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Pharmacological interrogation of a rodent forced ambulation model: leveraging gait impairment as a measure of pain behavior pre-clinically

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SUMMARY

Objective: The aim of this study was to investigate whether inflammogen-induced temporal and spatial gait changes in a rodent forced-ambulation paradigm were sensitive to pharmacological intervention with both clinically validated and novel analgesics.

Methods: Using the GaitScan (CleverSys Inc., Reston, VA) treadmill system, we identified four functional endpoints inspired by clinical literature and sensitive to unilateral joint injury induced by intra-articular Complete Freund's Adjuvant (CFA). These endpoints included: range of motion, normalized stance distance, stance/swing ratio, and paw print size as a measure of guarding; collectively, these measures are proposed to serve as a high fidelity index of joint pain. We then examined the ability of known analgesic mechanisms to attenuate gait impairment as measured by this index.

Results: Clinically efficacious opioids, Nonsteroidal anti-inflammatory drugs (NSAIDs), and the yet unapproved anti-NGF antibody dose-dependently attenuated the CFA)-induced gait deficits, while a TNF-alpha fusion protein blocker had no effect on gait, but did produce a reduction in swelling. As well, the time course for gait impairment in the model appears to be distinct from the traditional endpoint of tactile hypersensitivity, offering the potential to assess a novel functional pain phenotype.

Conclusions: In response to the call for more functional pain measures, we submit this composite gait score as a novel endpoint to interrogate joint pain pre-clinically. As the etiology of human osteoarthritis (OA) remains unclear, this model/endpoint cannot attempt to improve construct validity, but may provide an additional dimension to interrogate pain-induced gait deficits.

Introduction

Frequent knee pain affects approximately 25% of adults and has become a leading cause of disability, due to its impact on function, mobility, and quality of life[1]. Painful joints result from various causes, but osteoarthritis (OA) is the most common cause of knee pain in people 50 years or older[2]. The assessment of joint pain clinically includes radiographic measures to identify structural abnormalities, as well as patient-rated scales of joint pain and function. However, because the relationship between structural abnormalities and pain is poor, pain and functional impairment dominate the diagnostic scale[3,4]. Indeed, the vast majority of joint pain clinical trials have used the Western Ontario and McMaster Universities Osteoarthritis Index which includes 17 items of physical function, vs five pain items[5].

Critically, these assessments of joint pain and function are subjective, patient-reported evaluations. And while pain perception cannot be standardized against an objective measure and has no easily identifiable physical correlate[6], both gait deficits and the relationship between the perception of functional deficits and measurable gait limitations can be investigated. Significantly reduced walking speed, shortened stride length, prolonged stance time, and decreased range of motion at multiple joints have been observed in joint pain patients[7]. Interestingly, the limited investigation into the degree to which a patient's description of his/her own pain and disability relates to measurable limitations implicates these same gait deficits[8].

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Assessment of such gait endpoints pre-clinically is more complicated. It is not possible to dissociate changes in function from changes in pain state(s) in pre-clinical studies; these are inextricably linked in animals. Traditional pre-clinical models of pain rely on measures of evoked endpoints after some injury-producing insult. While these models are reasonable assessments of hypersensitivity and offer expediency in identifying the analgesic potential of a particular compound, they do not interrogate ongoing or movement-induced pain, nor its impact on joint function[4,9,10].

The pre-clinical pain field has recently begun supplementing the traditional evoked measures with a number of behavioral endpoints that more closely assess the impact of pain on function, including wheel running[11], weight bearing[12], locomotor activity[13,14], and limitedly, changes in gait performance[15–17]. While gait analysis has existed for several decades, leveraging gait impairment after a presumed painful insult as a reasonable surrogate of pain behavior is a relatively new trend. Systems which quantify gait on a treadmill apparatus have also shown the ability to detect impairment in rodent models of arthritis[15,16,18–25], and more importantly, a number of these studies have shown that observed gait impairments in rodent models of joint pain can be impacted pharmacologically. Thus, the aim of this study was to investigate whether Complete Freund's Adjuvant (CFA)-induced temporal and spatial gait changes in a forced-ambulation rodent gait paradigm were sensitive to pharmacological intervention with analgesics.

Methods

Animals

Female Sprague Dawley (SD) rats (Harlan, Indianapolis, IN, USA), weighing 200–240 g, were pair-housed in plexiglass cages with bedding and enrichment. Animals were maintained in temperature and humidity controlled rooms on a 12/12-h light/dark cycle and allowed *ad libitum* access to food and water until testing. Experimental protocols were reviewed and approved by the Lilly Animal Care and Use Committee. Naïve cohorts were used for each parametric and drug study due to the duration of impairment. Testing occurred between 7 a.m. and 3 p.m.

Intra-articular CFA injection

Rats were anesthetized with 4% isoflurane in oxygen and the rear right knee was shaved and sterilized with 70% ethanol. Injections were performed with a 27-gauge needle fitted with PE10 tubing such that 4 mm of the needle tip was exposed, thereby controlling injection depth. For the concentration response study, CFA (Sigma; 1 mg heat killed mycobacterium per 1 mL adjuvant) was administered between 5 and 50 μg *via* a Hamilton syringe. For the time course study, CFA was diluted in Incomplete Freund's Adjuvant (Sigma) and administered between 5 and 20 μg. For all pharmacology studies, rats received a 20 μg injection in 50 μL.

Drugs

Unless otherwise indicated, drugs were synthesized at Lilly Research Laboratories and dosed based on pharmacokinetic properties. Morphine sulfate (Sigma–Aldrich) was dissolved in saline and dosed subcutaneously (s.c.). Tramadol HCl (Teva Pharmaceuticals) tablets were sonicated in a 1% Hydroxyethylcellulose, 0.25% Tween 80% and 0.05% Dow antifoam (HEC) to form a 50 mg suspension of active drug. Rats were dosed per os (p.o.). Diclofenac sodium salt (Calbiochem) was suspended in the HEC vehicle at 5 mg/ml and administered p.o. Carprofen (Rimadyl™) was suspended in the HEC vehicle at concentrations of 5 and 15 mg/ml and dosed p.o. The EP4 receptor antagonist CJ-023,423[26] was suspended in 10% Acacia between 3.33 and 16.7 mg/ml and dosed p.o. Etanercept (Enbrel™, Immunex) was prepared at 5.5 mg/ml in saline and dosed intraperitoneally (i.p.). The anti-NGF and hIgG4 antibodies were prepared at 1 mg/ml, diluted to 0.1 mg/ml in saline and dosed i.p.

