Research Paper

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Peripheral brain-derived neurotrophic factor contributes to chronic osteoarthritis joint pain

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Abstract

Brain-derived neurotrophic factor (BDNF) and the high-affinity receptor tropomyosin receptor kinase B (TrkB) have important roles in neuronal survival and in spinal sensitization mechanisms associated with chronic pain. Recent clinical evidence also supports a peripheral role of BDNF in osteoarthritis (OA), with synovial expression of TrkB associated with higher OA pain. The aim of this study was to use clinical samples and animal models to explore the potential contribution of knee joint BDNF/TrkB signalling to chronic OA pain. Brain-derived neurotrophic factor and TrkB mRNA and protein were present in knee synovia from OA patients (16 women, 14 men, median age 67 years [interquartile range: 61-73]). There was a significant positive correlation between mRNA expression of NTRK2 (TrkB) and the proinflammatory chemokine fractalkine in the OA synovia. Using the surgical medial meniscal transection (MNX) model and the chemical monosodium iodoacetate (MIA) model of OA pain in male rats, the effects of peripheral BDNF injection, vs sequestering endogenous BDNF with TrkB-Fc chimera, on established pain behaviour were determined. Intra-articular injection of BDNF augmented established OA pain behaviour in MIA rats, but had no effect in controls. Intra-articular injection of the TrkB-Fc chimera acutely reversed pain behaviour to a similar extent in both models of OA pain (weight-bearing asymmetry MIA: −11 ± 4%, MNX: −12 ± 4%), compared to vehicle treatment. Our data suggesting a contribution of peripheral knee joint BDNF/TrkB signalling in the maintenance of chronic OA joint pain support further investigation of the therapeutic potential of this target.

Keywords: BDNF, TrkB, Osteoarthritis

1. Introduction

Osteoarthritis (OA) is an increasingly prevalent musculoskeletal disease affecting synovial joints. The most significant symptom is debilitating chronic pain. Existing pharmacological treatments often do not provide effective analgesia.[13] Association between joint inflammation and pain in people with OA[37] supports investigation of inflammatory factors in the knee joint that provide potential new therapeutic targets.[37]

Brain-derived neurotrophic factor (BDNF) is vital for neuronal growth and survival,[48] and is upregulated in the central nervous system in chronic pain states.[33,46] Brain-derived neurotrophic factor acts through the tropomyosin receptor kinase B (TrkB) receptor, which is also upregulated in some pain states.[33,46] Mechanisms by which BDNF-TrkB signalling facilitates spinal processing of noxious inputs include phosphorylation of NMDA subunits and downregulation of the potassium/chloride cotransporter, KCC2.[50,60] TrkB-Fc is a chimeric compound formed of the extracellular domain of TrkB and the Fc region of IgG1, which sequesters endogenous BDNF.[17,59] Hippocampal and spinal administration of TrkB-Fc chimera blocked effects of BDNF on synaptic plasticity in vivo[10] and spinal administration reversed pain behaviour in neuropathic pain models in rats and mice.[17,59]

Recent evidence supports a peripheral role of BDNF in arthritis pain. Brain-derived neurotrophic factor and TrkB are present in nerve fascicles in synovial tissue from both human OA and a murine model of inflammatory arthritis.[19,20,35] Human OA synovial fibroblasts and macrophages express BDNF, and release it in response to stimulation of the pronociceptive stimulus ATP-acting at P2X4 receptors.[29] Analysis of differentially expressed genes in the synovial tissue of patients with high or low OA pain revealed a pain-associated increase in the mRNA for TrkB.[9] Positive correlation between plasma levels of BDNF and reported OA pain[49] further supports a role of BDNF in OA pain.

Animal models of OA are important research tools to study mechanisms underlying chronic pain associated with cartilage damage, synovitis, and subchondral bone changes.[55] Two widely used rodent models of OA use intra-articular injection of monosodium iodoacetate (MIA)[22] or surgical transection of the medial meniscus (MNX).[5] Although these models do not mimic human OA aetiology, both exhibit key aspects of the joint pathology evident in human OA, alongside weight-bearing asymmetry and altered pain pressure thresholds.[5,44]

Sponsorships or competing interests that may be relevant to content are disclosed at the end of this article.

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Table 1

Characteristics of the study population for each group.

	Low inflammation	High inflammation
Sex (% female)	53%	53%
Age (median [IQR])	67 (59-73)	63 (61-73)
Joint-space narrowing (median [IQR])	5 (5-5)	5 (5-5)
Osteophytes (median [IQR])	8 (7-8)	8 (7-8)
Total radiographic score (median [IQR])	13 (12-14)	13 (12-13.5)

Preoperative posteroanterior radiographs for participants were graded by a blinded observer. Joint-space narrowing (JSN) and presence of osteophytes (OST) were graded by comparison with a line drawing atlas, with ranges from 0 to 6 (JSN) and 0 to 12 (OST) and higher scores indicating more severe pathology. The JSN scores and OST scores were combined for a total radiographic score.
IQR, interquartile range.

We hypothesised that peripheral knee joint BDNF contributes to OA pain. To test this, expression of BDNF and TrkB mRNA and protein in human OA synovial tissues was quantified in relation to expression of molecules present in synovial fluid and known to have proinflammatory roles in knee OA: IL-6,[16] TNFα,[27] MMP3,[11] and CX3CL1.[25] Using validated rat models of OA pain, we then confirmed the presence of TrkB and CX3CL1 in synovial tissue, quantified synovial fluid levels of BDNF, and investigated the contribution of peripheral BDNF to established OA joint pain. To this end, this study evaluated the effects of intra-articular injection of BDNF, or TrkB-Fc, on established pain behaviour and joint pathology in the MIA and MNX models of OA pain in the rat.

2. Methods

2.1. Chemicals and reagents

A TrkB-Fc chimera compound (R&D Systems, Minneapolis, MN: 688-TK) was used in these studies.[7] TrkB-Fc chimera is a divalent homodimer compound that contains the ligand-binding domain of TrkB and the Fc region of human IgG1. This chimera has been shown to be highly potent and selective for BDNF induced TrkB phosphorylation.[7] Previously, intrathecal injection of TrKB-Fc chimera was shown to attenuate neuropathic pain in mice[42] and rats.[34] Human IgG1 control was from R&D Systems (110-HG-100).

2.2. Clinical samples

Informed consent was obtained from patients before total knee replacement surgery, as per the Declaration of Helsinki, and the study was approved by the United Kingdom National Research Ethics Service (Nottingham Research Ethics Committee [05/Q2403/24]).

Human OA synovium was obtained from 30 patients (16 women, 14 men, median age 67 years [interquartile range: 61-73]) with knee OA (**Table 1** for more details), collected during total knee joint replacement surgery (arthroplasty). We did not have access to any quantitative data on patient-reported pain for these samples. Sections (4-μm thick) were taken from each of the samples and then stained with haematoxylin and eosin (H&E). Synovitis was measured according to the following scale: 0 = normal (synovial lining < 4 cells thick, sparse, cellular distribution, with few or no inflammatory cells), 1 = mild inflammation (synovial lining 4 or 5 cells thick, increased cellularity with some inflammatory cells), 2 = moderate inflammation (synovial lining 6 or 7 cells thick, dense cellularity with inflammatory cells but not lymphoid aggregates), and 3 = severe inflammation (synovial lining > 7 cells thick, dense cellularity and inflammatory cell infiltration, may contain perivascular lymphoid aggregates).[23] The 2 groups investigated were a low-inflammation group (a score of 0 or 1) and a high-inflammation group (a score of 3). Samples were gender- and age-matched.

2.3. Quantification of brain-derived neurotrophic factor & NTRK2 mRNA in human osteoarthritis synovia

Messenger RNA expression of BDNF and NTRK2 (which encodes TrkB) was quantified in human OA synovia. To further explore the inflammatory status of the human OA synovia samples, we also quantified the expression of a range of other inflammatory mediators that have previously been found in the knee joints of OA patients, the adipokine IL-6,[16] the cytokine TNFα,[27] the metalloprotease MMP3,[11] and the chemokine CX3CL1.[25] A 50-mg sample of each human frozen synovium was homogenised in 1 mL of ice-cold TRI reagent (T9424; Sigma Aldrich, St. Louis, MO). RNA was extracted according to the manufacturer's instructions. For cDNA synthesis, 500 ng of total RNA was reverse-transcribed using M-MLV reverse transcriptase (28025013; ThermoFisher Scientific, Waltham, MA) in a total reaction volume of 25 μL. Primers and probes for BDNF, NTRK2, IL-6, TNF-α, CX3CL1, and MMP3 were designed using Primer

Table 2

Forward, reverse primer, and probe sequences used for RT-PCR.

