Using in vivo calcium imaging and home cage behavioural analysis to study pain in mouse models of rheumatoid- and osteo-arthritis

George L Goodwin[1] PhD, Alina-Cristina Marin[1] PhD, Julia Vlachaki Walker[1] PhD, Carl Hobbs[1] & Franziska Denk[1] PhD

[1]Wolfson Sensory, Pain and Regeneration Centre (SPaRC), King's College London, SE1 1UL, UK

Corresponding author:

Dr George Goodwin

george.l.goodwin@kcl.ac.uk

+44 (0)2078486179

Wolfson Sensory, Pain and Regeneration Centre (SPaRC)

King's College London

London SE1 1U

The authors declare no conflicts of interest in relation to this work

Funding: F.D. and G.G. are funded by Advanced Pain Discovery Platform UKRI MRC grants (MR/W027518/1 & MR/W002426/1). A.C.M & J.V.W. are funded by a Wellcome Trust Collaborative Award (224257/Z/21/Z).

Abstract

Objective: The purpose of this study was to evaluate the use of *in vivo* calcium imaging to monitor activity in joint afferents and group-housed home cage monitoring for the assessment of pain-like behaviors in mouse models of rheumatoid- and osteo-arthritis.

Methods: Antigen induced arthritis (AIA) was used to model rheumatoid arthritis and partial medial meniscectomy (PMX) was used to model osteoarthritis. Group-housed home cage monitoring was used to assess behaviour in all mice, and weight bearing was performed in PMX mice. *In vivo* calcium with GCaMP6s was used to monitor spontaneous activity in L4 ganglion joint neurons retrogradely labelled with fast blue 2 days following AIA and 13-15 weeks following PMX. Cartilage degradation was assessed in knee joint sections stained with Safranin O and Fast Green in PMX mice.

Results: Antigen induced arthritis produced knee joint swelling and PMX caused degeneration of articular cartilage in the knee. In the first 46 hours following AIA, mice travelled less distance and were less mobile compared to their control cage mates. In contrast, these parameters were similar between PMX and sham mice between 4-12 weeks post-surgery. Joint neurons had increased spontaneous activity in AIA but not PMX mice. Spontaneous activity was mostly localized to medium-sized neurons in AIA mice and was not correlated with any of the home cage behaviors.

Conclusion: We conclude that group-housed home cage monitoring is a suitable technique for assessing joint pain behaviour in AIA mice. *In vivo* imaging of joint afferents revealed increased spontaneous activity in AIA but not PMX mice.

Introduction

Current analgesic treatments for arthritis pain are not sufficient. For instance, first line drugs for osteoarthritis (OA) pain are either ineffective and/or poorly tolerated[1]. Thus, we urgently need to better understand the mechanisms that lead to the development of pain in order to develop better analgesic medications. One limitation of this development is the comparatively low number of studies examining pain in preclinical models of arthritis. For instance, <10% of papers studying OA in mice examined pain between 2018-2023, and very few studies examine functional changes in neurons[2]. This omission may appear strange, given that pain has a huge impact on individuals living with arthritis[3]; this is until one is reminded of how challenging it is to study joint pain in rodents.

Functional changes to sensory neurons are particularly difficult to assess and traditionally have had to rely on highly specialized electrophysiological techniques[4-6]. These are very low-throughput and, in the context of arthritis, have to obtain recordings from the very small proportion of neurons that innervate joints e.g. only ~3-5% of lumbar sensory neurons project to the intra-articular space of the mouse knee[7]. *In vivo* calcium imaging is a relatively new approach for assessing sensory neuron function, which we and others have utilized to study models of arthritis[8-10]. However, thus far, only global changes in all types of sensory neurons within a given ganglion have been examined; important functional changes in joint afferents specifically may therefore have been 'diluted', seeing as this population is relatively small.

Another issue with studying pain in pre-clinical models of arthritis is the relative scarcity of robust and reproducible behavioural assays. Mice are now more commonly used than rats in pain behavioural studies[11]. However, they are a more challenging species in this regard, because they require longer habituation times and are more prone to experimenter induced stress compared to rats[11,12]. Static weight bearing behaviour is purported to be the 'gold-standard' for assessing joint pain in mice[13,14]. This test normally involves restraint, which can lead to stress-induced alterations of behavioural responses, such as modulation of nociception[15]. This might explain why the reproducibility of static weight bearing in surgical models of OA in mice is mixed: some groups find significant shifts in weight bearing at late

timepoints[14,16], whereas others do not[17,18]. Larger weight bearing shifts are seen in arthritis models that induce substantial knee swelling, like the Antigen-induced arthritis model (AIA)[18], however, these experiments are difficult to blind due to the visibly obvious phenotype induced by AIA.

Here, we set out to establish whether *In vivo* calcium imaging and group-housed home cage analysis could be used to overcome some of the challenges of studying joint pain in mice. Using the AIA model of rheumatoid arthritis and the partial medial meniscectomy (PMX) model of osteoarthritis in mice, we can record from multiple back-labelled joint afferents simultaneously. We observed increased spontaneous activity in AIA but not PMX mice. Home cage analysis showed significant reductions in activity following methylated bovine serum albumin (mBSA) injection in AIA mice, but not following PMX surgery.

Materials and Methods

Animals

Adult C57BL/6J male and female mice (n =69; Charles River, UK) were used in this study. Mice were housed on a 12/12 h light/dark cycle with a maximum of 4 mice per cage, with food and water available *ad libitum*. All experiments were performed in accordance with the United Kingdom Home Office Animals (Scientific Procedures) Act (1986). Animals undergoing behavioural experiments were housed in cages of 4 and were randomly assigned to treatment groups with 2 treatment and 2 controls in each cage. The experimenter assessing behaviour was blinded to the treatment group during testing and analysis.

Administration of calcium indicator

GCaMP6s was administered as previously described[9]. See Supplementary Methods for further details.

Retrograde labelling of knee joint afferents

10-12 week old mice were anesthetized using ketamine and xylazine (75mg/kg and 1mg/kg) and placed on a heating blanket. After shaving the knee and wiping it with 70% ethanol, the patellar tendon was identified and marked by running a 27g needle in the gap beneath the patella. A Hamilton syringe with a 30g needle attached was inserted through the patella tendon at a depth of 2-2.5 mm to deliver the retrograde

tracer fast blue (FB; 2 µl 2% in H2O; Polysciences) into the articular space. Mice were then left on a heating blanket for 1.5 hours to ensure that fast blue was up taken into joint afferents. After reversal of anesthesia with antisedan (1mg/kg), mice were allowed to recover for 1.5 hours in a heated chamber before being returned to their home cage.

Partial medial meniscectomy model

Twelve week-old male C57BL6/J mice (Charles River, UK) were randomized to undergo surgical destabilization by partial meniscectomy or sham surgery as previously described[19]. Briefly, animals were placed under general anesthesia by inhalation of isoflurane (Vetpharma, Leeds, UK); 3% induction, 2% maintenance in 1-1.5 L/min O2. 0.1 mg/kg buprenorphine (Vetergesic Alstoe Animal Health, UK) and 1mg/kg butorphanol (Zoetis,UK) were administered subcutaneously for peri- and post-operative analgesia, respectively. For PMX surgery, the knee joint was opened and the meniscotibial ligament was transected and approximately 1 mm of the medial meniscus removed. For the sham operation, the knee joint was opened to expose the meniscotibial ligament, then closed with sutures.

Antigen induced arthritis model

Mice were immunized using an emulsion of CFA (3.3 mg/ml; Sigma, UK) and mBSA (40 mg/ml; Sigma, UK), as described previously[20]. Briefly, mice were anesthetized using 2 % isoflurane, and 100 µl of the emulsion was subcutaneously injected at the base of the tail and in the right flank (50 µl each). Mice were then allowed to recover and were returned to their home cages. Seven days after immunization, mice were anesthetized with isoflurane, and 2.5 µl of mBSA (200µg) was injected into the left knee joint using a Hamilton syringe with a 30G needle. Two days post-injection, the size across ipsilateral and contralateral knee joints was measured using a digital caliper.

