


SCIENTIFIC REPORTS



OPEN

Differential contributions of peripheral and central mechanisms to pain in a rodent model of osteoarthritis

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The mechanisms underlying the transition from acute nociceptive pain to centrally maintained chronic pain are not clear. We have studied the contributions of the peripheral and central nervous systems during the development of osteoarthritis (OA) pain. Male Sprague-Dawley rats received unilateral intra-articular injections of monosodium iodoacetate (MIA 1 mg) or saline, and weight-bearing (WB) asymmetry and distal allodynia measured. Subgroups of rats received intra-articular injections of, QX-314 (membrane impermeable local anaesthetic) + capsaicin, QX-314, capsaicin or vehicle on days 7, 14 or 28 post-MIA and WB and PWT remeasured. On days 7&14 post-MIA, but not day 28, QX-314 + capsaicin significantly attenuated changes in WB induced by MIA, illustrating a crucial role for TRPV1 expressing nociceptors in early OA pain. The role of top-down control of spinal excitability was investigated. The mu-opioid receptor agonist DAMGO was microinjected into the rostroventral medulla, to activate endogenous pain modulatory systems, in MIA and control rats and reflex excitability measured using electromyography. DAMGO (3 ng) had a significantly larger inhibitory effect in MIA treated rats than in controls. These data show distinct temporal contributions of TRPV1 expressing nociceptors and opioidergic pain control systems at later timepoints.

Osteoarthritis (OA) is a common form of degenerative joint disease and a major cause of joint pain worldwide¹. Structural changes within the joint are well characterised, however the mechanisms of OA pain are less clearly defined^{2,3}. Patients with OA pain have symptoms consistent with hyperalgesia including altered lower limb reflexes⁴, lowered mechanical pain thresholds at sites distal to the diseased joint and facilitated temporal summation⁵. Around 30% of patients exhibit features consistent with central sensitisation mechanisms⁶. Synovitis is significantly associated with OA pain^{7,8} and increased levels of pro-inflammatory mediators in synovial fluid sensitise nociceptors⁹. These nociceptors are primary afferent sensory neurons which innervate multiple components of the joint and detect changes in the local environment, as well as being potential sources of nociceptive factors themselves. Intra-articular injection of the local anaesthetic lidocaine significantly reduced pain scores in OA patients¹⁰, supporting the importance of a peripheral drive to OA pain, however whether all sensory fibres, or just subsets, are sensitised in OA is unknown.

As well as primary afferent sensory input to the spinal cord and higher brain centres, supraspinal networks powerfully control spinal excitability and hyperalgesia. Preclinical studies support the contribution of supra-spinal mechanisms to the maintenance of chronic pain¹¹ including in models of OA pain¹². Descending control mechanisms arise from multiple supra-spinal sites, changes in neuronal activity in the rostral ventromedial medulla (RVM)¹¹ has a fundamental role in the modulating spinal dorsal horn excitability¹³. Descending modulation is altered in OA patients^{14,15}, however identifying the underlying structures and mechanisms responsible and the timecourse over which central mechanisms become clinically significant necessitates the use of translationally relevant animal models.

Although a range of OA pain models have been developed, given the complex nature of OA in humans there is no single 'gold standard' model which can replicate all aspects of human OA¹⁶. Recent reviews confirm the value of both surgical and chemical models¹⁷. The mono-sodium iodoacetate (MIA) model of OA has been used

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extensively to study structural features of OA¹⁸ and pain behaviour^{19,20} in the rodent. Intra-articular injection of MIA results in extensive cartilage degradation and subchondral bone changes at later timepoints¹⁹, which is dose and time dependent. Studies using an intermediate dose report structural changes to the joint commensurate with that seen in human OA joints²¹. Concomitant MIA-induced pain behaviour (weight bearing asymmetry (WB) and lowered paw withdrawal thresholds (PWT)) models aspects of activity-related pain and altered distal pain pressure thresholds in OA patients^{19,22}. This model is also associated with hallmarks of spinal^{20,23} and supraspinal²⁴ sensitization.

We hypothesised that there are differential contributions of the peripheral and central nervous system to MIA induced OA pain over time, which are relevant to the development of novel treatment approaches.

Results

Joint pathology was assessed post-mortem. Macroscopic scoring in six compartments of the knee in MIA and saline injected rats showed significantly higher scores in the treated group than in the controls (Supplemental Fig. 1). Further macroscopic scoring increased significantly over time.

Targeted inactivation of joint nociceptors attenuates pain evoked by intra-articular injection of NGF.

The first study confirmed whether targeted inactivation of joint nociceptors reversed pain behaviour evoked by a well studied sensitising agent. NGF is a key mediator of OA pain and is able to sensitise nociceptors in the joint. The ability of the membrane impermeable local anaesthetic QX314 with capsaicin to block acute joint pain responses following intra-articular administration of NGF was determined. Intra-articular injection of NGF (10 ug) evoked significant WB asymmetry ($p < 0.0001$; Fig. 1A) and a reduction in PWTs ($p < 0.001$; Fig. 1B) 2 hours post injection of NGF. Concomitant intra-articular injection of QX-314 (1 mg/50 ul) with capsaicin (1 ug/ul) significantly attenuated NGF-induced WB asymmetry ($p = 0.0004$; Fig. 1A), compared to all other intra-articular interventions. Intra-articular injection of QX-314 (1 mg/50 ul) with capsaicin (1 ug per ul) did not however alter NGF-induced changes in PWT ($p > 0.05$; Fig. 1B). Intra-articular injection of QX-314 (1 mg/50 ul), lidocaine (1 mg/50 ul), capsaicin (1 ug/ul; Sigma) or vehicle (5% EtOH, 5% tween-20, 90% saline 0.9%) did not alter NGF-induced WB asymmetry or PWTs. These data show that in an established model of joint hyperalgesia, targeted inactivation of TRPV1 expressing C-fibres are able to reverse WB asymmetry, but not lowered PWTs.

MIA-induced weight bearing asymmetry is dependent upon TRPV1 positive joint nociceptor activation at early time points.

Next we studied the contributions of nociceptors innervating the joint versus central pain facilitating pathways to hyperalgesia in a model of joint damage over time. Intra-articular injection of MIA (1 mg/50 ul) resulted in significant WB asymmetry at day 7 ($p < 0.0001$; Fig. 2A), day 14 ($p < 0.0001$; Fig. 2C) and day 28 ($p < 0.0001$; Fig. 2E) compared to baseline values. In our NGF study lidocaine failed to reverse changes in PWT or WB, whereas targeted inactivation of TRPV1 expressing nociceptors did. We therefore decided to investigate the contribution of these TRPV1 positive sensory nerve fibres in MIA induced pain behaviours.

