Title (20 words): **Anxiety enhances pain in a model of osteoarthritis and is associated with altered endogenous opioid function and reduced opioid analgesia**

Authors: Amanda Lillywhite[a b,*], Stephen G. Woodhams[a,b,*], David J. G. Watson[b], Li Li[a,b], James J. Burston[a,b], Peter R. W. Gowler[a,b], Meritxell Canals[b,c], David A. Walsh[a,d,e], Gareth J. Hathway[a,b,e], Victoria Chapman[a,b,e,f]

[*] These authors contributed equally to this work

Addresses:

[a] Pain Centre Versus Arthritis, University of Nottingham, Medical School, Queen's Medical Centre, Nottingham, United Kingdom

[b] School of Life Sciences, Medical School, Queen's Medical Centre, Nottingham, United Kingdom

[c] Centre of Membrane Proteins and Receptors, Universities of Birmingham and Nottingham, Midlands, UK.

[d] School of Medicine, University of Nottingham, Nottingham, United Kingdom

[e] NIHR Nottingham Biomedical Research Centre, University of Nottingham, Nottingham, United Kingdom

[f] Corresponding author. victoria.chapman@nottingham.ac.uk

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Abstract (239)

Chronic pain states such as osteoarthritis (OA) are often associated with negative affect, including anxiety and depression. This is, in turn, associated with greater opioid analgesic use, potentially contributing to current and future opioid crises. We utilise an animal model to investigate the neurobiological mechanisms underlying increased opioid use associated with high anxiety and chronic pain.

Combining a genetic model of negative affect, the Wistar Kyoto (WKY) rat, and intra-articular injection of monosodium iodoacetate (MIA; 1mg), our model of high anxiety and augmented OA-like pain behaviour mirrors the clinical problem. Effects of morphine (0.5-6mg.kg^{-1}) on pain behaviour and spinal nociceptive neuronal activity were determined in WKY rats, and normo-anxiety Wistar rats, 3 weeks after MIA injection. WKY rats developed augmented OA-like pain, and had blunted inhibitory responses to morphine, when compared to Wistar rats. Potential alterations in endogenous opioid function were probed via systemic blockade of opioid receptors with naloxone (0.1-1mg.kg^{-1}), quantification of circulating levels of β-endorphin, and determination of spinal expression of the mu-opioid receptor (MOR). These studies revealed increased opioidergic tone, and increased spinal desensitization of MORs via the master phosphorylation site at serine residue 375, in this model.

We demonstrate attenuated MOR function in the absence of previous exogenous opioid ligand exposure in our model of high anxiety and OA-like pain, which may account for reduced analgesic effect of morphine and provide a potential explanation for increased opioid analgesic intake in high anxiety chronic pain patients.

Significance Statement (116)

Chronic pain affects large numbers of people, and pain management often relies on poorly effective opioid analgesics, the iatrogenic effects of which are increasingly recognised. The endogenous opioid system - the target for exogenous opioid analgesics - plays key roles in emotional affective states and pain control, but the complex interplay between anxiety, chronic pain, and endogenous opioid system function is challenging to study in people. Here, we have addressed this using a clinically-relevant experimental model. Anxiety-like behaviour was associated with increased chronic arthritis-like pain behaviour, altered opioid receptor function, and reduced efficacy of opioid analgesics. We provide new evidence, which may explain why chronic pain patients with comorbid high anxiety have higher opioid analgesic use.

1. Introduction (628)

Opioid analgesics have formed the backbone of the management of moderate to severe pain for decades. Opioid drugs, such as morphine, produce their effects via μ-opioid receptors (MOR) at key sites in the spinal cord and brain (McMahon, 2013). Since the 1990s, there has been a shift in the prescribing of opioids from managing acute pain and pain in terminally ill patients, to more wide-spread prescribing for a wide range of long-term pain conditions (Curtis et al., 2019).

Both morphine's analgesia and its side-effects (tolerance, respiratory depression, euphoria, and dependence) are mediated by MOR. Clinical studies provide strong evidence that, although opioids are excellent analgesics for acute pain such as post-operative pain, long-term use of opioids is not associated with useful pain relief in most people (Colvin et al., 2019). This loss of analgesic benefit is attributed to MOR tolerance and the manifestation of opioid-induced hyperalgesia (Yang et al., 2019), both of which have major implications for pain trajectories *per se*, as well as post-operative outcomes and a contribution towards poorly-controlled pain (Colvin et al., 2019). Chronic pain alters endogenous opioid function, including increased release of endogenous opioid ligands, such as β-endorphin, which may lead to alterations at MOR with increased phosphorylation, opioid tolerance, and lower analgesic responsiveness to morphine (Petraschka et al., 2007; Bruehl et al., 2013).