Static weight bearing

Weight bearing behaviour was assessed using a Linton incapacitance tester. Mice were acclimatized to the experimenter, room and weight bearing apparatus on at least 3 separate occasions prior to baseline testing. For each test session, the force exerted over a 1s period by each hind paw was measured five times. Measurements from each paw were averaged, and the percentage of weight born on each side was

calculated. Weight bearing was assessed at baseline and at 2-week intervals after osteoarthritis surgery.

Home cage analyzer

Mice were microchipped in the lower flank (identichip, Animalcare Ltd, UK) whilst anesthetized for the fast blue administration. Groups of four mice (n=2 treatment and n=2 control for RA and OA models and n=4 for naïve controls) that were housed in their home cages (GM500, Tecniplast) were placed into a home cage analyzer (Actual Analytics, UK)[21], and recorded at baseline and different time points after induction of the arthritis models. Time spent mobile, distance travelled, time spent isolated - defined as being over 20cm away from nearest mouse, and average distance separated from nearest cage mate were analyzed. The percentage change from baseline data was calculated using the night phase period (19:00-07:00). We did not analyze the anxiety-related parameters, such as thigmotaxis, because the GM500 home cage is too small and thus not sensitive enough to detect changes in these parameters (communication from Actual Analytics).

***In vivo* imaging of sensory neuron activity using GCaMP6s**

In vivo imaging was performed as previously described [9]. Briefly, mice were anesthetized using a combination of drugs: 1-1.25 g/kg 12.5% w/v urethane administered intraperitoneally and 0.5-1.5 % isoflurane delivered via a nose cone. Body temperature was maintained close to 37°C. An incision was made in the skin on the back, and the muscle overlying the L3, L4, and L5 DRG was removed. Using fine-tipped rongeurs, the bone surrounding the L4 DRG was carefully removed. The exposure was then stabilized at the neighboring vertebrae using spinal clamps (Precision Systems and Instrumentation, VA, USA) attached to a custom-made stage. Finally, the DRG was covered with silicone elastomer (World Precision Instruments, Ltd) to maintain a physiological environment. Prior to imaging, we administered a subcutaneous injection of 0.25 ml 0.9 % sterile saline to keep the mouse hydrated. It was then placed under an Eclipse Ni-E FN upright confocal/multiphoton microscope (Nikon). See supplementary methods for microscope settings.

Imaging data analysis

The researcher analyzing the data was blinded to the treatment group. Timelapse recordings were concatenated and scaled to 8-bit in Fiji/ImageJ, Version 1.53. The image analysis pipeline Suite2P (v 0.9.2)[22] was utilized for motion correction, automatic region of interest (ROI) detection and signal extraction. Further analysis was undertaken with a combination of Microsoft Office Excel 2013, Matlab (2018a) and RStudio (Version 4.02). A region of background was selected, and its signal subtracted from each ROI. To generate normalized ΔF/F0 data, the ROMANO toolbox ProcessFluorescentTraces() function was utilized[23]. This function uses the calculation: ΔF/F0 = (Ft-F0)/F0, where Ft is the fluorescence at time t and F0 is the fluorescence average over the first 3 minutes of recording. ΔF/F0 is expressed as a percentage.

The area of each neuron in the L4 DRG was estimated by using the radius extracted with Suite2P. Cells were grouped into small to medium (≤750µm^2) and large-sized (>750µm^2) neurons. Knee joint afferents (FB+ GCaMP6s+) were identified using a z-stack max projection of blue and green channels in ImageJ. The area of FB+ neurons was measured precisely using the free hand selection tool in ImageJ. Cells that could not clearly be identified and had substantial overlap with neighboring cells were excluded from the analysis.

A positive response to capsaicin was taken if the max signal during the 5-minute post-capsaicin period was 10 SD greater than the 2-minute baseline period. Neurons with spontaneous activity were identified using a trained machine learning classifier[9].

Mechanical stimulation of sensory afferents

Neurons in the foot were activated by pinching, using a pair of serrated forceps. We performed this stimulation because ROIs are identified in Suite2p based on their activity, i.e. this maximized the number of automatically detected ROIs because the foot is densely innervated by neurons originating in the L4 DRG.

***In vivo* application of capsaicin and lidocaine**

The sciatic nerve was exposed at the level of the midthigh prior to drug application. In some experiments, 50µl capsaicin (Sigma, UK; 1mM in 0.2% ethanol 0.9% saline)

was applied to the nerve. Any residual capsaicin was then removed from the nerve and lidocaine hydrochloride (Sigma, UK; dissolved in 0.9 % w/v sodium chloride to a concentration of 74 mM) was applied to block activity.

Knee joint histology and analysis

Knee joint sections were processed, cut and stained with Saffarin O/Fast Green - see supplementary methods for details.

Quantification and Statistical analysis

Graphing and statistical analysis was undertaken with a combination of Excel 2013, R Studio (Version 4.02), GraphPad Prism (Version 10) and SPSS (Version 29.0). Details of statistical tests and sample sizes are recorded in the appropriate figure legend, while observed effects & sensitivity analyses are provided in Supplementary Table 1.

Data loss and exclusion

Information on animal losses and data exclusions can be found in the Supplementary Methods.

Results

Partial medial meniscectomy causes loss of articular cartilage and antigen induced arthritis induces joint swelling

Histopathological analysis of the knee joints from PMX mice showed substantial loss and damage of articular cartilage compared to sham controls (F (1, 17) [Treatment] = 45.1, P<0.001; repeated measures 1-Way ANOVA; n=9-10/group; Figure 1A-D). The mean OARSI score in PMX mice on the medial side (4.26 +/- 0.37; Figure 1B&C) was significantly greater than the medial side of sham mice (0.91 +/- 0.17; p= 0.0000004; Bonferroni post hoc comparison; Figure 1B&D). As expected, cartilage loss was mostly confined to the medial portion of the joint in PMX mice (F (1, 17) [Knee side*Treatment] = 32.0, P<0.001; repeated measures 1-Way ANOVA; Figure 1C): the mean OARSI score in the medial side (4.26 +/- 0.37) was significantly greater than the lateral side (1.69 +/- 0.26; p= 0.000001; Bonferroni post hoc comparison).

Both PMX and sham mice showed reduced red staining in the superficial cartilage, which may indicate proteoglycan loss[24]. As this was unexpected in sham mice based on previous histopathological findings[24] and was not observed in naïve mice (data not shown), we wondered whether the prior intraarticular injection of fast blue into the knee joint caused these changes. To test this idea, we injected fast blue into joints of naïve mice and found that there was indeed discoloration of the superficial cartilage 7 days following fast blue but not saline injection (Supplementary Figure 1 A&B): the blue and green intensity ratios in the superficial cartilage vs. deep layer was significantly increased in fast blue compared to saline-injected mice (F (1, 5) [Group] = 151.8, P=0.00006; repeated measures 1-Way ANOVA, n=3-4/group; Supplementary Figure 2 C).

In mice that were previously immunized with mBSA, 2.5 µl mBSA (200µg) injection into the knee joint caused a significant increase in swelling in the ipsilateral knee (Ipsilateral/contralateral size ratio = 1.21 +/- 0.03) compared to mice injected with 2.5 µl saline (Ipsilateral/contralateral = 1.04 +/- 0.01; p = 0.00008, n=11/group; unpaired t-test).

