasty and >400 cases collected post mortem [21]. Informed consent was obtained from TKR cases, or from the next of kin of PM cases. Protocols were approved by Nottingham 1 Research Ethics Committee 05/Q2403/24 and Derby Research Ethics Committee 1 11/H0405/2. Symptomatic chondropathy samples were from people fulfilling American College of Rheumatology classification criteria for OA [22] at the time of TKR.

Human sample processing

Formalin-fixed coronal sections of the middle third of medial tibial plateaux - MTP (key weight-bearing area characteristically affected by OA) were decalcified in 10% ethylenediaminetetraacetic acid (EDTA) in 10mM Tris buffer (pH 6.95, 4°C) prior to wax embedding. Samples used for CGRP-IR nerves staining were fixed by the method of Zamboni[23] (Supplementary text). Zamboni's fixed tissues were decalcified, then immersed and frozen at an optimal cutting temperature and stored at 80°C.

Macroscopic chondropathy score and radiographic OA severity score

Following tissue harvesting, articular surfaces of the MTP were evaluated on the extent and severity of loss of surface integrity by a single assessor[24]. Articular surface defects were graded 0 [normal], 1 [swelling and softening], 2 [superficial fibrillation], 3 [deep fibrillation] and 4 [subchondral bone exposure]. The proportion of articular surface area corresponding to each grade was allocated to each severity grade to calculate a macroscopic chondropathy score;

Macroscopic chondropathy score (0-100) = (Grade 1 x 0.14) + (Grade 2 x 0.34) + (Grade 3 x 0.65) + Grade 4[24].

Radiographic OA severity scores were derived using preoperative postero-anterior knee radiographs as previously described[24]. An atlas of line drawings of the knee joint was used to grade medial and lateral joint space narrowing and osteophytes[25]. Scores for tibiofemoral joint space narrowing (0–6) and osteophytes (0–12) were summed to provide a total radiographic OA severity score (0–18)[24].

Human histology and grading

Tibial plateaux sections (5µm) were stained with H&E, or Safranin-O and fast green. OA articular cartilage changes were graded using the Mankin scoring system[26] (Supplementary text). Subchondral bone marrow replacement was defined as replacement of bone marrow fat spaces with fibrovascular tissue, and assessed as either present or absent. Section width was measured by a digital electronic caliper (Mitutoyo, UK), and densities were calculated of osteochondral channels per mm in subchondral bone, calcified cartilage and non-calcified cartilage, and of channels breaching tidemark.

Immunohistochemistry and quantification of CGRP-IR nerve

Tibial plateaux sections (20µm) were blocked with 3% bovine serum albumin (BSA) for 1h at room temperature. The sections incubated in mouse anti-CGRP antibody (1:300 TA309091; Acris Antibodies, Herford, Germany) were diluted in goat blocking serum overnight in a humid chamber at 4°C. The next day, secondary detection was performed with goat anti-mouse IgG conjugated with Alexa 488 (1:100 A32723; ThermoFisher scientific, Mississippi, USA) for CGRP for 2h at room temperature. Before, between, and after each incubation step, the sections were washed three times for 5min in PBS. CGRP-IR sensory nerves were measured as a proportion (%) of osteochondral channels in each case that displayed CGRP-IR sensory nerves. One section per each knee joint was used for analysis of CGRP-IR nerves.

Animals and OA induction

Male Sprague-Dawley rat knee joints (Charles River, Kent, UK), n=30, were collected for this study from our previous experiment[8]. The rats were used in accordance with UK Home Office regulations and followed the guidelines of the International Association for the Study of Pain. Rats weighing 200–250 g were anaesthetized briefly with isoflurane (2% in O2) and underwent transection of the medial meniscus (MNX; n=20)[27]. Non-osteoarthritic (Sham-operated; n=10) rats were used as controls. Rats were randomized to 3 groups (sham plus vehicle, MNX plus vehicle and MNX plus AR786) using a computer program, and mixed within cages. Data presented in this paper extend behavioural data and macroscopic chondropathy scores that have been reported previously from these rats[8]. All outcome measurements were carried out by an experimenter blinded to randomized treatments.

TrkA inhibitor (AR786) administration

AR786 (Array Biopharma, Boulder, Colorado, USA) was administered in a preventive protocol based on previous data[28,29]. Oral doses (30 mg/kg) of AR786 or vehicle (5% Gelucire 50/13) were administered 1h prior to and 8h following OA induction, and twice daily until the end of the study (28 days after OA induction).

Rat knee joint pathology and quantification of CGRP-IR nerve

Rats were sacrificed by an overdose of pentobarbital (intraperitoneal) (day 28). Macroscopic chondropathy scores based on the Guingamp classification[30] have been previously published[8]. For the current report, histological assessment of cartilage and subchondral bone including osteophytes in medial tibial plateaux was undertaken based on the Osteoarthritis Research Society International recommendations[31]. Subchondral bone marrow replacement by fibrovascular tissue and osteochondral channel density were assessed in the same way as human samples.