Gene of interest	Sequence (5′ -> 3′)
Taqman	
huB-actin	Fwd: CCTGGCACCCAGCACAAT
	Rev: GCCGATCCACACACGGAGTACT
	Pro: ATCAAGATCATTGCTCCTCCTGAGCGC
huIL-6	Fwd: CGGGAACGAAAGAGAAGCTCTA
	Rev: AGGCGCTTGTGGAGAAGGA
	Pro: CTCCCCTCCAGGAGCCCAGCTATG
huTNF-a	Fwd: CCCAGGGACCTCTCTCTATACA
	Rev: GGTTTGCTACAACATGGGCTACA
	Pro: CTCTGGCCCAGGCAGTCAGATCATCT
huBDNF	Fwd: CGTGATAGAAGAGCTGTTGGATGA
	Rev: GACGTGTACAAGTCTGCGTCCTT
	Pro: ACCAGAAAGTTCGGCCCAATGAATGAAGAAAAC
huNTRK2	Fwd: ACGATGGTGCAAACCCAAAT
	Rev: CCGGTTTTATCAGTGACGTCTGT
	Pro: CACCACGAACAGAAGTA
SYBR green	
huCX3CL1	Fwd: AGATACCTGTAGCTTTGCTC
	Rev: TCTCGTCTCCAAGATGATTG
huMMP3	Fwd: GCAGTTAGAGAACATGGAG
	Rev: ACGAGAAATAAATTGGTCCC

BDNF, brain-derived neurotrophic factor; RT, room temperature.

Express v.3 (Applied Biosystems, Foster City, CA) and were synthesised by MWG Biotech (**Table 2** for more details). TaqMan PCR and SYBR Green PCR were performed using an Agilent AriaMx PCR system, and B-actin was used as a house-keeping gene.[47]

2.4. Protein isolation and Western blotting

Ten to 20 mg of human synovium was minced and homogenized in 500-μL modified RIPA lysis buffer containing 50-mM Tris hydrochloride pH 7.4, 150-mM sodium chloride, 1% TX-100, 0.5% sodium deoxycholate, 0.1% SDS, 1-mM EDTA, and protease inhibitor cocktail (EDTA-free complete, Roche, Basel, Switzerland), and then placed on ice for another 2 hours before collection by centrifugation. A total of 50-μg protein per sample was boiled for 5 minutes in a denaturing Laemmli buffer and then separated on a 9% polyacrylamide SDS-PAGE gel and transferred to a nitrocellulose blotting membrane (GE Healthcare, Chicago, IL) that was blocked in 5% milk blocking buffer. Membranes were then incubated with primary antibodies overnight at 4°C (pro-BDNF sc-65514, 1:200, Santa Cruz Biotechnology, Dallas, TX; TrkB J977.7, 1:500, ThermoFisher; CX3CL1 ab85034, 1:500, Abcam; β-actin ab8227, 1:1000, Abcam, Cambridge, United Kingdom). Antibody binding was visualized using 800- or 680-nm infrared dye–conjugated donkey anti-rabbit or goat anti-mouse secondary antibodies (1:5000; LiCor, Lincoln, NE). Immunoreactive bands were detected using LiCor Odyssey Imaging System (LiCor). Proteins levels were quantified by densitometry using Image Studio Lit version 5.2 software and normalized to loading control β-actin.

2.5. Animal models of osteoarthritis pain

All experiments using rats were performed in accordance with the United Kingdom Animals (Scientific Procedures) Act (1986), and reported in line with the ARRIVE guidelines. Adult male Sprague-Dawley rats (total n = 153, weight 175-199 g, Charles River, United Kingdom) were housed in conventional cages in temperature-controlled (20-23°C) rooms under a 12-hour light–dark cycle (7 AM-7 PM). Rats had access to standard rodent diet and water ad libitum.

2.6. Behavioural studies

Experimenters were blinded to the model and treatments throughout the study. Rats were randomly allocated to either model or control treatment by a third party, and then treatment groups were matched to ensure balancing of groups. Weight-bearing asymmetry between the left (ipsilateral) and right (contralateral) hind limbs was assessed using an incapacitance tester (Linton Instrumentation, Norfolk, United Kingdom) as previously described.[44] Hind paw withdrawal thresholds (PWT) were ascertained using von Frey monofilaments as previously described.[44]

All intra-articular injections were conducted under inhalational anaesthesia (isoflurane 2.5-3%, 100% O_2) through the infrapatellar ligament of the left (ipsilateral) or the right (contralateral) knee in areflexic rats.[44]

2.7. Intra-articular injection of brain-derived neurotrophic factor

Sprague-Dawley rats (n = 28) were anaesthetised with isoflurane before undergoing intra-articular injection of either 100-ng/50-μL BDNF (n = 6), 1-μg/50-μL BDNF (n = 7), 10-μg/50-μL BDNF (n = 7), or 50-μL 0.9% saline (n = 8). Weight-bearing asymmetry and paw withdrawal threshold were measured 1, 3, and 6 hours after injection, and then on day 1, 4, and 7 after injection.

A separate cohort of Sprague-Dawley rats (n = 29) were anaesthetised with isoflurane before receiving an intra-articular injection of either 1-mg/50-μL MIA (Sigma, Dorset, United Kingdom) (n = 13) or 50-μL 0.9% saline (n = 16). Weight-bearing asymmetry and paw withdrawal thresholds were measured for 21 days after injection. At 21 days after MIA model induction, rats were anaesthetised again with isoflurane and received intra-articular injection of either 1-μg/50-μL BDNF (n = 15) or 50-μL 0.9% saline (n = 14).

The final group sizes were: MIA + saline (n = 6), MIA + BDNF (n = 7), saline + saline (n = 8), and saline + BDNF (n = 8). Weight-bearing asymmetry and paw withdrawal thresholds were then measured at 1, 5, 10, and 14 days after injection.

2.8. Intra-articular injection of TrkB-Fc chimera

Sprague-Dawley rats (n = 30) underwent injection of either 1-mg/50-μL MIA (n = 20) or 50-μL 0.9% saline (n = 10) as described above. Weight-bearing asymmetry and paw withdrawal thresholds were measured for 21 days after injection. At 21 days, rats were anaesthetised and received intra-articular injection of either 100-ng/50-μL TrkB-Fc chimera (n = 15) or 100-ng/50-μL human IgG (n = 15). The final group sizes were: MIA + TrkB-Fc (n = 10), MIA + IgG (n = 10), saline + TrkB-Fc (n = 5), and saline + IgG (n = 5). Weight-bearing asymmetry and paw withdrawal thresholds were measured 1 and 3 hours after injection. A separate group of rats (n = 30) underwent injection of either 1-mg/50-μL MIA (n = 20) or 50-μL 0.9% saline (n = 10) at 21 days after MIA or saline, rats were anaesthetised and received intra-articular injection of either 100-ng/50-μL TrkB-Fc chimera (n = 15) or 100-ng/50-μL human IgG (n = 15). Therefore, the final group sizes were: MIA + TrkB-Fc (n = 10), MIA + IgG (n = 10), saline + TrkB-Fc (n = 5), and saline + IgG (n = 5). In these rats, the duration of the effects of TrkB-Fc was quantified up to 2 weeks after TrkB-Fc. Weight-bearing asymmetry and paw withdrawal thresholds were measured 24 hours, 1 week, and 2 weeks after injection.

To ensure that any effects of TrkB-Fc on joint pain were generalizable to OA and not just to MIA-induced joint pathology, the effects of TrkB-Fc were also studied in a surgical model of OA. Sprague-Dawley rats (n = 30) were anaesthetised with isoflurane before undergoing either transection of the medial meniscal (MNX) surgery (n = 20) or sham surgery (n = 10), as previously described.[5] Weight-bearing asymmetry and paw withdrawal thresholds were measured for 21 days after injection. Rats were then anaesthetised and received intra-articular injection of either 100-ng/50-μL TrkB-Fc chimera (n = 15) or 100-ng/50-μL human IgG (n = 15). The final group sizes were: MNX + TrkB-Fc (n = 10), MNX + IgG (n = 10), sham + TrkB-Fc (n = 5), and sham + IgG (n = 5). Weight-bearing asymmetry and paw withdrawal thresholds were then measured for 3 hours after injection. Rats were then euthanised before tissues were collected.