Osteoarthritis (OA) is the most common form of arthritis (Glyn-Jones et al., 2015) and the fastest growing cause of chronic pain worldwide (Vos et al., 2012; GBD_2013_DALYs_&_HALE_Collaborators et al., 2015). OA pain phenotypes reflect both nociceptive and neuropathic mechanisms of pain (Glyn-Jones et al., 2015; Perrot, 2015), and approximately 20% of people exhibit features of central

sensitization, including regional spread of pain (Suokas et al., 2012; Arendt-Nielsen et al., 2015). Despite the reportedly high rates of opioid-prescribing for OA pain (Bedson et al., 2016; Thorlund et al., 2019), opioids have no superior effect over non-opioid treatments over 12 months (Krebs et al., 2018), and long-term opioid use is associated with increased risk of adverse events (Bedson et al., 2016). Negative affect, including anxiety and depression, is associated with exacerbated chronic pain (de Heer et al., 2014), and is common in people with OA (Axford et al., 2010; Barnett et al., 2018). Previous studies reported complex relationships between endogenous opioid function and depressive symptoms and trait anxiety in people (Burns et al., 2017), and negative affect is associated with greater use of opioid analgesics in people with OA (Valdes et al., 2015; Barnett et al., 2018; Namba et al., 2018)· Substantial advances in the understanding of the mechanisms of pain and opioid-induced analgesia have been made using rodent models of disease and molecular and pharmacological studies of opioid receptor function (Pasternak, 2012, 2014). Inbred Wistar Kyoto (WKY) rats are used as an experimental model of anxiety-like behaviour (McAuley et al., 2009). Previously, we reported exacerbated pain behaviour in the monosodium iodoacetate (MIA) model of OA pain in WKY rats, including a spread of pain to the contralateral side and increased markers of astrocytic activation (Burston et al., 2019).

We hypothesised that heightened anxiety is associated with alterations in the endogenous opioidergic system and MOR function, which may counter the effects of exogenous morphine used to treat OA pain. Herein, we evaluated the degree of morphine-mediated analgesia and effects of naloxone-mediated blockade of the endogenous opioid system in a model of OA-like pain in high anxiety WKY and normo-anxiety Wistar rats, then investigated the underlying neurobiological

mechanisms. Circulating levels of β-endorphin were measured in Wistar and WKY rats, and potential changes in MOR function in the spinal cord were investigated. Levels of MOR protein and MOR receptor phosphorylation at serine residue 375, which is required for morphine-mediated desensitization (Schulz et al., 2004), were quantified in the dorsal horn of the spinal cord.

2. Materials & Methods

2.1 Experimental Animals

Studies were in accordance with UK Home Office Animals (Scientific Procedures) Act (1986) and ARRIVE guidelines (Kilkenny et al., 2012). A total of 183 male rats were used for this study; Wistar *n* = 97 (Charles River, Margate, United Kingdom), & Wistar Kyoto *n* = 86 (WKY; Envigo, Bicester, United Kingdom). Males only were used to reduce variability in the data, and to maintain consistency with previous studies characterising this model. Wistar rats were used as the most genetically similar control strain to WKY. Rats were group housed by strain in groups of 4 on a 12-hour light/dark cycle in a specific pathogen-free environment with *ad libitum* access to standard rat chow and water. Treatments were randomly assigned to rats, and experimenters were blinded to all treatment groups throughout the study. A total of 14 rats were excluded from the study (7.6%; see **Extended Data Table 1-1** for further details).

2.2 Induction of the MIA model of OA pain

All rats received a single intra-articular injection into the left knee, through the infra-patellar ligament using a 29-gauge hypodermic needle, under isoflurane anaesthesia

(3% 1Lmin^{-1} O2). Rats were randomly assigned to receive either 1mg/50µl MIA in 0.9% saline (Wistar *n* = 48, WKY *n* = 45) or 50µl 0.9% saline (Wistar *n* = 41; WKY *n* = 41). Health and welfare checks were performed immediately after anaesthetic recovery, then daily for the first 3 days, and weekly thereafter. Pain behaviour was assessed twice weekly from D3 to 21 post-model induction.

2.3 Behavioural Testing

All rats were habituated to the pain behaviour testing environment for 2 days prior to taking baseline measurements to minimise any exploratory behaviour during testing. Baseline measurements were taken in the morning prior to treatment (D0). Weightbearing (WB) asymmetry was assessed using an incapacitance tester (Bove et al., 2003) (Linton Instrumentation, Diss, UK). Healthy rats distribute their weight evenly between limbs, and a weight shift onto the contralateral limb indicates pain at rest in the ipsilateral knee joint (Bove et al., 2003). Referred pain at distal sites was assessed via determination of mechanical hindpaw withdrawal thresholds (PWTs) via von Frey hair (vFH) monofilaments using the up/down method for both ipsilateral and contralateral paws (Chaplan et al., 1994).