Gait analysis

The GaitScan gait analysis system was used to record and quantify gait features. The system consisted of a clear treadmill (ExerGait XL, Columbus Instruments, OH, USA) fitted with an angled mirror underneath. A high-speed camera (Basler, 100fps) beneath the treadmill belt recorded the ventral view of a moving rat. An opaque green plexiglass box with a semi-transparent viewing window was positioned above the treadmill to house the animal. Video was captured by the BCam software program at 2000 frames per animal for analysis in the GaitScan software. This color-based tracking system allowed maximal tracking of the paw, while excluding other body parts or shadows. Filters were applied to enable the analysis of multiple steps per animal. An *a priori* inclusion criterion of a minimum of four strides per limb was required for all studies.

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Prior to CFA administration, rats were habituated to the treadmill for 60 s. The treadmill was slowly increased from 0 to 2 cm/s. After the animal moved forward and away from back wall twice, the speed was slowly increased to 4 cm/s. Infrequently, a cotton swab was inserted into the front of the treadmill to re-orient a rat that was excessively rearing or exploring the chamber, effectively returning the rat to a normal walking pattern. As well, a rounded 'bumper' was placed on the back wall to minimize any negative impact of contact while the rat was learning the forced ambulation paradigm. Once the animal walked consistently, trial speeds were increased in increments of 2–3 cm/s until the animal successfully ran at or near 16 cm/s. The animal was then placed back in the home cage.

For test sessions, speeds were slowly ramped from 0 to the target speed. This range was 8–24 cm/s for parametric studies, 12–16 cm/s for CFA time course and pharmacology studies, depending on the ability of the individual rat. Filters in the analysis software were implemented to exclude instances of pausing, rearing, turning, riding back, or when the rat was touching the rear of the treadmill, thereby decreasing the variability in gait measures and allowing a more accurate representation of locomotion.

Parametric gait studies

The effect of different treadmill speeds on gait parameters was assessed in order to select the speed for pharmacology studies. Animals received a habituation session to the treadmill followed by a test session 24 h later. The optimal concentration of 20 μg of intra-articular CFA was determined after administering a concentration range of 5–50 μg vs saline.

Tactile hypersensitivity

von Frey monofilaments calibrated to incremental bending forces (0.3–15 g) were used to assess tactile hypersensitivity *via* the up-down method[27]. On test days, rats were placed in elevated observation chambers with wire mesh floors and allowed a 20-min acclimation. The tactile hypersensitivity threshold was determined by applying von Frey filaments at the base of the third and fourth digits by a blinded observer.

Fluoroscopy

Imaging of the knee joint and surrounding soft tissue was performed with a high resolution fluoroscope (LabScope™, Glenbrook Technologies Inc., New Jersey) to obtain a gross measure of swelling in a subset of animals. Immediately following euthanasia, the rear limbs were extended to expose the ventral knee surface for imaging the joint from a frontal plane at 28 kV. The contralateral limb was then crossed over the ipsilateral and positioned in front of the animal, placing the ipsilateral medial knee on a side plane for imaging. A small metal reference marker was placed into the field of view to confirm calibration of the analysis software. The Glenbrook software program GTI-2000 measured the frontal and lateral aspects of knee width.

Pharmacology studies

Compounds with known analgesic properties were administered to determine if an attenuation of CFA-induced gait impairment could be detected. Pre-treatment times for acute administration studies were determined based on each drug's pharmacokinetic properties in rat. Acute drug studies were always conducted 3 days post-CFA. For all subchronic administration studies, dosing was initiated 2 h post-CFA and continued once daily (QD) for the duration of the studies; however, diclofenac was administered twice daily (BID) based on its short half-life[28]. For all pharmacology studies, rats were randomly assigned to drug treatment.

Pathology

The structural impact of the intra-articular CFA injection was assessed histologically (see Supplemental methods) by a veterinary pathologist at Bolder BioPath, Inc.

Statistical analysis

The experimental unit for each study was one animal. Data were analyzed using JMP 8.0 (SAS Institute) by one-way or repeated measures multivariate analysis of variance (MANOVA) with two-tailed *t*-tests or Dunnett's *post hoc* tests for between group comparisons. Mean data are presented in the context of the 95% confidence interval as a measure of estimation uncertainty, and thus an alpha level of 0.05 was used to determine significance. For between group analyses within a repeated measures study, the alpha level was divided by the number of time points analyzed to account for the increased likelihood of a Type I error in repeated samples.

Results

Gait deficit-related functional endpoints

The GaitScan system outputs numerous standard gait features reflecting spatial and temporal aspects of gait. After analysis of the multiple dependent measures recorded by the CleverSys software, and though many of these measures are highly inter-correlated, we reported and mathematically combined four measures which provide a comprehensive representation of gait deficits in this model of joint pain: range of motion, stance/swing ratio, normalized stance distance and paw print size. The range of motion measure is unique to the GaitScan software. It provides a spatial measurement relative to the body and is calculated as the distance of the paw from the middle of the animal at the start of the stance phase to the distance from the paw to the middle of the body at the end of the stance phase. The temporal measure of stance/swing ratio was determined by dividing the stance time by the swing time. This ratio of both temporal features of the stride was incorporated into a single measure to capture gait changes that may arise from alterations in either measure, and is illustrated in Fig. 1(A). Similar to the reported measure of duty cycle[29], stance/swing ratios have been examined in both rodent[21,30] and human studies of gait[31]. The normalized stance distance measure incorporates both spatial and temporal features by multiplying the stride length by the percent time spent in the stance phase to give a representation of the distance traveled while that limb was in contact with the ground. The paw print size was calculated by taking the average paw print size in pixels across the stance phase, which has been previously reported to have a high correlation with dynamic weight bearing when compared to the contralateral paw print and reported as a guarding index[32]. Figure 1(B) illustrates the effect a dynamic weight-bearing imbalance on paw print size. It is important to note that while these four measures appear sensitive to changes in limb function in this model, they may not be as informative for other models, particularly in cases where rodents may utilize other strategies of compensation such as changes in coupling or sequencing.

For each measure, we then calculated the percent change from the contralateral limb to the CFA-injected limb, thereby creating an index measure. The index measure incorporated the reduced use of the injured limb as well as the compensation in the contralateral limb, yielding an overall index of gait behavior rather than the ipsilateral limb in isolation. For comparisons across studies, a composite gait score was then calculated by taking the sum of each index measure.

Intra-articular CFA-induced time course of gait impairment and tactile hypersensitivity

The onset of gait impairment was rapid, with significant impairment beginning 24-h post-CFA administration, but gradually improved over time, as measured by the range of motion index [Fig. 2(A)], Stance/Swing Index [Fig. 2(B)], Stance Distance Index [Fig. 2(C)] and Guarding Index [Fig. 2(D)]. The composite gait score [Fig. 2(E)] illustrates the cumulative effect of these measures in one unified gait measure. In contrast to the rapid onset of gait impairment, tactile hypersensitivity after intra-articular CFA was not observed until day 4 [Fig. 2(F)], but persisted to at least 21 days in this cohort. This may be a result of the distance from the insult to the tactile assessment in the hindpaw, but is in contrast to other reports[33]. Additional parametric studies (CFA concentration response, speed dependency) are included in the Supplemental material.