Immunohistochemistry and quantification of CGRP-IR nerve fibers in osteochondral channels in medial tibial plateaux were carried out in the same way as human samples. Width of the entire medial proximal tibial epiphysis was measured by a digital caliper and CGRP-IR nerve density per mm in the bone marrow space was calculated. Two sections containing weight-bearing area characteristically affected by OA per each knee joint were used for analysis of CGRP-IR nerves.

Behavioral measurements of OA pain

Pain behavior was assessed as weight-bearing asymmetry and as paw withdrawal threshold to punctate stimulation of the hind-paw. Baseline measurements were obtained immediately prior to intra-articular injection or surgery (day 0) and every 2–4 days from day 3 onwards to day 28 and have been previously reported[8] . Weight-bearing asymmetry was assessed as percent difference in weight distribution between hind-limbs[32].

Image analysis

All human and rat histological scoring and quantification of CGRP-IR nerve fibers were undertaken by a single observer (KA) who was blinded to the diagnostic group, using a Zeiss Axioscop-50 microscope (Carl Zeiss, Welwyn Garden City, UK).

Statistical analysis

Statistical analyses were performed with JMP, Version 10 (SAS Ins. Cary, NC), IBM SPSS version 26.0 software and IBM SPSS Bootstrapping (IBM Corp. Armonk, NY, USA). Data of age, gender, radiographic OA score, macroscopic chondropathy score, OA histopathology, CGRP sensory nerve and pain behaviours were analyzed using Kruskal-Wallis tests followed by post hoc Dunn’s comparisons. Estimates of mean differences of CGRP-IR nerve between groups with 95% confidence interval (CI) were derived from 2000 bootstrap resampling. Logistic regression was performed to adjust for

age. Spearman's rank correlation (r) assessed associations between pain behaviors and CGRP-IR nerve densities, macroscopic chondropathy score and OA istological changes in MNX plus vehicle and MNX plus AR786 models (n=20). The 95% CIs for Spearman's correlation were derived from 2000 bootstrap resampling. Bias-corrected and accelerated percentile method were used for estimation of CIs. $P<0.05$ indicated statistical significance.

Results

Patient characteristics and joint pathology

Demographics and sample details of cases selected for this study are shown in Table 1. The asymptomatic chondropathy group was older than the non-arthritic control and symptomatic chondropathy groups. As expected from our selection criteria, macroscopic chondropathy scores were similar in asymptomatic and symptomatic chondropathy groups; and both were higher than in non-arthritic controls. Histological chondropathy scores were higher in chondropathy cases than in non-arthritic controls (Table 1 and Figure 1 A, B, C). Channels were present at the osteochondral junction in each group (Figure 1, D). Increased numbers of osteochondral channels breaching the tidemark (Figure 1 E), and the percentage of cases with subchondral bone marrow replacement by fibrovascular tissue did not reach statistical significance in chondropathy groups compared to non-arthritic controls (Table 1).

CGRP-IR sensory nerve fibers in human medial tibial plateaux

CGRP-IR nerve profiles were localized to osteochondral channels and subchondral bone marrow spaces (Figure 1 F, G, H). The percentage of osteochondral channels containing CGRP-IR sensory nerves did not significantly differ between chondropathy and

non-arthritic control groups (median percentages (interquartile range (IQR)) of non-arthritic control, asymptomatic and symptomatic chondropathy were 1.2 (0, 2.9), 0 (0, 1.9) and 3.6 (2.5, 4.7)) (Figure 2). Bootstrap estimates of mean differences between asymptomatic or symptomatic chondropathy and non-arthritic control were 0.8% [95% CI: -0.6 to 2.4%] and 1.3% [95%CI: -0.4 to 2.9%], respectively. The percentage of osteochondral channels containing CGRP-IR sensory nerves in the symptomatic chondropathy group was higher than in asymptomatic chondropathy group and this difference remained significant after adjusting for age (aOR=3.9 [95% CI: 1.5 to 31.3], p=0.01) (Figure 2). The bootstrap estimate of mean difference between symptomatic and asymptomatic chondropathy was 2.5% [95% CI: 1.1 to 3.7%].

MNX-induced OA and pain behavior in rats

New data presented here extend previously published macroscopic chondropathy scores, paw withdrawal thresholds and weight-bearing asymmetry data from these experiments.[8] MNX surgery was associated with a greater OA structural change than was sham surgery (Table 2 and Figure 3A, B, C). Subchondral bone marrow replacement by fibrovascular tissue was observed in MNX- but not in sham-operated rats. Numbers of osteochondral channels did not differ between groups, and were not altered by AR786 treatment (Table 2 and Figure 3D, E, F, C). Asymmetric weight distribution and reduced paw withdrawal thresholds were more severe in MNX-operated rats treated with vehicle than in sham-operated rats at day 28 after surgery, and AR786 reversed the OA-induced pain behavior (Table 2).