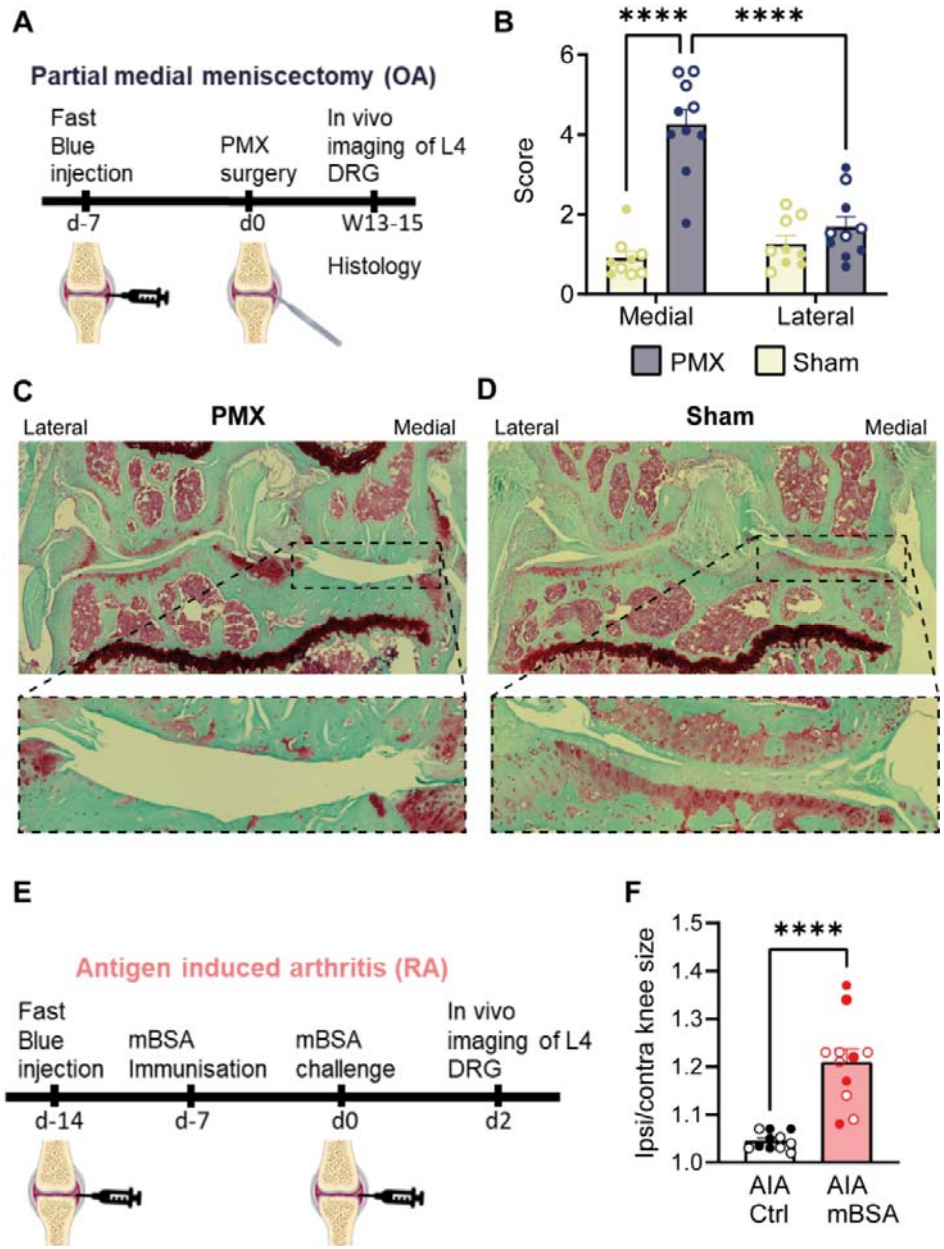

Figure 1. Partial medial meniscectomy causes loss of articular cartilage and mBSA injection causes joint swelling. A) Schematic showing experimental time course for the partial medial meniscectomy model of osteoarthritis. B) The average OARSI score to measure cartilage degradation for the medial and lateral compartments of the knee joint. Note that a minimum OARSI score of 0.5 was given to all samples because there was a lack of red staining in the superficial cartilage in all sections. C&D) Example images of Saffrin O & Fast Green-stained knee joint sections in PMX (left) and Sham (right) mice at 13-15 weeks post-surgery. E) Schematic showing experimental time course for the antigen induced arthritis model of rheumatoid arthritis. F) Ratio of the ipsilateral to contralateral knee size in AIA mice. Each point represents data from an individual animal. PMX: n= 9-10 mice/group AIA: n=11 mice/group. males = closed circles, females = open circles. PMX data: *** $p<0.001$ **** $p<0.0001$, repeated measures ANOVA. AIA data: $p<0.0001$, unpaired t-test.

Home cage analysis shows activity is reduced in antigen induced arthritis but not partial medial meniscectomy mice

We assessed whether group-housed home cage monitoring could be used to detect changes in activity following arthritis model induction (Figure 2A). As expected, mice were mostly quiescent during the light phase (07:00-19:00) and therefore we only analyzed the dark phase (19:00-07:00) during which mice are most active (Figure 2B&D and Supplementary figure 2A-B). Home cage data analysis in AIA mice revealed a main effect of treatment (F (1, 17) [Treatment group] = 10.9, P=0.0001; repeated measures 1-Way ANOVA; n=9-10/group; Fig 2A-E). Specifically, mBSA injected mice travelled less distance (between subject effect F (1, 17) [Distance] = 25.8, p=0.00009; Figure 2 B&C) and were less mobile (between subject effect F (1, 17) [Mobility] = 25.3, p=0.0001; Figure 2 D&E) compared to saline injected mice. Following mBSA injection, there was a reduction in distance (-44.0%, +/-5.3) and mobility (-21.6%, +/-5.0) during the second night phase, which was significantly different to the % change in saline injected mice at the same timepoint (distance = -22.2%, +/- 5.5; mobility = -0.7%, +/- 5.3; both p=0.011 Bonferroni post hoc comparison; Figure 2 C&E). In contrast, there were no significant changes to the amount of time spent isolated (between subject effect F (1, 17) [Isolation] = 2.4, p=0.14) and the average distance separated (between subject effect F (1, 17) [Separation] = 6.5, p=0.21; Supplementary figure 2 C-H).

In contrast to AIA mice, there was no significant effect of treatment in PMX OA experiments (F (1, 18) [Treatment group] = 0.5, P=0.72; repeated measures 1-Way ANOVA; n=10 group; Figure 3A-E), suggesting that changes to activity are not as severely impacted in this model. At 12-weeks following PMX surgery, the % change in distance (-12.8%, +/- 8.2) and mobility (-21.2 %, +/- 5.3) was similar to that of the sham group (distance = -16.4%, +/- 6.6; mobility = -17.8%, +/- 3.3; Figure 3 C&E). Similarly, the amount of time mice spent isolated and the average distance separated before and after surgery was comparable between PMX and Sham mice (Supplementary figure 3A-D).

Weight bearing was also assessed in the PMX mice. PMX mice began to shift weight from their ipsilateral to contralateral hind limbs at 8 weeks following surgery (Ipsilateral/contralateral ratio mean = 0.95 +/- 0.03, Figure 3F). By 12 weeks, the ipsilateral/contralateral ratio in PMX and sham mice was 0.91 (+/- 0.04, n=10) and 0.99 (+/- 0.02, n=10), respectively (Figure 3F). However, significant differences were not found at any timepoint (F (6, 108) [Treatment x Time] = 1, P=0.42; repeated measures 1-Way ANOVA; n=10 group).