In rats at 7 days following intra-articular injection of MIA, intra-articular injection of QX-314 (1 mg/50 ul) with capsaicin (1 ug/ μ l) significantly reduced WB asymmetry (Fig. 2B) compared to baseline. Intra-articular capsaicin significantly potentiated WB asymmetry 30 and 60 mins post application, compared to baseline (Fig. 2B). None of the other intra-articular manipulations had significant effects on WB in MIA treated rats at this time point compared to baseline. Comparisons of the mean response to these intra-articular manipulations between groups over the entire 180 min observation period demonstrated significant differences between QX-314 + capsaicin treated rats with those treated with just capsaicin ($p < 0.001$), or vehicle ($p < 0.05$; Table 1).

The same intra-articular drug applications were made in a separate cohort of rats 14 days after MIA injection. At this timepoint only QX-314 (1 mg/50 ul) with capsaicin (1 ug/ μ l) significantly reduced WB asymmetry compared to baseline, none of the other treatments altered WB asymmetry (Fig. 2D). QX314 and capsaicin co-administration had a significantly different effect to all other drug treatment groups at day 14 post-MIA over the entire 180 min observation period (see Table 1).

Finally at Day 28 post-MIA the same pharmacological treatments were also applied intra-articularly in another cohort of rats. At this timepoint administration of QX-314 (1 mg/50 ul) with capsaicin (1 ug/ μ l) had no effect upon WB asymmetry compared to baseline values. Capsaicin however was able to increase WB asymmetry at this timepoint.

Intra-articular injection of MIA (1 mg/50 ul) resulted in significant PWT asymmetry at day 7 ($p < 0.0001$; Fig. 3A), day 14 ($p < 0.0001$; Fig. 3C) and day 28 ($p < 0.0001$; Fig. 3E). Unlike WB, intra-articular administration of QX-314 with capsaicin, QX314, capsaicin or vehicle at each of these timepoints had no effect upon PWT when compared to each other or their respective baseline (pre-local anaesthetic) values (Fig. 3B,D–F).

MIA-induced central sensitization: facilitation of EMG responses.

To advance understanding of the contribution of central mechanisms to MIA-induced pain over time, EMG responses were recorded at different timepoints and the effects of altering the output of the RVM on these responses were quantified in separate groups of rats. Our EMG approach permits discrete pharmacological manipulation of brainstem structures, whilst preserving spinal reflexes and has been used to investigate opioidergic signalling in descending pain control systems previously²⁵. It also overcomes animal welfare concerns associated with supraspinal microinjections in freely-behaving rats. EMG activity is directly correlated with spinal excitability. Changes in spinal excitability are associated with reductions in thermal and mechanical threshold and central sensitisation²⁶.

At 14 and 28 days following model induction there was a significant reduction in the threshold for mechanically-evoked EMG responses in MIA-treated rats, compared to saline controls ($p = 0.0002$ and $p = 0.0071$ respectively; Fig. 4A,B). At day 14 in MIA-treated there was no significant difference in the overall magnitude

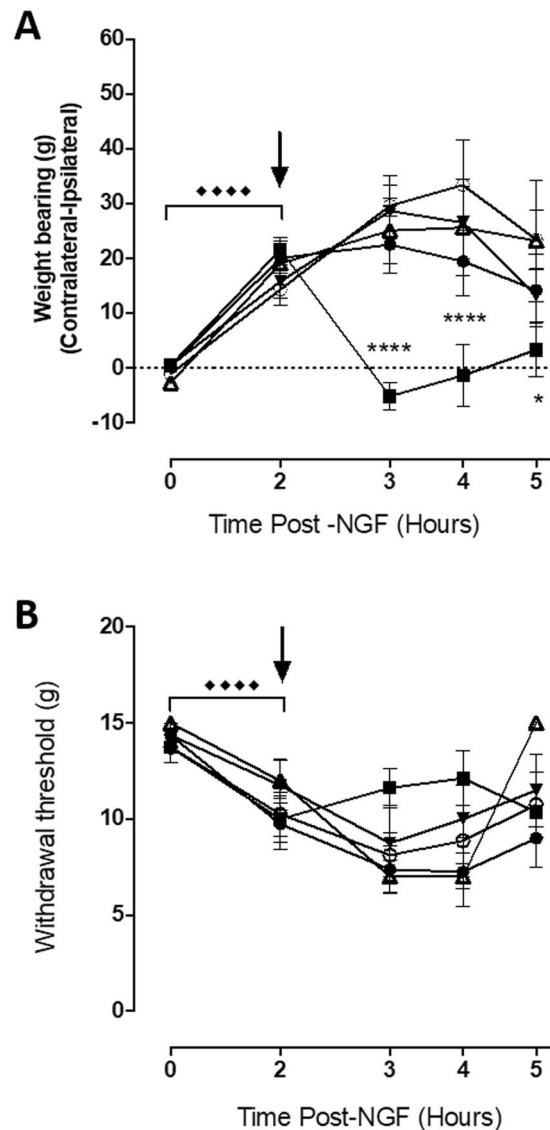


Figure 1. Intra-articular inactivation of TRPV1 expressing nociceptors reverses NGF induced pain. Intra-articular NGF (10 μ g/50 μ l) produced significant WB asymmetry (**A**) and reductions in PWTs (**B**) at 2 hours post-injection. Subsequent injection (indicated by the black arrow) of membrane impermeable local anaesthetic QX-314 alone (●) did not alter NGF-induced pain behaviour. Concomitant injection of QX-314 (1 mg/50 μ l) with capsaicin (1 μ g per μ l) (■) significantly reversed WB asymmetry up to 2 hours post-injection (**A**), but had no effect on PWTs (**B**). ● = QX-314, ■ = QX314 + capsaicin, △ = capsaicin, ▲ = lidocaine, ○ = vehicle. Changes in PWT or WB following NGF injection were performed using a 2-way ANOVA (treatment x time) with Dunnett's multiple comparison test comparing WB and PWT to the measurement. ♦♦♦♦ < 0.0001 (time 0 vs 2hrs post NGF). **p* < 0.05, ***p* < 0.01, ****p* < 0.001, *****p* < 0.0001 (Dunnett's; 2hrs postNGF vs pharmacological intervention). All data are presented as mean \pm SEM.

of EMG responses (EMG responses to all hairs) in MIA-treated rats, compared to saline rats (*p* = 0.9; Fig. 4C). However, by day 28 overall EMG responses in MIA rats were increased in magnitude (*p* = 0.025; Fig. 4D), compared to saline rats. Post-hoc analysis at Day 28 showed a significant difference in the mean EMG response evoked by the supra-threshold hair (*p* = 0.0025 Fig. 4D) in MIA rats, compared to saline rats. The duration of the EMG response was also quantified. No significant interaction was observed between groups at 14 days following intra-articular injection of MIA (*p* = 0.2484; Fig. 3E). At day 28 the EMG response duration was significantly longer in MIA-rats, compared to saline-treated rats (*p* = 0.0312; Fig. 4F). Post-hoc analysis revealed a significantly longer duration of supra-threshold evoked EMG responses in the MIA-rats, compared to saline-treated rats at this late timepoint (*p* = 0.003; Fig. 4F).