CGRP-IR nerve fibers in rat knee joints

CGRP-IR nerve profiles were localized to osteochondral channels and subchondral bone marrow spaces in rat knee joints (Figure 3G, H, K). The percentage of osteochondral

channels containing CGRP-IR sensory nerves was higher in MNX-operated knees from rats treated with vehicle than in sham-operated knees (median percentages (IQR) of sham plus vehicle and MNX plus vehicle were 2.8 (0.5, 7.4) and 10 (8, 13.7)) (Figure 4A). The bootstrap estimate of mean difference between sham plus vehicle and MNX plus vehicle was 7.8% [95% CI: 1.7 to 15.0%]. Treatment with AR786 prevented this increase (Figure 4A and Figure 3G, H, I, J). The bootstrap estimate of mean difference between MNX plus vehicle and MNX plus AR786 groups was 7.7% [95% CI: 2.5 to 14.4%]. CGRP-IR sensory nerve density in subchondral bone marrow spaces did not differ between groups (Figure 4B). The percentage of osteochondral channels containing CGRP-IR sensory nerves in knees from rats 28 days after MNX surgery, treated with vehicle or AR786, was significantly associated with weight-bearing asymmetry (Spearman's r=0.50 [95% CI: 0.07 to 0.77], p=0.04), and with paw withdrawal threshold (Spearman's r=-0.55 [95% CI: -0.82 to -0.08], p=0.02).

Discussion

We have identified CGRP-IR sensory nerves within osteochondral channels, associated with symptoms in human knee OA and pain behaviour in MNX-induced rat knee OA. These new data support the view that CGRP-IR sensory nerves invade the osteochondral channels from bone marrow spaces in joints with OA cartilage damage. In rats, blocking NGF activity by inhibiting TrkA prevented the OA-induced growth of CGRP-IR sensory nerves in osteochondral channels. This was associated with, and might contribute to, reduced pain behaviour. Our findings support the hypothesis that NGF-induced growth of sensory nerves at the osteochondral junction might contribute to chronic pain in knee OA.

In our previous studies on human tissues, we showed NGF-like immunoreactivity in

multinucleate osteoclasts adherent to bone, osteochondral channels and synovium (but not mRNA expression) was associated with OA pain in human OA[20,6,33]. In the mouse OA model induced by destabilization of the medial meniscus, increased NGF messenger RNA in knee joints was also associated with pain behavior[34]. Increased NGF expression by osteoclasts might induce the invasion by CGRP-IR sensory nerves into osteochondral channels. Indeed, nerve fibers are increased in channels under areas of most damaged articular cartilage in osteoarthritic mouse knees[35], and chondrocytes produce higher NGF levels in more severely damaged cartilage in human OA[36,37,38] and in surgically-induced mouse knee OA[39]. However, chondrocyte-derived NGF was not significantly associated with pain in late-stage OA[20]. These findings suggest a more important contribution to the generation of pain from NGF in osteochondral channels and synovium than from chondrocytes, particularly in late-stage OA. Here we demonstrate that inhibition of the NGF/TrkA pathway with a specific TrkA inhibitor reduced osteochondral innervation in the rat. These data extend previous findings that NGF-blocking antibodies can reduce pathological sensory innervation in bone[40] or skin[41], to show similar effects of TrkA inhibition in osteochondral channels. NGF pathway inhibition did not, however, have detectable effects on mature sensory innervation, consistent with a lack of effect on mature innervation in other tissues from NGF-blockade[42]. Subchondral bone marrow lesions detected by MRI have been associated with OA pain[1-3]. We speculate that sensitization of pre-existing nerves in subchondral bone marrow lesions might contribute to OA pain, and that generation of neurotrophic factors by BMLs[4] might contribute to osteochondral channel innervation.

Nerve growth into articular cartilage occurs within vascular channels. Penetration of channels into non-calcified articular cartilage has been associated previously with OA

disease, whereas total osteochondral channel densities in calcified and non-calcified cartilage differ little between OA and non-arthritic joints[5]. We found that CGRP-IR sensory nerve densities within osteochondral channels (but not osteochondral channel densities per se) were higher in symptomatic than in asymptomatic chondropathy. Also, channel innervation was significantly associated with weight-bearing asymmetry and paw withdrawal threshold in MNX-induced rat knee OA. These data suggest that rather than an increase in osteochondral channel densities, increased innervation contributes to OA pain. Increased NGF expression in osteochondral channels associated with symptomatic knee OA[20], might further contribute to OA pain by sensitizing these osteochondral nerves.

As previously reported[8], blocking NGF activity by oral administration of the specific TrkA inhibitor AR786 prevented OA-associated pain behaviours in these rats. Inhibiting the NGF/TrkA pathway reduces peripheral sensitization[43,44]. We now also show that AR786 administration prevented the increase in CGRP-IR nerves within osteochondral channels that otherwise follows OA induction by MNX surgery, and that lower CGRP-IR nerve densities were significantly associated with less OA-induced pain behavior.