Joint histopathology

Intra-articular CFA administration resulted in severe inflammation of the synovium, but minimal pannus, cartilage damage or bone resorption [Fig. 3(A)]. Minimal inflammation ipsilateral to the injection was observed at 3 h post-CFA, and primarily characterized by the infiltration of neutrophils into the synovium. Severe inflammation (e.g., neutrophils and mononuclear inflammatory cells) with minimal pannus, cartilage damage, and bone resorption [Fig. 3(A)] was observed at day three, which corresponds to acute pharmacology testing, and persisted to day 7 post-injection. Mild to moderate periosteal proliferation of osteoblasts was observed in all CFA-injected knees. Four of five knees showed moderate periosteal proliferation of osteoblasts and osteoid disposition. A representative micrograph across time points is shown in Fig. 3(B). Fluoroscopic quantification showed significant increases in soft tissue swelling [Fig. 3(A)] as represented in Fig. 3(C).

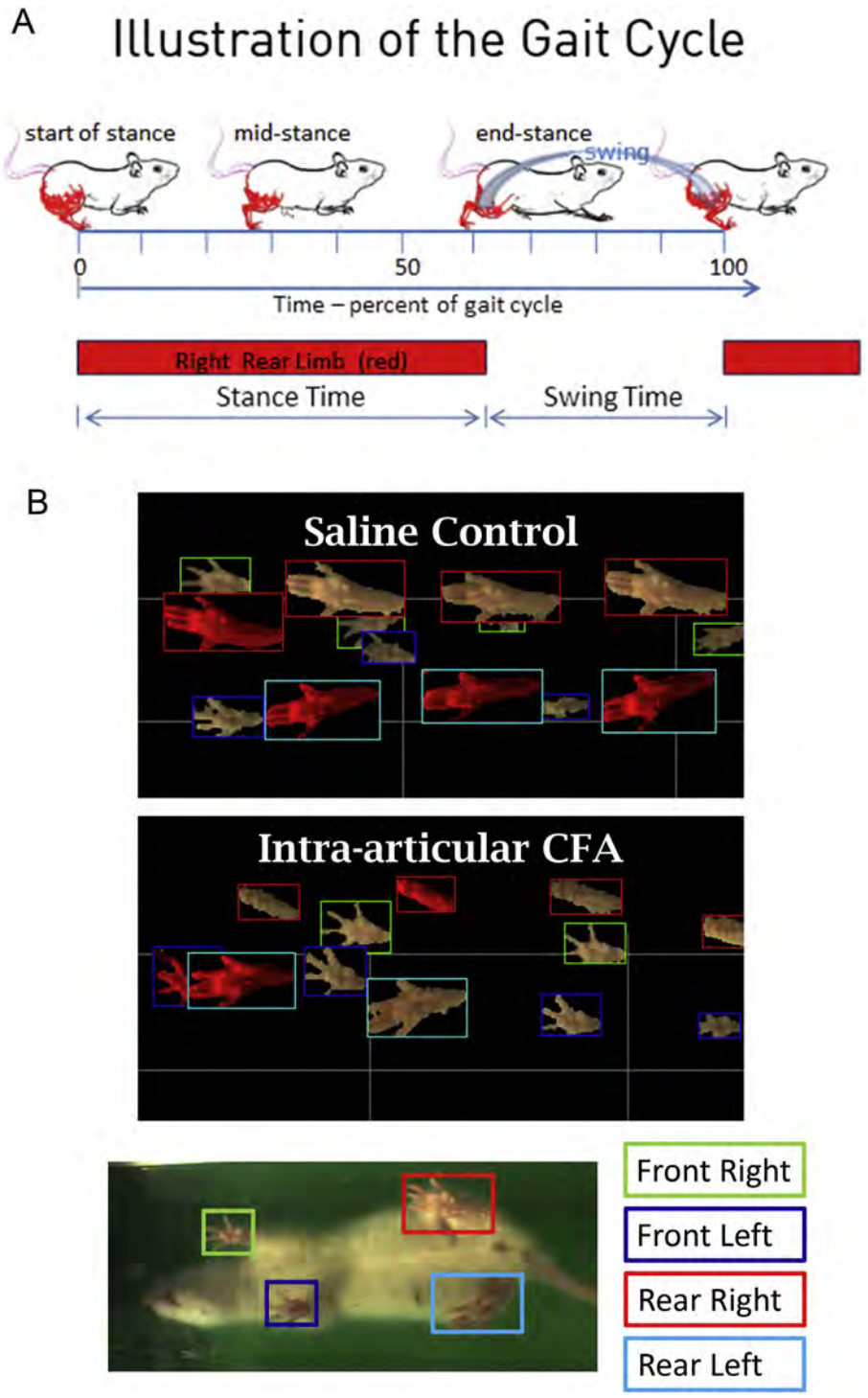

Fig. 1. Illustration of the temporal features of the gait cycle in rats. (A) The right rear limb in the rat rendering is highlighted in red, and the movement progression illustration depicts the stance phase across the start, mid and end of stance (highlighted as the red bar) below the scale. The swing phase, defined as the lifting and returning of the limb back to stance, is then illustrated and reflected as the empty bar below the scale. The stance and swing phases comprise the stride cycle. (B) A representative paw print capture of a rat after intra-articular saline (top panel) and intra-articular CFA (middle panel). Intra-articular CFA injection produces a smaller paw print (red box) as the affected limb makes little contact with the ground. In contrast, the contra-lateral paw (light blue box, illustrated in bottom panel) makes significant ground contact, and the entire paw, including toe spread, can be visualized.