To investigate whether the site of action of the effects of TrkB-Fc were peripheral, or mediated by sites other than the knee joint (including the central nervous system), a group of rats with MIA-induced pain were injected with TrkB-Fc into the contralateral knee joint. Sprague-Dawley rats (n = 6) were anaesthetised with isoflurane before undergoing intra-articular injection of 1-mg/50-μL MIA into the ipsilateral knee. Twenty-one days later, rats were anaesthetised with isoflurane before undergoing intra-articular injection of 100-ng/50-μL TrkB-Fc

into the contralateral knee joint. Weight-bearing asymmetry was measured before MIA injection, 21 days after MIA injection, and 3 hours after TrkB-Fc injection.

2.9. Macroscopic scoring of joint pathology

Knee joints were collected postmortem from rats 21 days after either injection of 1-mg MIA (n = 20), injection of 50-μL saline (n = 10), meniscal transection surgery (n = 20), or sham surgery (n = 10), and the various BDNF/TrkB-Fc treatments. Knee joints were disarticulated as previously described.[38] The following compartments were then scored for chondropathy: medial tibial plateau, lateral tibial plateau, medial femoral condyle, lateral femoral condyle, and the femoral groove using the scoring system described by Guincamp et al.[22] The sums of these individual scores were taken to give a total chondropathy score for each knee joint on a scale of 0 to 20.

2.10. TrkB expression in rat synovium

Rat synovia with patellae were dissected at 21 days after MIA or saline injection and embedded in optimal cutting temperature compound, and then snap-frozen in isopentane. Sagittal sections (3 per rat) of synovia from MIA- and saline-treated rats were prepared for immunohistochemistry for TrkB (MIA: n = 3 rats, saline: n = 3 rats) and CX3CR1 (MIA: n = 3 rats, saline: n = 2 rats). Sections were incubated in room-temperature (RT) distilled water to remove optimal cutting temperature, washed in 0.1-M phosphate-buffered saline, and incubated for 1 hour at RT in a blocking solution (5% serum and 0.5% Triton X-100). Sections were then incubated in 1:250 rabbit anti-TrkB (ab18987; Abcam) or 1:400 rabbit anti-CX3CR1 (ab8021; Abcam) primary antibodies overnight at RT. After washing, sections were then incubated with 1:400 Alexafluor 488 conjugated goat anti-rabbit secondary antibody for 2 hours at RT followed by 1:1000 4′,6-diamino-2-phenylindole (DAPI) for 20 minutes at RT to counterstain nuclei.

Images were taken with a Zeiss LSM Exciter wide-field microscope using a 20 × 0.5 NA objective lens. Resulting images were autothresholded using the Huang method,[26] and the fractional area of suprathreshold areas of labelling in the synovial section were quantified using ImageJ software.[39]

2.11. Enzyme-linked immunospecific assay measurement of brain-derived neurotrophic factor

A commercial enzyme-linked immunospecific assay (ELISA) kit was used to measure levels of BDNF in the rat synovial fluid according to manufacturer's instructions (Thermo Scientific: ERBDNF). To confirm the specificity of TrkB-Fc for BDNF, we determined the effects of adding TrkB-Fc to known quantities of BDNF. Different concentrations of TrkB-Fc (0.01×, 0.1×, 0×, 1×, 10×, and 100× the concentration of BDNF) were added to standard curves of BDNF (0, 12.79, 30.72, 76.8, 192, 480, 1200, and 3000 pg/mL), and levels of BDNF were quantified. To determine whether TrkB-Fc had off-target effects on nerve growth factor (NGF), the effects of TrkB-Fc on known quantities of NGF were determined. Standard curves of NGF (0, 20.58, 61.73, 185.2, 555.6, 1666, 5000, and 15,000 pg/mL) were made up according to the ELISA's manufacturer's instructions (Thermo Scientific: ERNGF). Different concentrations of TrkB-Fc (0×, 1×, and 10× the concentration of NGF) were then added to these known quantities of NGF before being quantified with the ELISA kit.

Synovial fluid was collected postmortem from a cohort of MIA rats 4 hours after injection of IgG using an established method.[56] Synovial fluid was collected by joint wicking, which involved disks of protein saver card being placed in the knee joint to absorb the synovial fluid.[56] Disks were dried overnight at RT and were then frozen and stored at −80°C until analysis. To elute out the proteins, disks were placed in 100 μL of distilled water for 24 hours. The eluted fluid was then diluted 1:2 before BDNF concentration was quantified with an ELISA kit according to the manufacturer's instructions.

2.12. Data analysis

Data were analysed and graphically presented using Prism 7 (Graphpad; San Diego, CA). Data were tested for normal distribution using the D'Agostino and Pearson normality test. If data were normally distributed, then parametric analyses were used. If data significantly differed from normal distributions, nonparametric analyses were used.

Differences in the expression levels of proteins in the human synovia were analysed using unpaired *t* test or Wilcoxon test. Correlation between mRNA and protein expression in the human synovia samples was analysed using Spearman's rho. Weight-bearing data are presented as the weight borne on the contralateral limb minus the weight borne on the ipsilateral limb divided by the total weight borne and multiplied by 100 (mean ± SEM). Log-transformed paw withdrawal thresholds are presented as the mean threshold ± SEM. Differences in both weight-bearing asymmetry and paw withdrawal threshold between MIA/MNX and sham animals over time were assessed by 2-way analysis of variance (ANOVA), with the model induction condition as the between-subjects factor and time after model induction as the within-subjects factor; multiple comparisons between-subjects at individual time points underwent Bonferroni correction. Differences between the area under the curve for log-transformed paw withdrawal thresholds at the time point after intra-articular injection of BDNF or TrkB-Fc were analysed using a one-way ANOVA. Differences between the macroscopic scores of cartilage damage between MIA/MNX and sham rats were tested using unpaired *t* test. An unpaired *t* test was also used to test differences in the levels of BDNF in plasma and synovial fluid between MIA- and saline-injected animals.

Analysis of the fractional area of TrkB and CX3CR1 was performed with SPSS V.25. A multilevel model, with each rat being the "top" level and each section nested within the appropriate rat, was used to analyse the effects of the model of OA on labelling of TrkB and CX3CR1. A random intercept was included in the multilevel regression.

3. Results

3.1. Expression of brain-derived neurotrophic factor and TrkB in human synovium

All human synovium samples were obtained during knee joint arthroplasty for OA pain (n = 30). These samples had varying levels of histologically evident synovial inflammation based on the quantification of cellular infiltration of the synovium, as previously described.[23] The OA samples were subdivided into 2 groups: those with OA pathology and high inflammation (a score of 3) and those with OA pathology and low inflammation (scores of 0-1) (**Fig. 1A**). Messenger RNA for both NTRK2 (the gene that encodes TrkB) and BDNF were present in human OA

synovium (**Figs. 1B and C**). Messenger RNA levels for IL-6, TNFα, MMP3, and CX3CL1 were also quantified to further explore the inflammatory status of the OA synovia samples (**Figs. 1D–G**). There was no significant difference between the expression of these mRNAs in synovia with macroscopic low- or high-grade inflammation. However, protein levels of CX3CL1 (also known as fractalkine), as measured by Western blot, were significantly higher in the high-inflammation group, compared with the low-inflammation group (**Figs. 2A and B**). To further evaluate the extent to which expression of NTRK2 and BDNF relates to the level of inflammation in the joint, we investigated the potential associations between BDNF and NTRK2 mRNA and expression of CX3CL1 mRNA in human OA synovia. There was a significant positive correlation between the mRNA expression of NTRK2 and CX3CL1 in the human OA synovia (**Fig. 3A**), but not between BDNF and CX3CL1 (**Fig. 3B**).

Using Western blotting, we were able to investigate the expression of the 2 isoforms of TrkB: the full-length isoform that is involved in intracellular signalling, and the truncated isoform that does not contain the intracellular domains.[57,58] Expression of the truncated isoform of TrkB was greater than that of the full-length isoform in OA synovia (**Figs. 4A and B**). There was a significant positive correlation between the truncated isoform of TrkB and CX3CL1 (**Fig. 4C**), but not between the full-length isoform of TrkB and CX3CL1 (**Fig. 4D**). The expression of BDNF protein in clinical OA synovia was also investigated. An antibody targeting the propeptide of BDNF was selected due to its ability to detect the precursor form of BDNF (pro-BDNF) and its propeptide that is present in equimolar ratios with mature BDNF, and as such can be used as a surrogate for BDNF.[15] The presence of both pro-BDNF and the propeptide was confirmed in OA synovia by Western blotting (**Figs. 5A–C**). Levels of the propeptide and CX3CL1 were positively correlated in these samples (**Fig. 5D**), supporting a potential relationship between BDNF and inflammation in the OA knee joint.

