

1 The stage specific plasticity of descending modulatory 2 controls in a rodent model of cancer induced bone pain

3 **Mateusz Wojciech Kucharczyk^{1,2,*}, Diane Derrien², Anthony Henry Dickenson² and Kirsty
4 Bannister^{1,*}**

5 1 Central Modulation of Pain Group, Wolfson Centre for Age-Related Diseases, King's College London,
6 London SE1 1UL, UK

7 2 Department of Neuroscience, Physiology and Pharmacology, University College London, Gower Street,
8 WC1E 6BT London, UK

9 * Correspondence: mateusz.kucharczyk@kcl.ac.uk; Tel.: +44 2078484617; Fax: +44 2078486806 (M.W.K.),
10 Address: Wolfson Centre for Age-Related Diseases, Guy's Campus, King's College London, London, SE1
11 1UL, UK.

12 **Simple Summary:**

13 The mechanisms that underlie pain resulting from metastatic bone disease remain elusive. This
14 translates to a clinical and socioeconomic burden; targeted therapy is not possible, and patients do
15 not receive adequate analgesic relief. Complicating matters is the heterogeneous nature of
16 metastatic bone disease. Early stage cancers are molecularly very different to their late stage
17 counterparts and so too is the pain associated with infant and advanced tumours. Thus, analgesic
18 approaches should differ according to disease stage. In this article we demonstrate that a unique
19 form of brain inhibitory control responsible for modulation of incoming pain signals at the level of the
20 spinal cord changes with the progression of bone tumours. This corresponds with the degree of
21 damage to the primary afferents innervating the cancerous tissue. Plasticity in the modulation of
22 spinal neuronal activity by descending control pathways reveals a novel opportunity for targeting
23 bone cancer pain in a stage-specific manner.

24 **Abstract:** Pain resulting from metastatic bone disease is a major unmet clinical need. Studying
25 spinal processing in rodent models of cancer pain is desirable since the percept of pain is influenced
26 in part by modulation at the level of the transmission system in the dorsal horn of the spinal cord.
27 Here a rodent model of cancer induced bone pain (CIBP) was generated following syngenic rat
28 mammary gland adenocarcinoma cell injection in the tibia of male Sprague Dawley rats. Disease
29 progression was classified as 'early' or 'late' stage according to bone destruction. Even though
30 wakeful CIBP rats showed progressive mechanical hypersensitivity, subsequent *in vivo*
31 electrophysiological measurement of mechanically evoked deep dorsal horn spinal neuronal
32 responses revealed no change. Rather, a dynamic reorganization of spinal neuronal modulation by
33 descending controls was observed, and this was maladaptive only in the early stage of CIBP.
34 Interestingly, this latter observation corresponded with the degree of damage to the primary
35 afferents innervating the cancerous tissue. Plasticity in the modulation of spinal neuronal activity by
36 descending control pathways reveals a novel opportunity for targeting CIBP in a stage-specific
37 manner. Finally, the data herein has translational potential since the descending control pathways
38 measured are present also in man.

39 **Keywords:** Cancer-induced bone pain (CIBP); Diffuse Noxious Inhibitory Controls (DNIC); Wide
40 Dynamic Range Neurons; Neuronal Damage; Tibial afferents; *In vivo* electrophysiology; Mechanical
41 hypersensitivity.

42 **1. Introduction**

43 The mechanisms that underlie pain resulting from bone cancer remain only partially understood.
44 This translates to a clinical and socioeconomic burden; targeted therapy is not possible and patients
45 do not receive adequate pain relief. Complicating matters is the heterogeneous nature of metastatic
46 bone disease. Not only does the individual's pain phenotype depend on genetic, emotional and
47 sensory factors, but also on the progression of the disease. Early stage cancers are very different to

48 their late stage counterparts and so too is the pain associated with infant and advanced tumours,
49 which may be primary or metastatic.

50 Animal models of bone cancer are essential to better understand the underlying mechanisms
51 that drive this distinct pain state. We have previously shown that injection of syngenic rat mammary
52 gland adenocarcinoma (MRMT-1) cells in the rat tibia, which manifests a pre-clinical model of cancer
53 induced bone pain (CIBP) [1], causes increased sensory input to the central nervous system
54 quantified as the recruitment and activation of normally mechanically insensitive nociceptors at day
55 14 post-injection [2]. As expected, progressive tumour burden also reflects plasticity in central (spinal)
56 events [3–6]. However, hitherto there is a dearth of data regarding the impact of disease progression
57 on the evoked activity of spinal cord deep dorsal horn wide dynamic range (DDH-WDR) neurons.
58 These neurons are of interest since they form a crucial component of spinal neuronal circuits that
59 receive sensory information from the periphery as well as modulation from descending
60 brainstem-origin pathways. In total, their activity reflects global changes in spinal nociceptive
61 processing.

62 Diffuse noxious inhibitory controls (DNIC) represent a unique top-down modulatory pathway that
63 acts to endogenously reduce the percept of pain via inhibition of DDH-WDR neuronal activity [7–9].
64 DNIC and its human counterpart conditioned pain modulation (CPM) is dysfunctional in rodent
65 models of chronic pain and chronic pain patients respectively [9–12]. We propose that investigating
66 the functionality of the DNIC pathway in CIBP rats is clinically relevant since pain phenotyping of
67 patients with bone cancer pain has begun to include a measurement of CPM (ClinicalTrials.gov
68 Identifier: NCT03908853) using a paradigm previously shown to translate between rodent and man
69 [13].

70 It is highly likely that the mechanisms underlying the development of pain in CIBP rats are
71 progressive and therefore representative of distinct molecular changes. We first aim to marry disease
72 progression to behavioural readouts. Thereafter, using an *in vivo* electrophysiological approach, we
73 will measure the evoked responses of DDH-WDR neurons. Is mechanical hypersensitivity in
74 response to threshold and suprathreshold stimulation evidenced behaviourally and
75 electrophysiologically, respectively? Does the top-down modulation of DDH-WDR neurons undergo
76 dynamic maladaptive plasticity, and does this occur in a stage specific manner? Finally, we will
77 investigate whether or not any behavioural and/or spinal changes correlate with a marker of cellular
78 stress in those afferents that innervate the cancer-bearing tibia, this in order to link peripheral and
79 central events.

80 **2. Results**

81 *2.1. Bone destruction in a rat model of cancer induced bone pain and early/late stage classification*

82 Following generation of a validated rat CIBP model using syngenic mammary gland carcinoma
83 cells [1] bone damage caused by cancer growth was evaluated using a high-resolution
84 micro-computer tomography technique (μ CT) at two different time points: days 7/8 and days 14/15.
85 Significant damage to the trabecular bone occurred in both stages, while cortical bone was mostly
86 impaired at days 14/15, suggestive of early versus late stage modelling of CIBP as classified in our
87 previous publication [2] (Fig. 1A).

