Peripheral Calcitonin Gene-Related Peptide Receptor Activation and Mechanical Sensitization of the Joint in Rat Models of Osteoarthritis Pain

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Objective. To investigate the role of the sensory neuropeptide calcitonin gene-related peptide (CGRP) in peripheral sensitization in experimental models of osteoarthritis (OA) pain.

Methods. Experimental knee OA was induced in rats by intraarticular injection of monosodium iodoacetate (MIA) or by transection of the medial meniscus (MMT). Single-unit recordings of joint-innervating nociceptors were obtained in MIA- and saline-treated rats following administration of CGRP or the CGRP receptor antagonist CGRP 8–37. Effects of CGRP 8–37 were also examined in rats that underwent MMT and sham operations. Protein and messenger RNA (mRNA) levels of CGRP receptor components in the L3–L4 dorsal root ganglion (DRG) were investigated following MIA treatment.

Results. In both the MIA and MMT groups, the mechanical sensitivity of joint nociceptors was enhanced compared to that in the control groups. Exogenous CGRP increased mechanical sensitivity in a greater proportion of joint nociceptors in the MIA-treated rats than in the saline-treated rats. Local blockade of endogenous CGRP by CGRP 8–37 reversed both the MIA- and MMT-induced enhancement of joint nociceptor responses. Joint afferent cell bodies coexpressed the receptor for CGRP, called the calcitonin-like receptor (CLR), and the intracellular accessory CGRP receptor component protein. MIA treatment increased the levels of mRNA for CLR in the L3–L4 DRG and the levels of CLR protein in medium and large joint afferent neurons.

Conclusion. Our findings provide new and compelling evidence implicating a role of CGRP in peripheral sensitization in experimental OA. Our novel finding of CGRP-mediated control of joint nociceptor mechanosensitivity suggests that the CGRP receptor system may be an important target for the modulation of pain during OA. CGRP receptor antagonists recently developed for migraine pain should be investigated for their efficacy against pain in OA.

Despite pain being the dominant clinical symptom of osteoarthritis (OA), the mechanisms that drive this pain are poorly understood. The activity-related pain and distal allodynia associated with OA (1) suggest that there are alterations in the peripheral and/or central nociceptive pathways. Joint replacement (2) and intraarticular local anesthetic (3) are analgesic, highlighting the importance of peripheral nociceptive mechanisms. The synovium, ligaments, joint capsule, and subchondral bone of the knee joint are richly innervated by nociceptors and could be sources of nociceptive input during

OA (4). Experimental evidence in humans suggests a sensitization of joint-innervating nociceptors in patients with symptomatic OA and that this "peripheral sensitization" makes an important contribution to pain severity (5). A better understanding of the mechanisms driving this sensitization would aid the development of improved analgesics.

Accumulating evidence implicates a role of the sensory neuropeptide calcitonin gene-related peptide (CGRP) in the peripheral mechanisms of OA pain (6–11). Multiple knee joint structures are richly innervated by CGRP-expressing sensory neurons (8,12). Synovial CGRP-immunoreactive nerve fiber density is greater in OA patients with pain as compared to asymptomatic controls (6), and the pain localizes to the joint compartment, exhibiting increased CGRP-immunoreactive fiber density (7). These clinical findings demonstrate an association between altered local levels of CGRP and OA pain. Whether CGRP directly contributes to the augmented peripheral nociceptive inputs that drive OA pain remains an important unanswered question that has direct relevance to the treatment of pain.

Intraarticular injection of the chondrocyte metabolism inhibitor monosodium iodoacetate (MIA) and surgical transection of the medial meniscus (MMT) are 2 commonly used rodent models of OA. Although these models have different triggering stimuli, both exhibit joint pathology and pain responses that mirror key aspects of knee OA in humans (13), providing an opportunity for furthering our understanding of the mechanistic aspects of nociception during OA. Weight-bearing asymmetry in MIA-injected rats is coincident with sensitized responses of joint C fiber and Aδ fiber nociceptors (14) and is associated with increased CGRP expression in joint afferents (8,9). The release of CGRP from primary afferents increases following MIA (15,16), and destruction of knee joint CGRP-expressing sensory terminals prevents the development of MIA-induced pain (10). These preclinical data together with the available human data suggest an association between local CGRP levels, peripheral sensitization, and the resultant pain of OA, providing strong rationale for further examining whether a direct relationship exists.

Although CGRP has established roles in central nociceptive transmission and neurogenic inflammation in peripheral tissues (17,18), its role in modulating peripheral nociception is poorly understood. In vivo, CGRP sensitizes cutaneous nociceptors to noxious stimuli, causing pain (19–21), a finding consistent with reports of CGRP sensitizing sensory neurons in vitro (22,23). The CGRP receptor consists of the G protein–coupled receptor (GPCR) calcitonin-like receptor (CLR), which requires 2 accessory proteins to fully function, receptor activity–modifying protein 1 (RAMP-1), which confers pharmacologic specificity, and an intracellular CGRP receptor component protein (CRCP), which couples CLR to intracellular signaling pathways (24). All 3 components are expressed in dorsal root ganglion (DRG) neurons (25,26). Whether they are expressed in joint afferents and whether their activation sensitizes joint nociceptors is unknown.

The aim of this study was to examine whether CGRP makes an important contribution to the maintenance of peripheral sensitization during experimentally induced OA. Given the recent success of CGRP receptor antagonists in trials of patients with migraine pain (27), studies aimed at answering this question are extremely timely (28). The ability of CGRP to sensitize mechanoreceptive joint-innervating nociceptors and the effects of blocking the action of local endogenous CGRP on established joint nociceptor sensitization was investigated in the MIA model. To assess the physiologic relevance of our findings, we also determined the effects of CGRP receptor blockade in the MMT model. The impact of MIA-induced OA on CGRP receptor component at the protein and messenger RNA (mRNA) levels in the L3–L4 DRG was determined.

MATERIALS AND METHODS

Drugs. Sodium pentobarbital (Sigma, UK) was dissolved in 10% ethanol, 20% propylene glycol, and 70% saline. MIA (Sigma), rat α-CGRP (Sigma), rat CGRP 8–37 (Tocris Bioscience), and 2% Fluoro-Gold (Fluorochrome) were dissolved in sterile saline. Antibodies were prepared in antibody diluent (Dako).

Animals and induction of OA. Procedures were carried out in accordance with the UK Animals (Scientific Procedures) Act 1986 and the Animals in Research: Reporting In Vivo Experiments (ARRIVE) guidelines (29). A total of 109 male Sprague-Dawley rats (Charles River UK) ages 5–7 weeks and weighing 152–289 gm were used in these experiments. Rats were anesthetized with 3% isoflurane in O_2, and OA was induced in the left knee joint by a single intraarticular injection of MIA (1 mg in 50 μl of sterile saline; n = 48 rats) or by transection of the medial meniscus (n = 8 rats) as described previously (30). Control rats received an intraarticular injection of saline (50 μl; n = 47 rats) or underwent sham surgery (n = 6 rats).