Effects of intra-RVM lidocaine and DAMGO on EMG responses in the MIA model. Intra-RVM microinjection sites were determined post-mortem and are shown in Supplemental Fig. 2. To understand the potential influence of descending control pathways in the MIA model of OA pain, we first investigated the

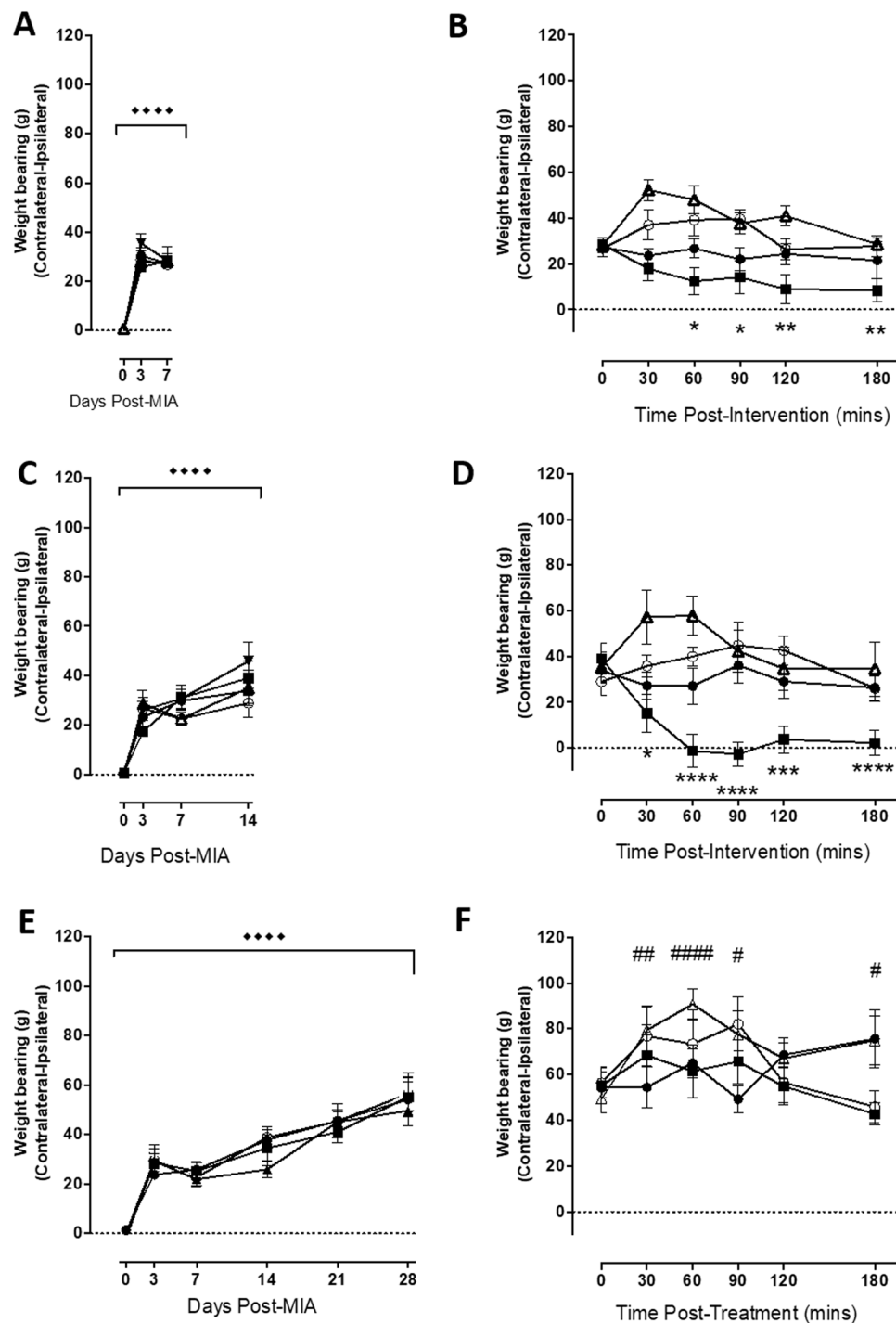


Figure 2. Inactivation of TRPV1 expressing nociceptors reverses MIA induced alterations in weight-bearing up to Day 14. Intra-articular injection of MIA significantly altered WB 7 (A), 14 (C) and 28 (E) days following administration (Dunnett's; **** $p < 0.0001$). At Days 7 (B) and 14 (C) co-administration of QX-314 and capsaicin reversed MIA induced alterations in WB asymmetry at Day 7 and 14 but not at Day 28 (F), compared pre-injection values. None of the other drugs significantly altered WB asymmetry at Day 7 or 14. However at Day 28, capsaicin significantly enhanced WB asymmetry compared to pre-drug, baseline values. ● = QX-314, ■ = QX-314 + capsaicin, △ + capsaicin, ○ = vehicle. Effects of interventions on WB asymmetry were performed using a two-way repeated measures ANOVA with Dunnett's post-hoc test. * $p < 0.05$, ** $p < 0.001$, *** $p < 0.0001$ (* QX-314 + capsaicin vs respective baseline value). # $p < 0.05$, ## $p < 0.01$, #### $p < 0.0001$ (# capsaicin alone vs respective baseline value). All data are presented as mean \pm SEM.