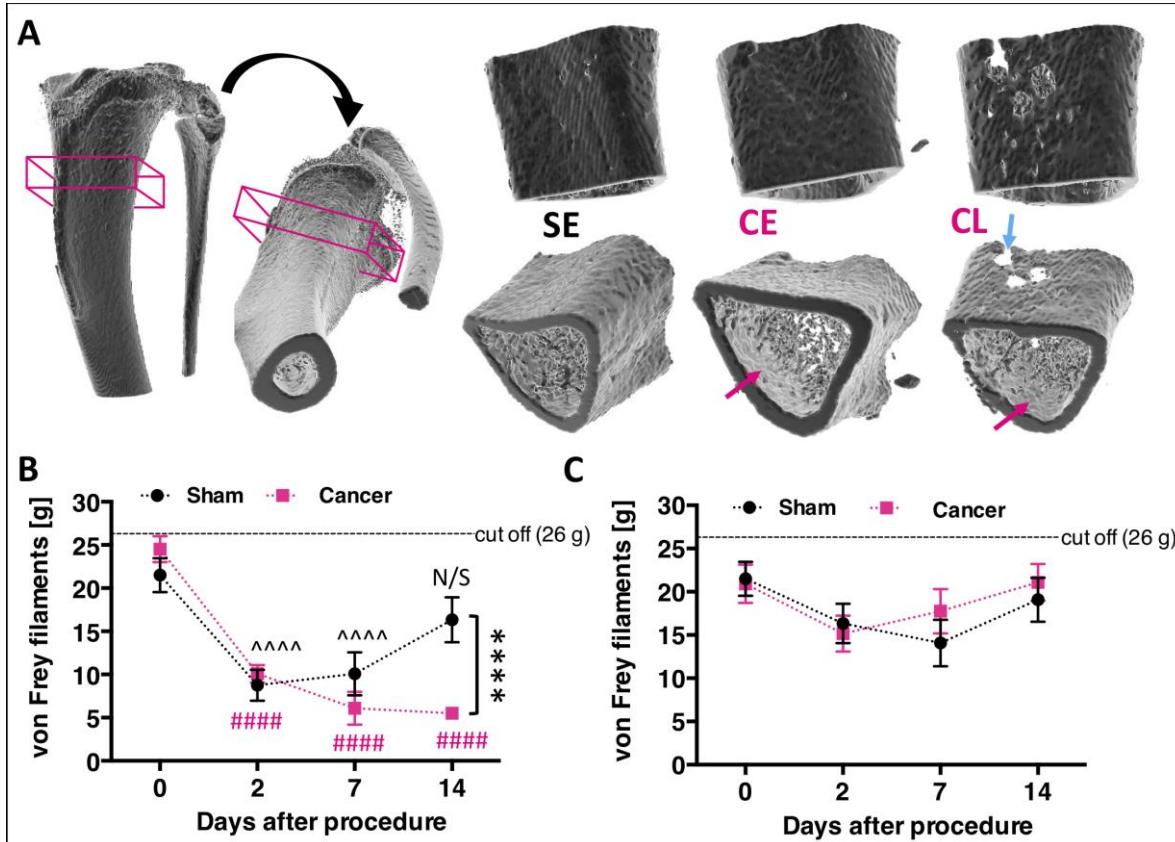


Figure 1. Progressive cancer-evoked bone destruction is reflected in the development of mechanical allodynia. (A) Example microcomputer tomography 3D-rendered rat tibia with corresponding orthogonal projections. The boxed area represents centre of the cancer-growth zone and constitutes of 114 z-scans taken every 34 µm. Example of the reconstructed cancer growth zone in sham early (SE), cancer early (CE, day 7/8), and cancer late (CL, day 14/15) stages are shown. Blue arrow points at cortical bone lesion, and red arrows point at trabecular bone lesions. See Movie 1 for 360° view. **(B)** Von Frey filaments-tested mechanical hypersensitivity progresses following bone surgery, substantially differing from sham-operated control by the late cancer stage (Two-Way ANOVA with Bonferroni post-hoc: ^~~~Sham, #####Cancer vs. day 0, p < 0.0001. Day 14 Sham vs. Cancer: ***p < 0.0001, n = 12 per group). **(C)** No changes in mechanical sensitivity were observed on the contralateral paw during the whole course of the experiment (Two-Way RM-ANOVA with Bonferroni post-hoc, p > 0.05, n = 12 per group). Results represent mean ± SEM.

101 2.2. CIBP rats exhibit secondary mechanical hypersensitivity

102 Since tumour progression is expected to relate to animal behaviour, rats were monitored up to 14 days post-surgery. While body weight gain remained stable in all groups, the behavioural data 103 demonstrate that CIBP rats manifest mechanical hypersensitivity. These results correspond to other 104 studies using similar rodent models of CIBP [1,5]. The presence of secondary mechanical 105 sensitization was assessed in sham operated (n = 12) and cancer bearing (n = 12) rats using the von 106 Frey test. CIBP rats exhibited mechanical allodynia on the side ipsilateral to cancer cell injection in the 107 late stage (from day 14 post-surgery) (Two-Way RM ANOVA [group]: $F_{[1, 11]} = 5.263$, p = 0.0425, with 108 Bonferroni post-hoc: day 0 – day 7: p > 0.05, day 14: p < 0.0001) (Fig. 1B). Both animal groups 109 experienced postsurgical pain in the week following surgery as revealed by a lowered mechanical 110 threshold for von Frey filaments that lasted up until day 7 (Two-Way RM ANOVA [time]: $F_{[3, 33]} = 24.05$, 111 p < 0.0001, Bonferroni post-hoc vs. day 0: Sham (day 2 and day 7): p < 0.0001, Cancer (day 2-14): p 112 < 0.0001). No differences were detected on the contralateral sites in either analysed group (Two-Way 113 RM ANOVA [group]: $F_{[1, 11]} = 0.22$, p = 0.648, Two-Way RM ANOVA [time]: $F_{[3, 33]} = 3.57$, p = 0.244, 114 Bonferroni post-hoc vs. day 0: all p > 0.05) (Fig. 1C).