Behavioral testing. Baseline pain behavior was assessed on day 0 (immediately prior to OA induction) and up to day 14 following MIA/saline injection or days 14–18 following MMT/sham surgery (immediately prior to electrophysiology or perfusion).

Effects on weight bearing in the ipsilateral and contralateral hind limbs were assessed using an incapacitance

tester (Linton Instrumentation) as described elsewhere (14). Data are expressed as ipsilateral weight bearing, which was calculated as a percentage of total hind limb weight bearing.

Surgical preparation for electrophysiology. The surgical preparation methods were similar to those described elsewhere (14). Fourteen days post-MIA/saline injection or 14–18 days post-MMT/sham surgery, anesthesia was induced with sodium pentobarbital (50–60 mg/kg intraperitoneally) or isoflurane (3% in O_2; 1.5 liters/minute) and maintained with sodium pentobarbital (20–35 mg/kg/hour intravenously). Core body temperature (36.5–37.5°C) was maintained. In some rats (CGRP experiments; n = 20), the left carotid artery was cannulated (SensoNor 840 cannula) for monitoring of the mean arterial blood pressure.

Extracellular recordings of knee joint nociceptor activity. Rats were prepared for joint nociceptor recordings as described previously (14). The skin and connective tissue overlying the knee joint were incised, and the medial aspect of the joint was exposed. Mechanosensitive knee joint nociceptors of the saphenous nerve were identified by probing the subcutaneous structures over and around the exposed knee joint, using a 15-gm von Frey monofilament. Nerve filaments were teased until 1–3 single units could be identified and sorted offline (CED Spike2 software); firing frequencies evoked by trains of von Frey monofilaments (0.16–15 gm for 5 seconds for each monofilament, with 5 minutes between trains) of the most reliable unit were quantified until stable; 1 fiber per rat was studied.

Drugs were then administered via a cannula into the contralateral femoral artery, with the tip placed at the bifurcation of the descending aorta for semilocal, close intraarterial injection into the ipsilateral hind limb. The effects of rat α-CGRP (0.5 or 1 μg/100 μl per unit; n = 20) or the CGRP receptor antagonist rat CGRP 8–37 (5 or 10 μg/100 μl per unit; n = 31) on mechanical evoked responses (0.16–15 gm for 5 seconds each at 5-minute intervals and at 60 minutes per dose) were recorded. In vehicle experiments (n = 12), two 100-μl injections of saline were administered; thus, the recording period was identical to that for the drug experiments.

Data were recorded using a Micro 1401 data acquisition unit (CED) and were captured by a computer running CED Spike2 software. Conduction velocities of recorded units were estimated from conduction latency values recorded between electrical monopolar stimulation (up to 30V for 0.5 msec at 0.3 Hz) of their receptive field and the onset of evoked action potentials, and conduction distances were estimated along the nerve between the stimulation and recording sites (31). C fibers and Aδ fibers had conduction velocities of <1.3 meters/second and 1.3–15 meters/second, respectively (31). KCl (100 μl of a 250 mM preparation) was administered intraarterially (contralateral femoral artery) to confirm that the drugs reached the peripheral afferent terminals; afferents not activated were excluded. The mechanical threshold was calculated as the minimum von Frey monofilament weight that evoked $\geq$2 action potentials.

TaqMan real-time quantitative polymerase chain reaction (qPCR) analysis. Total RNA was extracted from the L3–L4 ipsilateral DRG obtained on day 14 and 28 from MIA- or saline-treated rats, snap frozen, homogenized in 0.5 ml of ice-cold TRI Reagent (Sigma), and purified according to the manufacturer's instructions. For complementary DNA

(cDNA) synthesis, 100 ng of total RNA was reverse transcribed using an AffinityScript cDNA synthesis kit (Agilent) in a 20-μl total reaction volume. Reaction volumes were incubated for 10 minutes at 25°C followed by 1 hour at 50°C and terminated by incubation for 15 minutes at 70°C. Gene expression was quantified by the relative standard curve method using Taq-Man real-time qPCR, as described previously (32). Threshold cycles were normalized to those of the housekeeping gene β-actin to obtain relative expression levels. Primers and probes (FAM or TAMRA modified) were designed using Primer Express v3 software (Applied Biosystems) and synthesized by Eurofins MWG (Table 1).

Fluoro-Gold labeling of knee joint afferents. Seven days prior to MIA/saline injection, rats (n = 8 per group) were anesthetized with isoflurane (3% in O_2) and 5 μl of 2% Fluoro-Gold (Fluorochrome) was injected into the left knee joint. Fourteen days post-MIA/saline (21 days after Fluoro-Gold injection), rats were perfused transcardially with 4% paraformaldehyde (PFA) and L3 through L5 DRGs were collected, postfixed overnight in PFA, and cryoprotected in 30% sucrose overnight. DRGs were rapidly frozen over liquid nitrogen–cooled isopentane in OCT compound (VWR) and stored at −80°C. Ipsilateral L4 DRGs were cryosectioned (13 μm) onto Superfrost Plus slides (Fisher Scientific) and stored at −20°C. DRGs were serially sectioned over 8 slides; each slide contained 6–9 sections per DRG, each separated by 8 sections (104 μm), ensuring that each cell was counted once. One slide per DRG was used for immunohistochemical staining, and cell counts were performed on 5 sections per slide.

Immunohistochemistry. Ipsilateral L4 DRG sections from MIA- and saline-treated rats were blocked with 5% normal donkey serum then incubated with a mouse monoclonal (IgG1) anti-CLR antibody (1:250 dilution; Welcome Receptor Antibodies). Slides were incubated with a rabbit polyclonal anti–neuronal nuclei (anti-NeuN) antibody (1:300 dilution; Millipore) or an affinity-purified rabbit polyclonal anti-CRCP antibody NY1047 (1:500 dilution). Immunoreactivity of CLR, NeuN, and CRCP were determined by incubation with Alexa Fluor 488–labeled donkey anti-mouse IgG (1:1,000 dilution; Invitrogen) and Alexa Fluor 568–labeled donkey anti-rabbit IgG (1:1,000 dilution for NeuN and 1:600 dilution for CRCP; Invitrogen), respectively. Slides were mounted with Fluoromount (Sigma). Negative controls included omission of primary antibodies or incubation with a nonspecific mouse IgG1 for CLR (1:250 dilution; Dako).

Images were captured on a Leica DMIRB fluorescence microscope at 20× magnification and acquired using Openlab software (PerkinElmer) controlling a Hamamatsu Orca C4642-95 camera. Image analysis and cell counts were performed offline using SimplePCI (Hamamatsu). Somata measuring <30 μm, 30–40 μm, and >40 μm in diameter were considered small, medium, and large, respectively (9).