3.2. Synovial expression of TrkB and brain-derived neurotrophic factor in a rodent model of osteoarthritis

To confirm the utility of our preclinical models of OA pain, we next investigated whether TrkB and CX3CR1 were also present in the synovia of rats at 21 days after MIA injection. As was the case in the human OA synovia, TrkB was expressed in the synovia taken from the MIA-treated rats (supplementary Fig. 1A, available at http://links.lww.com/PAIN/A874), and there was a trend towards increased expression of TrkB compared with saline-treated rats (MIA: 5.73 [4.6-5.9], saline: 3.1 [1.3-3.3]) (supplementary Fig. 1B, available at http://links.lww.com/PAIN/A874). Multilevel model analysis of these data revealed a significant association between the model of OA pain and the expression of TrkB in the synovium ($P = 0.02$). Taken together, these data suggest that there is increased expression of TrkB in the joints of OA rats, compared with saline-treated controls. Using an ELISA, we then demonstrated that levels of BDNF in the synovial fluid of rats 21 days after induction of the MIA model were also significantly elevated compared with levels in the saline control group (supplementary Fig. 2A, available at http://links.lww.com/PAIN/A874). CX3CR1 protein was also detected in rat synovia; however, levels were comparable between MIA and saline control rats (supplementary Figs. 1C and D, available at http://links.lww.com/PAIN/A874).

3.3. Intra-articular brain-derived neurotrophic factor exacerbates pain behaviour in a model of osteoarthritis

The next series of experiments investigated the extent to which activation of TrkB in the knee joint may alter pain behaviour in the MIA rat model of OA pain. After intra-articular injection of MIA, rats exhibited significant weight-bearing asymmetry at 17 days after injection (supplementary Fig. 3A, available at http://links.lww.com/PAIN/A874), a time point at which structural features of OA are present in the injected joint.[44] There was also a reduction in ipsilateral PWTs after intra-articular injection of MIA (supplementary Fig. 3B, available at http://links.lww.com/PAIN/A874). Intra-articular injection of 1-μg BDNF at 21 days in the MIA model led to

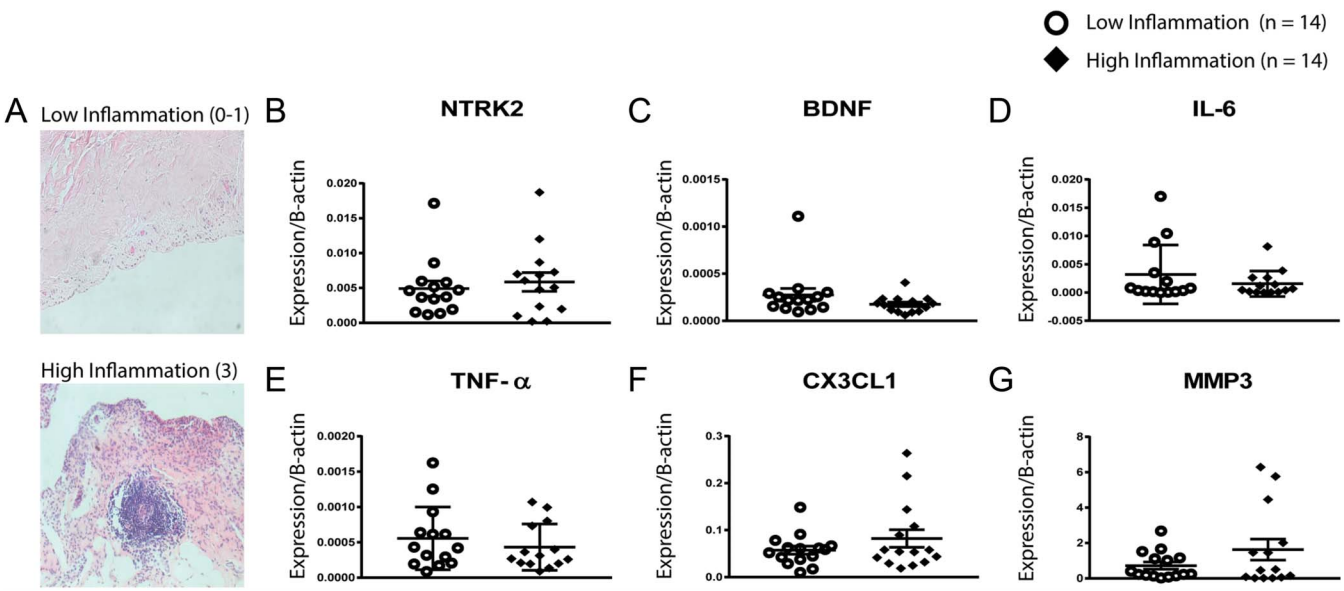

Figure 1. Representative images of human OA synovia with low-grade inflammation and human OA synovia with high-grade inflammation (A). Expression of NTRK2 (B), BDNF (C), IL-6 (D), TNF-a (E) CX3CL1 (F), and MMP3 (G) mRNA in human OA synovia with either low-grade inflammation (inflammation score of 0-1) or high-grade inflammation (inflammation score of 3) taken post-arthroplasty. Data were analysed using the Mann–Whitney *U* test. BDNF, brain-derived neurotrophic factor; OA, osteoarthritis.

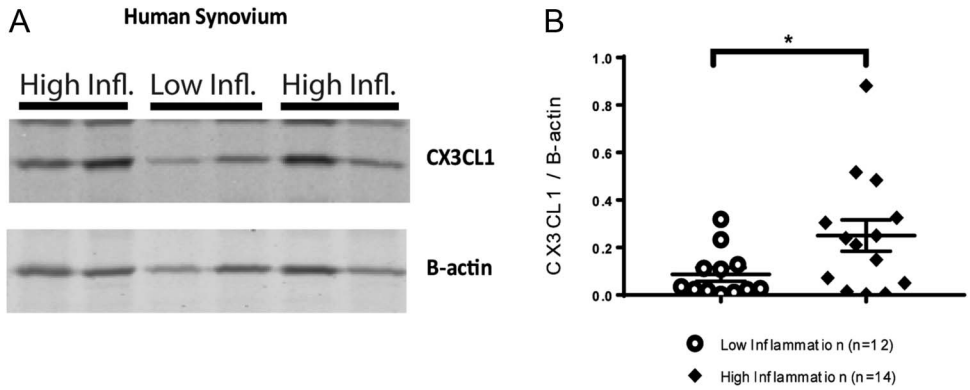

Figure 2. Representative images of Western blot quantification of CX3CL1 protein in human OA synovia taken after arthroplasty (A). Quantification of CX3CL1 protein in low (n = 12) and high (n = 14) inflammation human OA synovia (B). Levels of CXCL1 protein was significantly greater in the high-inflammation group compared with the low-inflammation group (*$P < 0.05$, unpaired *t* test). OA, osteoarthritis.

a further increase in weight-bearing asymmetry (**Fig. 6A**). At 5 days after BDNF treatment (**Fig. 6A**), there was a significantly increased asymmetry in MIA-injected rats, compared with MIA-injected rats that received intra-articular injection of saline. There was no significant effect of intra-articular injection of BDNF on this pain behaviour in saline-injected (non-OA) controls. Analysis of the area under the curve for weight-bearing asymmetry after injection (**Fig. 6B**) revealed a significant difference between groups ($F_{(3, 25)} = 10.37$, $P < 0.0001$, one-way ANOVA).

Intra-articular injection of 1-μg BDNF at 21 days in the MIA model led to a further decrease in PWTs (**Fig. 6C**). Analysis of these data revealed a significant effect of intra-articular BDNF on PWTs in the MIA model one day after injection (**Fig. 6C**). Area-under-the-curve analysis over the 14 days after BDNF injection showed that MIA-injected rats treated with BDNF had significantly lower PWTs, compared to MIA-injected rats treated with saline (**Fig. 6D**). The effects of intra-articular BDNF on established MIA-induced pain behaviour were persistent, being evident from 5 to 14 days after administration, when the study ended. Osteoarthritis pathology 35 days after MIA injection was, as expected, more severe than after saline injection (**Fig. 6E**). Single intra-articular injection of BDNF did not alter the severity of MIA-induced joint pathology as assessed at 35 days after MIA injection (**Fig. 6E**). Consistent with the lack of effect on pain behaviour in saline-treated control rats, intra-articular injection of BDNF (100 ng-10 μg/50 μL) did not alter weight-bearing asymmetry or ipsilateral hind paw withdrawal thresholds in naive rats (supplementary Fig. 4, available at http://links.lww.com/PAIN/A874).