Days Post-MIA	QX314 vs QX314 + capsaicin	Capsaicin vs QX314 + capsaicin	Lidocaine vs QX314 + capsaicin	Vehicle vs QX314 + capsaicin
7	—	****	**	*
14	*	***	****	**
28	—	—	—	—

Table 1. The effect of intra-articular pharmacological treatments at days 7, 14 and 28 on WB asymmetry in MIA pre-treated rats. Comparisons between QX-314 + capsaicin treated rats have been made with QX-314, capsaicin, lidocaine and vehicle treated rats. Comparisons were made with a repeated measure 2-way ANVOA with Sidak's post-test. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$.

effect of an intra-RVM injection of lidocaine on EMG responses in MIA rats. The RVM contains projections of at least two populations of neurons that are able to inhibit or facilitate pain responses at the spinal cord level. Intra-RVM lidocaine treatment had no significant effect on any parameter of the EMG responses in MIA-treated rats compared to saline controls (see Supplemental Fig. 3). Given the generalised effects of lidocaine on this mixed population of neurones in the RVM²⁷, the effect of a more specific intervention using a pharmacological tool that activates endogenous pain control systems was studied. The effects of injection of the mu-opioid receptor (MOR) agonist DAMGO into the RVM of MIA- and saline-treated rats on EMG thresholds and the magnitude of responses were compared to the effects of intra-RVM injection of saline in separate groups of rats. At day 14 in MIA rats, intra-RVM DAMGO (3 ng) did not alter the threshold of mechanically-evoked EMG responses (Fig. 4A), but 30 ng DAMGO significantly increased thresholds compared to MIA rats which received intra-RVM saline as a control ($p < 0.0001$) and MIA rats that received the low dose of intra-RVM DAMGO (3 ng) ($p < 0.0001$; Fig. 4A). In the Day 28 MIA group of rats, both 3 ng and 30 mg intra-RVM DAMGO significantly increased EMG thresholds in MIA rats, compared to intra-RVM-saline in MIA rats ($p < 0.0001$ in both comparisons Fig. 4B). These data show altered sensitivity of the RVM to activation of opioidergic signalling at later time points in the MIA model. Intra-RVM injection sites were confirmed at the end of each recording session (Supplementary Figure 1).

To further analyse the change in opioid sensitivity we performed post-hoc analyses of responses to threshold (Fig. 4C) and suprathreshold (Fig. 4D) mechanical stimuli in MIA and saline rats at Day 14 and 28 in separate groups of rats.

Threshold Responses. At day 14 intra-RVM DAMGO (3 ng) had the same effect in both saline and MIA treated rats. However at Day 28 3 ng DAMGO caused significantly greater analgesia in MIA treated rats than in saline controls ($p < 0.05$). Administration of 30 ng intra-RVM DAMGO to MIA treated rats at both day 14 ($p < 0.0001$) and day 28 ($p < 0.01$) produced significantly more analgesia than when the same concentration was applied to saline controls. In MIA treated rats low dose intra-RVM DAMGO (3 ng) had a significantly greater analgesic effect at day 28 compared to day 14 ($p < 0.05$).

Suprathreshold Responses. Responses to these higher intensity stimuli were less complex than to threshold stimuli. Low dose DAMGO (3 ng) had the same effect in MIA and saline treated rats at Day 14 but had a significantly greater effect in MIA treated rats at Day 28. DAMGO (30 ng) had a greater effect at Day 14 than 3 ng DAMGO ($p < 0.01$). Low dose of DAMGO was more efficacious in producing analgesia at Day 28 than it was at Day 14 ($p < 0.05$).

These data illustrate that the sensitivity μ -opioid receptor activated endogenous pain control systems in the RVM are increased at later time points in the MIA model of OA pain, indicative of alterations in endogenous opioidergic signalling.

Discussion

Here we provide new evidence for a differing contribution of peripheral inputs to the early versus late stages of MIA-induced OA pain and for an alteration in opioid mediated top-down control of spinal excitability and central sensitization in the late stage of the model. We have demonstrated that behavioural responses following the induction of acute inflammatory joint pain induced by NGF or the earlier stages of longer lasting OA-like joint pain induced by MIA were significantly attenuated by transient inactivation of TRPV1 expressing nociceptors in the affected knee using QX-314 plus capsaicin. The ability of QX-314 plus capsaicin to attenuate pain behavioural responses at early but not late stages of the MIA model suggests either a significant phenotypic switch in the peripheral input driving pain (i.e. within nociceptors), or an increased central contribution to the maintenance of this pain. Appreciating the presence and timecourse of this switch is important in targeting the pharmacological management of OA pain to the appropriate target (i.e. peripheral inflammation versus altered CNS function).

Intra-articular injection of NGF resulted in an acute WB asymmetry and lowered PWTs consistent with NGF-induced sensitisation of TRPV1 channels via activation of TrkA receptors, and facilitated responses of these nociceptors to capsaicin²⁸. Acute intra-articular injection of QX-314 plus capsaicin had a selective inhibitory effect on WB asymmetry, but not lowered PWTs. In the absence of the TRPV1 ligand capsaicin, QX-314 did not alter WB asymmetry, consistent with other studies using this pharmacological intervention to target TRPV1 positive sensory nerves²⁹. NGF-induced lowering of hindpaw withdrawal thresholds (distal allodynia), thought to arise from alterations in the central processing of nociceptive inputs, was unaffected by sensory specific blockade of joint nociceptors by QX-314 plus capsaicin. NGF-induced distal allodynia persists up to 3 days post-injection³⁰