Pharmacology

Opioids

A single administration of morphine produced a significant, dose-dependent attenuation of CFA-induced gait impairment, with 1 mg/kg as the no-effect dose [Fig. 4(A)]. Similarly, a single administration of tramadol also significantly attenuated CFA-induced gait impairment in a dose-dependent manner, with 20 mg/kg as the no-effect dose [Fig. 4(B)]. An advantage of using gait analysis for drug testing is that non-specific side effects such as sedation, which yields false positives in evoked measures, are

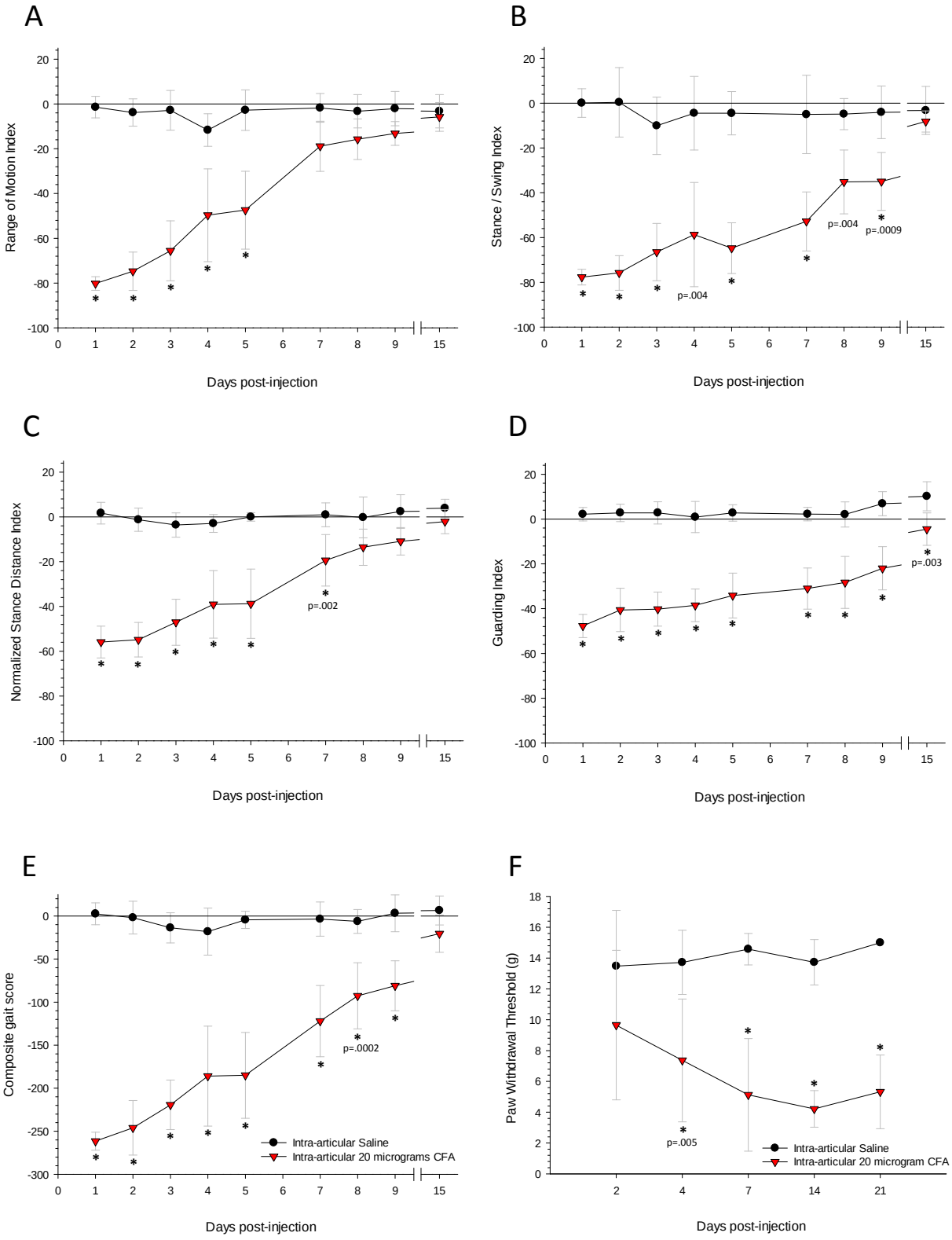

Fig. 2. The characterization of the CFA joint pain model on parameters of gait analysis. A time course of gait impairment reveals profound initial impairment over the first 3 days post-CFA with gradual improvement of performance and a return to near normal levels by day 15. A similar pattern of gait impairment is shown over time in each index measure for range of motion (A), stance/swing index (B), stance distance index (C) and guarding index (D). The four gait features are summed to create the multi-factorial gait index score (E) which allows a singular overview of gait performance. In contrast, paw withdrawal threshold (g) evaluated with the von Frey filament test of the ipsilateral hindpaw details increased hypersensitivity over an extended time course of at least 3 weeks, but with delayed onset relative to gait impairment (F). Data are presented as Mean with 95 % CI, $n = 8$ per group.*$P < .0001$ vs saline control (ANOVA) unless otherwise shown.