Statistical analysis. Data were analyzed using Graph-Pad Prism software version 5/6 and were tested for normality using the Kolmogorov-Smirnov test. Where appropriate, parametric statistics were used. Any data not normally distributed were analyzed using nonparametric tests. Two-way analysis of variance (ANOVA) with Bonferroni post hoc test was used to compare multiple groups and variables in the behavioral and electrophysiologic data. Mann-Whitney t-tests were used to compare mechanical thresholds, immunohistochemical data,

Table 1. Primer and probe sequences used for the TaqMan real-time quantitative polymerase chain reaction analyses

Gene, NCBI accession no.	Primer strand/probe sequence
CLR	
NM_012717	Forward: TGTACACTATGTTTTCTGTTTCTCTTGCT
	Reverse: TTTGGTTCGCGCCTTCTT
	Probe: CTTAATATGGCTCTCATCGCAGCAGAGTCG
RAMP1	
NM_031645	Forward: GCTGCTGGCTCATCATCTCTT
	Reverse: TGGTCTCCATGTCCTCTTTGAA
	Probe: TACTCTCATCCAGGAGCTGTGTCTCAGCC
RCP	
NM_053670	Forward: CCATCACCTACGAGACATTAAAATACA
	Reverse: TCTTCATTGCTGTGAGGAATTCTT
	Probe: AAACTCCATGTAAGAACCAGAGTCCGGCG
α-CGRP	
NM_001033955	Forward: TTGTCAGCATCTTGCTCCTGTAC
	Reverse: GCCTGGGCTGCTTTCCA
	Probe: TCCAGGCAGTTCCTTTGAGGTCAACC
β-actin	
NM_031144	Forward: GGCCATGTACGTAGCCATCCA
	Reverse: TCTCCGGAGTCCATCACAATG
	Probe: GTCCCTGTATGCCTCTGGTCGTACCAC

and mRNA expression data. Fisher's exact test was used to compare contingency tables (2 × 2). Data are expressed as described below. P values less than 0.05 were considered significant.

RESULTS

Pain behavior evoked by MIA injection and MMT surgery. Weight-bearing analysis confirmed OA pain development. As previously described (14,33,34), there was a significant reduction in weight bearing on the ipsilateral hind limb in MIA-injected rats compared to saline-injected rats on days 3, 7, and 14 ($P < 0.001$ by two-way ANOVA with Bonferroni post hoc test). Immediately prior to electrophysiology (day 14), ipsilateral weight bearing in MIA-treated rats was 39.29 ± 0.71% (mean ± SEM) compared to 49.09 ± 0.16% in saline-treated rats ($P < 0.001$ by two-way ANOVA with Bonferroni post hoc test).

Overall, MMT surgery resulted in a modest but significant reduction in ipsilateral hind limb weight bearing as compared to sham surgery ($P < 0.01$ by two-way ANOVA). On day 8 post-MMT, ipsilateral weight bearing was 43.68 ± 1.12%, as compared to 47.46 ± 0.65% in sham-operated rats ($P < 0.01$ by two-way ANOVA with Bonferroni post hoc test). Immediately prior to electrophysiology (days 14–18), there was no significant difference between the MMT and sham-operated groups (45.97 ± 0.42% versus 46.45 ± 0.90%); however, in both groups, weight bearing was

significantly reduced compared to day 0 (50.19 ± 0.22% [$P < 0.001$ by two-way ANOVA with Tukey's post hoc test] and 49.98 ± 0.25% [$P < 0.01$], respectively).

Mechanically sensitized joint nociceptors in OA models. Sixty-three joint nociceptors (n = 25 MIA-treated, n = 24 saline-treated, n = 8 MMT-operated, and n = 6 sham-operated rats) were studied. The range of conduction velocities was similar in MIA- and saline-treated rats (0.39–13.1 meters/second [median 1.4] and 0.3–13.6 meters/second [median 1.7]) as well as in MMT- and sham-operated rats (1.2–3.3 meters/second [median 1.93] and 0.99–7.67 meters/second [median 1.73]). All fibers were either C fibers (<1.3 meters/second) or Aδ fibers (1.3–15 meters/second). Conduction velocities were unidentified in 10 fibers.

Fourteen days post-MIA and 14–18 days post-MMT, mechanical evoked responses (0.16–15 gm mean firing rates) of joint nociceptors were significantly increased compared to control responses, with a mean ± SEM area under the curve (AUC) of 65.13 ± 5.94 in MIA-treated rats versus 41.36 ± 3.95 in saline-treated rats ($P < 0.01$ by unpaired t-test) and a mean AUC of 49.50 ± 4.62 in MMT rats versus 32.93 ± 2.37 in sham-operated rats ($P < 0.01$ by unpaired t-test). For example, firing rates evoked by 8 gm of mechanical stimulation in MIA-treated rats (mean ± SEM 11.96 ± 0.76 impulses/second) and by 10 gm of stimulation in MMT rats (8.53 ± 0.94 impulses/second) were significantly greater than those in control rats (9.89 ± 0.62
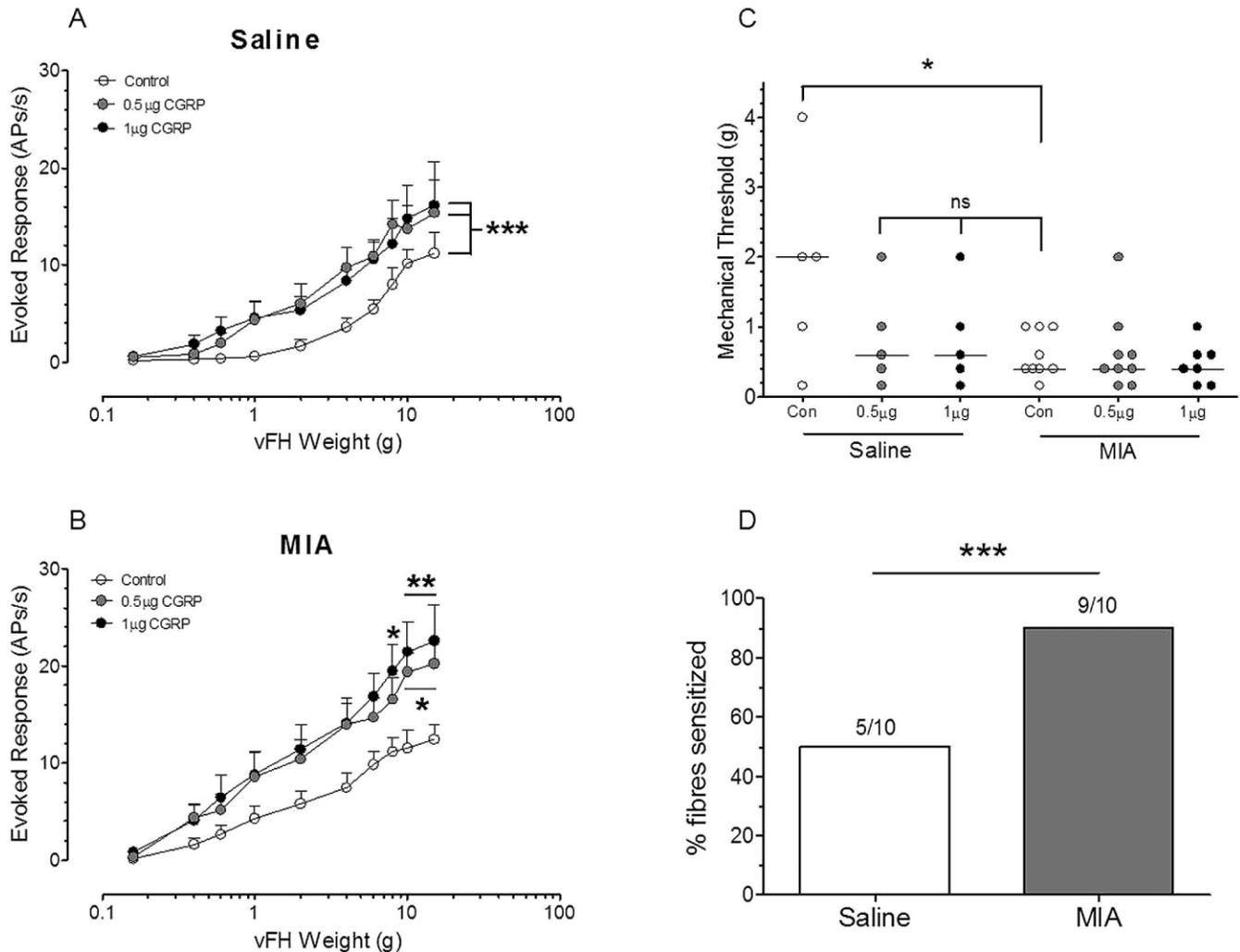
Figure 1. Calcitonin gene-related peptide (CGRP)–induced mechanical sensitization of an increased proportion of knee afferents following monosodium iodoacetate (MIA) injection. **A** and **B,** CGRP significantly increased the mechanical evoked firing rates (action potentials per second [APs/s]) of joint afferents in saline-treated (**A**) and MIA-treated (**B**) rats ($P < 0.001$ by 2-way analysis of variance with Bonferroni post hoc test). Values are the mean $\pm$ SEM maximum sensitizing effects of CGRP over each 1-hour recording period (n = 9 MIA-treated rats; n = 5 saline-treated rats). **C,** CGRP administration reduced mechanical thresholds (minimum stimulus evoking ≥ 2 action potentials) in saline-treated rats to a level not significantly different from that in MIA-treated rats ($P > 0.05$ for comparisons of 0.5 μg or 10 μg of CGRP versus controls, by Mann-Whitney t-test). Mechanical thresholds in MIA-treated rats were unaltered by CGRP administration. Each circle represents an individual rat (n = 7–9 MIA treated and n = 5 saline treated); horizontal lines show the median. **D,** The percentage of fibers sensitized by CGRP was greater in MIA-treated rats than in saline-treated rats ($P < 0.001$ by Fisher's exact test). ∗ = $P < 0.05$; ∗∗ = $P < 0.01$; ∗∗∗ = $P < 0.001$ by Mann-Whitney t-test. vFH = von Frey hair; NS = not significant.