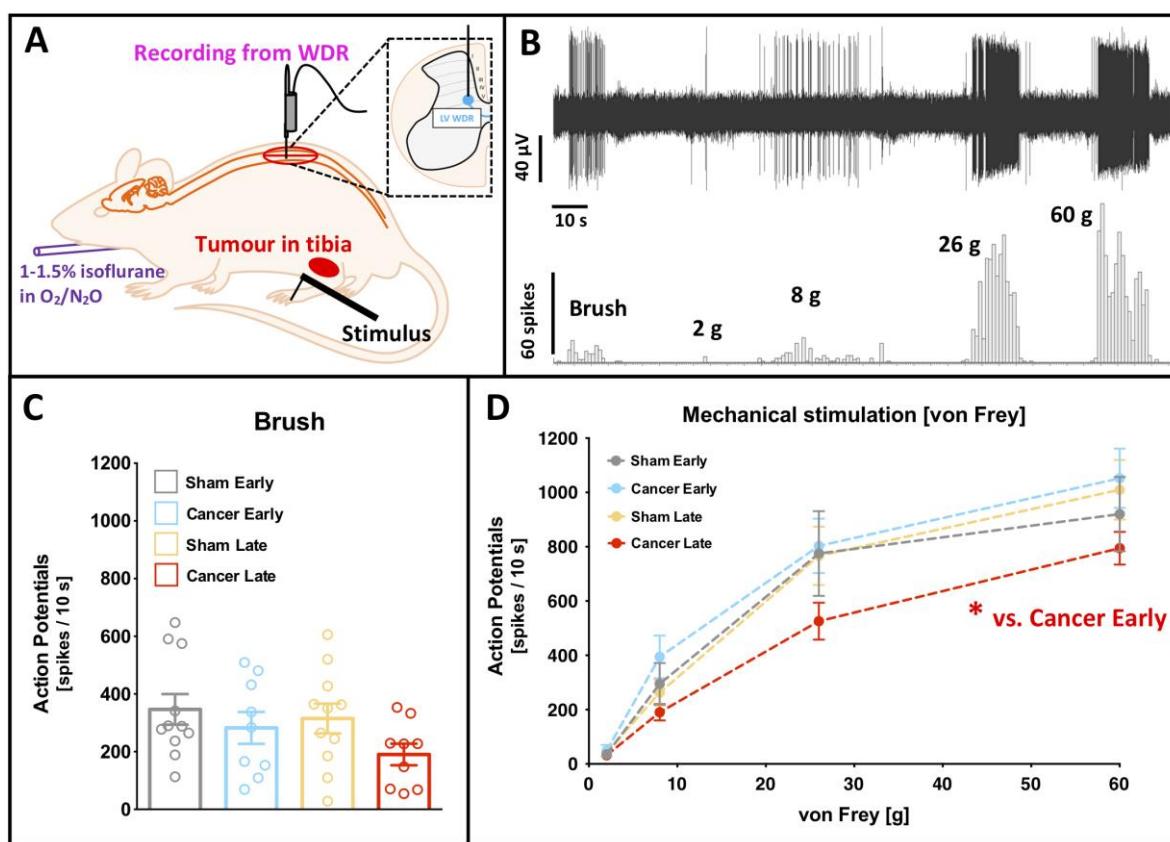


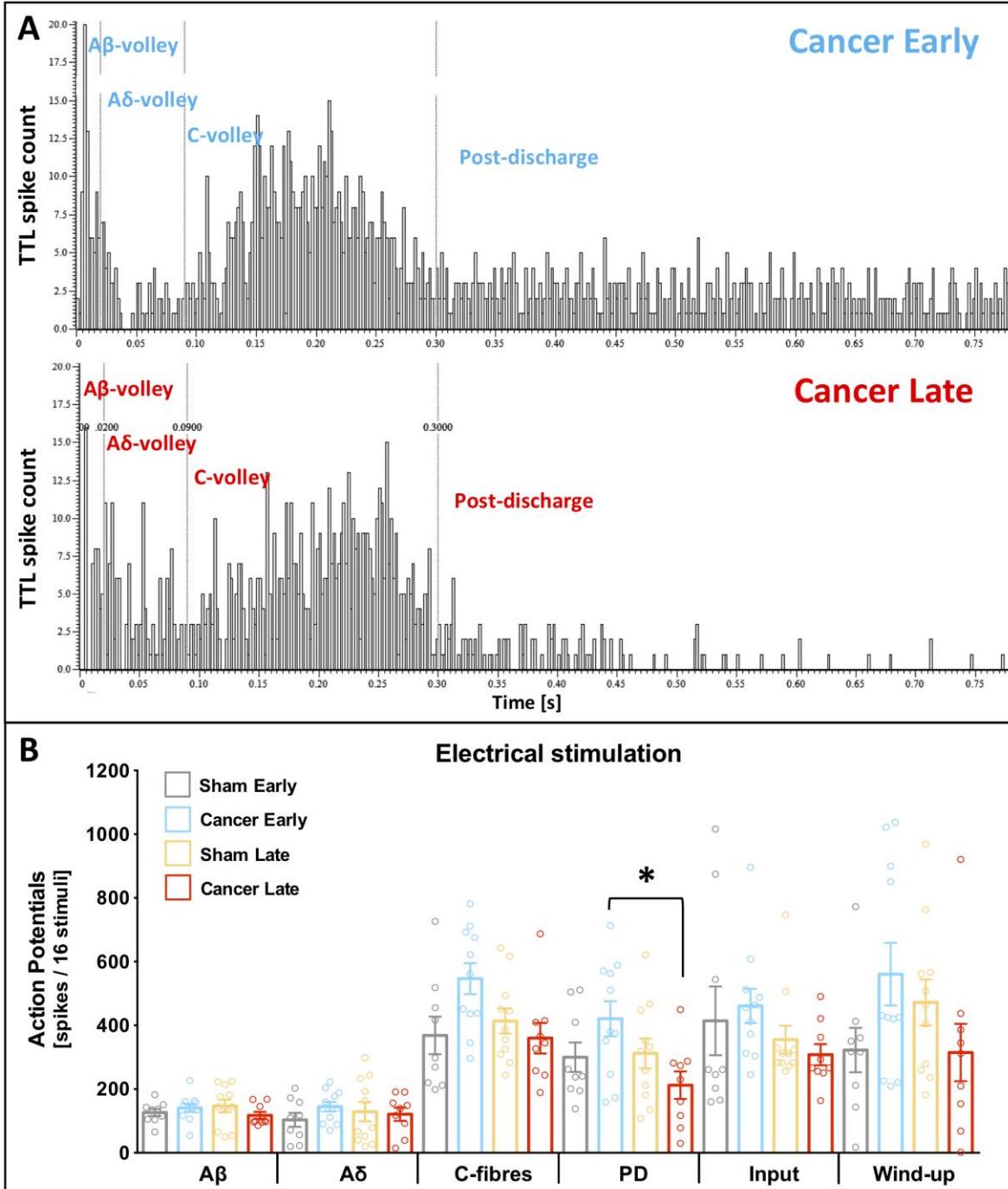
Figure 2. Deep dorsal horn wide dynamic range neurons are less excitable in the late cancer stage to noxious mechanical stimuli. (A) Schematic representation of the *in vivo* electrophysiological experiment. WDR, wide-dynamic range neurons. **(B)** Example of a single cell deep dorsal horn lamina V WDR neuronal responses to dynamic brushing and punctate mechanical stimulation (von Frey filaments) of the receptive field (paw ipsilateral to the cancer) in the late stage bone cancer rat. **(C)** Dynamic brushing-evoked responses of lamina V WDR in early (day 7-8) and late (day 14-16) cancer stage and corresponding sham-operated rats. Each dot represents one animal. One-way ANOVA with Bonferroni post-hoc: $p > 0.05$. **(D)** Von Frey-evoked responses of lamina V WDR neurons in early (day 7-8) and late (day 14-16) cancer stage and corresponding sham-operated rats. One-way ANOVA with Bonferroni post-hoc: * $p < 0.05$ cancer early vs. cancer late. All the data represent the mean \pm SEM from sham early ($n = 9$), cancer early ($n = 11$), sham late ($n = 11$) and cancer late ($n = 9$).

2.3. Deep dorsal horn wide dynamic range neurons are not hyperexcitable in CIBP rats at early or late stages

The activity of DDH WDR neurons was studied in rats under light isoflurane/ N_2O/O_2 anaesthesia (slight toe pinch reflex maintained) (Fig. 2A). *In vivo* electrophysiological recordings of DDH WDR neurons were used to study von Frey and brush-evoked firing rates. An example neuronal recording is shown (Fig. 2B). Stable baseline neuronal recordings from sham early (SE, $n = 9$), cancer early (CE, $n = 11$), sham late (SL, $n = 11$) and cancer late (CL, $n = 9$) were made. One neuron was studied per animal. Animals with cancer (early or late stage) showed no significant change in the basal firing rate of WDR neurons when compared to sham-operated WDR neuronal firing rates. Dynamic brushing of the receptive field (localised typically on the paw) revealed no significant difference between all analysed groups (univariate ANOVA [group]: $F_{3, 36} = 1.708$, $p = 0.183$) (Fig. 2C). Analysis of variance revealed no significant changes in the basal von Frey-evoked activity (Two-Way RM ANOVA [von Frey] $F_{[3, 108]} = 217.5$, $p < 0.0001$, [group] $F_{[3, 36]} = 1.257$, $p = 0.304$, Bonferroni post hoc: all $p > 0.05$) (Fig. 2D).

A train of 16 electrical impulses to the receptive field (localised on the hind paw toes ipsilateral to injury) was also applied to verify changes in the basal spinal coding and temporal summation. Example cumulative post-stimulus histograms generated after the delivery of 16 train stimuli from

146 cancer early and late stage rats are shown (Fig. 3A). Electrically evoked parameters: A β - ($F_{[3, 36]} = 0.815$, $p = 0.494$), A δ - ($F_{[3, 36]} = 0.543$, $p = 0.656$) and C- fibre ($F_{[3, 36]} = 3.251$, $p = 0.033$, Bonferroni post-hoc: all $p > 0.05$) evoked activity and input ($F_{[3, 36]} = 1.112$, $p = 0.357$) and wind-up ($F_{[3, 36]} = 2.005$, $p = 0.131$) were all unchanged between groups (all: univariate ANOVA [group]) (Fig. 3B). Interestingly, post-discharge was significantly reduced in the late stage cancer animals as compared to the early stage ones ($F_{[3, 36]} = 3.063$, $p = 0.040$, Bonferroni post-hoc: all $p > 0.05$ but CE vs. CL $p = 0.029$) (Fig. 3B).