OA is a multi-tissue disease involving many molecular mediators. Our cross sectional data from humans, and interventional studies in rats, suggest a contribution of NGF pathway-induced osteochondral innervation to OA pain. Further research should investigate whether osteochondral innervation might be a predominant cause of pain in some patients, and its relative importance compared to other pain mechanisms. CGRP-IR sensory nerves have also been localized to osteoarthritic synovium[45,46], possibly in higher densities than in asymptomatic knees[47], particularly in joint compartments displaying increased sensitivity[45]. Synovitis has also been associated with OA knee pain, both in humans[6] and in the MNX-induced rat model[48]. However, we previously showed that

AR786 did not significantly reduce either knee swelling or synovitis in rats with MNX-induced OA, and synovitis scores were not significantly associated with pain behaviors[8]. Other aspects of osteochondral pathology in OA might additionally contribute to OA pain. Loss of osteochondral integrity might increase osteochondral permeability, exposing subchondral nerves to chemical mediators from the cartilage or synovium and mechanical injury[49]. Osteoclast activity may also increase pain both by sensitizing osteochondral nerves and by increasing structural pathology[50]. Furthermore, NGF both influences nerve growth, as indicated by our findings, and quickly induces sensitization of peripheral nerves by multiple signalling pathways[14]. The rapid onset of analgesia associated with NGF blockade[15] or TrkA inhibition is likely attributable to reduced peripheral sensitization, rather than to reduced nerve growth, which is a slow process occuring over a period of weeks[51]. However, our data indicate that osteochondral innervation might contribute to OA pain, and suggest that nerve growth might be a key target for structural disease modification in OA. Other approaches for structural disease modification in OA have been largely unsuccessful, in part due to the prolonged treatment required to demonstrate clinically important structural modification, and a lack of symptomatic benefit. Targeting aspects of OA structural pathology such as aberrant osteochondral innervation with treatments that also more immediately reduce pain is an attractive proposition.

Limitations

Quantification of nerves is limited by sensitivity of the immunohistochemical method, and by the challenge of detecting changes in nerve density in a tissue which normally contains nerves. CGRP-IR was used as a well-established marker of unmyelinated sensory nerves which express TrkA[11]. Half of neurons innervating the subchondral bone expressed CGRP

and TrkA in normal rat knees, whereas all were isolectin B4-negative[52]. Sensitivity of CGRP to detect subchondral sensory nerves might be even higher in OA[53]. It is unclear whether CGRP is itself important for OA pain, and, unlike experience with NGF-blocking antibodies, an RCT of CGRP receptor blockade did not reveal clinically important benefit for OA pain[54]. However, different results might have been obtained using other neuronal markers, and we do not exclude biologically important changes in innervation in tissue compartments additional to osteochondral channels. We used non-parametric statistical methods in order to optimize validity depite inclusion of an outlying value for channel innervation in our per protocol analysis. Future research should seek to confirm our present findings.

Characteristics other than osteochondral innervation, some unmeasured, might explain symptomatic and asymptomatic chondropathy classification. However, the groups had similar chondropathy scores and OA histopathology. Ageing might also influence sensory innervation in mice[35,55], although differences in osteochondral innervation in our study persisted after adjustment for age. Some people in our `asymptomatic' chondropathy group might have experienced chronic knee pain without their relatives knowing. However, all people undertaking TKR report severe knee pain, and it is highly likely that people who have not undergone surgery have less knee pain than those who do. Samples were from the mid-coronal section of the medial tibial plateau, a key weight-bearing area with the greatest amount of cartilage loss, but findings could differ for other joint regions such as femoral condyles. We here focused on NGF at the osteochondral junction, and further systematic studies of other molecules and in other articular tissues might reveal additional pathways contributing to OA pain.

Conclusions

Our data indicate a possible role of osteochondral innervation and TrkA in structural pathology which contributes to OA pain. Previous attempts at structural disease modification in OA have focused on radiographic features such as joint space narrowing and osteophytosis, features which are only weakly associated with OA pain severity[56]. Osteochondral innervation might be a key structural change that contributes to human and rat OA pain. Most analgesic drugs alter sensory nerve function rather than structure. Inhibiting pathological nerve growth in osteochondral channels may reduce chronic OA pain and herald a step change for structural pain modification.

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Author Contributions

All authors approved the final version to be published. K.A. had full access to all of the data in the study and takes responsibility for the integrity of the data and the accuracy of the data analysis. K.A., D.M., L.N., V.C. and D.W. designed the experiments, analyzed and interpreted results, and wrote the manuscript. K.A. and M.S. did immunohistochemistry, histological analysis. R. H. and D. W. did human sample processing. L.N. did pain-related behavior tests and macroscopic chondropathy scoring in rats. K.A., D.M. and D.W. analyzed and interpreted the results.