impulses/second in saline-treated rats and 5.97 ± 0.28 impulses/second in sham-operated rats; $P < 0.05$ for each comparison, by unpaired t-test). Mechanical thresholds were significantly reduced in MIA-treated rats as compared to saline-treated rats (median 0.6 gm [range 0.16–4] and median 1.0 gm [range 0.16–6]; $P < 0.001$ by Mann-Whitney t-test). Although mechanical thresholds were reduced in MMT rats compared to sham-operated rats, the difference was not significant

(median 0.16 gm [range 0.16–2] versus median 0.5 gm [range 0.16–2]). Data in the MIA-treated rats were consistent with published data (14). This is therefore the first report of the sensitizing effect of MMT on the mechanical sensitivity of joint nociceptors.

CGRP sensitization of an increased proportion of joint nociceptors to mechanical stimuli following MIA treatment. To determine whether exogenous CGRP affects knee joint nociceptor mechanical sensitivity and,

if so, whether this is influenced by OA development, mechanically evoked responses were studied in MIA-treated and saline-treated rats following CGRP administration. CGRP significantly increased mechanical evoked responses of joint nociceptors to a similar degree in MIA- and saline-treated rats ($P < 0.001$ by two-way ANOVA) (Figures 1A and B). The lowered mechanical thresholds of joint nociceptors in MIA-treated rats were not significantly affected by CGRP, while mechanical thresholds in saline-treated rats were reduced to a level similar to those in the MIA-treated rats (Figure 1C).

Although the magnitude of increase in evoked firing rates caused by CGRP was similar in MIA- and saline-treated rats, the proportion of fibers affected was significantly greater following MIA treatment ($P < 0.001$ by Fisher's exact test) (Figure 1D). Injection of vehicle (saline) had no significant effect (data not shown). CGRP administration had a transient (<5 minutes) hypotensive effect that was equal in the MIA- and saline-treated rats (mean ± SEM reduction following 1 μg of CGRP 40.76 ± 2.77 mm Hg and 39.17 ± 7.84 mm Hg; $P > 0.05$ by Mann-Whitney t-test). Blood pressure returned to normal before mechanical stimulation was started (i.e., within 1 minute).

Reversal of OA-induced mechanosensitization of joint nociceptors by the CGRP receptor antagonist CGRP 8–37. We next examined whether endogenous CGRP contributes to the OA-induced increase in mechanical sensitivity of joint nociceptors. The effects of the CGRP receptor antagonist CGRP 8–37 on enhanced mechanical evoked responses in the MIA and MMT models were investigated. Figure 2 illustrates the inhibitory effects of CGRP 8–37 on 8–15-gm evoked responses in an MIA-treated rat as compared to a saline-treated rat. It is noteworthy that the inhibitory effects were more pronounced following MIA treatment.

Overall, CGRP 8–37 had no significant effects on evoked responses of joint nociceptors in control rats, whether saline-treated (Figure 3A) or sham-operated (Figure 3D), but significantly and dose-dependently inhibited responses in MIA-treated (Figure 3B) and MMT-operated (Figure 3E) rats ($P < 0.001$ for each comparison, by 2-way ANOVA).The lowered mechanical thresholds in MIA-treated rats were significantly increased by CGRP 8–37 ($P < 0.05$ by Mann-Whitney t-test) (Figure 3C) to a similar level as those in the joint nociceptors in saline-treated rats. Thresholds in saline-treated rats were unaltered by CGRP 8–37 (Figure 3C). Although mechanical thresholds in MMT-operated rats showed a trend toward being increased by CGRP 8–37, this did not reach significance (Figure 3F), and thresholds were unaltered in sham-operated rats (Figure 3F).
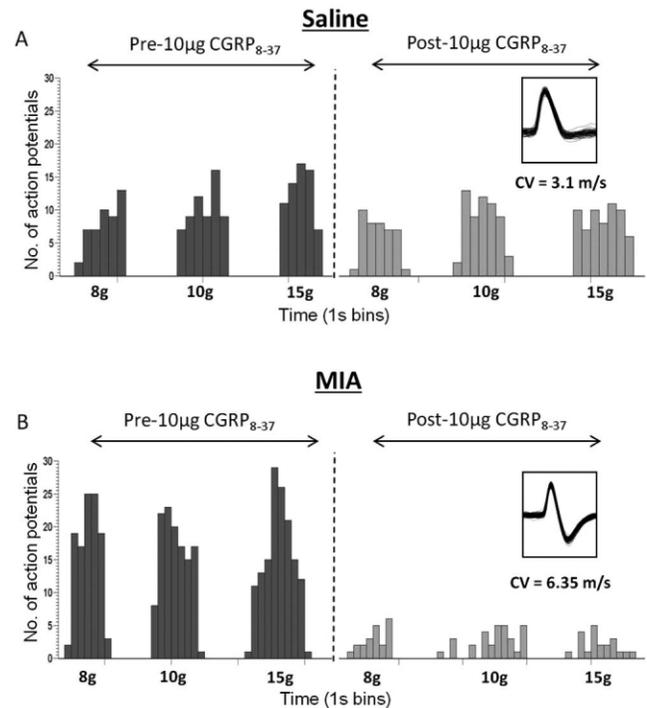
Figure 2. Inhibition of knee afferent mechanical evoked firing by the calcitonin gene-related peptide (CGRP) receptor antagonist CGRP 8–37 in monosodium iodoacetate (MIA)–treated rats. Representative examples of knee afferent mechanical evoked (8-, 10-, or 15-gm von Frey monofilaments) response data recorded before and after administration of 10 μg of CGRP 8–37 in a saline-treated rat (**A**) and an MIA-treated rat (**B**) are shown. The inhibitory effects of CGRP 8–37 were more pronounced following MIA treatment. Results are represented as rate histograms (1-second bins) of action potential firing of knee afferents (single units) in response to stimulation at the indicated von Frey monofilament level. **Insets** show an overlay of a representative fiber recording with the corresponding conduction velocity (CV; in meters/second), demonstrating single-fiber recordings; both are Aδ fibers.