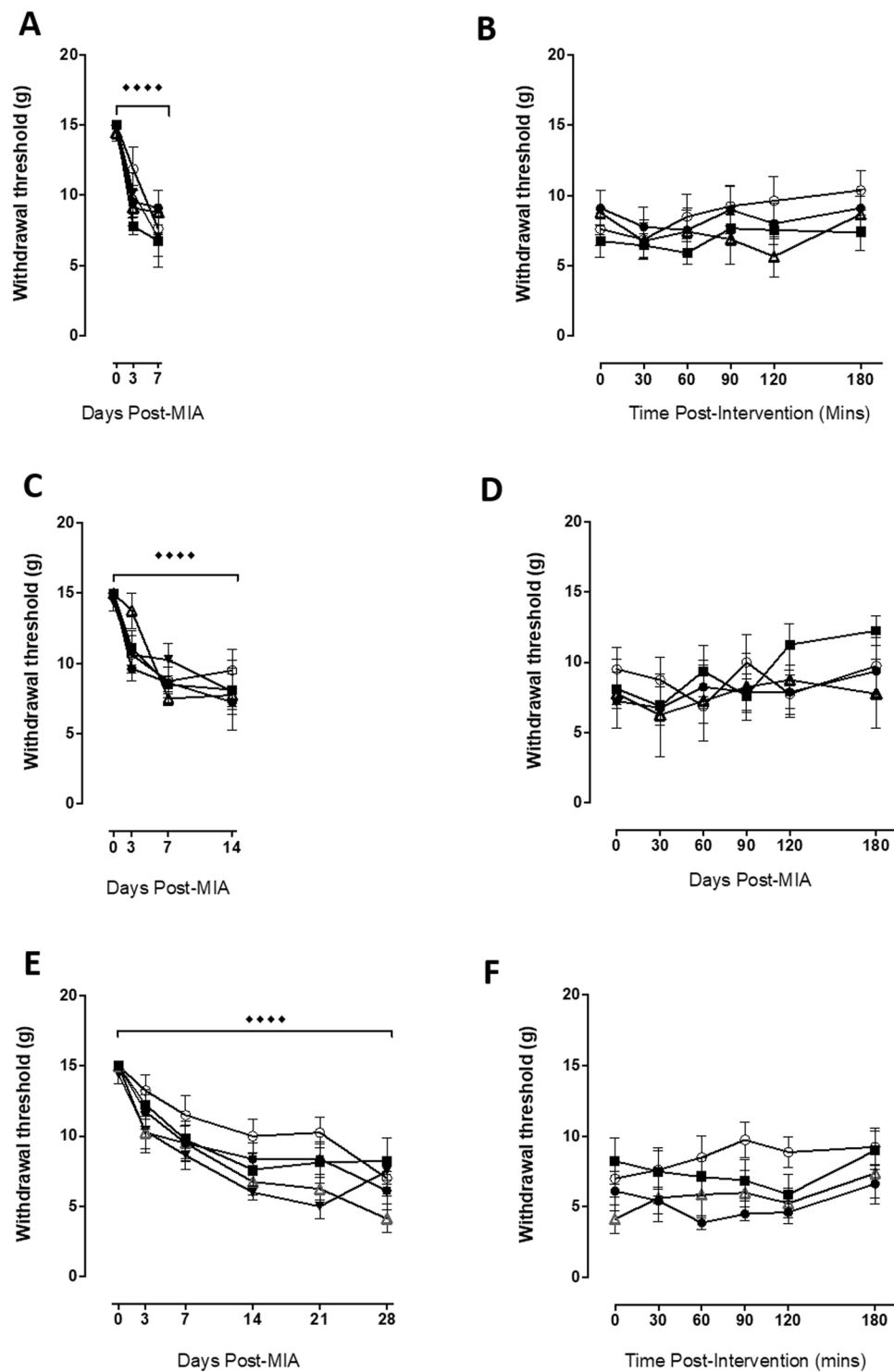


Figure 3. QX-314 + capsaicin is unable to effect MIA induced distal allodynia. Intra-articular injection of MIA significantly lowered PWTs 7 (A), 14 (C) and 28 (E) days following administration (Dunnett's; **** $p < 0.0001$). Subsequent pharmacological treatments (intra-articular injection) were without effect at any of the post-MIA timepoints. ● = QX-314, ■ = QX314, △ + capsaicin, ○ = vehicle. All data are presented as mean \pm SEM.

and the continued presence of distal allodynia following the blockade of TRPV1 expressing sensory input from the joint at 2 hours following NGF injection, suggests that relatively short term activation of sensory nerves by NGF in the joint is sufficient to induce long lasting central changes³¹ that are not reversed by the removal of peripheral input.