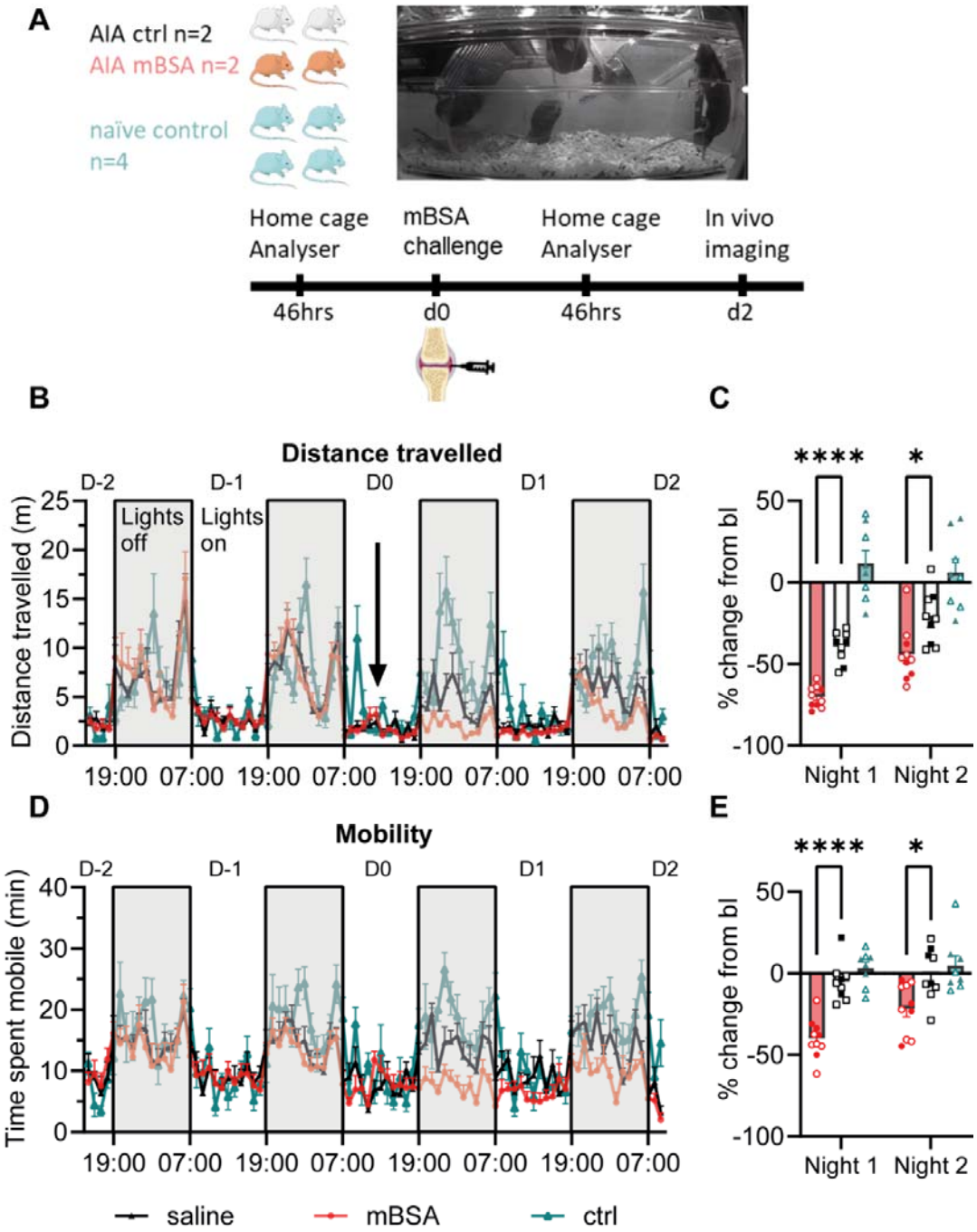

Figure 2. Home cage analysis shows that antigen induced arthritis mice are less active following mBSA injection. A) Schematic showing time course for the home cage analyzer experiments in AIA mice. AIA mice were grouped n=2 mBSA and n=2 saline, whereas naïve ctrl mice were in groups of 4. B-D) Graphs showing the total distance travelled (B&C) and time spent mobile (D&E) before and after intraarticular injections (indicated by arrow in B). Data in B) and D) are presented as mean +/- SEM in 1hr intervals. Data in C) and E) show the percentage change from baseline for the night phase (19:00-07:00). Grey bars represent the night phase. mBSA: n = 10, saline: n = 9. Males = closed circles, females = open circles. ****p<0.0001 *p<0.05, repeated measures 1-way ANOVA with Bonferroni adjusted post hoc comparisons.

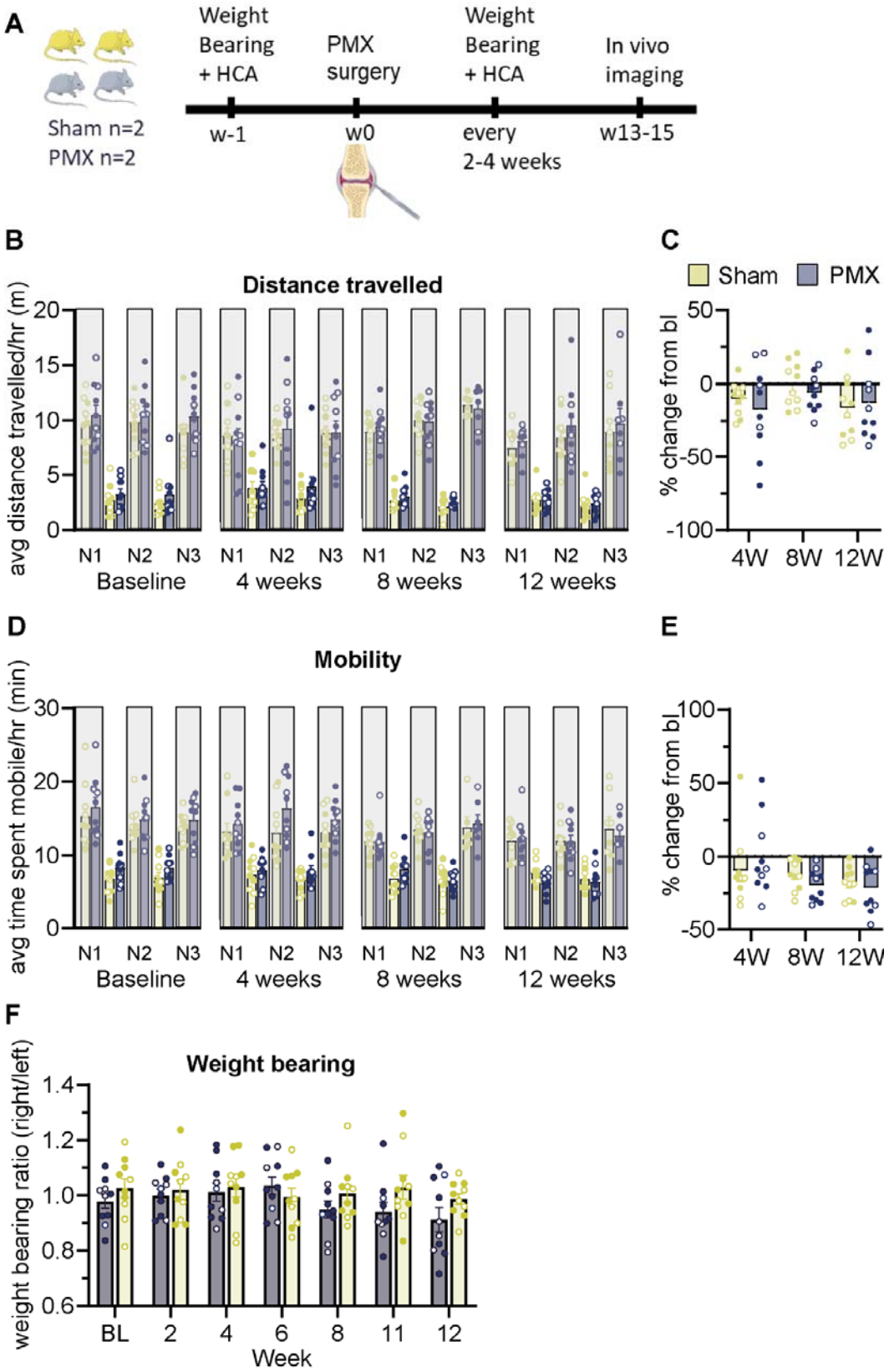

Figure 3. Home cage analysis shows that activity was similar in partial medial meniscectomy and sham mice.

Legend for Figure 3. A) Schematic showing time course for the home cage analyzer experiments in OA mice. OA mice were grouped n=2 PMX and n=2 sham. B-D) Graphs showing the total distance travelled (B&C) and time spent mobile (D&E) at baseline and following surgery. Data in B) and D) are presented as mean +/- SEM in 12hr intervals. Data in C) and E) show the percentage change from baseline for the night phase (19:00-07:00). Grey bars represent the night phase. F) Graph showing the weight bearing ratio (right/left hind limb) over time. Data are presented as mean +/- SEM. PMX: n = 10, sham: n = 10. Males = closed circles, females = open circles.

In vivo imaging of retrogradely labelled joint afferents reveals increased spontaneous activity in mBSA injected mice but not PMX mice.

We optimized our *In vivo* imaging approach for recording from fast blue retrogradely labelled joint afferents and used it to assess spontaneous activity in AIA and PMX models (Figure 4A and Supplementary Figure 4). We focused on spontaneous activity because reports indicate that it is the likely neurophysiological correlate of unpredictable or 'spontaneous' pain[25,26], which has a significant impact on quality of life in those with chronic arthritis pain[27].