3.4. Sequestering knee joint brain-derived neurotrophic factor attenuates pain behaviour in models of osteoarthritis

To explore whether endogenous BDNF in the knee joint contributes to OA pain, the effects of sequestering BDNF using TrkB-Fc chimera were studied. We first confirmed that TrkB-Fc reduced the quantity of BDNF detected by ELISA (supplementary Fig. 2B, available at http://links.lww.com/PAIN/A874) and that TrkB-Fc did not alter the quantity of NGF detected by ELISA (supplementary Fig. 2C, available at http://links.lww.com/PAIN/A874), supporting the selectivity of TrkB-Fc for BDNF.

Intra-articular injection of TrkB-Fc chimera (100 ng/50 μL) acutely reversed weight-bearing asymmetry in both the MIA chemical model and the MNX surgical model of OA pain, when compared with treatment with IgG control (**Fig. 7**). Inhibitory effects of TrkB-Fc chimera on pain behaviour in the MIA model were significant at 1 hour after treatment and still evident at 3 hours (**Fig. 7A**). In the MNX model, TrkB-Fc chimera treatment was associated with slight decrease in weight-bearing asymmetry at 1hour, which was significantly different to IgG controls by 3 hours (**Fig. 7B**). Area-under-the-curve analysis of weight-bearing asymmetry after TrkB-Fc injection in the MIA (**Fig. 7C**) and the MNX (**Fig. 7D**) models also identified a significant difference between TrkB-Fc-injected rats and the IgG controls, supporting

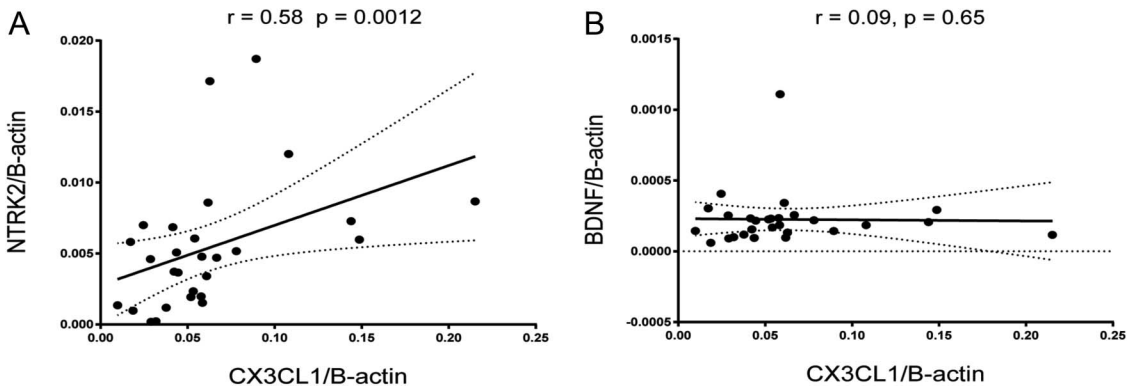

Figure 3. Correlations between expression of NTRK2 mRNA and CX3CL1 mRNA (A) or BDNF mRNA and CX3CL1 mRNA (B) in human OA synovia. (Spearman's rho analysis). BDNF, brain-derived neurotrophic factor; OA, osteoarthritis.

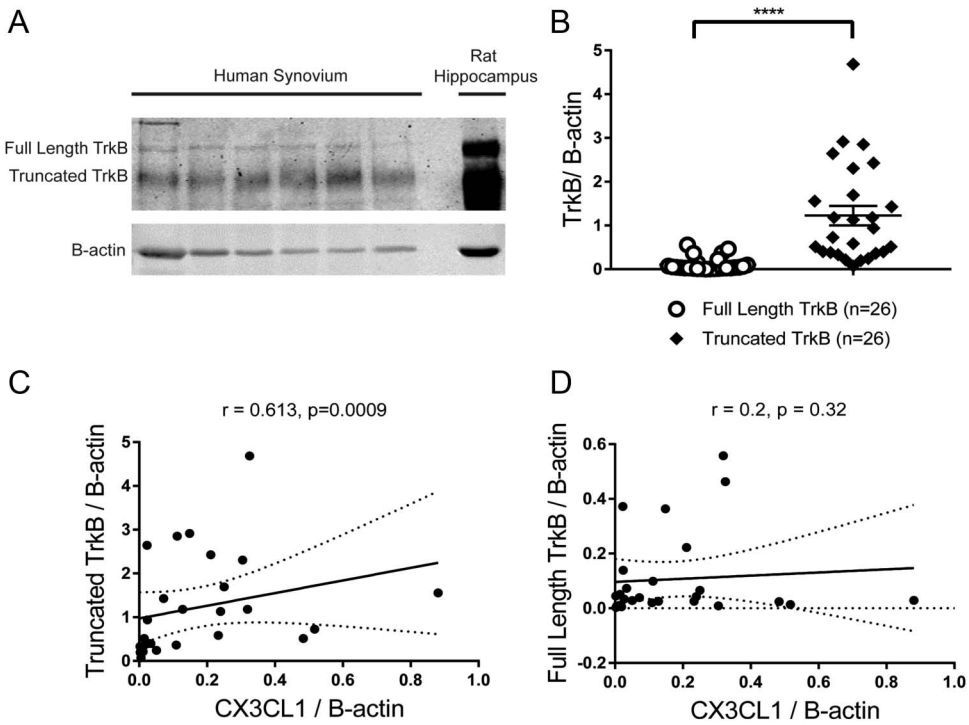

Figure 4. Representative image of Western blots of TrkB protein in human OA synovia after arthroplasty (A). Quantification of full-length and truncated TrkB protein in human OA synovia after arthroplasty (B, Wilcoxon test, $^{****}P < 0.0001$). Correlation between expression of truncated TrkB and CX3CL1 (C) or full-length TrkB and CX3CL1 protein (D) in human OA synovia. (Spearman's rho analysis). OA, osteoarthritis.

these findings. Intra-articular injection of TrkB-Fc chimera also partially reversed the lowering of ipsilateral paw withdrawal thresholds in both the MIA and MNX models, compared with intra-articular injection of IgG control. In MIA-injected rats, lowered PWTs were partially, but not significantly, reversed at 1 and 3 hours after injection of TrkB-Fc chimera (**Fig. 8A**), as evidenced by a slight increase in the area under the curve (**Fig. 8B**). In the MNX model, lowered PWTs were also partially, but not significantly, reversed by the TrkB-Fc chimera compared with IgG injected controls (**Fig. 8C**), further supported by area-under-the-curve analysis (**Fig. 8D**).

To confirm that the site of action of the inhibitory effects of intra-articular injection of TrkB-Fc were localised to the ipsilateral knee joint, a control experiment was conducted in which a group of MIA-treated rats received injection of TrkB-Fc into the contralateral knee joint. This treatment did not significantly alter increases in weight-bearing asymmetry present at 21 days after MIA model induction (supplementary Fig. 5, available at http://links.lww.com/PAIN/A874), suggesting that effects of intra-articular administration are restricted to the knee joint. Acute intra-articular injection of TrkB-Fc chimera did not significantly alter MIA- or MNX-induced joint pathology (supplementary Fig. 6, available at http://links.lww.com/PAIN/A874). To determine the duration of the effects of TrkB-Fc on pain behaviour, a separate group of MIA-injected rats received a single intra-articular injection of TrkB-Fc and the effects on pain behaviour were followed up to 14 days after injection. At 24 hours, 7 days, and 14 days after injection, there was no difference in weight-bearing asymmetry or ipsilateral paw withdrawal thresholds in rats injected with TrkB-Fc and those injected with IgG control (supplementary Figs. 7A and 7B, available at http://links.lww.com/PAIN/A874). These data suggest that the effects of intra-articular injection of TrkB-Fc into the knee joint on OA pain behaviour are not sustained longer than 1 day.