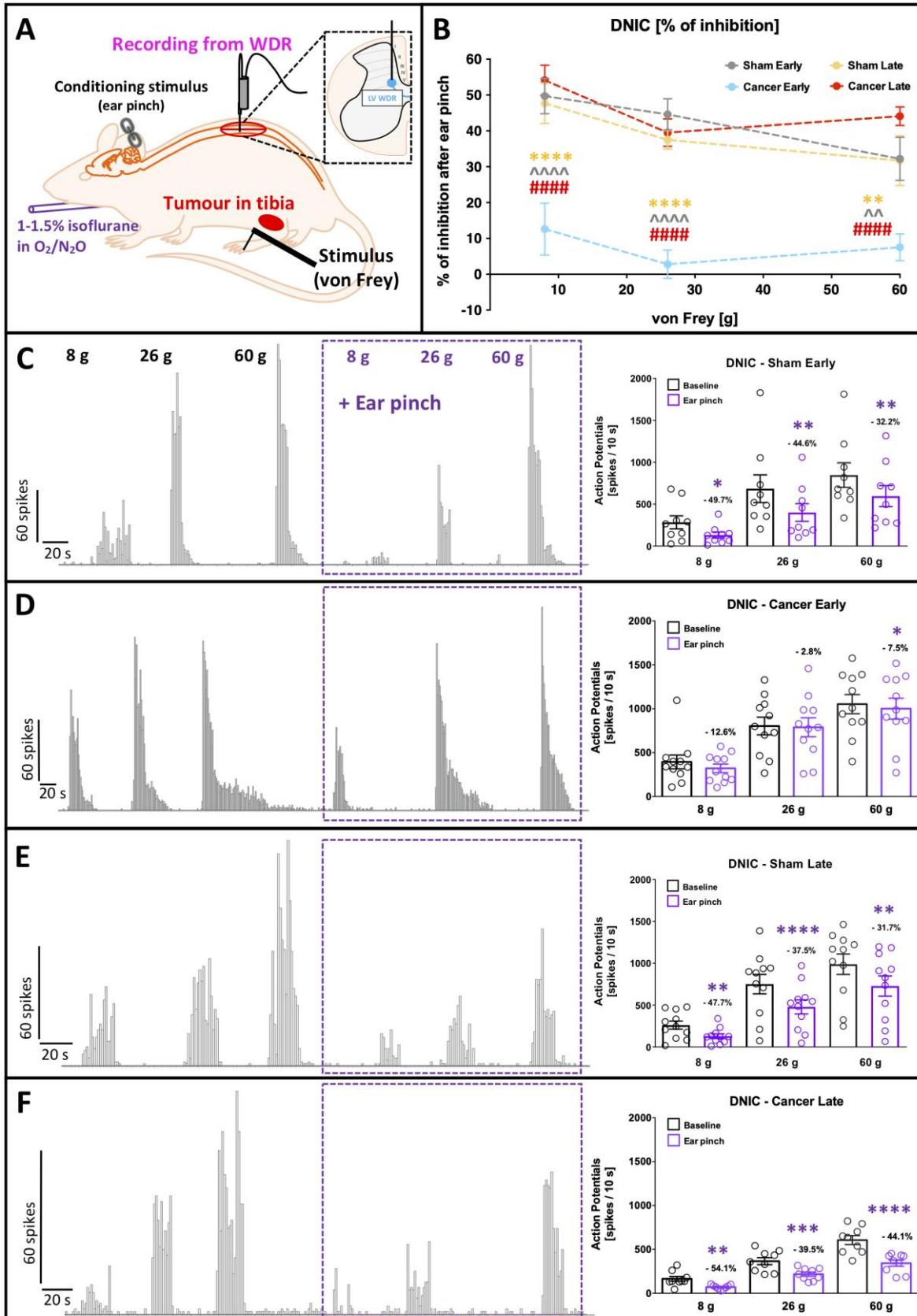
153
154 **Figure 3. Deep dorsal horn wide dynamic range neurons exhibit shortened post**
155 **discharge in the late cancer stage. (A)** Examples of the post stimulus histograms generated from a
156 **single cell deep dorsal horn lamina V wide-dynamic range (WDR) neuronal responses recorded in**
157 **vivo from anaesthetised cancer early (day 7/8, top panel) and late (day 14/15, bottom panel) rats.**
158 **Rats received subcutaneous injection of the current to the peripheral receptive field located on the**
159 **ipsilateral paw: train of 16 stimuli, 0.5 Hz, 2 ms pulse width, 7.8 ± 1.2 mA mean current. (B)**
160 **Electrically-evoked responses of lamina V WDR neurons in early (day 7-8) and late (day 14-16)**

161 cancer stage and corresponding sham-operated rats. Each dot represents one animal. One-way
162 ANOVA with Bonferroni post-hoc: all $p > 0.05$, but post discharge (PD) * $p < 0.05$ cancer early vs.
163 cancer late. All the data represent the mean \pm SEM from sham early ($n = 9$), cancer early ($n = 11$),
164 sham late ($n = 11$) and cancer late ($n = 9$).

165 *2.4. Diffuse noxious inhibitory controls are dysfunctional in the early, but not late, stage of disease*

166 Previous research has shown that descending modulation of DDH-WDR neurons by the
167 inhibitory DNIC pathway is compromised in rodent models of nerve injury and inflammation [9,10]. In
168 the present study, despite the manifestation of behavioural hypersensitivity, the baseline
169 evoked-activity of DDH-WDR neurons was comparable between sham operated and cancer rats.
170 Therefore, we sought to investigate whether or not descending modulation of those same neurons
171 was dysfunctional during disease progression. DNIC expression was studied in the four
172 aforementioned experimental groups under light isoflurane/ N_2O/O_2 anaesthesia (slight toe pinch
173 reflex maintained). Terminal electrophysiological recordings of DDH WDR neurons were used to
174 study the von Frey-evoked firing rate changes upon simultaneous ipsilateral application of noxious
175 conditioning stimulus (ear pinch) to evoke DNIC (Fig. 4A). One neuron was studied per animal. DNIC
176 were expressed in SE, SL and CL animals, resulting in around 50%, 40% and 30% inhibition of the
177 evoked action potentials to 8 g, 26 g and 60 g von Frey application, respectively (Two-Way RM
178 ANOVA [von Frey] $F_{[2, 72]} = 6.887$, $p = 0.0018$) (Fig. 4B). Interestingly, DNIC expression was impaired
179 in CE rats (Two-Way RM ANOVA [group] $F_{[3, 36]} = 34.66$, $p < 0.0001$, Bonferroni post hoc: [8 g] CE vs.
180 all $p < 0.0001$, [26 g] CE vs. all $p < 0.0001$, [60 g] CE vs. SE and SL $p < 0.01$, CE vs. CL $p < 0.0001$)
181 (Fig. 4B).

182 In SE animals, DDH WDR neuronal activity was significantly inhibited after ear-pinch when
183 compared to baseline for all von Frey filaments (RM ANOVA: $F_{[3, 6]} = 7.669$, $p = 0.018$; Bonferroni post
184 hoc [8 g] $p = 0.019$, [26 g] $p = 0.003$, [60 g] $p = 0.001$) (Fig. 4C). In contrast, DDH WDR neuronal
185 responses in CE rats were not inhibited (RM ANOVA: $F_{[3, 8]} = 2.606$, $p = 0.124$) (Fig. 4D). In SL rats
186 DNIC was expressed (RM ANOVA: $F_{[3, 8]} = 13.766$, $p = 0.002$; Bonferroni post hoc [8 g] $p = 0.004$, [26
187 g] $p = 0.000047$, [60 g] $p = 0.00106$) (Fig. 4E), as well as in CL animals (RM ANOVA: $F_{[3, 6]} = 37.513$,
188 $p = 0.00028$; Bonferroni post hoc [8 g] $p = 0.001872$, [26 g] $p = 0.000204$, [60 g] $p = 0.000004$) (Fig.
189 4F).