These data indicate that local CGRP receptor blockade reverses established OA-induced mechanical sensitization of joint nociceptors.

Expression of mRNA for the CGRP receptor component in L3–L4 DRGs from MIA-treated and saline-treated rats. Since our electrophysiologic data indicated an enhanced sensitivity of the OA joint to local CGRP, we examined the expression of transcripts for CGRP and its receptor components (CLR, RAMP-1, and CRCP) in the L3–L4 DRG following MIA treatment (14 and 28 days). Using TaqMan quantitative reverse transcription–PCR (qRT-PCR) analysis (32), mRNA for CGRP, CLR, RAMP-1, and CRCP were detected in L3–L4 DRGs from MIA- and saline-treated
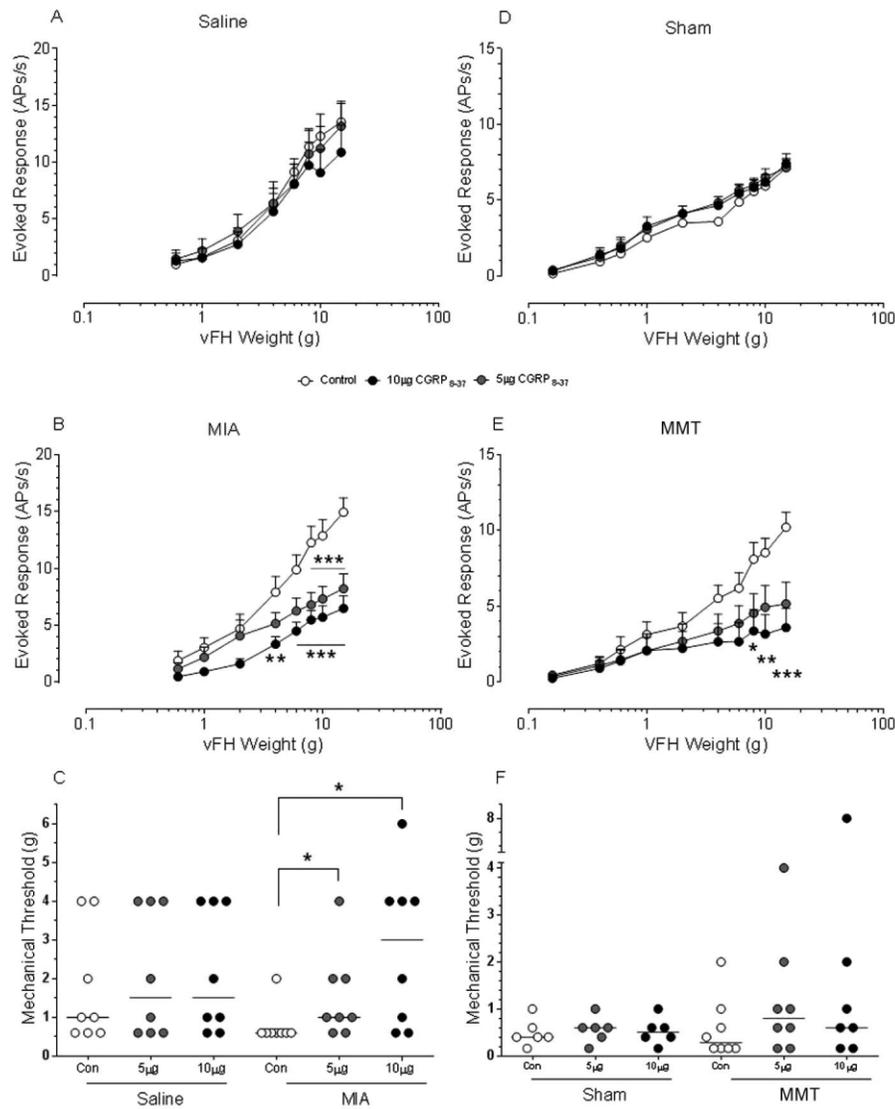
Figure 3. Ablation of monosodium iodoacetate (MIA)–induced and medial meniscus transection (MMT)–induced mechanical sensitization of joint afferents by the calcitonin gene-related peptide (CGRP) receptor antagonist CGRP 8–37. CGRP 8–37 had no effect on mechanically evoked responses (action potentials per second [APs/s]) of joint afferents in saline-treated (**A**) and sham-operated (**D**) rats, whereas it significantly inhibited mechanically evoked responses in MIA-treated (**B**) and MMT-operated (**E**) rats ($P < 0.0001$ for each comparison versus its corresponding control, by 2-way analysis of variance with Bonferroni post hoc test). Values are the mean ± SEM responses over each 1-hour recording period (n = 8 saline-treated, 6 sham-operated, 9 MIA-treated, and 8 MMT-operated rats). CGRP 8–37 had no effect on mechanical thresholds (minimum stimulus evoking ≥2 action potentials) in saline-treated (**C**) or sham-operated (**F**) rats. However, in MIA-treated (**C**) and MMT-operated (**F**) rats, thresholds in the presence of 10 μg of CGRP 8–37 were increased, reversing the osteoarthritis-induced reduction of mechanical thresholds. Each circle represents an individual rat (n = 6–9 per group); horizontal lines show the median. ∗ = $P < 0.05$; ∗∗ = $P < 0.01$; ∗∗∗ = $P < 0.001$, by Mann-Whitney t-test. vFH = von Frey hair.