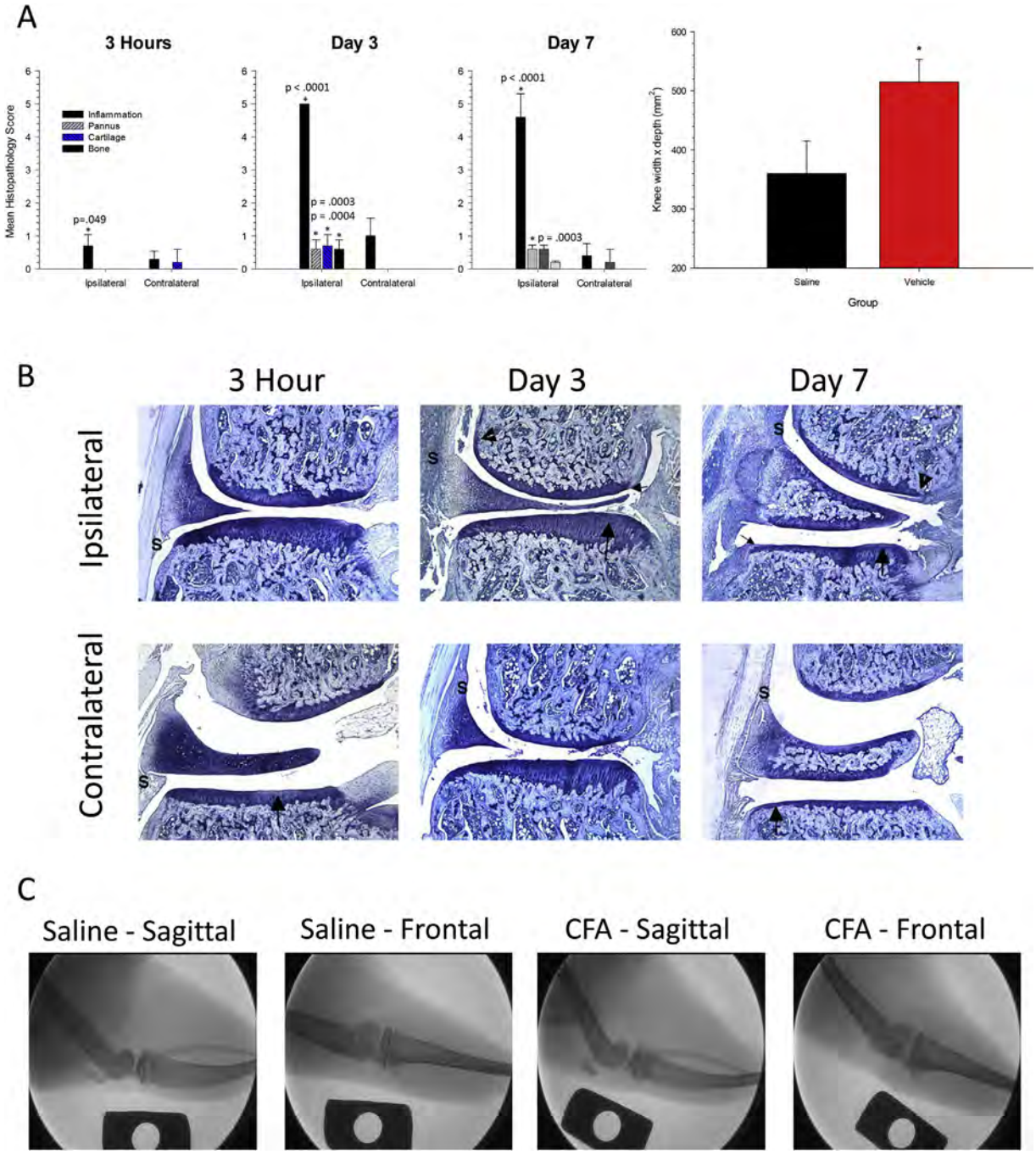

Fig. 3. Histopathology analysis of CFA-injected knee joints. Assessment of knee joints at 3 h, 3 days and 7 days post-CFA (A, left panel). Complementary fluoroscopic analysis of the knee joint diameter I shown in the left panel. Representative femorotibial joint sections stained with Toluidine Blue illustrate synovitis in the ipsilateral knee joints (B, top row images) compared to representative images from contralateral knee joints (B, bottom row images). Severe inflammation (S), with minimal pannus (small arrow), cartilage damage (large arrow), and bone resorption (arrowhead) are shown ipsilateral to CFA injection vs the contralateral knee. Representative fluoroscope images of a saline injected knee and CFA treated knee from the two measured orientations are also shown (C). Statistical significance shown on figures compared to controls. Mean pathology scores are presented in the context of the 95% CI.

readily detected by the system. Although rats treated with 7 and 10 mg/kg of morphine were able to walk on the treadmill, walking speeds were slower (Supplemental Fig. 3).

Nonsteroidal anti-inflammatory drugs (NSAIDs)

The analgesic effects of diclofenac and carprofen were determined following sub-chronic administration, whereas CJ-023,423 was tested acutely on day 3. Figure 5(A) shows that acute administration of diclofenac at 5 mg/kg slightly improved gait performance, but was not statistically significant ($P = .07$ compared to CFA vehicle control); however this same dose administered BID produced a profound attenuation of CFA gait impairments at every time point tested ($P < 0.0001$ for effect of group). Similarly, QD dosing of 5 and 15 mg/kg of carprofen significantly improved gait performance in CFA treated rats after sub-chronic dosing ($P < .0001$