The elevated plus maze (EPM; Walf and Frye, 2007) was used to measure anxiety in Wistar and WKY rats. Rats were placed into the centre of the arena with their nose pointing into an open arm and activity was monitored for a period of 10 minutes using Ethovision software (Noldus Information Technology, Netherlands). Latency to enter the open outer arm and total time spent in closed versus open arms was then determined. Some exploratory behaviour in the open arms of the maze is expected in normo-anxiety animals, whilst restriction of activity to the closed arms is considered a surrogate indicator of anxiety-like behaviour.

To control for any strain differences in locomotor activity in a non-anxiogenic environment, the number of beam breaks per hour was assessed in an activity box (39.5cm x 23.5cm x 24.5cm, 4 x 8 photobeam array; Photobeam Activity System, San Diego Instruments, USA) in animals of both strains at baseline and 19-21 days after model induction (Pezze et al., 2014).

2.4 Pharmacological Interventions

2.4.1 Systemic naloxone/morphine behavioural study:

To assess differences in sensitivity to systemic opioids, and potential alterations in endogenous opioidergic tone, behavioural responses to cumulative systemic doses of morphine, naloxone, or vehicle (0.9% saline) were determined in Wistar and WKY rats 21 days after model induction in separate groups of rats, respectively. Pre-drug pain behaviour was assessed via WB and vFH in MIA or saline-treated rats, then following 3 cumulative systemic doses of morphine (0.5, 2, & 3.5 $mg.kg^{-1}mL^{-1}$, s.c.; Wistar/MIA n = 10, WKY/MIA n = 10) or vehicle (Wistar/saline n = 8, WKY/saline n = 8), or naloxone (0.1, 0.3, & 1 $mg.kg^{-1}mL^{-1}$, s.c.; Wistar/saline n = 12, Wistar/MIA n = 12, WKY/saline n = 10, WKY/MIA n = 11). Pain behaviour was assessed at 15, 30, and 60 mins after each dose.

2.4.2 In vivo *Spinal Electrophysiology*

To assess potential differences in spinal sensory network activity and responses to systemic morphine between strains, in the presence and absence of OA-like pain, single unit extracellular recordings were obtained from wide dynamic range (WDR) neurons in the deep dorsal horn, as previously described (Urch and Dickenson, 2003). Briefly, a laminectomy was performed under isoflurane anaesthesia (surgery:

192 3%, maintenance: 1.5%) to expose lumbar L4 6 spinal cord, and a WDR neurone
193 was located with a receptive field in the toes of the ipsilateral hindpaw via a glass-
194 coated tungsten microelectrode (Wistar/saline *n* = 10, Wistar/MIA *n* = 15;
195 WKY/saline *n* = 12, WKY/MIA *n* = 12). Once identified, WDR neurones were
196 characterised via electrical stimuli delivered to the receptive field via bipolar
197 electrodes. WDRs exhibit responses to electrical stimulation at Aβ, Aδ, and C fibre
198 latencies, and wind up in response to a repeated noxious electrical stimulation (16 x
199 50ms, 0.5Hz, delivered at 3-fold C fibre threshold). The degree of wind up can be
200 used as a proxy of central sensitization (D'Mello and Dickenson, 2008). Mechanical
201 responses to hindpaw stimulation with 8, 10, 15, and 26g vFH (10s application, 10s
202 inter stimulus interval) were recorded at baseline, and then every 10 mins following
203 cumulative systemic doses of morphine sulfate (0.5, 2.5, & 6 $mg.kg^{-1}$, s.c., 60 min
204 intervals).
205
206 **2.5 Assessment of Opioid Function in *Ex Vivo* Tissues**
207 ***2.5.1 Western Blotting***
208 To determine potential differences in spinal opioid receptor expression, fresh spinal
209 cord tissue was collected from WKY and Wistar rats 21 days after intra-articular
210 injection of MIA or saline (*n* = 4/strain). Rats were killed via overdose with sodium
211 pentobarbital (Euthatal, 2mL, i.p.), decapitated, and spinal cord tissue rapidly
212 collected via hydraulic extrusion. The lumbar enlargement of the spinal cord was
213 then hemisected down the midline, snap-frozen in liquid nitrogen, and stored at -
214 80°C until processed. Tissue was homogenised in RIPA buffer with added protease
215 and phosSTOP inhibitor cocktails (Sigma Aldrich, Gillingham, UK) to prevent
216 degradation of proteins and preserve their phosphorylation sites. An equal amount of

protein (150µg) from each sample was then separated via SDS-PAGE, transferred onto nitrocellulose membranes, and probed for expression of total MOR (rabbit anti-mu opioid receptor, Neuromics, RA10104, 1:500), P-ser375 MOR (rabbit anti-mu opioid receptor Ser375, BIOSS-Stratech, bs-3724R, 1:500), and β-actin (mouse anti-β-actin, Sigma, A5441, 1:5000) via overnight incubation in 5% milk at 4°C. Secondary antibodies were IRDye donkey anti-rabbit 800CW and donkey anti-mouse 680RD (1:5000 in 5% milk, RT, 1.5hr), and resulting fluorescent signal imaged via Licor Odyssey system (LI-COR Biosciences, Cambridge, UK) and resulting bands quantified via densitometry measurements in Image Studio Lite version 5.2 (LI-COR Biosciences). Data are expressed as expression level relative to β-actin.