Ethics approval

Nottingham 1 Research Ethics Committee [05/Q2403/24] and Derby Research Ethics Committee 1 [11/H0405/2].

Conflict of interest

D.A. Walsh: Grants from Arthritis Research UK, while the study was being conducted; grants from Pfizer Ltd, other from Pfizer Ltd, personal fees from GlaxoSmithKline, outside

the submitted work.

D. F. McWilliams: grants from Pfizer Ltd.

The remaining authors have no conflicts of interest to declare.

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Figure 1: Histopathologic features in cartilage and subchondral bone from humans

A; non-arthritic control. B; Asymptomatic chondropathy. C; Symptomatic chondropathy Osteochondral channels were found in the subchondral bone plate in sections from non-arthritic control cases (D). Osteochondral channels breaching the tidemark and entering non-calcified cartilage in sections from symptomatic chondropathy cases (E). CGRP-IR nerves were found in osteochondral channels under the areas of damaged cartilage (asterisk) in sections from symptomatic chondropathy cases (white arrow head) (F, G). CGRP-IR sensory nerves (arrow) were found in bone marrow space (arrow) (H). (I)

explains where these images are located within the knee joint. Black arrow heads indicate tide mark. CGRP-IR; calcitonin gene-related peptide-immunoreactive. Scale bars = 100 μm

Figure 2: Percentage of osteochondral channels containing CGRP-IR sensory nerves in non-arthritic control, symptomatic and asymptomatic chondropathy cases.
Scatterplots illustrate the differences among non-arthritic control, symptomatic and asymptomatic chondropathy cases. Lines represent medians and IQR. Data were analysed using Kruskal-Wallis test followed by post hoc Dunn's comparison. *p=0.007 versus asymptomatic chondropathy.

Figure 3: Histopathologic features in cartilage and subchondral bone from rats
A; Sham + vehicle. B; MNX + vehicle. C; MNX + AR786
Osteochondral channels (black arrow head) were found in the subchondral bone plate in sections from Sham + vehicle (A, D), MNX + vehicle (B, E) and MNX + AR786 group (C, F). CGRP-IR sensory nerves invading osteochondral channels from bone marrow space (white arrow head) under areas of damaged cartilage (asterisk) in MNX + vehicle group (G, H). The increase in CGRP-IR nerves within osteochondral channels under areas of damaged cartilage (asterisk) was prevented in MNX + AR786 group (I, J). CGRP-IR sensory nerves (arrow) were found in bone marrow space (K). MNX; meniscal transection, CGRP-IR; calcitonin gene-related peptide-immunoreactive. Scale bars = 100 μm

Figure 4: Percentage of osteochondral channels containing CGRP-IR sensory nerves and nerve density in bone marrow space from sham plus vehicle, MNX plus vehicle and MNX plus AR786 models.
Lines represent medians and IQR. *p=0.02 versus Sham + vehicle and *p=0.03 versus MNX + AR786. Data were analysed using Kruskal-Wallis test followed by post hoc Dunn's comparison. MNX; meniscal transection, CGRP; calcitonin gene-related peptide-immunoreactive, IR; immunoreactive.

	Non-arthritic control	**Asymptomatic chondropathy**	**Symptomatic chondropathy**
Age	50 (47, 65)	86 (78, 89)	61 (58, 73)
Gender (Male, %)	70	50	67

Macroscopic chondropathy score (0-100)	20 (17, 26)	68 (62, 83) *****	73 (66, 79) ****
Total radiographic OA severity score (0-18)	NA	NA	12 (10.5 13)
Tibiofemoral JSN score (0-6)	NA	NA	5 (5, 5)
Medial tibiofemoral JSN score (0-3)	NA	NA	3 (3, 3)
Osteophyte score (0-12)	NA	NA	7 (5.5, 8)
Medial tibial osteophyte score (0-3)	NA	NA	2 (1.5, 2)
Total Mankin score (0-14)	6 (5, 8)	9 (6, 11)	11 (9, 12) *
Loss of cartilage surface integrity (0-6)	3 (2, 3)	5 (3, 6) ***	6 (4, 6) **
Chondrocyte appearance (0-3)	2 (2, 3)	3 (3, 3)	3 (3, 3)
Loss of tidemark integrity (Yes, %)	45	70	70
Proteoglycan loss (0-4)	1 (1, 1)	2 (1, 2)	2 (2, 2)
Subchondral bone marrow replacement (Yes, %)	45	67	64
Density of channels breaching tidemark (/mm)	0.00 (0.00, 0.00)	0.03 (0, 0.10)	0.07 (0.00, 0.13)
Total osteochondral channel density (/mm)	4.4 (3.9, 4.7)	3.7 (3.0, 5.0)	4.1 (3.3, 6.6)

Table 1: Details of demographics, radiographic OA severity and OA pathology

Data displayed as median (IQR). Total radiographic OA severity score is a summation of tibiofemoral joint space narrowing (JSN) and osteophyte scores. Tibiofemoral JSN score is a summation of medial and lateral tibiofemoral JSN scores. Osteophyte score is a summation of medial and lateral tibial and femoral osteophyte scores. Data were analysed using Kruskal-Wallis test followed by post hoc Dunn's comparison. *p=0.01, **p=0.007, ***p=0.006, ****p=0.003, *****p=0.0002 versus non-arthritic control. JSN; joint space narrowing, NA = Not available.