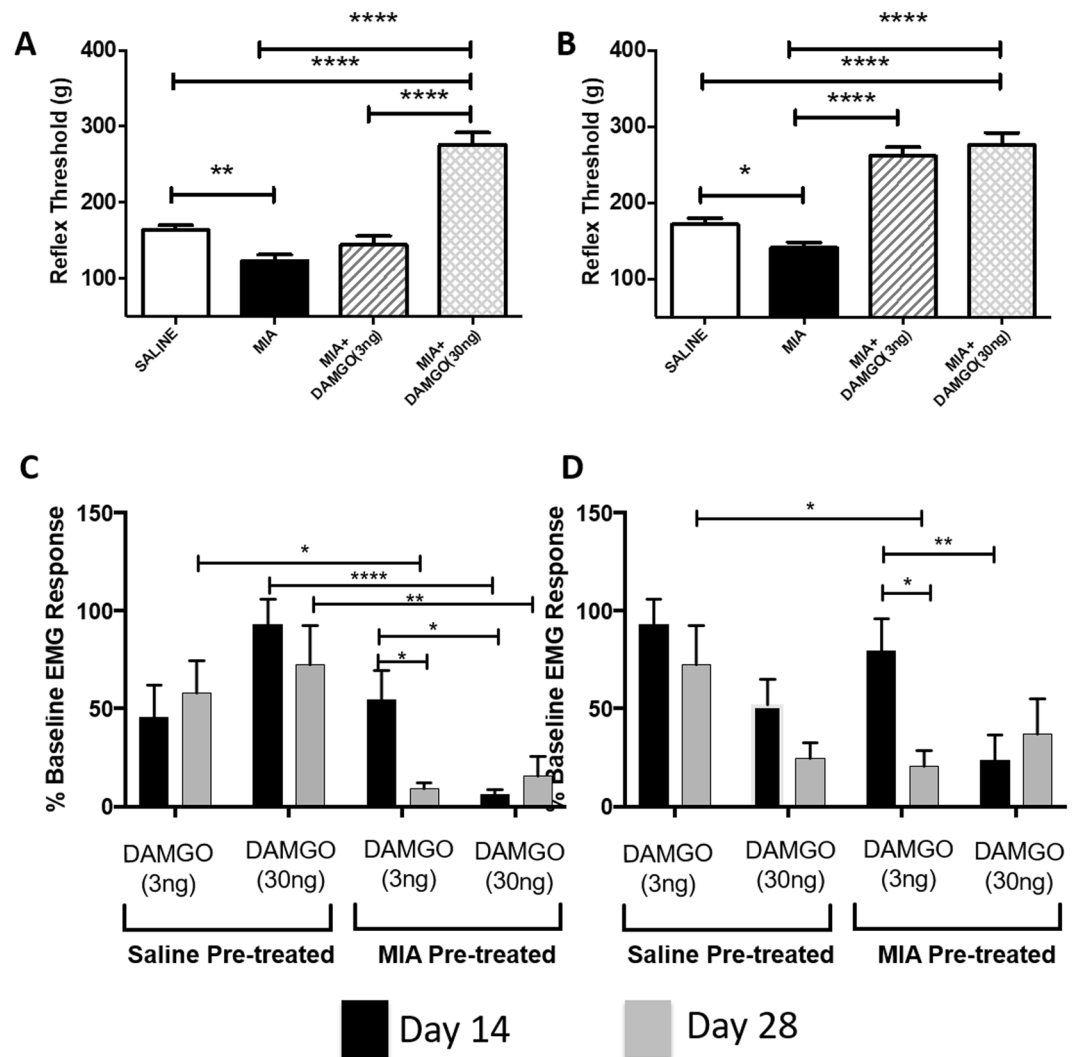


Figure 4. MIA leads to an increased sensitivity of endogenous opioid signaling within the RVM. EMG characterization of the nociceptive withdrawal reflexes in rats. Thresholds for mechanically evoked EMGs were significantly reduced in MIA-treated rats compared to saline rats at 14 (A) and 28 days (B) following intra-articular injection of MIA. Intra-RVM administration of 3 ng DAMGO did not alter MIA induced reductions in EMG threshold at Day 14 (A), but did reverse this reduction at Day 28 in MIA rats (B). Intra-RVM administration of DAMGO (3 ng) significantly inhibited EMG response magnitude to threshold vFh stimulation in saline (white bars) and MIA (black bars) treated rats at Day 14 compared to baseline (pre-drug) values. Low dose DAMGO (3 ng) was reduced EMG responses to threshold stimuli (C) in MIA treated rats at both Day 28 compared to saline treated controls but high dose DAMGO (30 ng) reduced responses at both Day 14 and 28. A similar but not identical pattern was seen following DAMGO to responses to suprathreshold stimuli (D). Threshold determinations were compared using a one-way ANOVA with Bonferroni post-test. EMG magnitude and duration were compared between groups using a two-way repeated measures ANOVA with Sidak post-hoc test $**p < 0.01$, $***p < 0.001$, $****p < 0.0001$. Day 14: MIA (n = 27 rats); Saline (n = 29 rats). Day 28: MIA (n = 50 rats); Saline (n = 38 rats). All data are presented as mean \pm SEM.

In the MIA model of joint pain, rats exhibited significant pain behaviour (WB asymmetry and lowered PWTs) at the earlier timepoints studied (7 and 14 days post-injection). At these two timepoints, intra-articular injection of QX-314 plus capsaicin significantly inhibited WB asymmetry, consistent with the known peripheral joint inflammation, increased spontaneous firing of nociceptive afferents at 14 days in the MIA model³² and that local blockade of TRPV1 in the joint reversed established MIA-induced pain behaviour³³. Consistent with our findings in the acute NGF model, QX314 plus capsaicin treatment had no effect on lowered PWTs in MIA rats at day 7 or day 14. Overall these data support a major contribution of TRPV1 positive sensory joint fibres to the WB asymmetry, but not lowered PWTs, at the early stages of the MIA model of OA pain. Recently it has been shown that chemogenetic inhibition of Nav 1.8 expressing neurons blocks hyperalgesia in early experimental knee osteoarthritis³⁴ which supports our data that TRPV1 expressing nociceptors (a subset of Nav 1.8 expressing C-fibres) underpin the development of joint pain.

The lack of efficacy of intra-articular injection of lidocaine in reversing either NGF or MIA mediated WB asymmetry is surprising. The concentration of lidocaine that was used in the studies is the same that has been used previously³⁵. We consider a period of at least 30 mins is necessary after the intra-articular injection of lidocaine (under general anaesthesia) is necessary before further behavioural assessments can be made to mitigate against anaesthetic impacting upon behaviour. Previous studies which have shown a positive effect of lidocaine upon pain behaviour in this context and the lack of efficacy in our studies of lidocaine may well reflect the fact that compared to these studies the volume used was smaller³⁵. Similarly intra-RVM lidocaine failed to alter descending control in our study. Although the concentration and volume of lidocaine microinjected in our study has been used successfully by others³⁶ it is at the lower end of range used in other studies³⁷ and this may explain the lack of efficacy in our hands.

The data demonstrate an effect of QX-314 + capsaicin upon WB but not on VF evoked withdrawal thresholds. This would indicate that the selective inactivation of TRPV1 + ve C-fibres only effects MIA induced primary hyperalgesia, and has no effect upon the distal allodynia observed by reduced mechanical withdrawal thresholds evoked by stimulating the ipsilateral plantar footpad. C-fibres therefore drive weight bearing asymmetry but play little to no role in the maintenance of central sensitisation that are thought to underpin allodynia.

In stark contrast to day 7 and 14, intra-articular injection of QX-314 plus capsaicin did not alter WB asymmetry at 28 days in the MIA model. These data suggest that at this timepoint factors other than TRPV1 expressing sensory nerves play a greater contribution to this pain phenotype. Day 28 represents a timepoint at which all behavioural changes associated with experimental OA have become fully developed³⁸. The lack of efficacy of QX-314 + capsaicin may represent the recruitment of additional/different sensory nerves, or a greater contribution of spinal/supra-spinal mechanisms to WB asymmetry. Previous work from our group has demonstrated markers of central sensitization indicative of the transition from acute to chronic pain mechanisms at this timepoint in the MIA model. Specifically, ipsilateral spinal expression of GFAP immunofluorescence is significantly elevated in this model at 28 days²⁰, which is linked to hyperalgesia in other models of chronic pain. Previously MIA has been shown to result in increased afferent drive that is refractory to the anti-inflammatory actions diclofenac or the TRPV1 antagonist HC030031³⁹. This work concluded that afferent drive is essential at 14 days post MIA for pain but that TRPV1 did not play a role. Our data add to this by illustrating that afferent drive from TRPV1 expressing neurons is essential in the early stages of MIA induced pain, including day 14, these are not essential in later stages and that whilst the TRPV1 receptor may not be essential in MIA induced WB and PWT the neurons they are expressed on are.