190

191

Figure 4. Diffuse noxious inhibitory controls expression is compromised in early but not

192

in the late cancer stage. (A) Schematic representation of the *in vivo* electrophysiological experiment. WDR, wide-dynamic range neurons. Activation of diffuse noxious inhibitory controls (DNIC) is quantified as a decrease in von Frey-evoked spinal WDR neuronal firing before (baseline; testing stimulus) and after concomitant application of noxious ear-pinch (DNIC; conditioning stimulus). **(B)** Magnitude of DNIC expression quantified as a percentage of WDR neuron inhibition

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197 following ear-pinch application in early (day 7-8) and late (day 14-16) cancer stage and corresponding
198 sham-operated rats. One-way ANOVA with Bonferroni post-hoc: ***p < 0.0001 cancer early vs. all
199 other groups. **(C-F)** Example of deep WDR neuron responses to increasing bending force of von Frey
200 filaments before and after ear pinch application in sham early **(C)**, cancer early **(D)**, sham late **(E)** and
201 cancer late **(F)**. Individual neuronal responses are quantified in the right panel. Values over each bar
202 represent percentage of change to the respective baseline. Each cell values represent averaged
203 responses from 3 consecutive trials and one cell was recorded per animal (shown as a single dot).
204 2-way RM-ANOVA with Bonferroni post-hoc: *p < 0.05, **p < 0.01, ***p < 0.001, ****p < 0.0001 vs.
205 corresponding baseline. All the data represent the mean \pm SEM from sham early (n = 9), cancer early
206 (n = 11), sham late (n = 11) and cancer late (n = 9).

207 *2.5. Damage to primary afferents innervating the cancerous tissue was evident in early but not late*
208 *stage CIBP rats*

209 Based on our fast blue (FB) tracing, the rat tibia is innervated by 100.7 ± 15.7 lumbar 2-5 DRG
210 neurons, which corresponds to $4.47 \pm 0.34\%$ of all neurons (based on Tubulin- β III positivity) therein.
211 In health, the majority of those tibia-projecting cells are located within the L3 DRG (Fig. S1A, and
212 [16]).

213 Damage to afferents innervating cancerous tissue was quantifiable using activating transcription
214 factor 3 (Atf3), a protein induced by cellular stress [17]. Representative micrographs of the L3 DRG
215 ipsilateral to the injury site clearly demonstrated the characteristic nuclear expression pattern of Atf3
216 in bone and other afferents (Fig. 5A). This is especially evident in early stages of our CIBP model (Fig.
217 5A, B). Interestingly, by the late stage, Atf3 positivity normalises in both groups, with almost no
218 occurrence in late-stage sham animals, suggesting full postsurgical recovery (One Way ANOVA
219 [group]: $F_{[3, 8]} = 17.29$, p = 0.0007, Tukey post-hoc: CE vs. SL p < 0.001, CE vs. CL p < 0.01, SL vs. SE
220 p < 0.01) (Fig. 5B, S1B). Bone afferents are more likely than other afferents to express Atf3 at early
221 disease stages, suggesting higher levels of stress in this population (One Way ANOVA [group]: $F_{[3, 8]} = 10.52$, p = 0.0038, Tukey post-hoc: SE vs. CE p < 0.01, CE vs. SL p < 0.01, CE vs. CL p < 0.05) (Fig.
222 5C). Moreover, there is a visible shift in the expression pattern of Atf3+/FB+ from L3 to L4 DRG
223 between early and late CIBP (Fig. S1C).

224 Interestingly, no changes were observed in the total number of FB+ neurons between groups
225 when DRG were pooled (One Way ANOVA [group]: $F_{[3, 8]} = 3.69$, p = 0.0062) (Fig. 5D), however, there
226 was a shift in tibial innervation from L3 to L4 DRG in cancer-bearing rats (One Way ANOVA [group]:
227 L3 DRG: $F_{[3, 8]} = 7.065$, p = 0.0012, Tukey post-hoc: CE vs. SE p < 0.05, CL vs. SL p < 0.05, L4 DRG:
228 $F_{[3, 8]} = 9.01$, p = 0.0061, Tukey post-hoc: CE vs. SE p < 0.05, CL vs. SL p < 0.05, CL vs. SE p < 0.01)
229 (Fig. 5E, 5F, S1A). Such a shift is suggestive of either degenerative changes in the L3 DRG afferents
230 and/or sprouting of the L4 DRG afferents towards the tumour mass, and/or leakage of the FB tracer
231 outside the bone cavity via cancer-induced perforations of the cortical bone.

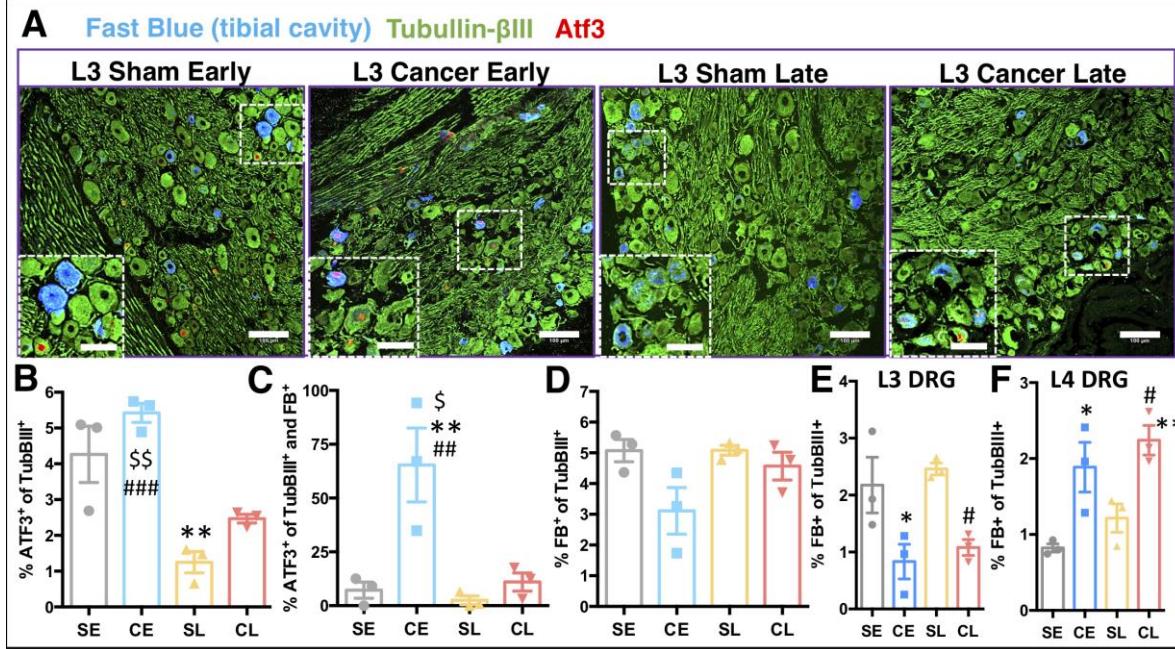


Figure 5. Cancer progression affects bone innervation. (A) Representative confocal scans selected from lumbar 3 DRG of immunohistochemical analysis of cellular stress factor Atf3 and Tubullin- β III protein expression in the Fast Blue (FB) traced tibial afferents. FB was injected a week before the cancer cells or vehicle (sham) implantation. Main scale bars are 100 μ m, and zoomed inclusions' scale bars are 50 μ m. (B) Quantification of all Atf3 $^{+}$ afferents within ipsilateral L2-5 DRG, analysed as a percentage of all neurons (Tubullin- β III) therein in cancer early (CE, day 7/8) and cancer late (CL, day 14/15) stage groups with corresponding sham groups (early – SE and late – SL). On average 4-20 10 μ m sections were counted per DRG. Data represent the mean \pm SEM and each dot represent a separate animal (n = 3). One Way ANOVA with Tukey post-hoc test. * vs. SE, # vs. SL, \$ vs. CL. *p < 0.05, **p < 0.01, ***p < 0.001. (C) Quantification of Atf3 $^{+}$ afferents within ipsilateral L2-5 DRG analysed as a percentage of all FB traced neurons. Analysed as in (B). (D) Total number of FB traced neurons within ipsilateral L2-5 DRG analysed as a percentage of all neurons (Tubullin- β III) therein. On average 100.6 \pm 15.7 L2-5 DRG neurons innervate tibia. No FB positivity was noticed in the contralateral lumbar DRG (not shown). (E) Total number of FB traced neurons in the ipsilateral L3 DRG analysed as a percentage of all neurons (Tubullin- β III) therein. Analysed as in (B). (F) Total number of FB traced neurons in the ipsilateral L4 DRG analysed as a percentage of all neurons (Tubullin- β III) therein. Analysed as in (B). See also Figure S1 for detailed analysis.