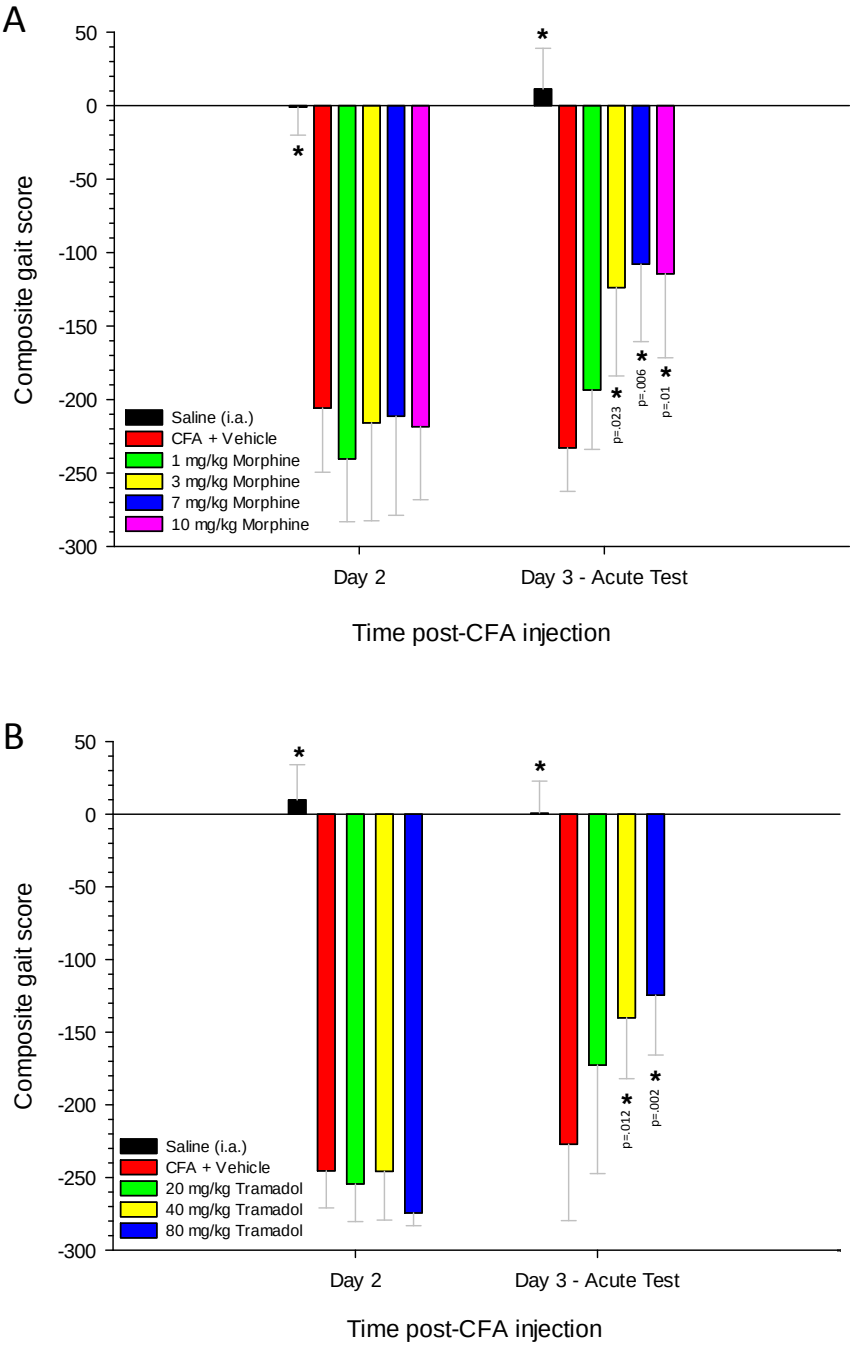

Fig. 4. The effects of opiates on CFA-induced gait impairment. (A) Baseline impairment is shown 2 days after CFA injection (left set of bars above Day 2). A single dose of morphine sulfate was administered at 1, 3, 7 and 10 mg/kg s.c. in a 1 mL/kg dose volume. Gait assessment was conducted 30 min after dosing, consistent with a Tmax across this dose range by the s.c. route of administration of 0.25–0.36 h and a half-life range of 2.5–5.3 h (right set of bars above Day 3 – Acute Test). *$P < .05$ vs CFA/vehicle controls, $n = 8$/group. Data presented as mean composite gait score with 95% CI. See also Supplemental Fig. 3 for an effect on walking speeds. (B) A single dose of Tramadol HCl was administered at 20, 40, and 80 mg/kg PO in a dose volume of 4 mL/kg. Gait assessment was conducted 1 h after dosing, consistent with a Tmax between 1 and 2 h and a T1/2 of 6–8 h by the p.o. route of administration. *$P < .05$ vs CFA/vehicle controls, $n = 8$/group. Data presented as mean composite gait score with 95% CI.

for effect of Group, $P < .0001$ for interaction of Group × Time), as did the positive control tramadol [Fig. 5(B)]. We also showed that blocking a downstream target of PGE2, the EP4 receptor with acute administration of CJ-023,423 dose-dependently attenuated gait impairment [Fig. 5(C), $P < .0001$ for effect of group].

TNF-α and anti-NGF antibodies

The anti TNF-α fusion protein etanercept had little effect on CFA-induced gait impairment [Fig. 6(A)]. Subchronic administration significantly improved gait performance 3 h post-CFA, but did not significantly improve performance at any other time point. Acute administration also failed to improve gait performance ($P = .14$ for effect of group excluding saline controls). The 80 mg/kg dose of the positive control tramadol significantly improved gait performance after acute treatment on day 3. Interestingly, even though gait performance was not improved, etanercept did significantly reduce knee swelling compared to vehicle controls as measured by high resolution fluoroscopy [Fig. 6(B), $P < .0001$ for effect of group including saline controls].

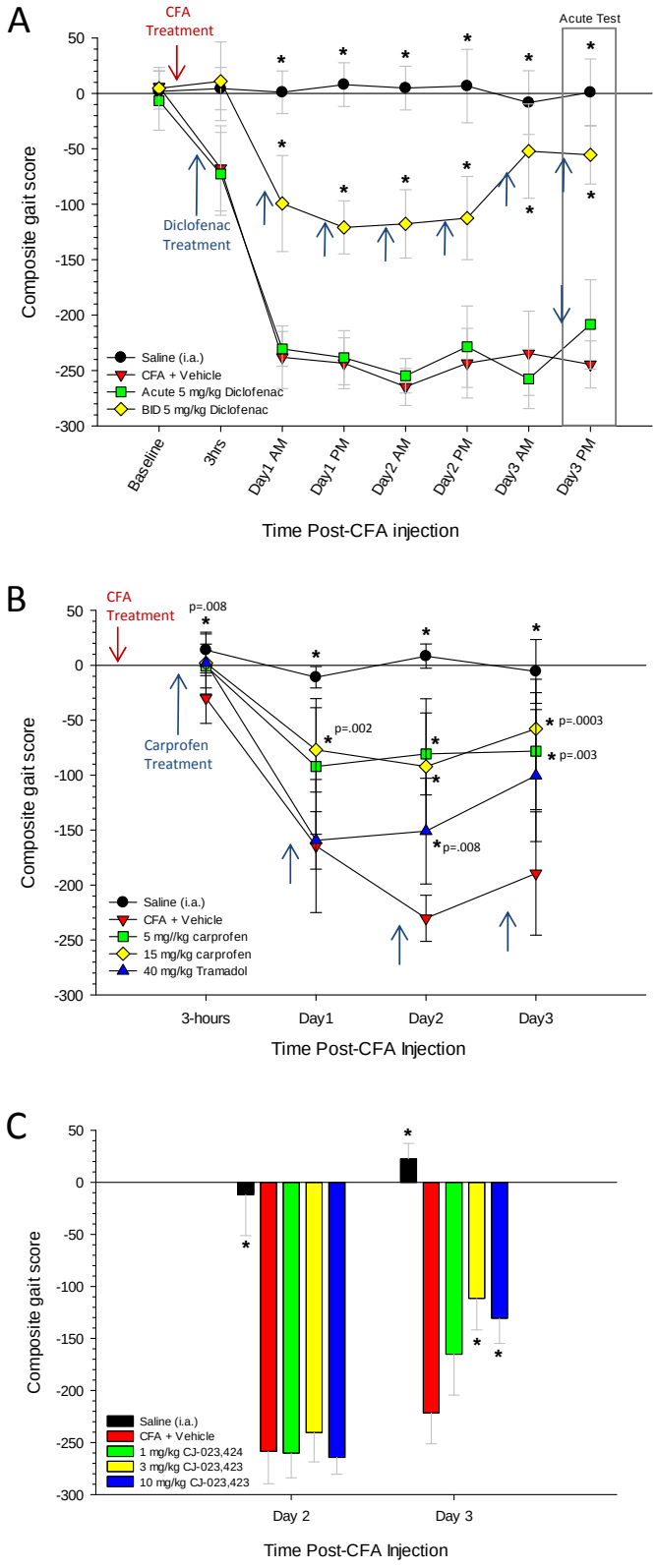

Fig. 5. The effects of COX1/2 inhibition on CFA-induced gait impairment. (A) Time course effects of diclofenac. Two diclofenac administration paradigms were as follows: 5 mg/kg BID beginning 2 h after CFA administration, and out to Day 3 for a total of seven doses (yellow diamonds) or a single administration of 5 mg/kg on Day 3 (prior to the afternoon gait assessment, green squares). The dose volume for both paradigms was 1 mL/kg. Gait assessment occurred 1 h after each administration in the BID arm, and 1 h after the single administration on Day 3, consistent with a Tmax of 0.5–1 h and a T1/2 of 1–2 h. (B) Time course effects of carprofen. 5 and 15 mg/kg of carprofen was administered QD beginning 2 h after CFA administration, and to Day 3 for a total of four doses. The dose volume for both doses was 1 mL/kg. Gait assessment occurred 1 h after each administration consistent with a Tmax of 1–3 h and a T1/2 of 5–7 h. (C) The effects of an EP4 antagonist on CFA-induced gait impairment. A single dose of CJ-023,423 was administered at 10, 30, and 50 mg/kg p.o. in a 3 mL/kg dose volume. Gait assessment was conducted 1 h after dosing, consistent with a Tmax 1–2 h of and a T1/2 of 7–9 h by the p.o. route of administration. *$P < .05$ vs CFA/vehicle controls, $n = 8$/group. Data presented as mean composite gait score with 95% CI.