2.5.2 β-endorphin ELISA

To determine any strain differences in circulating endogenous opioid peptides, levels of β-endorphin were measured via a commercial ELISA in blood obtained from naïve male Wistar (*n* = 8) and WKY (*n* = 7) rats. Rats were humanely killed via overdose of sodium pentobarbital (Euthatal, 2mL, i.p.) and exsanguinated to generate blood samples. Whole blood was collected in ice cold tubes containing 200µl heparin and processed within one hour. Samples were centrifuged at 13,000 rpm, 4°C for 20 mins, and the supernatant plasma collected and stored at -80°C prior to assay. Plasma samples were assayed for β-endorphin in duplicate using a commercially available EIA kit (Phoenix Pharmaceuticals, Burlingame, CA, USA) according to the manufacturer's instructions.

241 **2.6 Assessment of joint pathology**

242 At the end of each study, rats were euthanised via overdose with sodium
243 pentobarbital (Euthatal, 2ml, i.p.) and whole knee joints collected and fixed in 10%
244 neutral buffered formalin (Guingamp et al., 1997). Knee joints were disarticulated,
245 and cartilage damage quantified by a blinded experimenter via established
246 macroscopic scoring methods (Guingamp et al., 1997). 7.6% of rats were excluded
247 from the study due to inconsistent joint pathology (see **Extended Data Table 1-1** for
248 further details).

249

250 **2.7 Experimental design & statistical analyses**

251 **2.7.1 Behavioural Data:** To correct for strain differences in total bodyweight,
252 weightbearing asymmetry was calculated as the percentage of weight borne on the
253 ipsilateral hindlimb compared to the total of both hindlimbs [ipsilateral g/(ipsilateral
254 g+contralateral g)]. Similarly, locomotor activity was assessed as the number of
255 beam breaks per minute per kilogram bodyweight. Mechanical PWTs are reported as
256 raw vFH values, or as change in the number of vFHs from baseline when pooling
257 between different cohorts of animals (see Figure 1). For response to
258 pharmacological interventions, % analgesia was calculated for both weightbearing
259 and PWTs. 100% analgesia constitutes total normalisation of weightbearing
260 asymmetry [ipsilateral g/(ipsilateral g+contralateral g) = 50%] or a normalisation of
261 PWT to pre-model baseline values. Anxiety-like behaviour was determined for each
262 animal via Ethovision software as the area under the curve (AUC) of the time spent
263 in the open arms of the elevated plus maze in 1 minute bins for the entire 10 min
264 assessment period, and latency to enter the open outer arm in seconds.

265

2.7.2 Spinal electrophysiology: Degree of wind up was determined from the total number of spikes recorded in response to each stimuli in a train of 16 delivered at 3 x C-fibre threshold. Responses corresponding to each fibre type were binned according to post-stimulus latency, and wind up calculated from those falling in the C-fibre (90-300ms) and post-discharge (300-1000ms) latency range. Responses to mechanical stimuli were recorded as average firing rate (Hz) in response to 10s stimulation with each vFH. To determine effects of pharmacological interventions, mean maximal inhibition (MMI) for each dose was calculated as maximal % change versus baseline, with peak inhibition occurring 40-60 mins after administration. MMI data for each dose were then plotted for each strain and treatment, and the AUC calculated and compared.

2.7.3 Statistical Analyses

Group sizes were calculated based on previous similar studies using the MIA model to give sufficient statistical power whilst minimising animal usage. Data were analysed using Prism 8.3.1 software (GraphPad, La Jolla, USA). Data distributions were assessed via D'Agostino & Pearson normality testing, and subsequently treated as parametric or non-parametric, as appropriate. Differences in pain behaviour data were analysed via two-way ANOVA, with strain and treatment as the independent variables and Tukey's *post-hoc* test for multiple comparisons. For all other comparisons one-way ANOVAs with Holm-Sidak multiple comparisons, or Kruskal-Wallis tests with Dunn's multiple comparison *post-hoc* tests were used to analyse data with 3 or more groups, whilst Mann-Whitney *U* tests or Wilcoxon signed rank tests were used for comparisons between strains. Data are stated as mean ± SEM, or median with interquartile range (IQR), as appropriate.

Full experimental data are available from the authors upon request.