3. Discussion

In the present study neuronal activity in the deep dorsal horn of the spinal cord of male Sprague Dawley CIBP rats was investigated. Disease progression was classified as early (day 7-8 post MRMT-1 cells injection) or late (day 14-16) stage according to trabecular and cortical bone destruction. Despite the fact that, upon stimulation of the hind paw, wakeful CIBP rats demonstrated ipsilateral mechanical hyperalgesia in the early stage and mechanical allodynia in the late stage of disease (suggestive of central sensitization), deep dorsal horn wide dynamic range (DDH WDR) neuronal firing upon mechanical stimulation of the same hind paw was unchanged when measured using *in vivo* electrophysiological techniques. This result was unexpected not only due to the mismatch between behavioural and electrophysiological outcomes but also because spinal cord superficial lamina I neurons were previously consistently described as hyperactive in the CIBP model [3-5,18,19]. The divergence in responsiveness of superficial versus DDH WDR neurons could be partly explained based on the anatomy of primary afferents. Lamina I neurons receive direct input from A δ - and C-fibres as well as silent nociceptors [20]. These afferents predominantly innervate tibiae [2,20-23]. In contrast, DDH WDR neurons receive direct inputs from large A β - and small A δ -myelinated fibres and indirect polysynaptic inputs from C-fibres from distal dendrites that extend into superficial laminae [24]. The internal spinal circuitry is highly plastic and hugely heterogeneous

268 [25], and the disease state may lead to dysfunctionality in transmission circuits that include inhibitory
269 mechanisms [26], leading to a knock on 'adaptive' effect on the evoked activity of DDH WDR neurons
270 as observed in the present study.

271 The superficial dorsal horn is the origin of a spino-bulbo-spinal loop and prior investigation of
272 potential alterations in descending modulatory controls was conducted. Rodriguez et al. showed that
273 in CIBP rats both superficial and DDH WDR neurons undergo ongoing facilitatory control (mediated
274 by spinal 5-HT₃ receptors), suggestive of an enhanced descending serotonergic drive [4]. Our
275 observation that DDH WDR neurons are not hyperexcitable in the CIBP model could indicate
276 plasticity in ongoing (tonic) inhibitory controls.

277 Multiple descending inhibitory control pathways exist and activation of one such pathway, diffuse
278 noxious inhibitory controls (DNIC), gives rise to the 'pain inhibits pain' phenomenon, whereby
279 application of a noxious stimulus to one part of the body inhibits pain perception in a remote body
280 region. DNIC inhibitory controls are largely driven by α_2 -adrenergic receptor (α_2 -AR)-mediated
281 responses to inhibit the activity of DDH WDR neurons [9]. Notably during our *in vivo* spinal
282 electrophysiology studies, we observed a dynamic reorganization of descending inhibitory controls
283 during progression of the bone tumour, quantified as dysfunctional expression of DNIC. Intriguingly,
284 DNIC was abolished only in the early stages of the disease and they were functionally expressed
285 once again in the late stage of CIBP. The dynamic reorganization of spinal neuronal modulation by
286 descending controls corresponds with prior research demonstrating that facilitatory descending
287 controls are altered in CIBP rats [4].

288 The translational potential of the current study may be considered when discussing the
289 expression of an equivalent naturally occurring analgesic pathway in man, which is measured using
290 the human psychophysical paradigm conditioned pain modulation (CPM; [27]). Pharmacotherapies
291 that act to enhance the descending inhibitory pathways whose functionality is assessed with CPM
292 psychophysics have already shown promise in enabling chronic pain patients to harness their
293 endogenous pain-relieving mechanisms and thus reduce their pain experience [12]. The presence or
294 absence of CPM is proposed to be a reliable, simple diagnostic measure in terms of personalised
295 pain pharmacotherapies in particular pain types. Recently, several clinical paradigms have been
296 developed for a quantification of the inhibitory impact of CPM [28]. Analogically to DNIC, CPM is
297 pan-modal and it requires test and conditioning stimulus [29] and the conditioning stimulus must be
298 noxious [11]. Recently, a novel approach utilises two pressure cuffs controlled by a fully automated
299 algometer [30] and there is evidence that a comparable noxious pressure paradigm activates the
300 unique endogenous inhibitory control pathway in rat and man [13].

301 Abolished DNIC expression in the early stage CIBP rats could serve as an early indicator of the
302 bone cancer pain phenotype development; pushing the story further, it indicates that the expression
303 status of this unique naturally occurring descending-inhibitory pathway could perhaps be used
304 clinically as a diagnostic tool to tailor pain pharmacotherapy in patients suffering from (metastatic)
305 bone cancer. This type of research, the coupling of preclinical and clinical observations, has the
306 potential to identify new analgesic targets. For example, pain therapies utilising
307 noradrenaline-reuptake inhibitors in the early cancer phase, which has diminished DNIC expression,
308 could be therapeutically advantageous as observed in other chronicities where endogenous pain
309 inhibitory pathways are otherwise dysfunctional [12]. Interestingly, CIBP is not the only pain state
310 where dynamics in descending controls sub-serving DNIC/CPM have been recorded. CPM is not
311 expressed in patients with cluster headache in the active phase, and yet is restored in remission [31].

312 The sensitization of primary afferent fibres that innervate cancer-bearing bones in the early stage
313 of disease [2,16,32–35] was linked in the present study with a high expression of cellular stress
314 marker activating transcription factor 3 (Atf3). In the late cancer stage, the activation of Atf3 appeared
315 completely resolved in line with the resolution of DNIC functionality. Multiple mechanisms may
316 contribute to the reduction of Atf3 in the late stages of cancer. For example, it is possible that CIBP
317 afferents are undergoing cell death as a result of tumour invasion and toxic local conditions.
318 Supporting this hypothesis is an observed shift in the expression pattern of Atf3+/FB+ from L3 to L4
319 DRG between early and late CIBP, further indicating the presence of a degenerative mechanism in L3
320 tibial afferents, and consecutive sprouting and/or activation of L4 afferents. Indeed, the number of L3
321 afferents decreases in the cancer groups, as compared to the corresponding sham controls.

322 Over the past decade, studies of CIBP have revealed that neurons and cancer cells are engaged
323 in bi-directional crosstalk. For instance, cancer causes a reorganization of 'normal' anatomy, driving
324 neurons to sprout and more densely innervate the tumour-bearing bone [36–38]. This sprouting
325 process was shown to be mediated via tyrosine kinase A (TrkA) receptor activation by nerve growth
326 factor (NGF) released from both cancer and stromal cells [39–41]. Conversely, neurons release
327 factors which support tumour growth and vascularization [42–44]. This complex dialogue involves
328 numerous mediators and different local cells, including fibroblasts, osteoclasts and newly recruited
329 immune cells [43,45].

330 Our previously published research revealed important new information about bone afferent
331 expression patterns, including the fact that they encode mechanical stimuli. Thus, we provided a
332 potential functional mechanism explaining the recruitment of additional afferents from the outside of
333 the tibial cavity that could contribute to the CIBP phenotype. Interestingly, in the presence of the
334 tumour, tibial cavity afferents were not hyperexcitable, a result that was recently confirmed by an
335 independent study on murine model of CIBP, where femur cavity neurons were not sensitised by the
336 presence of Lewis lung carcinoma tumours after both intraosseous pressure and acid stimulation
337 [32].