rats (Figures 4A–D) confirming that mRNA for CGRP and its receptor components are expressed in DRGs at levels that contain the cell bodies of knee joint nociceptors. At 14 days, the levels of mRNA for CGRP, CLR, and RAMP-1 (Figures 4A–C) were not appreciably altered in the MIA-treated group as compared to the saline-treated group, whereas the levels of CRCP mRNA (Figure 4D) were significantly decreased. On day 28, CGRP mRNA levels were significantly decreased, CLR and CRCP were increased, and RAMP-1
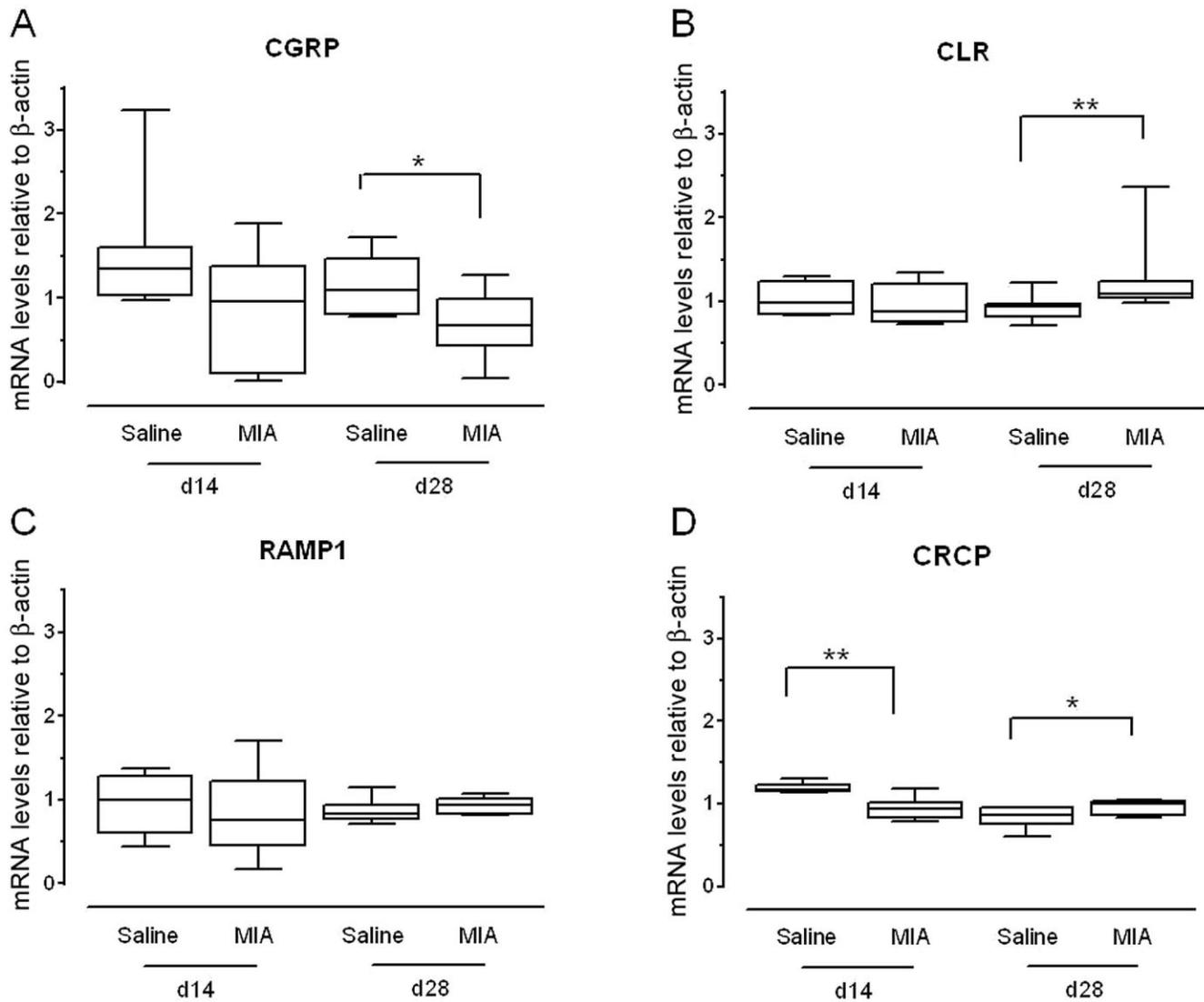
Figure 4. Expression of mRNA for calcitonin gene-related peptide (CGRP) receptor components in knee joint–innervating L3–L4 dorsal root ganglia (DRGs) and modulation of the levels following treatment with monosodium iodoacetate (MIA). **A,** Expression of mRNA for CGRP was significantly reduced 28 days after MIA treatment. **B,** Calcitonin-like receptor (CLR) expression was increased 28 days after MIA treatment. **C,** Levels of receptor activity–modifying protein 1 (RAMP-1) were unaltered, both on day 14 and on day 28 after MIA treatment. **D,** Levels of mRNA for CGRP receptor component protein (CRCP) were reduced 14 days after MIA treatment. There was a small but significant increase in CRCP expression 28 days after MIA treatment. Results were normalized to β-actin expression (which was unchanged between groups and treatments). Data are shown as box plots. Each box represents the 25th to 75th percentiles (n = 6–8 rats per group). Lines inside the boxes represent the median. Lines outside the boxes represent the 10th and 90th percentiles. $*$ = $P < 0.05$; $**$ = $P < 0.01$ by Mann-Whitney t-test.

mRNA levels were unaltered in MIA-treated rats compared to saline-treated rats. These data indicate that following the development of OA, the expression of CLR and CRCP in L3–L4 DRGs is regulated.