Acute administration of an anti-NGF antibody 2 h post-CFA produced a profound improvement in CFA gait impairment [Fig. 6(C)]. Rats treated with 1 mg/kg anti-NGF showed nearly significant improvement on day 1, which persisted to a complete reversal of impairment by day 4. The lower dose of 0.1 mg/kg showed a slower onset, but never reached statistically significant improvement. Despite the robust improvement in gait performance, anti-NGF did not affect joint swelling [Fig. 6(D)].

Discussion

Leveraging an intra-articular CFA injection as a unilateral injury model, we have reported four gait measures which encompass both temporal and spatial gait features *via* the CleverSys video tracking system. Subsequently, we combined these four measures into a composite gait index score which reflected an overall gait impairment measure. While initially biased towards measures of gait deficits that had been identified in the clinical literature, these four features were also the most reliable and consistent rodent gait deficits observed. Transforming these measures into an index score served to normalize gait measures, removing individual animal differences and speed dependency effects on the measure. It further allowed for a measure of compensation by including the contralateral limb function.

While some of the reported measures are similar to previous gait parameters, there are unique aspects to these forced ambulation endpoints. In contrast to the range of "joint motion" typically reported in the literature such as degree of knee flexion, we describe the range of motion as the distance the paw travels in relation to the center of mass. This measure is novel in rodents, but routinely measured in humans, potentially offering a direct translation of results. Our temporal measure of Stance/Swing ratio has a high correlation with the commonly reported duty cycle measure, but we find that duty cycle is limited mathematically for comparison to the contralateral limb. When the duty cycle $(x/x + y)$ is used, the maximal effect is 50% compared with the contralateral limb, whereas the stance/swing ratio in the index measure has a signal window up to a true 100% change from contralateral. Similarly, we report a measure of guarding similar to others[15,17,20] which is highly correlated with dynamic weight bearing in rats[34], and as a measure of movement-evoked pain, is more encompassing than measures of static weight bearing[15].

While the method used to produce the unilateral injury was not the focus of this study, a discussion of our choice of CFA to produce gait deficits is warranted. Several animal models of OA have been described in terms of their modeling of disease pathology, but no single model effectively mimics the human condition[35]. Specifically, there exists a poor correlation between joint pathology in animal models and impairments in mobility. In the MIA model, gait impairment is reported primarily using dynamic weight bearing measures across both the inflammatory and late phases of the model[17,24] but temporal measures of gait are either not affected[36] or only modestly altered in the late phase when joint pathology should be greatest[23]. Surgical models of OA also show disconnects in gait impairment as anterior cruciate ligament transection combined with medial meniscus tear (ACL + MMt) fail to produce gait