3. Results

3.1 WKY rats exhibit a basal anxiety-like phenotype

To confirm the anxiety-like phenotype of WKY rats, behaviour in the anxiogenic environment of the elevated plus maze (EPM) was assessed in both strains at baseline. WKY rats spent significantly less time in the open arms when compared to Wistar rats (Figure 1A, median AUC 17.40, IQR 4.92 – 31.68 versus 33.60, IQR 19.2 – 57.87, p=0.003), and had a significantly greater average latency to enter the open outer arm (600 versus 298, IQR 86-600 seconds, p=0.0077, Mann-Whitney *U* test, Figure 1B). Collectively, these data confirm that the WKY rats used in this study had an anxiety-like behavioural phenotype.

3.2 WKY rats exhibit exacerbated pain behaviour in the MIA model of OA

WKY and Wistar rats had comparable hindpaw mechanical sensitivity at baseline and weight was borne equally on both hindpaws (Table 1). Consistent with our previous work, intra-articular injection of MIA resulted in a significant shift in weightbearing from the injured side (weightbearing asymmetry) in both strains of rats from day 3 onwards, which persisted for the duration of the study (Figure 1C). The degree of asymmetry following injection of MIA was similar in both strains of rats, although slightly less pronounced in WKY rats. Both strains of rats also exhibited a lowering of the ipsilateral mechanical hindpaw withdrawal threshold (PWT, Figure 1D) which was evident from day 10. Ipsilateral PWTs were lowered to a greater extent in the WKY strain when compared to Wistar rats (Table 1; mean change in vFH from baseline Wistar = -1.68, WKY = -3.22), indicating an exacerbated OA-like

316 pain phenotype, as previously reported (Burston et al., 2019). We also observed a
317 reduction in contralateral PWTs in WKY rats (**Extended Data Figure 1-1**), which is
318 consistent with a centralised pain phenotype in this model (Burston et al., 2019).
319
320 **3.3 Anxiety-like behaviour and OA-like joint pathology**
321 To determine whether induction of OA-like pain behaviour altered anxiety-like
322 behaviours, behavioural responses in the EPM were reassessed 19-21 days after
323 intra-articular injection of saline or MIA. No significant differences were observed
324 between MIA and saline-injected rats when comparing within strains (Figure 1E),
325 however an anxiety-like phenotype was present in the WKY strain when compared to
326 Wistar rats at this time point (Figure 1F, AUC for time spent in open arms 1.50 0.03 –
327 6.48 versus 20.10 IQR 5.98 – 41.61, **** $p<0.0001$, one-tailed Mann Whitney U test).
328 This is in agreement with our previous work demonstrating that presence of OA-like
329 pain behaviour does not further increase anxiety-like behaviour in either strain
330 (Burston et al., 2019). Locomotor activity at baseline and at D18-21 was comparable
331 between the two strains of rats (Figure 1G), demonstrating no differences in
332 exploratory activity in the absence of an anxiogenic environment.
333 To ensure the marked increase in MIA-induced pain behaviour in the WKY strain
334 was not due to an alteration in joint pathology, cartilage damage in the injected knee
335 was assessed via macroscopic scoring. MIA administration was associated with
336 significant increases in cartilage damage compared to the appropriate saline control,
337 in both WKY and Wistar rats (Figure 1H). It is noteworthy that, despite the
338 significantly greater pain behaviour in MIA-treated WKY rats, this strain actually had
339 slightly less cartilage damage when compared to Wistar rats.
340

3.4 Exacerbated pain behaviour in the WKY/MIA model is associated with enhanced excitability of spinal neurones

To determine whether the altered behavioural phenotype in the MIA model of OA-like pain behaviour in WKY rats was encoded in the spinal cord dorsal horn, a key site in neuronal nociceptive circuitry, we then performed *in vivo* single-unit recordings of spinal WDR neurons in both WKY and Wistar rats 21-27 days after model induction. No differences were observed in baseline characteristics of recorded neurones (depth, Aβ and C-fibre latencies and thresholds, see **Extended Data Table 2-1**). However, wind up, a proxy of central sensitization (Li et al., 1999), did significantly vary across strains and treatments (Figure 2A). In the absence of a pain state, wind-up was significantly greater in WKY rats when compared to their Wistar counterparts ($p<0.05$ for stimuli 5 & 6, 2 way ANOVA with Tukey multiple comparisons *post-hoc* testing), and was further increased 21 days after MIA-treatment in the WKY strain ($p<0.05$, stimuli 3-6, 2 way ANOVA with Tukey multiple comparisons *post-hoc* testing). The response to the first stimulus in the train was also notably larger in the WKY strain (Figure 2A), particularly responses occurring at Aδ and C fibre latencies in WKY rats (**Extended Data Figure 2-2**). Despite a marked lowering of ipsilateral PWTs in the MIA model in both strains, there were no differences in the magnitudes of innocuous and noxious mechanically-evoked responses of WDR neurons between any of the groups (Figure 2B). Taken together, these data demonstrate enhanced spinal sensitivity to nociceptive input in the WKY strain, with further enhanced excitability to overt nociceptive stimuli in the presence of an OA-like pain state.