4. Discussion

The aim of this study was to investigate the potential contribution of peripheral BDNF in mediating chronic OA joint pain. Herein, we report the presence of BDNF and TrkB at the mRNA and protein level in human OA synovia collected after knee joint arthroplasty. These results corroborate earlier reports of BDNF protein in OA synovial fluid.[49] To support and advance our clinical data in the setting of well-validated preclinical models of OA pain, we demonstrated the presence of higher levels of BDNF in the synovial fluid from the model of OA compared with the control group, and increased expression of TrkB protein in the synovium in the rat model of OA pain. The presence of BDNF in the OA knee joint seemed to be of functional significance because intra-articular injection of BDNF in a model of OA exacerbated established pain responses, and local sequestration of BDNF in the knee joint significantly reversed established OA pain behaviour. It should be noted that these findings were demonstrated in male rats, and therefore may not be generalizable to females.

We have demonstrated the presence of BDNF and TrkB mRNA and protein in human OA synovium. Future comparative studies might compare expression levels between OA and healthy synovium to determine whether BDNF/Trk pathways may be active from the onset of disease, or are upregulated and therefore play a greater role with disease progression. Our data support earlier reports of BDNF in OA synovial fluid[49] and ATP-induced release of BDNF from fibroblasts taken from people with OA.[29] Despite differences in the experimental design of these studies, collectively, they support a role of BDNF in the knee joint of people with OA. To further probe links between BDNF/TrkB and OA pain, potential associations between this signalling pathway and pain behaviour were studied in the MIA model. Although there was

only a trend towards increased expression of TrkB in synovia from MIA rats, compared with saline controls, generation of a multilevel model identified a significant association between the MIA model and synovial expression of TrkB. The cellular location of BDNF/TrkB in the synovium remains to be determined, however.

In our study, we provide evidence for the presence of CX3CL1 (fractalkine) mRNA and protein in the synovium of OA patients, and that the protein levels of CX3CL1 are significantly higher in samples with higher inflammatory cellular infiltration. Protein expression of soluble CX3CL1 has been reported in the synovial fluid of OA patients[43] and the cognate receptor (CX3CR1) is expressed in synovial membrane from OA patients.[36] CX3CL1/CX3CR1 is a proinflammatory signalling pathway that has been shown to promote MMP-3 production by human OA synovial fibroblasts and induce cellular migration of these cells[25,30]; nevertheless, whether CX3CL1 directly contributes to OA-induced synovitis requires further study. It is of note that CX3CR1 is expressed by macrophages, dendritic cells, as well as fibroblasts in inflammatory rheumatoid arthritis.[8,36] In this study, levels of TrkB mRNA were significantly correlated with mRNA expression of CX3CL1, and expression of truncated TrkB protein was correlated with CX3CL1 protein. Previous work suggests that the BDNF-truncated TrkB complex is subject to endocytosis by the cell to form an intracellular reservoir of BDNF for later release, which could aid in maintaining extracellular levels of BDNF.[3] The correlation between the truncated TrkB and CX3CL1 may reflect a mechanism to potentiate BDNF signalling under conditions of high inflammation.

The functional consequences of elevated knee joint BDNF levels were confirmed by demonstrating that sequestration of BDNF acutely reversed OA pain behaviour, and that intra-articular injection of BDNF further exacerbated pain responses in a rat model of OA pain. To the best of our knowledge, this is the first report of effects of peripheral administration of TrkB-Fc on established pain. The inhibitory effects of TrkB-Fc were more robust on weight-bearing asymmetry compared to lowered ipsilateral paw withdrawal thresholds, which are at least partly mediated by changes in spinal processing of sensory inputs. We confirmed that the site of action of TrkB-Fc was predominantly localised to the knee joint because contralateral injection of TrkB-Fc did not significantly alter pain behaviour in the model of OA pain. Our results suggest that peripheral BDNF contributes to established knee joint–driven nociceptive output to the spinal cord in the model of OA pain, which at least partly underpins lowered paw withdrawal thresholds. Although roles of spinal BDNF were not a focus of our study, it is important to note that intrathecal injection of TrkB-Fc reversed pain behaviour in the spared nerve injury models of neuropathic pain in rats and mice,[51] and pretreatment with TrkB-Fc also attenuated the development of neuropathic pain.[34,42,59]

Intra-articular injection of BDNF in naive rats did not alter weight-bearing asymmetry or hind paw withdrawal thresholds, suggesting little role of knee joint TrkB receptor under non-pathological conditions. Thus, there seem to be differing roles for BDNF in the periphery vs spinal cord, where intrathecal administration of BDNF leads to acute increases in pain behaviour in naive mice.[21] Injection of BDNF into the knee joint in the established model of OA pain augmented existing pain responses after injection for up to 14 days after administration. However, this was the end point of the study and this augmented pain response may have lasted past this time point. It seems likely that this novel effect of knee joint BDNF in OA pain models is due to either

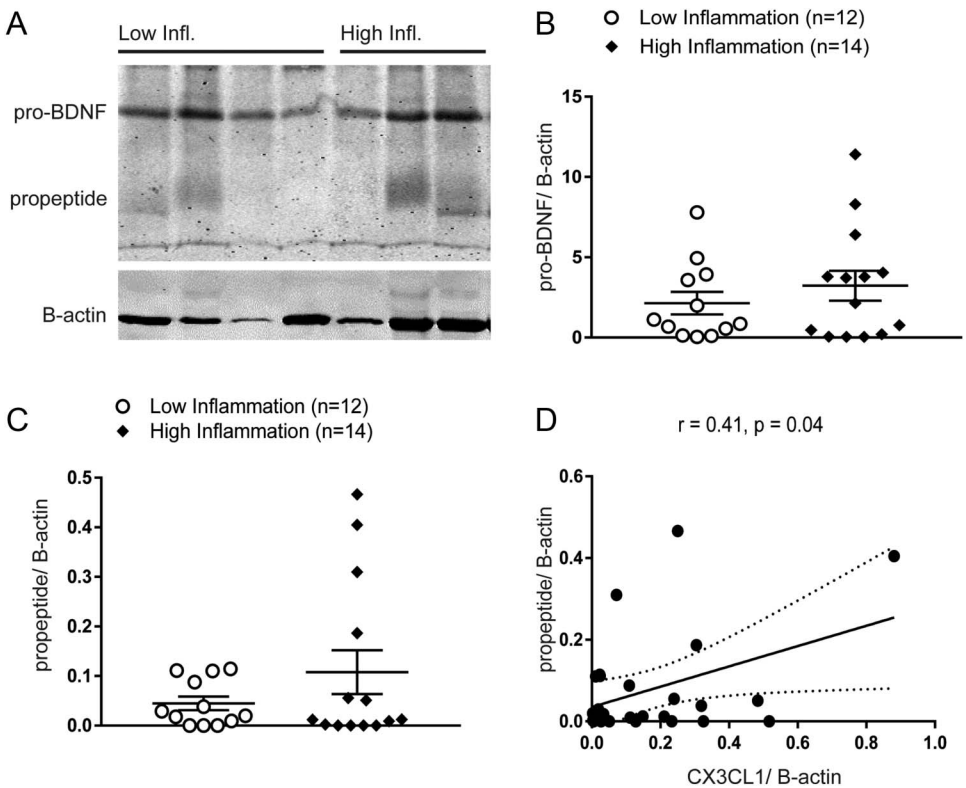

Figure 5. Representative image of Western blot of pro-BDNF and propeptide protein in human OA synovia after arthroplasty (A). Quantification of pro-BDNF (B) and propeptide (C) protein expression in OA synovia with low (n = 12) vs high (n = 14) inflammation. Correlation between the expression of the propeptide and CX3CL1 (D) in human synovia (Spearman's rho analysis). BDNF, brain-derived neurotrophic factor; OA, osteoarthritis.