Coexpression of CLR with CRCP in joint afferents and increased expression following MIA treatment. Our qRT-PCR data demonstrated that the expression of mRNA for CGRP and its receptor components is regu-
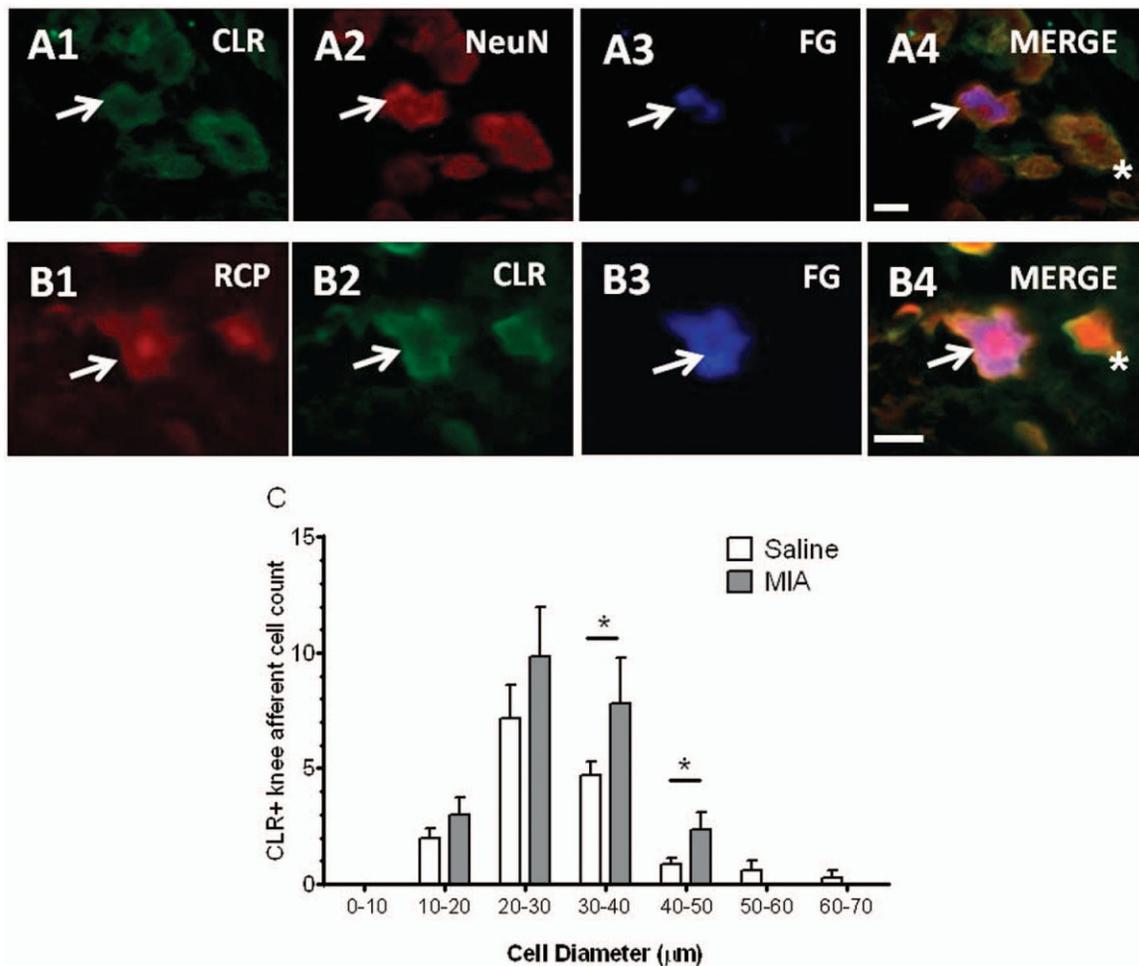
Figure 5. Coexpression of the calcitonin gene-related peptide (CGRP) receptor components calcitonin-like receptor (CLR) and receptor component protein (CRCP [RCP]) by knee afferent neurons and increased CLR expression following treatment with monosodium iodoacetate (MIA). **A1–A4,** CLR is expressed on knee afferents (**arrows**) and non–knee afferent neuronal cell bodies (∗) in ipsilateral L4 dorsal root ganglia (DRGs). Slides incubated with anti–neuronal nuclei (anti-NeuN) antibodies or with Fluoro-Gold (FG) are also shown. Bar = 34 μm. **B1–B4,** CRCP is coexpressed with CLR on knee afferent neurons (**arrows**) and non–knee afferent neurons (∗). Bar = 40 μm. **C,** The numbers of medium (30–40 μm in diameter) and large (40–50 μm in diameter) CLR-positive knee afferents were significantly increased in MIA-treated rats. CLR-positive knee afferent cell counts were analyzed according to diameter ranges similar to those described in a previously published report (9). Values are the mean ± SEM of 7 rats per group. ∗ = $P < 0.05$ by Mann-Whitney t-test.

lated in knee-innervating DRGs during OA. Fluoro-Gold retrograde labeling of knee afferents (9) combined with immunohistochemistry were used to investigate protein expression, specifically, whether joint afferents express CLR and CRCP and whether CLR expression by knee afferents is altered following MIA treatment. The numbers of Fluoro-Gold–labeled knee afferent cell bodies were consistent with published data (35) and were not significantly different between MIA-treated and saline-treated rats (mean ± SEM percentage of whole ganglia 3.47 ± 0.28% and 3.01 ± 0.61%). No

contralateral Fluoro-Gold–labeled cell bodies were observed, indicating that there was no systemic leakage of dye.

Consistent with CLR expression in trigeminal ganglia (36), CLR immunoreactivity was detected primarily in the plasma membrane and cytoplasm of DRG cell bodies expressing the neuronal marker NeuN (Figures 5A1 and A2). Occasional CLR-immunoreactive cells were also Fluoro-Gold–labeled cells (Figure 5A3), and frequent Fluoro-Gold–labeled cells were CLR immunoreactive, indicating robust expression of CLR pro-

tein in knee afferents (Figure 5A4). Consistent with previously published data (25), CRCP immunoreactivity was detected in the cytoplasm, plasma membrane, and nucleus of L4 DRG neurons (Figure 5B1). As with CLR, CRCP-expressing cells were Fluoro-Gold–labeled (Figure 5B3), indicating expression of CRCP protein in knee afferents (Figure 5B4).

Due to the complex nature of the CGRP receptor, expression of CLR alone does equate to the presence of functional CGRP receptors; thus, we investigated whether CLR is coexpressed with CRCP. Fluoro-Gold–labeled cells showed both CRCP immunoreactivity and CLR immunoreactivity (Figures 5B1 and B2), indicating coexpression in knee afferent neurons (Figure 5B4). These qualitative observations were made in L4 DRGs from MIA- and saline-treated rats; no quantification was attempted in this experiment.

In assessing the impact of experimentally induced OA on joint afferent expression of CGRP receptor components, we focused on CLR, the GPCR component of the CGRP receptor. The percentage of total cells (whole ganglion) expressing CLR was not altered following MIA treatment (mean ± SEM 53.63 ± 6.18% versus 59.55 ± 4.39% following saline treatment). The percentage of Fluoro-Gold–labeled knee afferents expressing CLR showed a tendency to increase following MIA treatment, although this increase did not quite reach significance (66.40 ± 7.92% versus 58.98 ± 4.23% in following saline treatment; $P = 0.091$ by unpaired t-test). When the numbers of knee afferent neurons showing CLR staining were analyzed based on cell diameter (cell diameter being related to function), a significant increase in the numbers of medium (30–40 μm) and large (>40 μm) knee afferents expressing CLR was observed following MIA treatment ($P < 0.05$ by Mann-Whitney t-test) (Figure 5C).

DISCUSSION

CGRP is synthesized and stored in sensory neurons and has known actions as a potent vasodilator and neuromodulator, in particular playing a key role in neurogenic inflammation (37). CGRP is implicated in the generation of migraine pain (28) and inflammatory joint pain (11), and there are now strong clinical and preclinical associations between the levels of CGRP in joints and pain during OA. Whether a direct relationship or integrated, causal mechanism exists is unknown.

Animal models of OA feature several common histopathologic features of human OA (14,32) and respond to the analgesics commonly used to treat OA pain

in humans (14,30,38). To this end, rat models of OA represent important translational research tools with which to investigate the pathophysiologic mechanisms of OA pain, and such models provide an opportunity for the investigation of CGRP-mediated pain mechanisms that cannot be achieved in humans.