	SHAM + Vehicle	MNX + Vehicle	MNX + AR786
Macroscopic chondropathy score	0 (0, 0.8)	3 (3, 3)**	3 (3, 4)****
Cartilage damage score (0-15)	0 (0, 0)	5 (3, 8)	6 (5, 10)*
Osteophyte score (0-3)	0 (0, 0)	1 (0, 3)	1 (0, 2)
Osteochondral channel density (/mm)	3.1 (2.9, 3.3)	2.5 (2.2, 3.6)	3.5 (2.5, 4.6)
Subchondral bone marrow replacement (%)	0	50	66.7*
Paw withdrawal threshold (g)	15 (11, 15)	6 (5, 6) ***, #	13 (10, 15)
Weight-bearing asymmetry (%)	1.2 (0.1, 1.9)	25.2 (20.6, 27.4) ***, ##	1.5 (0.6, 3.8)

Table 2: Histology and pain behavior 28 days after knee surgery in rats

Data displayed as median (IQR) and 95% confidence interval (CI) for median. Data were analysed using Kruskal-Wallis test followed by post hoc Dunn's comparison. *p=0.003, **p=0.002, ***p=0.0001, ****p<0.0001 versus SHAM+Vehicle. #p=0.003, ##p=0.002 versus MNX + AR786. MNX; meniscal transection. Weight-bearing asymmetry is given as percent difference in distribution between hindlimbs.

¥; Macroscopic chondropathy score, paw withdrawal threshold and weight-bearing asymmetry have been previously published[8].

Supplementary text

Method of Zamboni[23]

Samples were fixed using a solution of 2% (w/v) paraformaldehyde, 15% (v/v) picric acid in phosphate buffer (pH 7.3, 4°C) overnight, and then transferred to 15% (w/v) sucrose in phosphate buffered saline (4°C) solution for 5 days.

Mankin scoring system[26]

Cartilage surface integrity (0 [normal] to 6 [complete disorganisation]), tidemark integrity (0 [intact] or 1 [crossed by vessels]), chondrocyte morphology (0 [normal] to 3 [hypocellular]) and proteoglycan loss (0 [normal, no loss of Safranin-O stain] to 4 [complete loss of stain]).