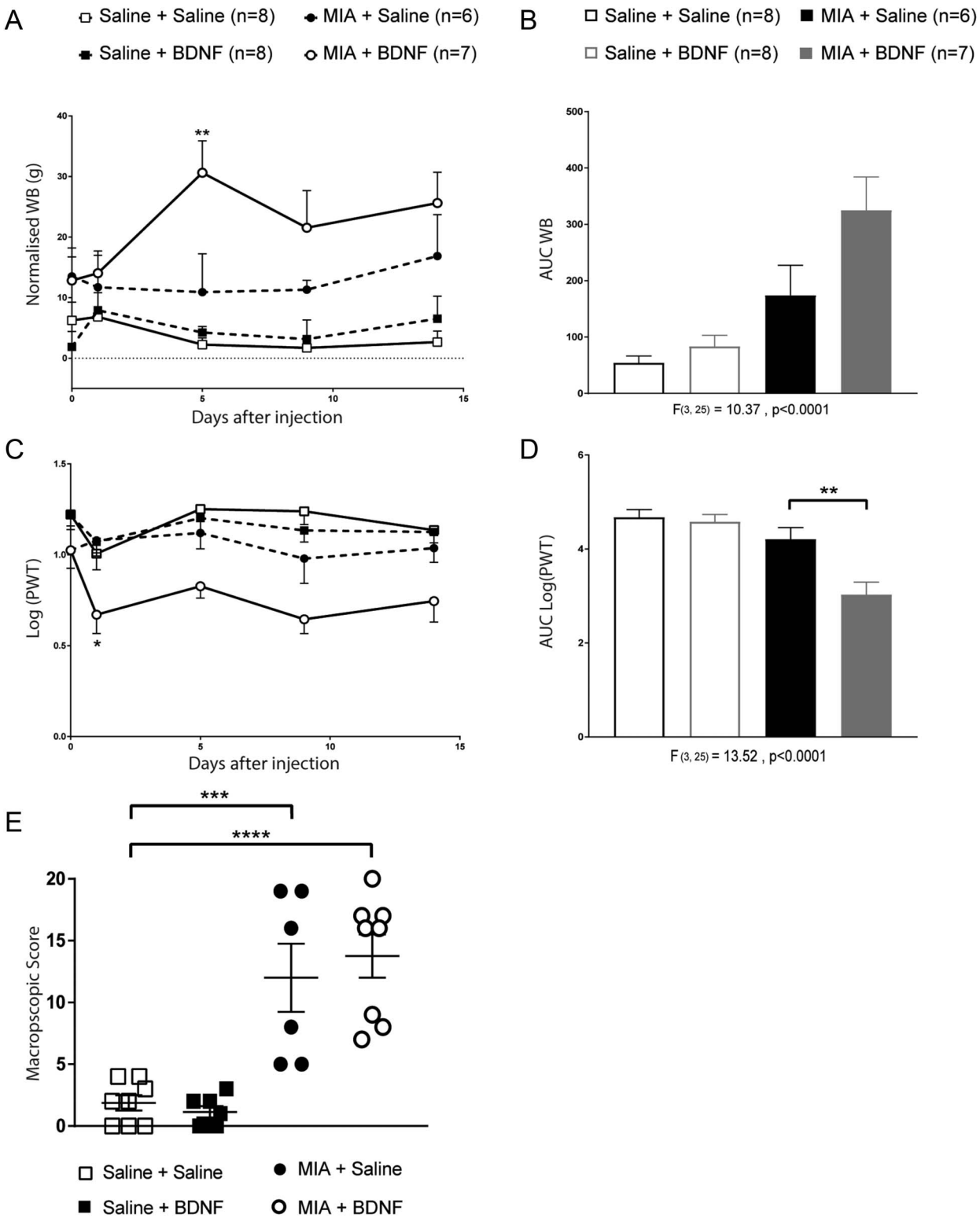

Figure 6. Effects of intra-articular injection of BDNF (1ug) on MIA-induced weight-bearing (WB) asymmetry (A). Data were analysed using 2-way ANOVA with Bonferroni-corrected multiple comparisons: $^{*}P < 0.05$ MIA + saline vs MIA + BDNF. The difference between the area under the curve for post-BDNF or vehicle injection weight-bearing asymmetry (B) were analysed using one-way ANOVA with Bonferroni-corrected multiple corrections: $^{*}P < 0.05$ MIA + Saline vs MIA + BDNF. Effects of intra-articular injection of BDNF (1ug) on MIA-induced ipsilateral hind paw withdrawal threshold (C). Data were analysed using 2-way ANOVA with Bonferroni-corrected multiple comparisons: $^{*}P < 0.05$ MIA + Saline vs MIA + BDNF. The difference between the area under the curve for post-BDNF or vehicle injection ipsilateral hind paw withdrawal thresholds (D) were analysed using one-way ANOVA with Bonferroni-corrected multiple corrections: $^{*}P < 0.05$ MIA + Saline vs MIA + BDNF. The effects of intra articular injection of BDNF on macroscopic cartilage damage (E). Data were analysed using one-way ANOVA with Bonferroni-corrected multiple corrections: $^{***}P < 0.001$ when compared with Saline + Vehicle, $^{****}P < 0.0001$ when compared with Saline + Vehicle. ANOVA, analysis of variance; BDNF, brain-derived neurotrophic factor; MIA, monosodium iodoacetate.

a change in the local cellular environment, or changes in the expression of TrkB by the sensory nerves innervating the OA joint. During OA, there is a substantial infiltration of $CD68^+$ macrophages into synovial tissue,[6] and monocyte-derived macrophages are known to express BDNF and TrkB.[45] In vitro, BDNF significantly increases IL-1β secretion from macrophages, and both BDNF and TrkB are required for macrophage phagocytic activity.[4] There is a significant increase in the numbers of activated macrophages in the knee joint of rats from 2 weeks after MIA injection,[40] suggesting that changes in the immune cell populations in the OA knee may be a regulatory factor in knee joint levels of BDNF. Recent studies of the neurotrophin NGF revealed a neuroimmune interaction in the knee joint whereby NGF-induced release of PGD_2 from mast cells resulted in mechanical hypersensitivity.[52] Osteoarthritis knees have greater sensitivity to NGF when compared to healthy knees,[5] in line with the increased sensitivity after injection of intra-articular BDNF reported here. Collectively, these data support a role of BDNF in regulating neuroimmune interactions within the OA knee joint.

Effects on other cell types may also contribute to proalgesic actions of BDNF in knee OA. The role of the BDNF/TrkB axis in regulating osteoclastogenesis in multiple myeloma[1] is of particular relevance to OA because subchondral bone remodelling and the activation of osteoclasts and formation of osteophytes are associated with chronic joint pain.[53] Antisense inhibition of BDNF reduced osteoclastogenesis in multiple myeloma in vivo through downregulation of RANKL.[2] In this study, we did not observe any acute effects of BDNF, nor TrkB-Fc, on OA structural damage. Future studies could explore effects of more sustained blockade of BDNF on osteoclastogenesis in models of OA.

Brain-derived neurotrophic factor also has a well-characterised role in sensitizing NMDA receptors in the dorsal horn of the spinal cord.[12,28] Human articular chondrocytes express functional NMDA receptors (NMDA-Rs),[41] and blockade of knee joint NMDA-R reduced collagenase induced joint pain and pathology progression.[32] Although BDNF may modulate chondrocyte NMDA-R function, this is unlikely to account for the rapid effects of BDNF reported herein.

Based on the expression patterns of TrkB by cutaneous sensory afferents, BDNF is likely to directly activate sensory afferents. TrkB is primarily expressed in low-threshold cutaneous mechanoreceptors.[54] Optogenetic activation of TrKB-expressing sensory afferent fibres evoked pain behaviour in a model of neuropathic pain, and inducible ablation of TrKB-expressing sensory afferent fibres prevented neuropathic mechanical hypersensitivity, but not inflammatory pain responses.[14] Although the expression of TrkB by sensory afferents innervating the knee joint is unknown, approximately 50% of retrograde labelled knee joint afferents express a marker for neuronal myelination (NF200), indicating the presence of low-threshold mechanoreceptors innervating the joint.[18] Bearing in mind potential species differences, it is plausible that TrkB-positive mechanoreceptors