338 In the current study, FB traced tibial cavity neurons were not sensitised in the late stage cancer,
339 which corresponds to the normalised Atf3 level in this group. A decrease in Atf3 levels in the late
340 stage cancer group may, together with the lack of hyperexcitability in this group [2], suggest an
341 ongoing tumour-induced neurodegenerative change in this neuronal population. Supporting this
342 hypothesis, there is a decrease in the total count of FB positive neurons in the L3 DRG in cancer
343 groups as compared to the sham-operated ones, pointing at an ongoing cellular death of this
344 population. A subsequent increase in the number of FB L4 neurons in the cancer groups may result
345 from either neuronal sprouting of the L4 afferents towards the tumour mass (NGF induced:
346 [36,40,46]) and/or leakage of the FB tracer via cancer-induced perforations in the cortical bone or
347 internal bone compartments resulting in labelling of additional L4 afferents. In fact, since in rats L3
348 DRG neurons innervate predominantly the medullary cavity and periosteum, and L4 DRG cells
349 innervate epiphysis and since the distal epiphysis and the medullary cavity are not in continuity [23], it
350 is likely that in our model cancer-evoked erosion of the medullary cavity towards the epiphysis would
351 result in labelling of additional L4 afferents. It remains to be established whether the long bones CIBP
352 phenotype differs depending on in which bone compartment the tumour grows, i.e. would the CIBP
353 phenotype differ if the tumour invaded the epiphysis to the point where the tumour encompassed the
354 medullary cavity or periosteum?

355 It is likely that peripheral hyperactivity drives plasticity in central pain controls, a view supported
356 by the aforementioned study demonstrating an increase in descending facilitatory controls
357 (orchestrated via spinal 5HT₃ receptors) in the CIBP rat dorsal horn neuronal activity [4]. Taking this
358 further, some clusters of primary afferents are modality specific, which superimposes the existence of
359 different synapses on the central (spinal) sites [25,47,48]. Further, the action of descending
360 monoamines (released from the terminals of the descending modulatory pathways investigated) in
361 the cord is rather diffuse, allowing for a broad (inhibitory or excitatory) control of multiple modalities
362 [49–51].

363 CIBP is unique in that the sufferers experience tonic and spontaneous pain as well as the type of
364 mechanically-evoked pain studied here. Probing the descending modulatory control of spinal
365 neuronal activity should ultimately include all three types of pain. Background (tonic) pain intensity
366 typically increases with the progression of the disease while spontaneous and movement-evoked
367 types of pain being mechanoceptive in nature are difficult to manage in mobile subjects; by definition,
368 they 'break-through' the barriers of analgesia [45]. Since they are also unpredictable, it is extremely
369 challenging to suit sufficient therapies without adverse effects of high doses of painkillers being
370 continuously administered. Such mechanistic studies were beyond the scope of this paper, but we
371 are aware of these challenges that face preclinical researchers who investigate mechanisms of CIBP.

372 **4. Materials and Methods**

373 **4.1. Cell lines**

374 Syngeneic rat mammary gland adenocarcinoma cells (MRMT-1, Riken cell bank, Tsukuba,
375 Japan) isolated from female Sprague-Dawley rat, were cultured in RPMI-1640 medium (Invitrogen,
376 Paisley, UK) supplemented with 10% FBS, 1% L-glutamine and 2% penicillin/streptomycin
377 (Invitrogen, Paisley, UK). All cells were incubated at 5% CO₂ in a humidity-controlled environment
378 (37°C, 5% CO₂; Forma Scientific).

379 *4.2. Animals*

380 Male Sprague-Dawley rats (UCL Biological Services, London, UK or Charles-River, UK) were
381 used for experiments. Animals were group housed on a 12:12-hour light–dark cycle. Food and water
382 were available ad libitum. Animal house conditions were strictly controlled, maintaining stable levels
383 of humidity (40-50%) and temperature (22±2°C). All procedures described were approved by the
384 Home Office and adhered to the Animals (Scientific Procedures) Act 1986. Every effort was made to
385 reduce animal suffering and the number of animals used in accordance with IASP ethical guidelines
386 [14].

387 *4.3. Cancer-induced bone pain model*

388 On the day of surgery, MRMT-1 cells were released by brief exposure to 0.1% w/v
389 trypsin-ethylenediaminetetraacetic acid (EDTA) and collected by centrifugation in medium for 5 min at
390 1000 rpm. The pellet was washed with Hanks' balanced salt solution (HBSS) without calcium,
391 magnesium or phenol red (Invitrogen, Paisley, UK) and centrifuged for 5 min at 1000 rpm. MRMT1
392 cells were suspended in HBSS to a final concentration of 300,000 cells/ml and kept on ice until use.
393 Only live cells were counted with the aid of Trypan Blue (Sigma) staining. Cell viability after incubation
394 on ice was checked after surgery, and no more than 5-10% of cells were found dead after 4 h of
395 ice-storage.

396 Sprague-Dawley rats weighting 120-140 g (for late-stage CIBP, 14 days post-surgery) or
397 180-200 g (for early-stage CIBP, 7 days post-surgery), following complete induction of anaesthesia
398 with isoflurane (induction 5%, maintenance 1.5-2%) in 1 l/min O₂ and subcutaneous perioperative
399 meloxicam injection (50 µl 2 mg/kg, Metacam®, Boehringer Ingelheim, Berkshire, UK), were
400 subjected to the surgical procedure of cancer cell implantation into the right tibiae [1]. Briefly, in
401 aseptic conditions, a small incision was made on a shaved and disinfected area of the tibia's
402 anterior-medial surface. The tibia was carefully exposed with minimal damage to the surrounding
403 tissue. Using a 0.7 mm dental drill, a hole was made in the bone through which a thin polyethylene
404 tube (I.D. 0.28 mm, O.D. 0.61 mm; Intramedic, Becton Dickinson and Co., Sparks, MD, USA) was
405 inserted 1-1.5 cm into the intramedullary cavity. Using a Hamilton syringe, either 3 × 10³ MRMT-1
406 carcinoma cells in 10 µl HBSS or 10 µl HBSS alone (Sham) was injected into the cavity. The tubing
407 was removed, and the hole plugged with bone restorative material (IRM, Dentsply, Surrey, UK). The
408 wound was irrigated with saline and closed with Vicryl 4-0 absorbable sutures and wound glue
409 (VetaBond 3M, UK). The animals were placed in a thermoregulated recovery box until fully awake.

410 *4.4. Von Frey behavioural testing*

411 Behaviour was assessed 2-4 hours before surgery (day 0) and at 2, 7 and 14 days following
412 cancer cells injection. Testing was preceded by a 30 min acclimatisation period. Rooms conditions
413 used for behavioural testing were strictly controlled, maintaining stable levels of humidity (40-50%)
414 and temperature (22±2°C). Mechanical hypersensitivity was assessed by application of increment
415 von Frey filaments starting from 0.16 g up to 26 g – cut off (Touch-test, North Coast Medical Inc., San
416 Jose CA, USA). Each hair was applied 5 times to the plantar surface proximal to the digits of the
417 ipsilateral and contralateral hind paws. Withdrawal responses and whole paw lifts elicited by von Frey
418 hairs were scored as positive remark. Five subsequent positive responses to the same filament were
419 considered as the overall positive reaction, the force of the filament noted, and further testing with
420 higher force filaments abandoned. Results are presented as a mean ± SEM.