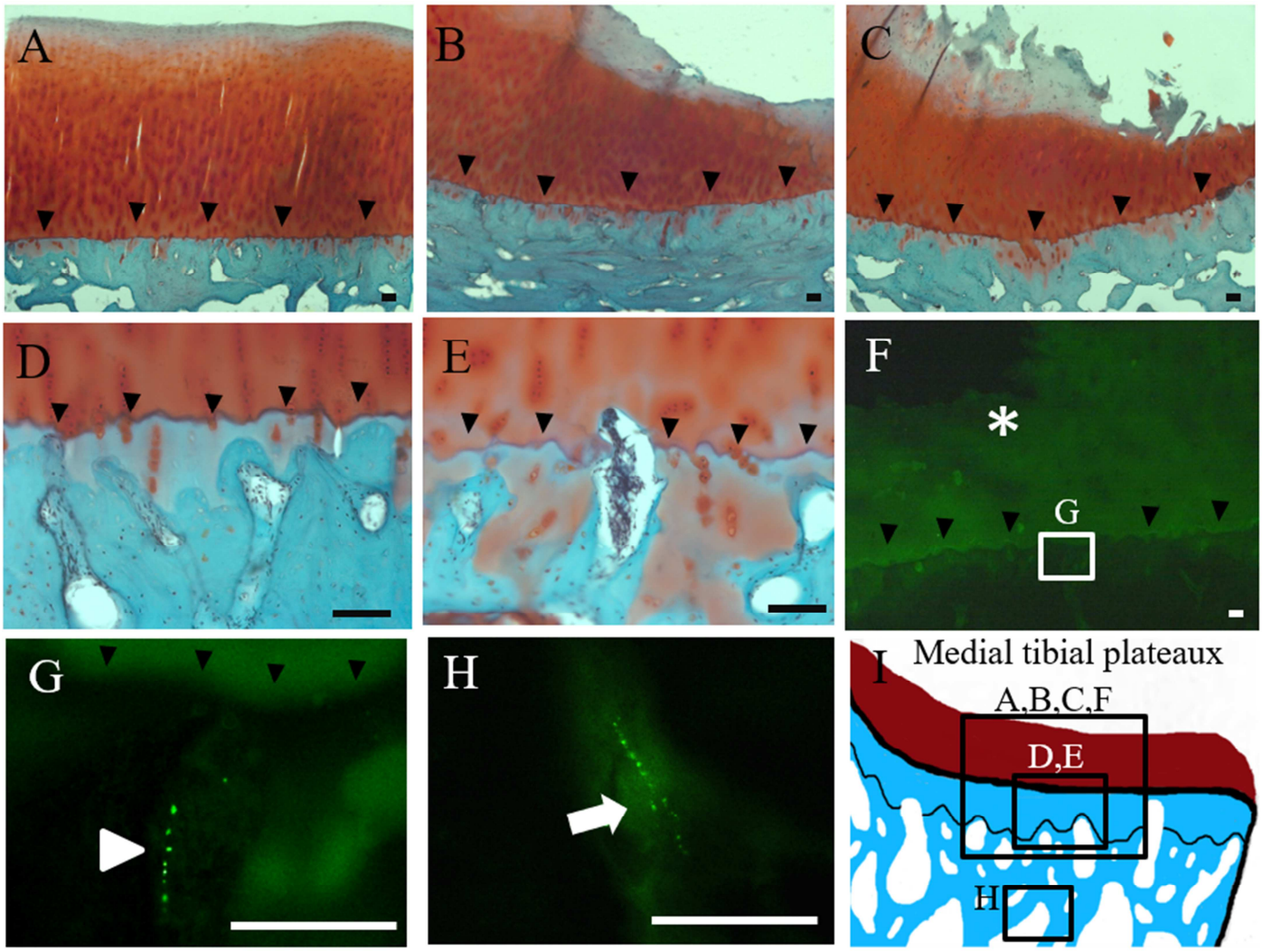

Figure 1: Histopathologic features in cartilage and subchondral bone from humans

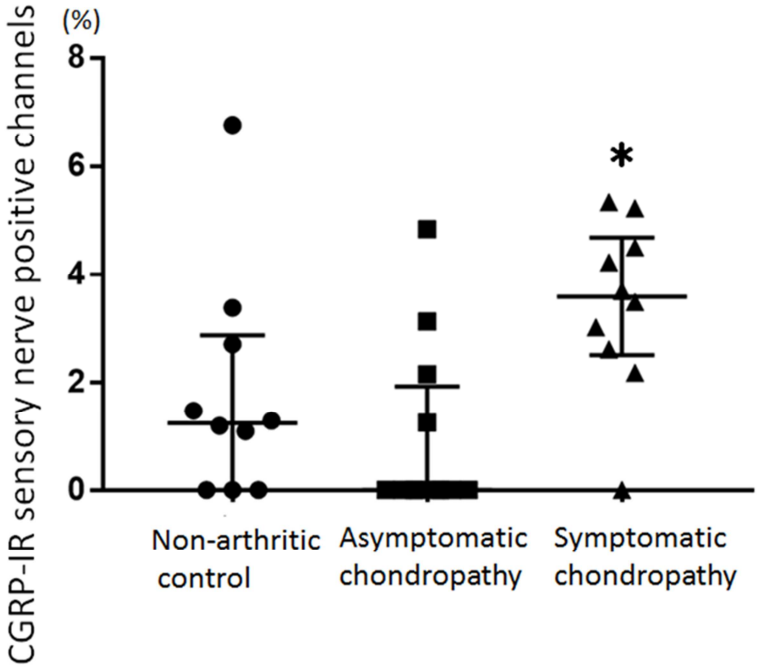

Figure 2: Percentage of osteochondral channels containing CGRP-IR sensory nerves in non-arthritic control, symptomatic and asymptomatic chondropathy cases.