3.4 Reduced effects of systemic morphine in the WKY/MIA model of high anxiety & OA-like pain behaviour

366 At 21 days following induction of the MIA model of OA pain, subcutaneous (s.c.)
367 injection of the opioid receptor agonist morphine (0.5, 2.5, & 6 mg.kg^{-1} cumulative
368 dose) produced a significant, dose-related reduction in weightbearing asymmetry in
369 Wistar rats (Figure 3A). In contrast, only the highest dose of morphine significantly
370 inhibited weightbearing asymmetry in MIA-treated WKY rats (Figure 3A). Similarly,
371 morphine had a significantly blunted effect on ipsilateral PWTs in MIA-treated WKY
372 rats when compared to the Wistar strain (Figure 3B). All three doses of morphine
373 significantly reversed PWTs in MIA-treated Wistar rats, whereas only the highest
374 dose of morphine produced a significant reversal in ipsilateral PWTs in WKY rats
375 (Figure 3B). This highest dose of morphine significantly reversed the lowered
376 contralateral PWT evident in MIA treated WKY rats (Figure 3C).
377 At the level of the spinal cord, the inhibitory effect of cumulative systemic (s.c.) doses
378 of morphine on evoked neuronal responses was also significantly blunted in MIA-
379 treated WKY rats when compared to Wistar rats (Figure 3D-I). Although the mean
380 maximal inhibitory (MMI) effect of the lowest dose (0.5 mg.kg^{-1}) was similar across
381 the range of noxious and non-noxious mechanical stimuli studied, there was little
382 additional inhibition following successive increased doses of morphine in the WKY
383 MIA rats. This was particularly evident with the lower force filaments (8 & 10g, Figure
384 3D-E, & G-H). These data provide further evidence of reduced inhibitory effects of
385 systemic morphine in rats with an anxiety-like phenotype and OA-like pain.
386

387 **3.5 Evidence for altered endogenous opioid signalling in WKY rats**

388 We hypothesised that the reduced inhibitory effects of morphine on pain behaviour
389 and spinal neuronal responses in WKY rats may arise as a result of changes in
390 opioid receptor function and circulating levels of endogenous opioids. To test this,

plasma levels of β-endorphin were measured by ELISA. Significantly higher levels of β-endorphin were detected in WKY rats when compared to Wistar rats (Figure 4A). We then focused on MOR expression at the level of the dorsal horn of the spinal cord. Western blotting revealed no significant differences in total spinal MOR expression in any group (Figure 4B&C). There was, however, an increase in the proportion of MOR phosphorylated at the master phosphorylation site (serine residue 375; P-ser375) in the dorsal horn of the spinal cord in MIA-treated WKY rats, when compared to saline-treated WKY rats (Figure 4D, $p = 0.0143$). By contrast, the proportion of phosphorylation at P-ser375 in the dorsal horn of the spinal cord was not altered in MIA-treated Wistar rats compared to saline-treated Wistar rats, and levels were comparable in the saline-treated WKY rats.

As our data so far point towards altered opioidergic function in WKY rats, the effects of blocking the μ-opioid receptor with the antagonist naloxone on pain behaviour were assessed in the MIA model in both strains of rats (Figure 5). Naloxone (0.1-1 $mg.kg^{-1}$ s.c.) did not alter PWTs in Wistar rats 21 days after intra-articular injection of saline, suggesting no overt basal endogenous opioidergic tone in these rats. However, all three doses of naloxone (0.1-1 $mg.kg^{-1}$) significantly lowered ipsilateral PWTs in MIA-treated Wistar rats, supporting the presence of endogenous opioid tone following induction of the model of OA pain in this strain of rats. In WKY rats, systemic naloxone (0.1-1 $mg.kg^{-1}$) produced a significant bilateral lowering of PWTs (Figure 5A-D) which was similar in both saline- and MIA-treated WKY rats. These data further support the presence of an elevated endogenous opioid tone in WKY rats in the absence of the pain model, consistent with the increased levels of β-endorphin demonstrated herein. The endogenous opioid tone did not appear to be further increased in the presence of the model of OA pain.

Discussion (1138)

Clinically, people with high anxiety and chronic pain, including OA pain, have greater opioid consumption compared to people with comparable pain but less anxiety (Barnett et al., 2018). Here, we demonstrate reduced morphine-mediated analgesia in a rodent model of heightened anxiety and OA-like pain, providing an experimental model to further investigate the underlying neurobiological mechanisms.