421 *4.5. Spinal cord *in vivo* electrophysiology*

422 In vivo electrophysiology was performed on animals weighing 250-300 g as previously described
423 [15]. Briefly, after induction of anaesthesia, a tracheotomy was performed, and the rat was maintained
424 with 1.5% of isoflurane in a gaseous mix of N₂O (66%) and O₂ (33%). A laminectomy was performed
425 to expose the L3-L5 segments of the spinal cord. Core body temperature was monitored and
426 maintained at 37°C by a heating blanket unit with rectal probe. Using a parylene-coated, tungsten
427 electrode (125 µm diameter, 2 MΩ impedance, A-M Systems, Sequim, WA, USA), wide dynamic
428 range neurons in deep laminae IV/V (~650–900 µm from the dorsal surface of the cord) receiving
429 afferent A-fibre and C-fibre input from the hind paw were sought by periodic light tapping of the
430 glabrous surface of the hind paw. Extracellular recordings made from single neurones were
431 visualized on an oscilloscope and discriminated on a spike amplitude and waveform basis. Sampling
432 parameters were set as follows: 30-40k amplification (preamplifier+amp), band-pass filtering between 1k
433 and 3k Hz and the signal was digitalised at 20 kHz sampling rate. HumBag (Quest Scientific, Canada)
434 was used to remove low frequency noise (50-60 Hz). Electrical stimulation (NeuroLog system,
435 Digitimer, UK) was given via two tuberculin needles inserted into the receptive field and a train of 16
436 stimuli was given (2 ms pulse duration, 0.5 Hz at three times C-fibre threshold: on average 7.8 ± 1.5
437 mA). Responses evoked by A_β-, A_δ-, and C-fibres were superimposed and separated according to
438 latency (0–20 ms, 20–90 ms and 90–300 ms, respectively), on the basis that different fibre types
439 propagate action potentials at different conduction velocities. Neuronal responses occurring after the
440 C-fibre latency band of the neuron were classed as post-discharge, a result of repeated stimulation
441 leading to wind-up neuronal hyperexcitability. The “input” (non-potentiated response) and the
442 “wind-up” (potentiated response, evident by increased neuronal excitability to repeated stimulation)
443 were calculated. Input = (action potentials evoked by first pulse at three times C-fibre threshold) ×
444 total number of pulses. Wind-up = (total action potentials after 16th train stimulus at three times C-fibre
445 threshold) – input. Natural mechanical stimuli, including brush and von Frey filaments (2 g, 8 g, 26 g
446 and 60 g), were applied to the receptive field for 10 s per stimulus. For each stimulus, the evoked
447 responses were recorded and quantified as the number of neuronal events counted during the 10 s
448 duration of a given stimulation. Data were captured and analysed by a CED 1401 interface coupled to
449 a PC with Spike 2 software (Cambridge Electronic Design, Cambridge, UK; peristimulus time
450 histogram and rate functions). Stabilization of neuronal responses to the range of electrical and
451 natural stimuli was confirmed with at least three consistent recordings (<10% variation in the action
452 potential) to all measures.

453 4.6. Diffuse Noxious Inhibitory Controls

454 Diffuse Noxious Inhibitory Controls (DNIC) were induced analogically to previously published
455 methodology [9]. Briefly, extracellular recordings were made from 1 WDR neuron per animal by
456 stimulating the hind paw peripheral receptive field and then repeating in the presence of the ear pinch
457 (conditioning stimulus - DNIC). The number of action potentials fired in 10 seconds was recorded for
458 each test. Baseline responses were calculated from the mean of 3 trials. Each trial consisted of
459 consecutive responses to 8, 26, and 60 g von Frey filaments applied to the hind paw. This was then
460 followed by consecutive responses to the same mechanical stimuli (8, 26, and 60 g von Frey
461 filaments) in the presence of DNIC. Precisely, DNIC was induced using a noxious ear pinch (15.75 x
462 2.3 mm Bulldog Serrefine; InterFocus, Linton, United Kingdom) on the ear ipsilateral to the neuronal
463 recording, whilst concurrent to this, the peripheral receptive field was stimulated using the von Frey
464 filaments listed. Diffuse noxious inhibitory control was quantified as an inhibitory effect on neuronal
465 firing during ear pinch. A minimum 30 s non-stimulation recovery period was allowed between each
466 test in the trial. A 10-minute non-stimulation recovery period was allowed before the entire process
467 was repeated for control trial number 2 and 3. The procedure was repeated 3 times and averaged
468 only when all neurons met the inclusion criteria of 10% variation in action potential firing for all
469 mechanically evoked neuronal responses.

470 4.7. Immunohistochemistry

471 For tracing of intratibial afferents, rats weighing 60-70 g were anaesthetised using isoflurane
472 (1.5-2% in oxygen, Piramal, UK) and the left tibia was injected with 5 µl of 4% Fast Blue neuronal
473 tracer (Polysciences Inc., Germany). After a 7-day recovery period, animals were randomly divided

474 into two groups – sham and cancer. 7 or 14 days after cancer cells inoculation (for early and late stage
475 respectively), animals were sacrificed by the overdose of pentobarbital and transcardially perfused
476 with cold PBS followed by 4% PFA in phosphate buffer (pH 7.5). Next L2-L5 ipsi/contra DRG were
477 collected, post-fixed in 4% PFA, cryo-sectioned and incubated with primary antibodies against Atf3
478 (rabbit, 1:200, Santa Cruz, (C-19): sc-188, US) and Tub β III (mouse, 1:1000, G712A, Promega, UK).
479 Slides were then incubated with the appropriate fluorophore-conjugated secondary antibodies.
480 Representative samples were imaged with a LSM 710 laser-scanning confocal microscope (Zeiss)
481 using 10x (0.3 NA) and 20 x (0.8 NA) dry objectives and analysed with Fiji Win 64. For quantification,
482 samples were imaged with 20x dry objective on Zeiss Imager Z1 microscope coupled with AxioCam
483 MRm CCD camera. The acquisition of images was made in multidimensional mode and the MosaiX
484 function was used to construct the full view. 3 DRG were imaged per lumbar region. Cell counting was
485 carried out on the Fiji Win 64 utilising cell counter plugin. For Atf3 analysis, cells were counted as
486 positive only when the cell's nucleus was stained. The percentage of Atf3 positive cells relative to the
487 total number of neurons (Tub β III) and FB positivity was calculated. On average, 4-20 DRG sections
488 (depending on the DRG size) were imaged for quantification. 3 rats per group were used for those
489 experiments and no other procedure was performed on those animals to prevent unspecific activation
490 of Atf3.

491 *4.8. Micro-computed tomography of cancer-bearing legs*

492 Rat tibiae, cleared of excess muscle and soft tissue, were placed into a micro-computed
493 tomography scanner (μ CT, Skyscan1172) with Hamamatsu 10 Mp camera. Recording parameters
494 were set as follows: source voltage at 40 kV, source current at 250 μ A, rotation step at 0.600 deg, with
495 2 frames averaging and 0.5 mm aluminium filter. For reconstruction NRecon software (version:
496 1.6.10.4) was used. In total, over 500, 34 μ m thick virtual slices were collected per bone. Cancer
497 growth encompassed an area proximal to the tibial knee head and 114 scan planes covered the
498 majority of the tumour mass (for analysis details see [2]). Representative visualisations were
499 prepared with Fiji with 3D viewer plugin.

500 *4.9. Quantification and statistical analysis*

501 Statistical analyses were performed using SPSS v25 (IBM, Armonk, NY). All data plotted in
502 represent mean \pm SEM. Throughout the manuscript 'n' refers to the number of animals tested.
503 Detailed description of the number of samples analysed and their meanings, together with values
504 obtained from statistical tests can be found in each figure legend. Symbols denoting statistically
505 significant differences were also explained in each figure legend. Main effects from ANOVAs are
506 expressed as an F-statistic and p-value within brackets. Throughout, p-value below 0.05 was
507 considered significant. Behaviour: Two-Way RM-ANOVA with Bonferroni post-hoc test was used to
508 analyse behavioural data for von Frey. Electrophysiology: One-way ANOVA with Bonferroni post hoc
509 test was used to assess significance for baseline electrical and brush. Von Frey responses were
510 assessed with RM-ANOVA with the Bonferroni post-hoc. Statistical differences in the neuronal
511 responses observed after ear pinch were determined using a 2-way repeated-measures analysis of
512 variance (RM-ANOVA) with Bonferroni post hoc test. One-way ANOVA with Tukey post-hoc
513 performed in the GraphPad Prism was used to analyse immunohistochemical data.

514 **5. Conclusions**

515 The overarching aim of the present study was to link previous reports of peripheral sensitization
516 to central (spinal) events. Changes in the peripheral nervous system reflect a notable impact on
517 spinal neuronal responses in the early stage of CIBP, and this is mechanistically linked to
518 dysfunctionality of the descending inhibitory 'DNIC' pathway. The data herein provide insight
519 regarding the stage specific plasticity in central modulatory processes that underlie the pain
520 phenotype in this particular rodent model of CIBP.

521 **Supplementary Materials: Figure S1:** Detailed Atf3 quantification in separate dorsal root ganglia, **Movie S1:**
522 3D-rendered micro-computed tomography scans of rat's tibiae in health and in the presence of bone cancer
523 (early and late stage). Scale bar 1 mm.

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