The proportion of double positive fast blue (FB+)/GCaMP6s+ cells among all GCamP6s labelled neurons was similar between AIA (1.7% +/-0.1) and PMX cohorts (2.1% +/- 0.2; Supplementary figure 4B). There was no spontaneous activity in FB+ joint neurons of naïve mice (0/15 neurons, n=3 mice) and only one active neuron in saline injected mice (1/19 neurons, median = 0%, n=3 mice), suggesting that the injection does not cause substantial abnormal firing in joint afferents. In AIA mice, mBSA injection caused a significant increase in the proportion of FB+ neurons with spontaneous activity (14/63 joint neurons, median = 26.8, IQR = 35.7), compared to saline injected controls (1/61 joint neurons, median = 0, IQR = 0; P = 0.009, Kruskal Wallis test; n=10-11/group; Figure 2B&C and Supplementary table 2). In contrast, the proportion of spontaneously active neurons in all L4 neurons was similar between mBSA and saline injected mice for small to medium- and large-sized neurons (Figure 4B and Supplementary table 2).

PMX was unlike AIA, with the levels of spontaneous activity in FB+ joint neurons at 13-15 weeks following PMX injury (2/75 neurons, median = 0 IQR = 1.9) similar to that of sham mice (2/55 neurons median = 0 IQR = 7.2; p =0.11, Kruskal Wallis test; n=10/group; Figure 2D and Supplementary table 2).

In some experiments, we aimed to determine if spontaneously active neurons express TRPV1 by applying the agonist capsaicin (1mM) to the sciatic nerve. In TRPV1 expressing neurons, we anticipated that there would be depolarization i.e., increased calcium due to channel opening, followed by block[28,29], which is largely thought to be dependent on TRPV1 activation[29,30]. However, some spontaneously active neurons were blocked without any prior depolarization (Supplementary figure 5 and supplementary table 3), suggesting that the concentration of capsaicin applied (1mM) may have caused block independent of TRPV1, such as through inhibition of voltage gated sodium channels[31]. We were therefore unable to verify the proportion of spontaneously active joint neurons that express TRPV1.

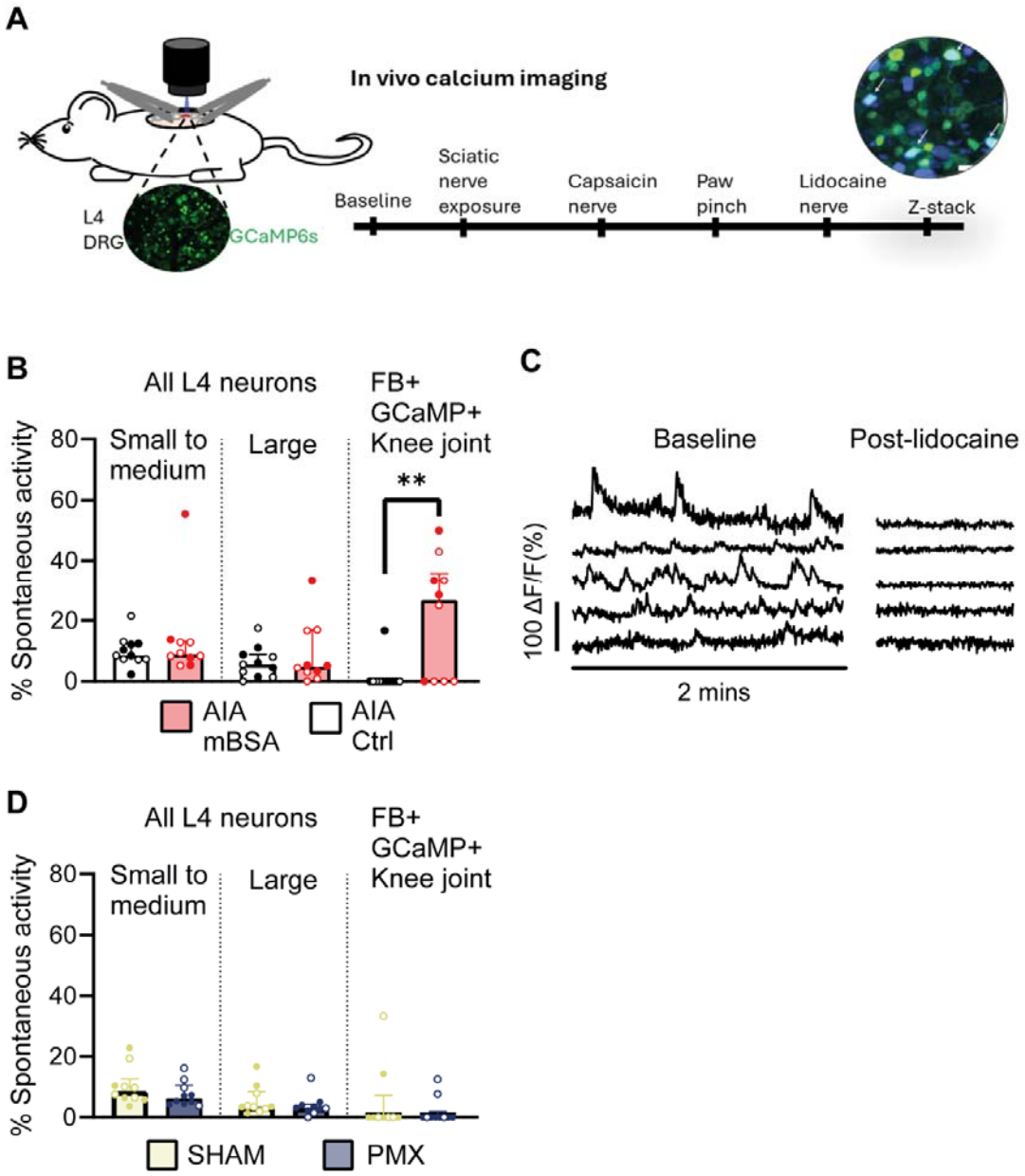

Figure 4. Increased spontaneous activity in joint afferents in mBSA injected mice but not in partial medial meniscectomy mice. A) Schematic showing *In vivo* calcium imaging experimental paradigm. B&D) Graphs showing the average proportion of neurons with spontaneous activity for small to medium, large and fb+ knee joints afferents in AIA (B) and OA (D) experiments. C) Example calcium traces from spontaneously active neurons in mBSA injected AIA mice before (left) and after (right) lidocaine. Small to medium at <750µm² and large at ≥750 µm². Each point represents data from an individual animal. OA weeks 13-15: n= 9-10 mice/group; AIA day 2: n=10-11 mice/group. males = closed circles, females = open circles. AIA data: ** p<0.01 Kruskal-Wallis test.

Spontaneously active neurons are predominantly medium-sized in mBSA injected-AIA mice

Retrogradely labelled joint neurons were mostly small to medium in size in AIA and PMX cohorts (Supplementary figure 6), as expected based on previous reports[32,33]. The only FB+ joint neuron with spontaneous activity in one of the saline-injected mice was small-sized (area = 237 μm^2). Conversely, spontaneous activity in small-sized (<400 μm^2) joint neurons was only recorded in one mBSA injected mouse out of a group of n = 6. In that animal, we recorded three small-sized spontaneously active FB+ neurons and unusually high overall levels of spontaneous activity (52.9% of all L4 neurons). Most other FB+ joint neurons with spontaneous activity following mBSA injection were medium-sized (9/14 neurons from n=6 mice; Figure 5 A&B, Supplementary video 1). The median area of spontaneously active neurons was 483 μm^2 (IQR = 225). We under-sampled from the smallest sized neurons (<350 μm^2) in AIA mBSA injected mice and therefore cannot rule out the possibility that there was also increased activity within this subgroup (Supplementary figure 6).

Most spontaneously active neurons were silent after application of lidocaine to the sciatic nerve (n = 10/11 neurons; Figure 5C).

We explored whether spontaneous activity in joint neurons was correlated with any of the home cage analyzer behaviour parameters in AIA mBSA injected mice. No significant correlations could be detected (Figure 6).