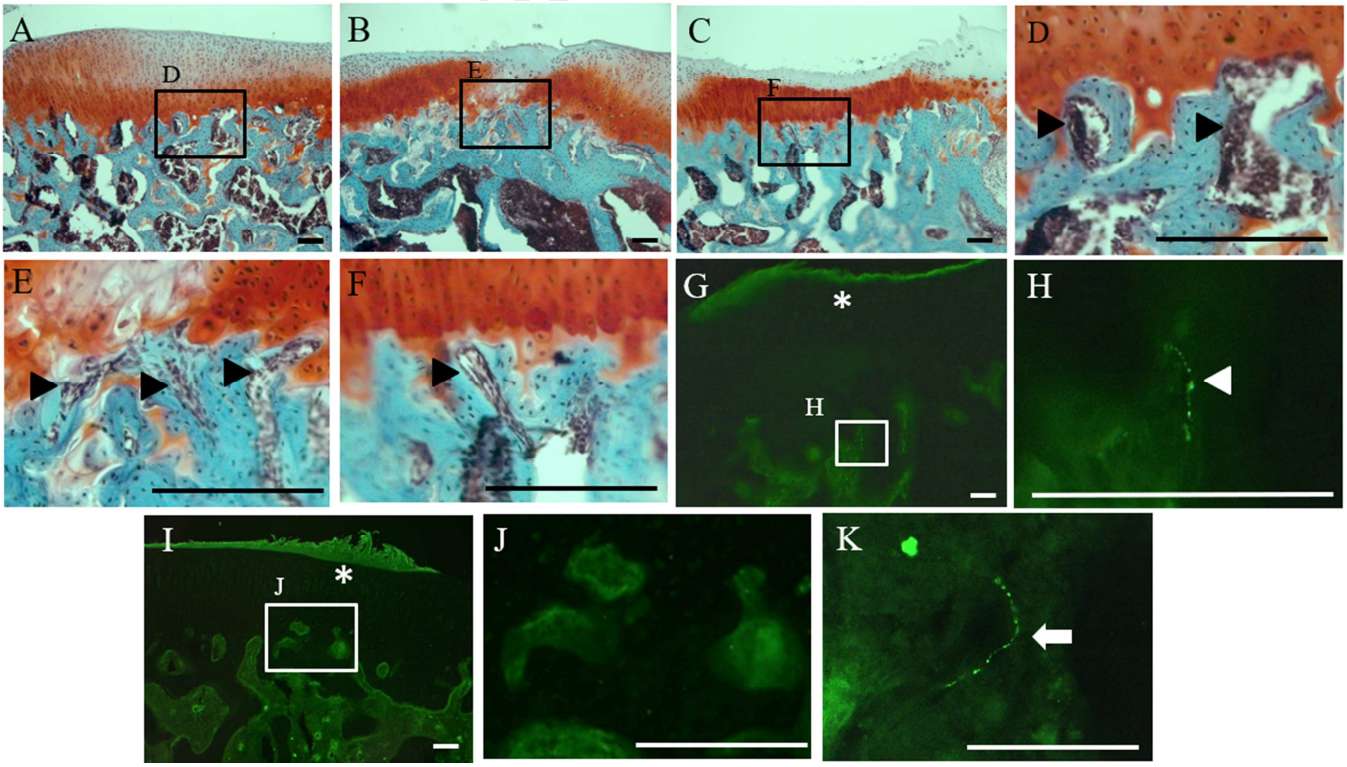

Figure 3: Histopathologic features in cartilage and subchondral bone from rats

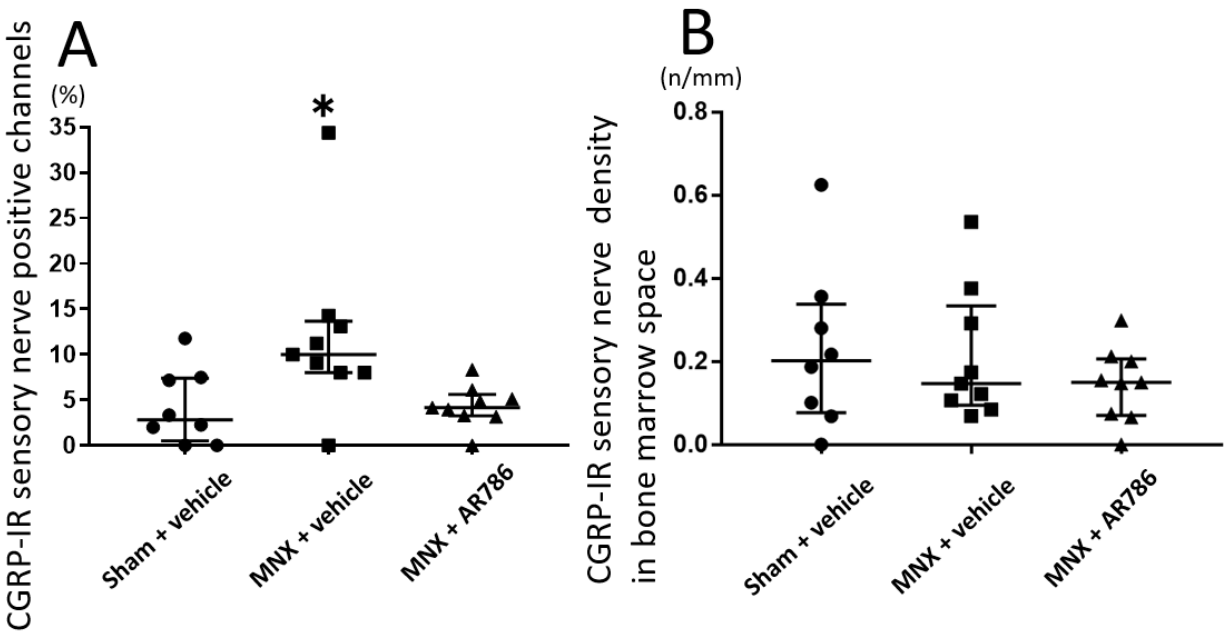

Figure 4: Percentage of osteochondral channels containing CGRP-IR sensory nerves and nerve density in bone marrow space from sham plus vehicle, MNX plus vehicle and MNX plus AR786 models.