Systemically-administered morphine produced a robust inhibition of behavioural pain responses following OA-induced joint pathology in Wistar rats. However, in WKY rats with a heightened anxiety-like phenotype, the effects of morphine on OA-induced pain behaviour were significantly blunted. In particular, the lowest dose of morphine significantly reversed weightbearing asymmetry and hindpaw withdrawal thresholds in OA-treated Wistar, but not WKY, rats. Since the highest dose of systemic morphine produced comparable inhibitory effects on MIA-induced pain behaviour in both strains of rats, it is likely that this results from reduced efficacy of opioid signalling in WKY rats. Reduced efficacy of morphine was previously reported in acute pain tests in WKY rats (Hestehave et al., 2019). We provide the first evidence of blunted opioid analgesia in a clinically-relevant model of chronic OA pain in this genetic strain of rats, providing a platform to interrogate the underlying neurobiology.

Our experimental data are consistent with clinical evidence that anxiety in people with joint pain is associated with the greater use of prescription opioids (Barnett et al., 2018), and prolonged use of opioids following total joint replacement for persistent joint pain (Namba et al., 2018). Catastrophizing has also been associated with increased prescription opioid use 4 years following total joint replacement (Valdes et al., 2015). Interestingly, recent work reported that the relationship

441 between anxiety and prescription opioid use for chronic pain is stronger in males
442 than females (Rogers et al., 2020), supporting our strategy of using male rats for this
443 preclinical experimental model system.
444 Electrophysiological recordings of spinal cord dorsal horn WDR neurones in OA-
445 treated WKY rats compared to OA-treated Wistar rats also revealed subtle changes
446 in MOR function. Specifically, the effects of systemic morphine on WDR responses
447 to low- and high-force mechanical stimulation of the hindpaw were blunted in WKY
448 OA-treated rats, supporting the notion that MOR function is altered in these animals.
449 Clearly, these changes in morphine responsiveness may reflect alterations in MOR
450 function at multiple levels of the neuraxis. We hypothesised that these blunted
451 inhibitory effects of exogenous morphine may arise due to a loss or desensitization
452 of MOR.
453 The C-terminus of MOR has a number of phosphorylation sites that contribute to
454 receptor desensitization and internalisation. Of the residues that undergo agonist-
455 dependent phosphorylation, residues 375 to 379 (STANT) have a critical role in
456 endogenous opioid-induced acute desensitization, recovery from desensitisation,
457 and internalisation of MOR (Arttamangkul et al., 2019; Kliewer et al., 2019). In our
458 study, phosphorylation of the serine-375 residue of MOR was significantly elevated
459 in the ipsilateral dorsal horn of the spinal cord of OA-treated WKY rats. These data
460 suggest that MOR tolerance in the dorsal horn spinal cord may account, at least in
461 part, for the loss of analgesic effect of morphine in these rats. However, the spinal
462 cord is unlikely to be the only site and changes in MOR expression and/or
463 phosphorylation at other sites in the brain may also contribute.
464 Previous studies have reported complex relationships between endogenous opioid
465 function, depressive symptoms, and trait anxiety in people (Burns et al., 2017). In our

study, a small, but significant, elevation in plasma β-endorphin was detected in WKY rats. This finding, alongside the demonstration of a reduced analgesic effect of systemic morphine in the model of OA pain, is consistent with the clinical evidence that greater endogenous opioid function is associated with lower morphine analgesic responsiveness (Bruehl et al., 2013). A limitation of our study is that plasma levels of β-endorphin were not measured in MIA-treated WKY rats with OA-like pain, nor were levels of other endogenous opioids determined. Nevertheless, based on previous studies of the MIA model in mice (Aman et al., 2019), we predict that β-endorphin levels are likely further elevated in the model of OA pain.

Functional evidence for changes in endogenous opioid tone in WKY rats was provided by our demonstration that systemic naloxone lowered hindpaw withdrawal thresholds in Wistar rats in the presence of the model of OA pain, but not in pain free saline-treated Wistar rats. These data are consistent with the engagement of the endogenous opioidergic systems in models of chronic pain previously demonstrated in clinical pain states (Levine et al., 1978; Kayser and Guilbaud, 1990; reviewed in Fields, 2004). Under these conditions, the endogenous opioidergic systems acts to counter the increased pro-nociceptive signalling and the manifestation of pain behaviour and experience. In the WKY strain, naloxone significantly lowered hindpaw withdrawal thresholds in the absence of the model of OA pain, consistent with the elevated levels of plasma β-endorphin in WKY rats demonstrated herein. The presence of the model of OA pain in WKY rats did not further increase the effect of naloxone on hindpaw withdrawal thresholds, suggesting that the presence of the model of OA pain could not further engage the endogenous opioidergic system in the WKY rats, as it did in the Wistar rats. This loss of chronic pain-induced engagement of the opioidergic inhibitory pathways, alongside our evidence for increased