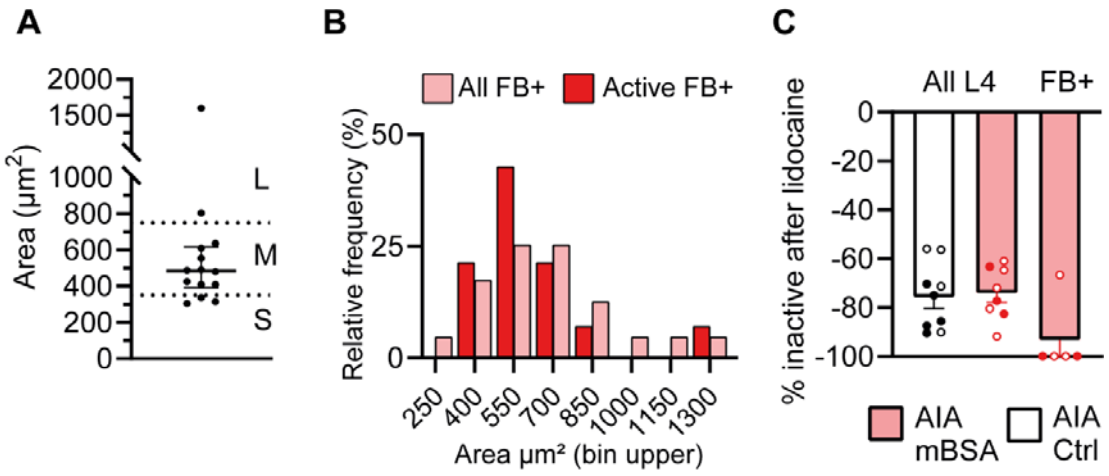

Figure 5. Most spontaneously active joint neurons are medium-sized and not active after peripheral lidocaine. A) Graph showing the size of the spontaneously active FB+ joint neurons in mBSA injected AIA mice. B) Size histogram showing the relative frequency of all FB+ joint neurons in mBSA injected mice (n=63) and those with spontaneous activity (n=14). Bin widths = 150 µm². The size of FB+ joint neurons was determined from z-stacks taken at the end of the recording. C) Graph showing the proportion of neurons that are not active following lidocaine application to the sciatic nerve.

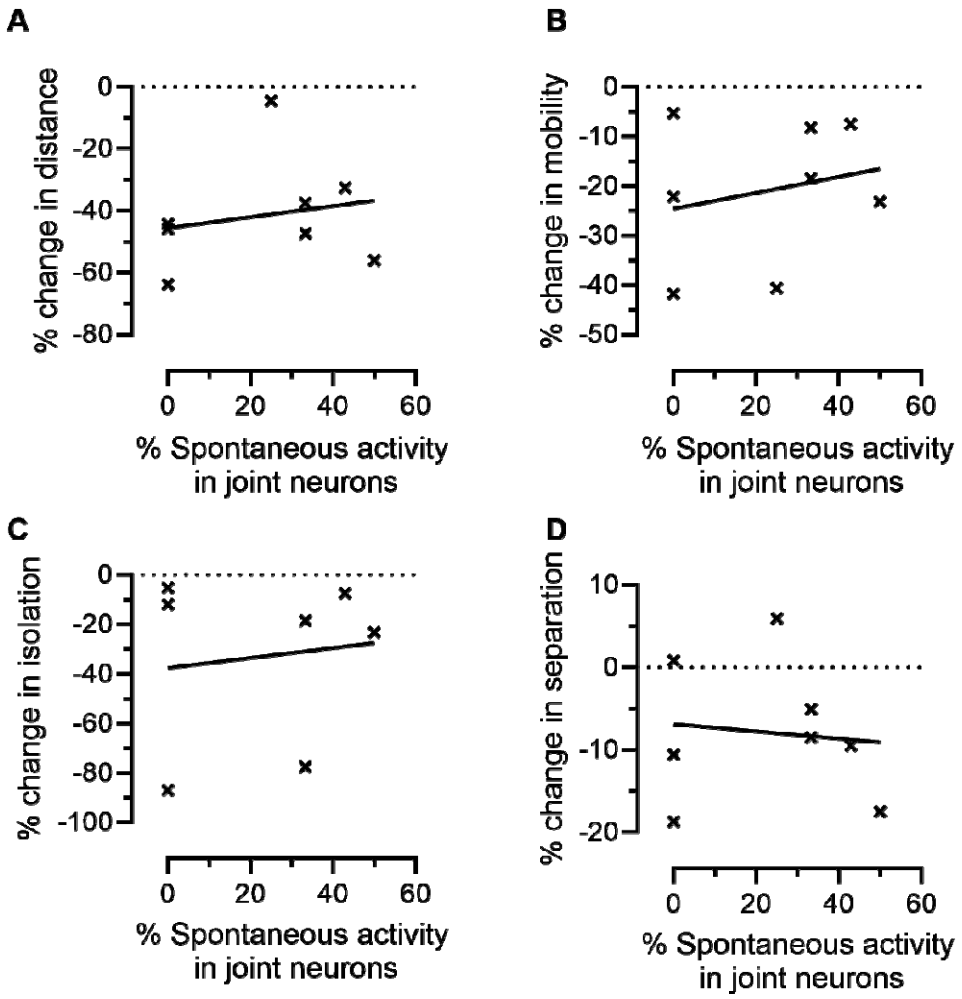

Figure 6. No correlations between joint neuron spontaneous activity and home cage analyzer behaviour parameters in antigen induced arthritis mice. A-D) Graphs showing the relationship between the % of joint neurons with spontaneous activity and the % change in Distance (A), mobility (B), isolation (C), separation (D) on night 2 in mBSA injected AIA mice. Pearson's correlations were as follows for distance (R^2 =0.04, P=0.31), mobility (R^2 =0.05, P=0.29), separation (R^2 =0.01, P=0.4) and isolation (R^2 =0.02, P=0.39). Data from n=8 mice.

Discussion

In this study, we set out to determine if *In vivo* calcium imaging and group-housed home cage monitoring could be used for studying joint pain in models of RA and OA. We have shown that *In vivo* imaging can be used to monitor activity in multiple retrogradely labelled joint afferents (~6/experiment) in the L4 DRG, which is greater than that sampled in a typical electrophysiology experiment (~1-2/experiment[4]). In the AIA model of RA, we found increased spontaneous activity in joint afferents 2-days following intraarticular injection of mBSA, which is predominantly localized to medium-sized neurons. In contrast, spontaneous activity was not elevated in joint afferents at 13-15 weeks in the PMX model of OA. Group-housed home cage monitoring showed AIA mice were less active than their control cage mates in the first 46 hours following mBSA injection, whereas no differences were found between PMX and sham mice between 4-12 weeks post-surgery.

While previous studies have used *In vivo* imaging to assess neuronal activity globally in entire sensory ganglia in models of arthritis[8-10], our study, is the first to monitor activity in retrogradely labelled joint afferents. Fast blue labelled joint afferents are likely those that innervate the synovium and ligaments[34,35], but not bone[32]. We found increased levels of spontaneous activity in joint afferents in AIA mice, which is consistent with a previous electrophysiological study[36], and suggests that these afferents are involved in driving pain, in this model. We found that spontaneously active neurons were mostly medium-sized, which suggests they might be thinly myelinated Aδ-fibres. This appears to contrast the C-fiber spontaneous activity reported by Qu et al.'s electrophysiological experiments[36]. However, some medium-sized neurons are unmyelinated C-fibers[37], making it possible that our work agrees with their findings.