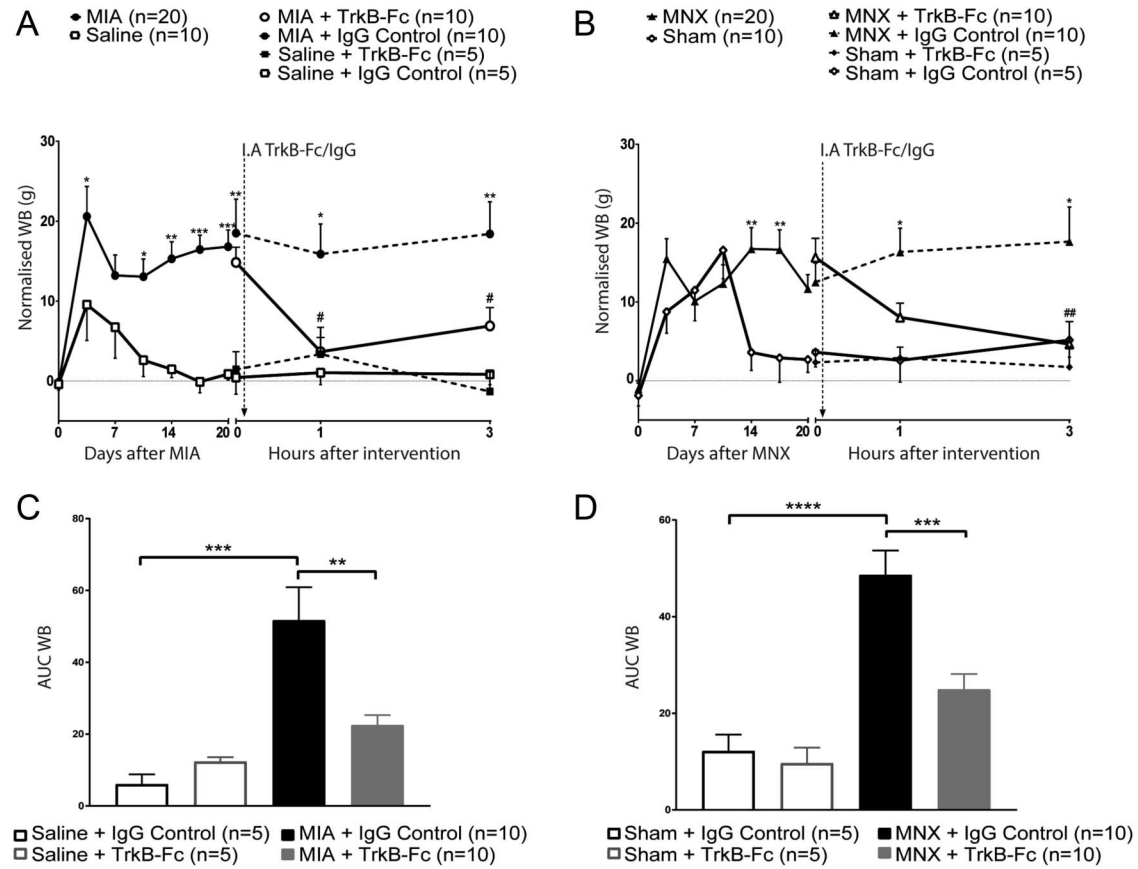

Figure 7. Effects of intra-articular injection of TrkB-Fc (100 ng) on MIA-induced weight-bearing (WB) asymmetry (A) and MNX-induced weight-bearing asymmetry (B). Data were analysed using 2-way ANOVA with Bonferroni-corrected multiple corrections. $^*P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$, $^{****}P < 0.0001$ MIA/MNX vs Saline/Sham. $\#P < 0.05$, $\#\#P < 0.01$ MIA/MNX + Human IgG vs MIA/MNX + TrkB-Fc. Differences in the area under the curve for post TrkB-Fc injection weight-bearing asymmetry in MIA animals (C) and MNX animals (D) were analysed using one-way ANOVA with Bonferroni-corrected multiple corrections: $^{**}P < 0.01$ MIA + IgG vs MIA + TrkB-Fc, $^{***}P < 0.001$ MIA + IgG vs Saline + IgG, $^{****}P < 0.0001$ MNX + IgG vs Sham + IgG, $^{***}P < 0.001$ MNX + IgG vs MNX + TrkB-Fc. ANOVA, analysis of variance; MIA, monosodium iodoacetate.

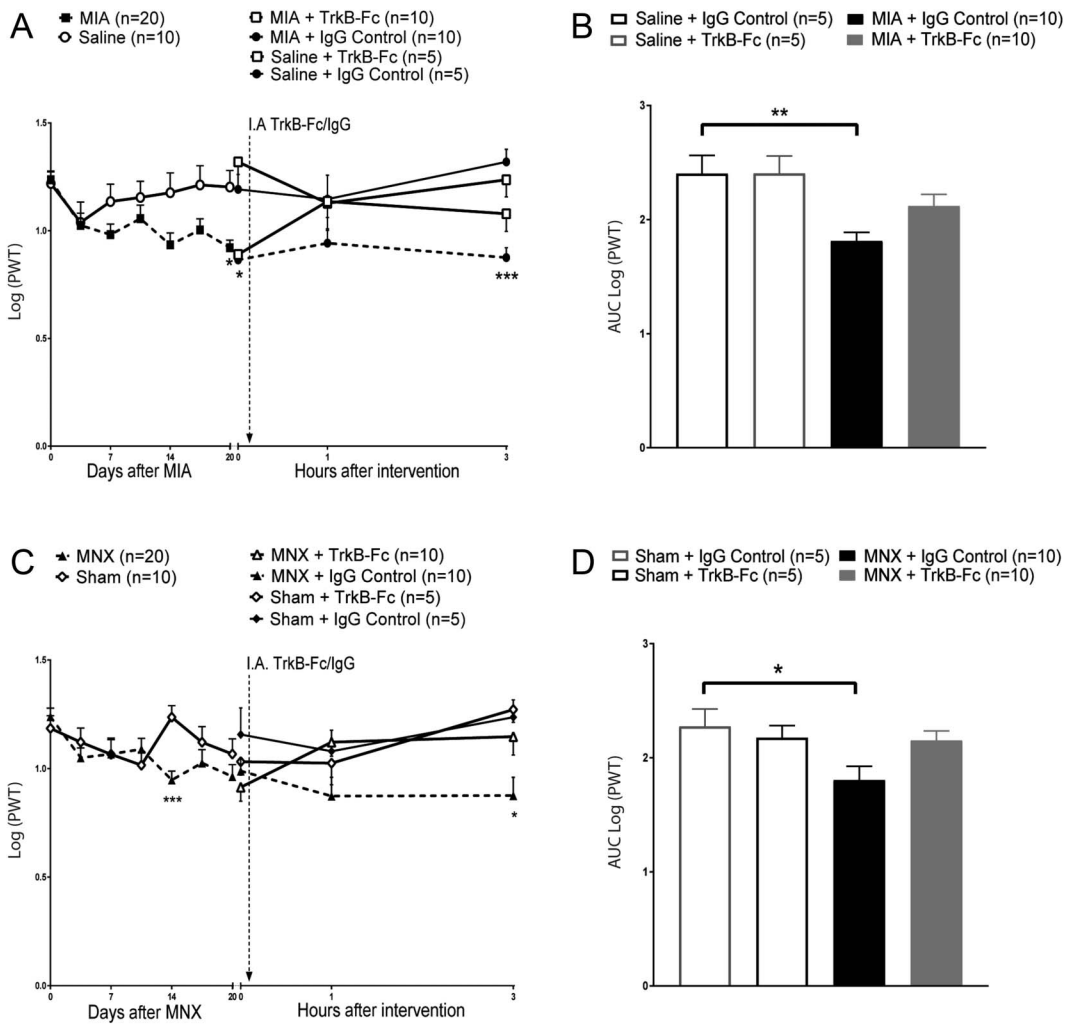

Figure 8. Effects of intra-articular injection of TrkB-Fc (100 ng) on MIA-induced ipsilateral hind paw withdrawal threshold (A) and MNX-induced ipsilateral hind paw withdrawal threshold (B). Data were analysed using 2-way ANOVA with Bonferroni-corrected multiple corrections. $^{*}P < 0.05$, $^{***}P < 0.001$MIA/MNX vs Saline/Sham. Differences in the area under the curve for post TrkB-Fc paw withdrawals threshold in MIA animals (C) and MNX animals (D) were analysed using one-way ANOVA with Bonferroni-corrected multiple corrections. $^{**}P < 0.001$, MIA + IgG vs Saline + IgG, MNX + IgG vs Sham +IgG, $^{*}P < 0.05$. ANOVA, analysis of variance; MIA, monosodium iodoacetate.

innervating the knee joint are a target for the elevated levels of BDNF in the models of OA and may directly contribute to the pain response.

In the search for novel therapeutics for chronic OA joint pain, monoclonal antibodies targeting NGF have been shown to be highly efficacious.[31] However, there were reports of some adverse effects in the joint, especially with concomitant dosing with nonsteroidal anti-inflammatory drugs.[24] Here, we show that another neurotrophin, BDNF, has an important role in the maintenance of OA pain at the level of the knee joint. Our data suggest that inhibition of peripheral BDNF could represent an exciting new therapeutic target for the treatment of OA pain.

Conflict of interest statement

The authors have no conflicts of interest to declare.

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Appendix A. Supplemental digital content

Supplemental digital content associated with this article can be found online at http://links.lww.com/PAIN/A874.

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