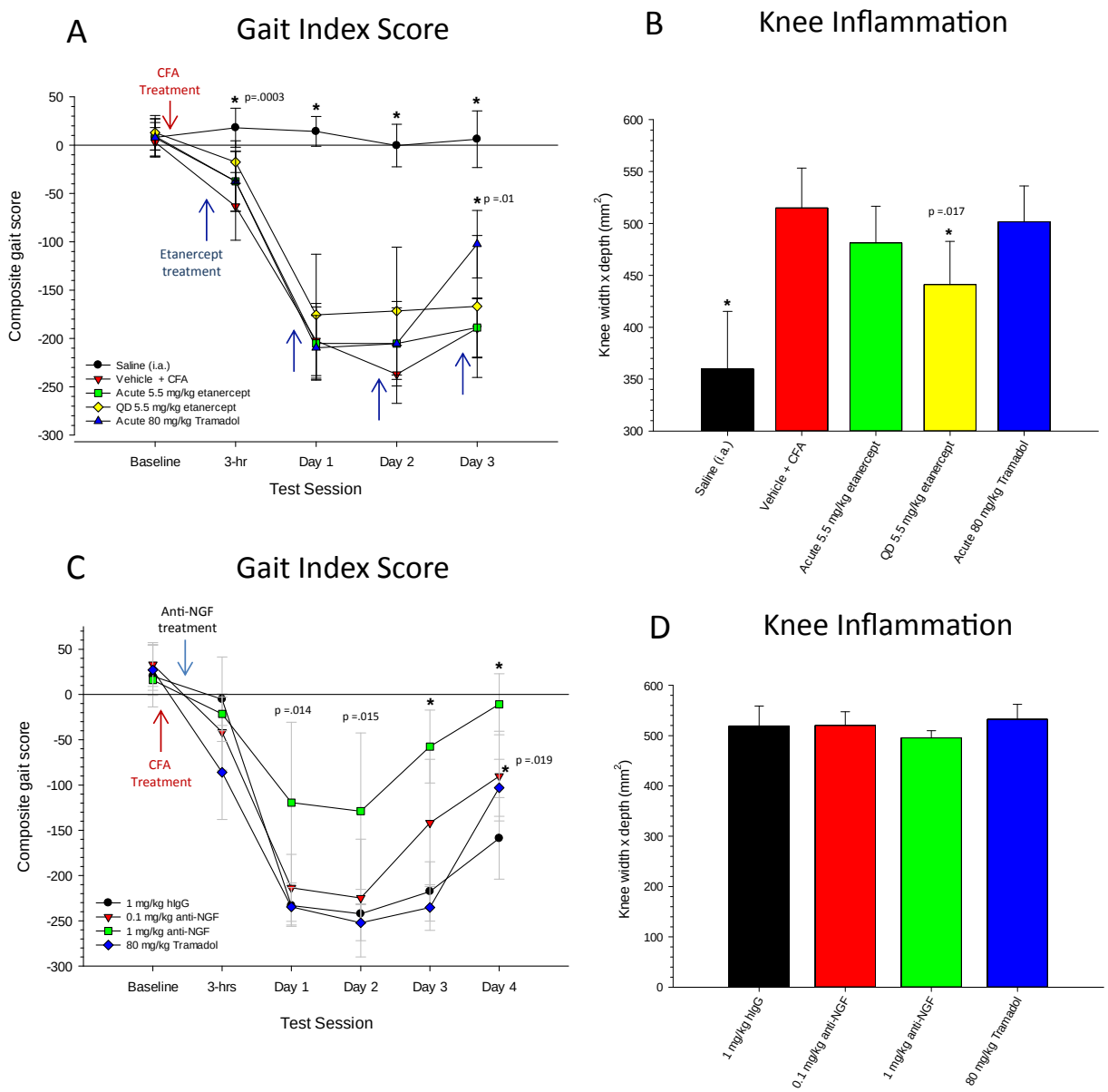

Fig. 6. The effects of the anti-TNF alpha fusion protein etanercept and an anti-NGF Ab on CFA induced gait impairment. (A) Time course effects of etanercept. Two etanercept administration paradigms were as follows: 5 mg/kg BID beginning 2 h after CFA administration, and out to Day 3 for a total of seven doses (yellow diamonds) or a single administration of 5 mg/kg on Day 3 (prior to the afternoon gait assessment, green squares). Tmax is 2 h and T1/2 is 3–9 h by the i.p. route of administration. The dose volume for both paradigms was 1 mL/kg. Gait assessment occurred 1 h after each administration in the BID arm, and 1 h after the single administration on Day 3. (B) Joint swelling as measured by fluoroscope imaging. (C) Time course effects of an anti-NGF antibody. A single dose of the anti-NGF antibody was administered 2 h after CFA at 0.1 and 1 mg/kg and tested each day of the study out to Day 3. The Tmax for this antibody was 16 h and the T1/2 96–112 h by the i.p. route of administration. The antibody produced a significant and dose-dependent improvement in gait performance ($P < .0001$ for effect of group, $P < .0001$ for interaction of group × day, see also supplemental video). (D) Joint swelling was not affected by anti-NGF treatment as there were no significant differences in the volume of the knee as measured by the fluoroscope ($P = 0.38$ for effect of group). Data presented as means ± SEM, $n = 8$ per group, $^{*}P < .05$ compared to CFA/vehicle or IgG controls. $^{*}P < .05$ vs CFA/vehicle controls, $n = 8$/group. Data presented as mean composite gait score with 95% CI.

impairment in rats[23]; however, mild gait imbalance was reported in a MMt model[18]. This same disconnect is observed in the human condition, where poor correlations exist between reported pain and radiographic imaging. However, a recent MRI study found strong associations between synovitis, bone lesions and reported pain[9], supporting the role of inflammatory mediators in OA pain signaling. Nonetheless, our findings with intra-articular CFA have confirmed and extended the findings of others[15,20] in that the inflammatory joint pain induced by CFA alters gait similar to compensatory gait patterns observed in human OA patients[1,37].

That said, the model limitations are important to consider. We induced a contrived and specific inflammation which does not share the etiology or prolonged time course of joint pain in humans. The impairment after a single injection of CFA is not long-lived; further studies will be necessary to determine if other animal models are capable of producing chronic gait impairment, similar to OA patients. As such, pharmacological intervention in the model must occur at the maximum signal window (day 3 post-CFA), which is quite proximal to the pain-producing stimulus. This is much earlier than the typical pharmacological intervention in the

time course of human joint pain. Additionally, ascribing gait impairment to joint pain after a pro-inflammatory insult is potentially confounded if the observed impairment is driven by physical limitations of the joint due to swelling or other physical changes. To mitigate this risk, we used a high-resolution fluoroscope to semi-quantify the knee area and to examine drug effects on joint swelling, in addition to their effects on gait deficits. The finding that anti-TNFα treatment could reduce swelling but not attenuate gait impairment suggests that the observed effects on gait indices are not merely a function of the swelling in the joint.

Further, a number of studies suggest that gait impairment may serve as an objective measure of joint pain in animal models by showing that analgesics are sufficient to improve gait function[15–17,25,38,39]. Our studies also support this tenet across two clinically validated analgesic classes of opioids and NSAIDs used to treat joint pain. In each case, the representative compounds dose-dependently attenuated the CFA-induced gait impairment. The one clinically approved agent for joint pain and swelling that was not detectable in this model was the TNF-alpha fusion protein etanercept[40], but had previously been demonstrated to improve some measures of gait and pain in a rat adjuvant induced arthritis model[16]. While the failure to detect this compound may be attributable to lack of an autoimmune induction of pain, the duration of gait impairment, methods of gait analysis, and frequency of dosing were also different between models. Given the attenuation of gait impairment after a single dose of the positive control diclofenac in the same study, it remains an open question as to why etanercept treatment did not improve gait performance in this study. Interestingly, two unapproved mechanisms that have demonstrated efficacy either pre-clinically or clinically were detected in this gait system. We demonstrated that a selective EP4 receptor antagonist attenuates gait impairment, consistent with a recent report in arthritic rodent pain models[41]. Further, we have shown that an NGF sequestration mechanism provides near restoration of gait impairment, consistent with the remarkable improvement in pain scores in OA patients which have been demonstrated with an anti-NGF antibody[42], and two recent reports using different pain induction and gait detection methods[43,44]. Interestingly, this effect develops over time in our model, and similar to the anti-nociceptive effects reported with anti-NGF in a rat nerve injury pain model[45].

Given the sensitivity to pharmacological intervention observed in our system, we feel confident that it detects joint pain-induced changes in gait. An obvious next question is whether the model is informing us of something beyond what is already gleaned from traditional measures. Though direct comparisons to evoked endpoints were not systematically evaluated, we conducted pilot studies to determine if gait added another dimension of movement-evoked pain to the "pain battery" of pre-clinical models. Using the same cohort of CFA-treated animals, initial results suggest that gait impairment and tactile hypersensitivity appear to have distinct time courses [Fig. 2(E) and (F)]. Similarly, it has been reported that while inflammatory and nerve injury models of pain in rodents produce impairments in gait, only the inflammatory mediated gait impairments appear sensitive to analgesic drug treatment, suggesting motor neuron damage may drive impairment in some models[25]. Unpublished data from our lab corroborates this observation as we failed to detect gait impairment in a spared nerve ligation model but robust gait impairment in a chronic constriction injury of the sciatic nerve model, where motor neurons are differentially affected. Taken together, these data suggest that the assessment of movement-evoked pain may be detecting a different pain phenotype than traditional evoked measures and may offer an added dimension to the interrogation of pre-clinical pain.

Author contributions

B.L. Adams contributed to the conception and design of the study, acquisition of data, and analysis and interpretation of data. He also contributed to the drafting of the manuscript. W. Guo and R. Gors contributed to the acquisition of data, and analysis and interpretation of data. K.L. Knopp contributed to the conception and design of the study and analysis and interpretation of data. She also revised, finalized and approved the version of the manuscript submitted.

Conflict of interest statement

All of the authors are employees of Eli Lilly & Company.

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Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.joca.2016.05.022.

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