We studied the PMX model at a late timepoint because spontaneous pain, which is associated with increased spontaneous activity[25,26], is reported to become more severe in the later stages of OA in humans[27]. It was therefore expected that spontaneous activity in joint afferents would be increased at a 'late' timepoint, I.e week 13-15, in the PMX model. However, in contrast to AIA mice, intraarticular afferents did not have increased spontaneous activity at a 'late' timepoint following PMX, which is consistent with reports from an electrophysiological study in the

monoiodoacetate (MIA) model of OA[4]. Contrastingly, there are reports of increased spontaneous activity at earlier timepoints in OA models[4,6,38]. Together, these data suggest that this form of peripheral sensitization contributes towards pain signaling in joint afferents at early, but not late stages of the disease. Is there increased spontaneous activity in other afferent populations, such as bone, at late-stage OA models? Our AIA data suggest that differences in spontaneous activity in a small group of neurons is missed when all neurons are examined collectively; since only ~2% of L4 neurons innervate bone[39], retrogradely labelling bone afferents is required to answer this question. A recent report found that there is no spontaneous activity in bone afferents at a late stage of the MIA model[4].

Group-housed home cage monitoring removes many out-of-cage confounding factors, such as changes to environmental parameters and handling, allowing for a more unbiased assessment of behaviour. Here, we used it to assess behavioural changes in models of arthritis. Our results indicate that the paradigm is sensitive enough to detect changes in activity-related parameters in the AIA model of RA, but not in the PMX model of OA (with n=10 mice/group). AIA mice are presumed to be less active because they are in pain i.e. forces generated from behaviors that are normally regarded as innocuous, such as moving about, activate sensitized joint mechanical nociceptors. We found no correlation between spontaneous activity and behavioural activity, suggesting that this form of peripheral sensitization is not sufficient to inhibit self-directed movement in mice. Our AIA data are consistent with previous studies using the LABORAS system in models of RA[14,40], showing that activity is a reproducible measure of behaviour change due to experimental RA. Meanwhile, our PMX home cage results mirror those of other studies using surgical models of OA in mice in the LABORAS system[14,41], but contrast others[42,43]. Together, these data suggest that overall locomotor activity is not a reproducible indicator of pain in surgical models of osteoarthritis. However, as most pre-clinical arthritis studies are only powered to detect large effect sizes (with n≤10 mice/group), it is possible that all studies so far have been underpowered.

Surprisingly, we also observed decreases in activity in our control mice for both models. It is not clear whether these changes were directly due to the control treatment i.e. the intraarticular injection or the sham surgery, or whether they were

mediated through a social interaction effect i.e. they move about less because their arthritic cage mates do.

Although we found a slight shift in weight bearing in PMX mice, it was not significantly different to the sham group, which is consistent with what others have reported[17,18]. The inconsistency of these results with the positive results that others have reported[14,16] could stem from the restraint required for the test, which causes stress. A recent report found that less variable results can be produced when mice are unrestrained[44]. Moreover, there could once again be a problem of statistical power: effects might not be large enough to be consistently detectable with the sample sizes commonly employed in the field.

We conclude that *In vivo* calcium imaging can be used together with retrograde labelling to assess functional changes in multiple joint neurons simultaneously. Changes in spontaneous activity are easily detectable at an acute timepoint in AIA mice, but not observed at a late timepoint in PMX mice. Similarly, we observed changes in home cage activity levels in the AIA model, but not the PMX model.

Acknowledgements

F.D. and G.G. are funded by Advanced Pain Discovery Platform UKRI MRC grants (MR/W027518/1 & MR/W002426/1). A.C.M & J.V.W. are funded by a Wellcome Trust Collaborative Award (224257/Z/21/Z). We thank Dr Jadwiga Zarebska, Dr Bryony Scott and Dr Vicky Batchelor for providing training on the PMX model, weight bearing behaviour and histology. We thank Dr Luke Pattison for assistance with the intra articular injection technique

This research was funded in whole or in part by UK Research & Innovation and the Wellcome Trust. For the purpose of Open Access, the author has applied a CC BY public copyright licence to any Author Accepted Manuscript (AAM) version arising from this submission.

Data will be shared in via the SPARC portal with accession information specified within the article.

Author contributions

Author contributions: G.G. conceived, designed, and performed experiments, analyzed data, and wrote the manuscript. F.D. provided conceptual input to the manuscript and edited the manuscript. C.H. performed histology. A.C.M and J.W. analyzed histological data and reviewed manuscript.

Data availability: data will be uploaded to the SPARC portal

References

1 da Costa, B. R. *et al.* Effectiveness and safety of non-steroidal anti-inflammatory drugs and opioid treatment for knee and hip osteoarthritis: network meta-analysis. *BMJ* **375**, n2321, doi:10.1136/bmj.n2321 (2021).

2 Vincent, T. L. & Miller, R. E. Molecular pathogenesis of OA pain: Past, present, and future. *Osteoarthritis Cartilage* **32**, 398-405, doi:10.1016/j.joca.2024.01.005 (2024).

3 Arthritis, V. The State of Musculoskeletal Health 2023. (2023).

4 Morgan, M., Thai, J., Nazemian, V., Song, R. & Ivanusic, J. J. Changes to the activity and sensitivity of nerves innervating subchondral bone contribute to pain in late-stage osteoarthritis. *Pain* **163**, 390-402, doi:10.1097/j.pain.0000000000002355 (2022).

5 Schaible, H. G. & Schmidt, R. F. Time course of mechanosensitivity changes in articular afferents during a developing experimental arthritis. *Journal of Neurophysiology* **60**, 2180-2195 (1988).

6 Kelly, S. *et al.* Spontaneous firing in C-fibers and increased mechanical sensitivity in A-fibers of knee joint-associated mechanoreceptive primary afferent neurones during MIA-induced osteoarthritis in the rat. *Osteoarthritis Cartilage* **20**, 305-313, doi:10.1016/j.joca.2012.01.002 (2012).

7 Chakrabarti, S. *et al.* Intraarticular Adeno-Associated Virus Serotype AAV-PHP.S–Mediated Chemogenetic Targeting of Knee-Innervating Dorsal Root Ganglion Neurons Alleviates Inflammatory Pain in Mice. *Arthritis & Rheumatology* **72**, 1749-1758, doi:https://doi.org/10.1002/art.41314 (2020).

8 Miller, R. E. *et al.* Visualization of Peripheral Neuron Sensitization in a Surgical Mouse Model of Osteoarthritis by In Vivo Calcium Imaging. *Arthritis Rheumatol* **70**, 88-97, doi:10.1002/art.40342 (2018).

9 Ingram, S. *et al.* Assessing spontaneous sensory neuron activity using in vivo calcium imaging. *Pain*, doi:10.1097/j.pain.0000000000003116 (2023).

10 Zhu, S. *et al.* Subchondral bone osteoclasts induce sensory innervation and osteoarthritis pain. *J Clin Invest* **129**, 1076-1093, doi:10.1172/jci121561 (2019).

11 Sadler, K. E., Mogil, J. S. & Stucky, C. L. Innovations and advances in modelling and measuring pain in animals. *Nat Rev Neurosci* **23**, 70-85, doi:10.1038/s41583-021-00536-7 (2022).

12 Callahan, B. L., Gil, A. S., Levesque, A. & Mogil, J. S. Modulation of mechanical and thermal nociceptive sensitivity in the laboratory mouse by behavioral state. *J Pain* **9**, 174-184, doi:10.1016/j.jpain.2007.10.011 (2008).

13 Batchelor, V., Miotla-Zarebska, J. & Vincent, T. L. REFINING METHODS TO MEASURE SPONTANEOUS PAIN BEHAVIOUR IN SURGICALLY INDUCED MURINE OSTEOARTHRITIS. *Osteoarthritis and Cartilage* **30**, S381, doi:10.1016/j.joca.2022.02.513 (2022).

14 von Loga, I. S. *et al.* Comparison of LABORAS with static incapacitance testing for assessing spontaneous pain behaviour in surgically-induced murine osteoarthritis. *Osteoarthr Cartil Open* **2**, 100101, doi:10.1016/j.ocarto.2020.100101 (2020).

15 Miczek, K. A., Thompson, M. L. & Shuster, L. Opioid-like analgesia in defeated mice. *Science* **215**, 1520-1522, doi:10.1126/science.7199758 (1982).

16 Gowler, P. R. W. *et al.* Interplay between cellular changes in the knee joint, circulating lipids and pain behaviours in a slowly progressing murine model of osteoarthritis. *Eur J Pain* **26**, 2213-2226, doi:10.1002/ejp.2036 (2022).