We report herein new evidence that CGRP plays a critical role in peripherally driven pain mechanisms in experimental models of OA. Important experimental outcomes of our study were the demonstration that CGRP was able to acutely sensitize the responses of joint nociceptors to mechanical stimulation of the joint and that local blockade of the effects of CGRP completely reversed the mechanical sensitization caused by MIA injection. Importantly, the chemical MIA and surgical MMT models responded similarly to local CGRP receptor antagonism. Thus, despite their different precipitants/etiologies, our data suggest a common pathophysiologic pathway involving CGRP that contributes to the enhancement of nociceptive drive from the OA joint in these models. A recent study in the MIA model demonstrated a reversal of weight-bearing asymmetry following systemic administration of the nonpeptide CGRP antagonist BIBN4096BS (11); pharmacokinetic data implicated a peripheral site of action. Our data suggest that the periphery is a critical site for CGRP receptor blockade–induced analgesia. Accumulating evidence is supportive; rat and mouse models of OA pain are associated with increased joint afferent expression of CGRP (8) and increased CGRP release (15,16), changes coincident with our demonstration of OA-induced mechanical sensitization of joint nociceptors (14).

Functional deactivation of knee joint CGRP-expressing sensory terminals prevents the development of MIA-induced pain behavior (10). Although these reported effects may not be solely attributable to CGRP, we have provided substantive evidence that local CGRP plays a pivotal role in the sensitization of joint nociceptors in 2 distinct models of OA pain. Acute blockade of joint CGRP receptors by CGRP 8–37 normalized the enhanced mechanical evoked responses of joint nociceptors in the MIA and MMT models, with no effects in the respective control groups. Activation of local CGRP receptors by exogenous CGRP sensitized a significantly greater number of joint nociceptors in MIA-treated rats than in saline-treated rats.

Our data suggest that the OA joint exhibits increased sensitivity to the pronociceptive effects of CGRP and that this increased CGRP receptor signaling acts to maintain joint nociceptor sensitization. This

prompted us to investigate whether CGRP receptor expression is regulated at this level in the MIA model. The CGRP receptor comprises the GPCR CLR and the accessory proteins RAMP-1 and CRCP; however, reliable and well-characterized antibodies against RAMP-1 for use in immunohistochemical analysis are not available. The strategy adopted herein was to investigate CGRP receptor expression in L3–L4 DRGs by use of qPCR to analyze CLR, CRCP, and RAMP-1 transcript levels and by use of immunohistochemistry, with colocalization of staining with antibodies against CLR and CRCP.

The expression of mRNA for CGRP, CLR, RAMP-1, and CRCP was demonstrated in saline- and MIA-treated rats. Relative to the reference gene β-actin, significant decreases in the levels of CGRP and CRCP mRNA were observed in L3–L4 DRGs following MIA treatment. Although decreased CGRP mRNA expression is at odds with the reported increases at the protein level (8,9), a decrease in CGRP mRNA levels has been reported in MIA and surgical OA models (39). This may reflect facilitated transport of these transcripts along the primary afferent axon, with subsequent increased intraaxonal protein expression (40,41) and increased expression at the peripheral terminal. The observed MIA-induced changes in mRNA levels were small in magnitude, likely reflecting discrete and localized changes specifically in knee afferents, a small but important population of cells that are not easily detected with this method. This is supported by our observation of a significant increase in CLR protein expression in the L4 DRG that was restricted to cells that innervated the joint.

Immunohistochemical analysis combined with retrograde labeling of knee afferents enabled us to identify colocalization of CLR and CRCP immunoreactivity specifically in these neurons, raising the possibility of functional CGRP receptors at this level. An increase in the numbers of medium and large afferents in the knee, demonstrating CLR immunoreactivity, was observed following MIA, a finding that is consistent with our demonstration of increased CLR mRNA levels in MIA-treated rats. MIA treatment has previously been shown to increase the number of medium and large knee afferents that express CGRP (9). Together with our data, these findings suggest that MIA increases CLR and CGRP expression in an overlapping population of knee afferents, an intriguing finding given the proposed autoreceptor role of the CGRP receptor (23,28).

Although coexpression with RAMP-1 is necessary to unequivocally demonstrate the presence of func-

tional CGRP receptors, our demonstration of RAMP-1 mRNA expression in L3–L4 DRGs partially addresses this issue. Given our electrophysiologic findings of an increase in the proportion of knee afferents sensitized by CGRP following MIA treatment, we hypothesize that this enhanced CLR expression represents, at least in part, the formation of additional functional CGRP receptors in joint nociceptors that would be expected to exacerbate joint nociceptive inputs. Given the high percentage of knee afferents expressing CGRP (up to 76%) (8) and the CGRP receptor being expressed solely on CGRP-positive neurons (23), it is highly likely that knee afferents coexpressing CLR and CRCP also express CGRP.

Our qPCR and immunohistochemistry data suggest that a direct neuronal mechanism contributes to CGRP-mediated peripheral sensitization via modulation of the excitability of joint nociceptor peripheral terminals. Potential cellular mechanisms involved may include phosphorylation of tetrodotoxin-resistant (TTX-R) voltage-gated sodium channels (Na_V). CGRP enhances TTX-R Na_V currents in DRG neurons in vitro (22,23,42), lowering the threshold for action potential generation, a hallmark of peripheral sensitization (43,44). These in vitro findings are supported by our in vivo data demonstrating an acute CGRP-evoked reduction of joint nociceptor mechanical thresholds and a reversal by CGRP 8–37 of the MIA-induced reduction in thresholds. This afferent sensitization may in part account for the acute pronociceptive effects of peripheral CGRP (37). Injection of CGRP into a joint increases the expression of phosphorylated forms of the MAP kinases p38 and ERK in peripheral ganglia, sensitizing nociceptors and enhancing the evoked release of CGRP (45). Thus, CGRP can initiate acute increases in sensitivity to sensory stimuli as well as long-term changes in protein expression in sensory neurons.

It is important to note that CGRP receptors are also expressed on non-neuronal cells at the level of the joint within the synovium (46) and on immune and endothelial cells (47). CGRP plays an important vasodilatory role in the joint (48) and is a known immune cell recruiter, modulating their release of proinflammatory mediators that are able to directly excite and/or sensitize sensory neurons (49). Thus, an indirect mechanism involving these cells types is also likely, although the relative contribution of neuronal/non-neuronal cells is unknown.

Our demonstration of a critical involvement of CGRP in driving the sensitization of the joint in experimental models of OA, along with published clinical

evidence of an association between joint expression of CGRP and symptomatic OA in humans emphasizes the relevance of CGRP to OA pain. Our novel experimental evidence of an increased functional role of the CGRP receptor at the level of the joint and our demonstration that blockade of joint CGRP receptors ablates joint nociceptor sensitization in models of OA pain support future targeted site-specific investigations of CGRP antagonists in the control of pain associated with OA in humans.

AUTHOR CONTRIBUTIONS

All authors were involved in drafting the article or revising it critically for important intellectual content, and all authors approved the final version to be published. Dr. Kelly had full access to all of the data in the study and takes responsibility for the integrity of the data and the accuracy of the data analysis.

Study conception and design. Bullock, Wookey, Bennett, Mobasheri, Dickerson, Kelly.

Acquisition of data. Bullock.

Analysis and interpretation of data. Bullock, Wookey, Bennett, Mobasheri, Dickerson, Kelly.

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