It is increasingly recognised that EMG responses are sensitive to disease related alterations in CNS processing^{23,40} and have great utility for the assay of central sensitisation associated with chronic pain in both animals and humans. At 14 and 28 days in the MIA model, rats exhibited reduced reflex thresholds, and at day 28 rats had significantly enhanced reflex magnitude. These findings are consistent with a previous animal study of OA²³ and clinical studies in OA patients⁴ reporting the presence of central sensitization using EMG as an assay of excitability. EMG reflex responses are sensitive to the inhibitory effects of analgesics which act either directly in the spinal cord, or within supraspinal centres that innervate the spinal cord^{41,42}. The RVM is the major source of both descending inhibitory and facilitatory influences over DH excitability⁴³. RVM neurophysiology is driven by three types of neurone: ON cells which promote chronic pain states and are the only RVM cell type to be directly inhibited by MOR agonists⁴⁴; OFF cells which decrease their basal activity just before a noxious stimulus; NEUTRAL cells show no consistent change in their firing patterns to noxious stimulation. Only ON cells express MOR and these receptors suppress the excitability and therefore produce a direct inhibition of descending facilitation [39]. However ON and OFF cells are reciprocally connected and inactivation of ON cells will also increase activity in OFF cells therefore amplifying DAMGO mediated descending inhibition of DH excitability. In the present study low dose DAMGO (3 ng) was able to inhibit spinal excitability in both MIA and saline treated rats in response to threshold, but not suprathreshold stimuli. This effect of DAMGO in saline treated rats is unsurprising as the drug will have inhibited low level basal descending facilitation and enhanced descending inhibition. Thus the low dose of DAMGO can provide enough descending inhibitory drive to overcome the increased excitatory input from the MIA effected knee combined with the larger vFh evoked noxious input emanating from the foot. Our major finding was that a lower dose of DAMGO had significant inhibitory effects on EMG thresholds and magnitude of evoked EMG responses at 28 days in the MIA rats, but not in MIA rats at the earlier timepoints studied. The fact that the higher dose of DAMGO (30 ng) significantly inhibited EMG responses in both groups at both times whereas the lower dose was most efficacious in MIA rats at Day 28 indicates increased RVM contributions to MIA induced pain at the later timepoint and/or increased opioid sensitivity in late stage OA pain. These data extend previous indirect evidence for a contribution of supraspinal sites to MIA-induced pain provided by studies of the effect of transection of the spinal cord at a thoracic level on pain behaviour²³ and that blockade of spinal cord 5-HT₃ receptors reversed MIA-induced hypersensitivity of spinal dorsal horn neurones²⁴. There may appear to be an incongruity between the DAMGO and QX-314 + capsaicin data in as much as vFh evoked activity was significantly effected by the former by unaffected by the latter. This difference is completely logical when it is considered that QX-314 + capsaicin only impacted upon sensory neurones that innervated the joint and a limited somatotopic region within the DH, whereas RVM administered DAMGO will have decreased DH facilitation and increased descending inhibition throughout the entire DH as the RVM has no somatotopic map and RVM neurons are known to respond to stimulation across the body.

Our demonstration of a significant change in the opioidergic system sensitivity in the RVM in this model of OA pain has considerable significance, both in terms of understanding the neurophysiological substrates driving OA pain and the importance of targeting central mechanisms of pain for treatment. OA patients exhibit alterations in supraspinal responses to pain as evidenced by increased activity within the cingulate cortex, thalamus and amygdala⁴⁵. In addition, atrophy of the thalamic⁴⁶ and cortical gray matter⁴⁷ that can be reversed by arthroplasty. Although, changes in these centres do not directly modulate spinal pain processing they will alter activity within

brain centres that do impact upon DH excitability⁴⁸. Altered diffuse noxious inhibitory control⁴⁹ (indicating alterations in descending inhibition) and increased activation of the periaqueductal gray⁵⁰ (a structure that mediates its effects on DH excitability indirectly via the RVM) have both been demonstrated in OA patients. The extent to which opioidergic systems in the brain are altered in OA patients has yet to be reported. The contribution of a supraspinal drive of central sensitization, mediated by the opioidergic system, in established OA pain may reflect a functional up-regulation of opioid receptors in the RVM and/or a result of a loss of endogenous mu opioid ligand, but this needs experimental confirmation.

In conclusion we have shown that there are specific periods during the development of pain in a model of OA in which peripheral input by TRPV1 positive nociceptors innervating the affected joint are a pre-requisite for pain on loading of the joint. At later timepoints TRPV1 positive nociceptors are involved to a much lesser extent, but neuromodulatory influences from the RVM appear to play a greater role in mediating the increased excitability of the DH associated with this model of OA pain. This time-dependent role of the peripheral and central nervous system in the development and maintenance respectively of chronic pain states is in agreement with that seen in other experimental pain states³⁷. Further investigation is required to elucidate how afferent sensory input leads to changes in brainstem pain modulation.

Materials and Methods

Animals. Male Sprague-Dawley rats (180–200 g; Charles River, UK) were used and kept on a 12 h dark/light cycle, group housed with free access to food and water. All procedures were performed in accordance with and specifically licenced under the Animals (Scientific Procedures) Act 1986 following ethical review by The University of Nottingham Animal Welfare Ethical Review Body and the UK Home Office. All studies took place in accordance with the ARRIVE guidelines. At the end of experiments, rats were killed by overdose of anaesthetic followed by cervical dislocation.

Model induction and behavioural testing. Rats were anaesthetised with isoflurane (3% in 99% O₂) and received a single injection through the infra-patellar ligament of the left knee, of one of the following: 1) recombinant rat beta-nerve growth factor [NGF; 10 ug/50 ul; R&D Systems, dose based on³⁰] to model acute joint pain, or 2) mono-sodium iodoacetate (MIA; 1 mg/50 ul; Sigma U.K.)³³ to model chronic joint pain. Pain behaviour was assessed as changes in hindlimb weight-bearing (WB) and changes in mechanical hindpaw withdrawal thresholds (PWT) as previously described²¹ by an experimenter blinded to all treatments. Baseline behavioural measurements were taken prior to intra-articular injections. In the NGF model, behaviour was tested pre and 2 hrs post injection. Following intra-articular injection of MIA, pain behaviour was assessed at day 7, 14, 21 and 28.

Intra-articular drug treatments. Following single injection of NGF or MIA, rats received a second pharmacological intervention at the timepoints as follows: NGF-treated rats at 2 hours post model induction; MIA-treated rats at 7, 14 or 28 days post model induction. The treatment was one of the following as an intra-articular injection in the ipsilateral knee under isoflurane anaesthesia: lidocaine (1 mg/50 ul; Sigma U.K.); QX-314 (N-Ethylidocaine bromide, 1 mg/50 ul; AbCam); capsaicin (1 ug per ul; Sigma U.K.); QX-314 (1 mg/50 ul) with capsaicin (1 ug per ul); or vehicle (5% EtOH, 5% tween-20, 90% saline 0.9%) (see Fig. 1). Effects of treatments on WB asymmetry and PWTs were assessed at 30, 60, 90, 120, and 180 mins post treatment. The local anaesthetic QX314 is membrane impermeable, however, co-administration with a low dose of the TRPV1 ligand capsaicin selectively inactivates the TRPV1 positive subset of sensory neurones²⁹.