17 Knights, C. B., Gentry, C. & Bevan, S. Partial medial meniscectomy produces osteoarthritis pain-related behaviour in female C57BL/6 mice. *Pain* **153**, 281-292, doi:https://doi.org/10.1016/j.pain.2011.09.007 (2012).

18 Zaki, S., Smith, M. M. & Little, C. B. Pathology-pain relationships in different osteoarthritis animal model phenotypes: it matters what you measure, when you measure, and how you got there. *Osteoarthritis Cartilage* **29**, 1448-1461, doi:10.1016/j.joca.2021.03.023 (2021).

19 Blease, A. *et al.* Studying Osteoarthritis Pathogenesis in Mice. *Curr Protoc Mouse Biol* **8**, e50, doi:10.1002/cpmo.50 (2018).
20 Weiss, M. *et al.* IRF5 controls both acute and chronic inflammation. *Proceedings of the National Academy of Sciences* **112**, 11001-11006, doi:10.1073/pnas.1506254112 (2015).
21 Bains, R. S. *et al.* Analysis of Individual Mouse Activity in Group Housed Animals of Different Inbred Strains using a Novel Automated Home Cage Analysis System. *Front Behav Neurosci* **10**, 106, doi:10.3389/fnbeh.2016.00106 (2016).
22 Pachitariu, M. *et al.* Suite2p: beyond 10,000 neurons with standard two-photon microscopy. *bioRxiv*, 061507, doi:10.1101/061507 (2017).
23 Romano, S. A. *et al.* An integrated calcium imaging processing toolbox for the analysis of neuronal population dynamics. *PLOS Computational Biology* **13**, e1005526, doi:10.1371/journal.pcbi.1005526 (2017).
24 Glasson, S. S., Chambers, M. G., Van Den Berg, W. B. & Little, C. B. The OARSI histopathology initiative – recommendations for histological assessments of osteoarthritis in the mouse. *Osteoarthritis and Cartilage* **18**, S17-S23, doi:https://doi.org/10.1016/j.joca.2010.05.025 (2010).
25 Namer, B. *et al.* Pain relief in a neuropathy patient by lacosamide: Proof of principle of clinical translation from patient-specific iPS cell-derived nociceptors. *EBioMedicine* **39**, 401-408, doi:10.1016/j.ebiom.2018.11.042 (2019).
26 Kleggetveit, I. P. *et al.* High spontaneous activity of C-nociceptors in painful polyneuropathy. *Pain* **153**, 2040-2047, doi:10.1016/j.pain.2012.05.017 (2012).
27 Hawker, G. A. *et al.* Understanding the pain experience in hip and knee osteoarthritis--an OARSI/OMERACT initiative. *Osteoarthritis Cartilage* **16**, 415-422, doi:10.1016/j.joca.2007.12.017 (2008).
28 Petsche, U., Fleischer, E., Lembeck, F. & Handwerker, H. O. The effect of capsaicin application to a peripheral nerve on impulse conduction in functionally identified afferent nerve fibres. *Brain Research* **265**, 233-240 (1983).
29 Su, X., Wachtel, R. E. & Gebhart, G. F. Capsaicin sensitivity and voltage-gated sodium currents in colon sensory neurons from rat dorsal root ganglia. *Am J Physiol* **277**, G1180-1188, doi:10.1152/ajpgi.1999.277.6.G1180 (1999).
30 Liu, L., Oortgiesen, M., Li, L. & Simon, S. A. Capsaicin inhibits activation of voltage-gated sodium currents in capsaicin-sensitive trigeminal ganglion neurons. *J Neurophysiol* **85**, 745-758, doi:10.1152/jn.2001.85.2.745 (2001).
31 Isaev, D., Yang, K. S., Shabbir, W., Howarth, F. C. & Oz, M. Capsaicin Inhibits Multiple Voltage-Gated Ion Channels in Rabbit Ventricular Cardiomyocytes in TRPV1-Independent Manner. *Pharmaceuticals (Basel)* **15**, doi:10.3390/ph15101187 (2022).
32 Aso, K. *et al.* Nociceptive phenotype of dorsal root ganglia neurons innervating the subchondral bone in rat knee joints. *Eur J Pain* **18**, 174-181, doi:10.1002/j.1532-2149.2013.00360.x (2014).
33 Ivanavicius, S. P., Blake, D. R., Chessell, I. P. & Mapp, P. I. Isolectin b4 binding neurons are not present in the rat knee joint. *Neuroscience* **128**, 555-560 (2004).
34 Freeman, M. A. & Wyke, B. The innervation of the knee joint. An anatomical and histological study in the cat. *J Anat* **101**, 505-532 (1967).
35 McDougall, J. J., Bray, R. C. & Sharkey, K. A. Morphological and immunohistochemical examination of nerves in normal and injured collateral ligaments of rat, rabbit, and human knee joints. *Anat.Rec.* **248**, 29-39 (1997).
36 Qu, L. & Caterina, M. J. Enhanced excitability and suppression of A-type K(+) currents in joint sensory neurons in a murine model of antigen-induced arthritis. *Scientific reports* **6**, 28899-28899, doi:10.1038/srep28899 (2016).
37 Ruscheweyh, R., Forsthuber, L., Schoffnegger, D. & Sandkühler, J. Modification of classical neurochemical markers in identified primary afferent neurons with Aβ-, Aδ-, and C-fibers after chronic constriction injury in mice. *Journal of Comparative Neurology* **502**, 325-336, doi:https://doi.org/10.1002/cne.21311 (2007).

38 O'Brien, M. S. & McDougall, J. J. Comparison of nociceptor properties using electrophysiology in preclinical models of osteoarthritis. *Neuroscience Letters* **840**, 137950, doi:https://doi.org/10.1016/j.neulet.2024.137950 (2024).
39 de Clauser, L., Luiz, A. P., Santana-Varela, S., Wood, J. N. & Sikandar, S. Sensitization of Cutaneous Primary Afferents in Bone Cancer Revealed by In Vivo Calcium Imaging. *Cancers (Basel)* **12**, doi:10.3390/cancers12123491 (2020).
40 Zaki, S. *et al.* Characterisation of pain-related behaviours in association with joint pathology in an 8-week antigen-induced arthritis model. *Osteoarthritis and Cartilage* **22**, S36-S37, doi:10.1016/j.joca.2014.02.086 (2014).
41 Sambamurthy, N. *et al.* Chemokine receptor-7 (CCR7) deficiency leads to delayed development of joint damage and functional deficits in a murine model of osteoarthritis. *J Orthop Res* **36**, 864-875, doi:10.1002/jor.23671 (2018).
42 Miller, R. E. *et al.* CCR2 chemokine receptor signaling mediates pain in experimental osteoarthritis. *Proceedings of the National Academy of Sciences of the United States of America* **109**, 20602-20607, doi:10.1073/pnas.1209294110 (2012).
43 Inglis, J. J. *et al.* Regulation of pain sensitivity in experimental osteoarthritis by the endogenous peripheral opioid system. *Arthritis Rheum* **58**, 3110-3119, doi:10.1002/art.23870 (2008).
44 Obeidat, A. M. *et al.* Piezo2 expressing nociceptors mediate mechanical sensitization in experimental osteoarthritis. *Nature Communications* **14**, 2479, doi:10.1038/s41467-023-38241-x (2023).
45 Chen, T. W. *et al.* Ultrasensitive fluorescent proteins for imaging neuronal activity. *Nature* **499**, 295-300, doi:10.1038/nature12354 (2013).
46 Wang, F. *et al.* Sensory Afferents Use Different Coding Strategies for Heat and Cold. *Cell Reports* **23**, 2001-2013, doi:https://doi.org/10.1016/j.celrep.2018.04.065 (2018).
47 Xu Yang, V. C., Alberto Carli, Matthew B. Shirley, & Olufunmilayo Adebayo, M. C. H. v. d. M., F. Patrick Ross, Mathias P.G. Bostrom. 2016 Annual Meeting of the Orthopaedic Research Society Orlando, FL March 5-8, 2016 - Refining Intra-articular and Intramedullary Injection Techniques to Establish Joint, Bone and Periprosthetic Joint Infection in Mice. *J Orthop Res* **34 Suppl 1**, S1, doi:10.1002/jor.23247 (2016).