EMG recordings and RVM Microinjections. EMG responses and the influence of modulating the output from the RVM on these responses was characterised at different stages of the model of OA pain. These studies were performed in anaesthetised rats as this permits responses to a range of sub- and suprathreshold stimuli to be assessed. Additionally this permits more accurate microinjection of pharmacological tools and has a significantly reduced burden of suffering on the animals compared to freely-behaving studies where animals have to be fitted with guide cannulae for a number of days prior to testing. EMG recordings of this type have been used to assess central changes in pain processing previously^{25,41}. Rats were anaesthetised with isoflurane (3% in 99% O₂) and a tracheotomy performed. Rats were artificially ventilated (Harvard Apparatus, UK) via an endotracheal cannula. Body temperature was maintained at 37 °C using a homeothermic heated blanket (Harvard Apparatus). Rats were mounted on a stereotaxic frame and craniotomies performed over the RVM (anterior-posterior [A-P] –11.0 mm, medial-lateral [M-L] 0.0 mm, dorsal-ventral [D-V] –10.5 mm). A custom-built bipolar concentric EMG electrode was inserted into the belly of the biceps femoris muscle ipsilateral to the MIA/saline injection. The electrode was connected to a NeuroLog head-stage (module NL100AK; Digitimer, Welwyn Garden City, UK), signals amplified x2000 (module NL104A), band-pass filtered between 10–1000 Hz (module NL125) before being sampled at 2 kHz using Spike2 V.7 software via a micro1401 data acquisition unit (CED, Cambridge, UK) as previously described⁴¹. Isoflurane concentration was reduced stepwise to a final concentration of between 1.0–1.3%, which was determined as being the highest concentration that permitted reproducible pedal reflex responses. Spinally mediated nociceptive paw withdrawal reflexes were evoked by the application of von Frey hairs to the plantar surface of the ipsilateral hindpaw. Mechanical threshold was defined as the minimum mechanical force in grams that evoked an increased EMG response at least 10% above basal activity. Once the threshold mechanical force was determined, von Frey hairs were presented in ascending order of bending force, with two below threshold, one at threshold and one suprathreshold. The magnitude of EMG responses to subthreshold, threshold and supra-threshold stimuli was determined. Each hair was presented 3 times, and 3 evoked baseline responses were measured at 10 minute intervals. EMG recording and pharmacological manipulation of the RVM was performed as previously described⁵¹. RVM microinjections consisted of administering 200 nl of [D-Ala², N-MePhe⁴, Gly-ol]-enkephalin (DAMGO (Tocris; UK): 3 ng or 30 ng per microinjection) or lidocaine (10 ng, to inactivate

neuronal excitability in all cells in the entire NRM) slowly over 5 mins into the RVM, specifically the nucleus raphe magnus. more information see Supplementary Information.

Following both behavioural and EMG studies animals were humanely killed and knees were harvested for histological examination. Animals in the MIA group that did not have verified pathological changes in the injected knee indicative of OA-like processes were removed from all data analyses.

Macroscopic confirmation of osteoarthritis. Rats were overdosed with sodium pentobarbital (1 ml, intra-peritoneal) and transcardially perfused with saline (0.9%) until exsanguinate ran clear. Infusate Perfusate was then switched to 4% paraformaldehyde (PFA; Sigma, U.K) made up in phosphate buffered saline (PBS). Whole knees (both ipsilateral and contralateral) were dissected and disarticulated and processed in accordance with procedures published previously¹⁸. Macroscopic scoring of the chondral surfaces was used to confirm the presence of MIA-induced lesions to corroborate behavioural and electrophysiological findings. Macroscopic lesions were graded as follows: 0 = normal appearance; 1 = slight yellowish discoloration of the chondral surface; 2 = little cartilage erosions in load-bearing areas; 3 = large erosions extending down to the subchondral bone; and 4 = large erosions with large areas of subchondral bone exposure [scoring system as used in²². Each of the chondral surfaces, medial and lateral femoral condyles, medial and lateral tibial plateaus and the femoral groove, was graded separately by 2 observers. Scores for each of the 5 regions were combined (maximum possible score of 20) and the mean determined for each group.

Group Sizes and Statistical Analysis. Allocation and blinding: prior to any intervention baseline behavioural measures, WB and PWT, were taken and animals assigned to either MIA/NGF or saline groups so that each group had equivalent means. Within these two groups, rats were assigned into groups for further pharmacological interventions on the basis of their WB and PWT values post the initial intervention so that mean values of these parameters within each group (MIA/NGF or saline) were comparable. Drugs used in this study were prepared by the authors and then anonymised and encoded. The instructions for which coded drug solution was given to each coded animal was then provided to the experimenter. Allocation and blinding was performed by a third party researcher who had no part in the study and is not an author on this publication.

Group sizes for studies were determined using Power Calculations using data obtained in our laboratories with the same model of OA (behavioural studies) or using the EMG recording to assess the impact of supraspinal pharmacological manipulations. The minimum group size required for studies with a systemic pharmacological intervention on pain behaviour (mean difference between two groups (drug versus vehicle) was 48.98 (SD 25.05)) was 4 rats per group to detect the difference between intervention and vehicle with 90% power and less than 5% type I error. In these studies experimentors were blind to both initial treatment (MIA or saline) as well as subsequent intra-articular pharmacological challenge. For EMG recordings the minimum group size for studies comparing reflex magnitude after a supraspinal pharmacological intervention in MIA and saline treated rats (mean difference between groups (baseline versus post-drug) was 49 (SD 36)) with 90% power and less than 5% type I error was 6.

Changes in PWT or WB following MIA or NGF injection were performed using a one-way ANOVA with Dunnett's multiple comparison test comparing WB and PWT to the baseline (0) measurement. For comparisons between treatment groups after the second intra-articular drug administration (in either the NGF or MIA studies) a 2-way ANOVA (treatment x time) was performed with mean responses of each treatment over the entire 180 post-drug period compared between groups with a Dunnett's multiple comparisons test. Analyses were carried out using Prism 6.0 software (Graphpad, La Jolla, USA). Raw EMG data was extracted using custom-written scripts in MATLAB (R2012a; MathWorks, Natick, MA, USA). Extracted data were imported into Prism and the magnitude and duration of baseline evoked EMG responses was compared between MIA and saline groups at different timepoints. EMG thresholds were compared using a one-way ANOVA with Tukey's multiple comparison post-test). The effect of each pharmacological intervention on the magnitude of the EMG response was expressed as a percentage of its pre-drug baseline response. Normalised post-drug responses and macroscopic knee scores were compared directly between treatments, interventions and timepoints using two-way ANOVA with Sidak post-tests.

Data Availability Statement. The datasets generated during and/or analysed during the current study are available from the corresponding author on reasonable request.

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Acknowledgements

This work was supported by Arthritis Research UK [grant number 19594].

Author Contributions

G.H., V.C. and A.H. designed studies, A.H. performed studies, A.H., G.H. and V.C. analysed data, G.H. and V.C. prepared the manuscript.

Additional Information

Supplementary information accompanies this paper at <https://doi.org/10.1038/s41598-018-25581-8>.

Competing Interests: The authors declare no competing interests.

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