491 phosphorylation of serine-375 of MOR in WKY MIA treated rats, may account for the
492 reduced effectiveness of the lower doses of morphine in WKY MIA treated rats.
493 A consideration of this work is the use of an inbred rat strain to model anxiety-like
494 behaviour, and whether this may confound our findings. Differences in MOR gene
495 expression between WKY and Sprague Dawley rats have been reported for some
496 brain regions (Burke et al., 2019). Although no differences in expression were
497 observed in the reward-associated nucleus accumbens (Dennis et al., 2016), and the
498 report of reduced acquisition of morphine-induced conditioned place preference in
499 WKY rats, supports our evidence for dysfunctional responses to exogenous opioids
500 in this strain. Alongside altered opioid function, WKY rats have lower basal levels of
501 limbic serotonin and dopamine, resulting in a blunted serotonergic and noradrenergic
502 response to acute stress (De La Garza and Mahoney, 2004; Yamada et al., 2013)
503 which may be relevant to the clinical situation. Given the key roles for supraspinal
504 monoamines in descending modulation of pain signalling (Bannister and Dickenson,
505 2016), it is likely that both opioidergic and monaminergic dysfunction contribute to
506 augmented OA- like pain responses in WKY rats.
507
508 Our study highlights the functional significance of the combination of anxiety, chronic
509 pain, and elevated opioidergic tone, which leads not only to increased pain
510 behaviour, but also decreased efficacy of opioid analgesia. Broader implications of
511 our work include caution in the prescription of opioids to manage chronic pain in OA
512 patients with comorbid high anxiety.

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658

659

Figure Legends

Table 1 – Summary of animal numbers and behavioural data

Data from all 3 cohorts of animals utilised in this study (M = morphine study, N = naloxone study, E = electrophysiology study). Data are expressed as mean ± SD, or median (IQR). Significance assessed via 2 way ANOVA with Tukey multiple comparison *post-hoc* testing, Kruskal-Wallis test with Dunn's multiple comparison *post-hoc* testing, or Mann-Whitney *U* tests as appropriate.

^^ p<0.01, ^^^^ p<0.0001 versus Wistar/saline & Wistar/MIA

# p<0.05, #### p<0.0001 versus Wistar/saline

* p<0.05, **** p<0.0001 versus WKY/saline

+ p<0.05, ++ p<0.01, +++ p<0.001, ++++ p<0.0001 versus Wistar/MIA.

Figure 1 – Anxiety-like phenotype & exacerbated OA-like pain in the WKY-MIA model

Basal anxiety-like phenotype in WKY rats in the elevated plus maze (EPM). WKY rats spent significantly less time in the open arms of the maze (**A**), and showed an increased latency to enter the most anxiogenic area of the arena; the open outer arms (**B**). Bars indicate median values, error bars represent IQR. ** p<0.01 versus Wistar rats, one-tailed Mann-Whitney *U* test.

MIA-treated rats had a similar degree of weight-bearing asymmetry in both the Wistar and WKY strains (**C**), with pain behaviour evident from day 3 onwards and maintained until post-injection day 21 (D21). Saline administration did not effect weightbearing in either strain. Data are mean ± SEM % weight borne on the

683 ipsilateral hindlimb, #### p< 0.001 versus Wistar saline, **** p< 0.0001 versus WKY
684 saline, 2 way ANOVA with Tukey mutiple comparison *post-hoc* testing.

685 MIA-treated rats had lowered ipsilateral PWT in both strains, though this occurred
686 earlier and to a greater extent in WKY rats when compared to Wistar rats (**D**). Data
687 are mean ± SEM change in vFH compared to baseline, * p<0.05, ** p<0.01, versus
688 WKY saline, # p<0.05, ## p<0.01 versus Wistar saline, + p<0.05, versus Wistar MIA,
689 2 way ANOVA with Tukey multiple comparison *post-hoc* testing.

690 Measurement of anxiety-like behaviour in the EPM at D21 after model induction
691 revealed no significant differences between MIA- and saline-injected rats for the two
692 strains (**E**), but there remained a significant anxiety-like phenotype in the WKY strain
693 when compared to the Wistar strain (**F**). Data represent AUC of time spent in the
694 open arm for each minute of the 10 min trial, individual data points are shown with
695 bars indicating the median value for each group, and error bars representing the
696 IQR. ## p<0.01 versus Wistar saline, Kruskal-Wallis test with Dunn's multiple
697 comparison *post-hoc* test, ++++ p<0.001 versus Wistar rats, one-tailed Mann-
698 Whitney *U* test.

699 To ensure any behavioural differences observed in the EPM did not result from strain
700 differences in locomotion, locomotor activity was assessed over a 1 hour period at
701 baseline, and 18-21 days after model induction (**G**). No significant differences
702 between strains were observed at either time point. Data are expressed as mean ±
703 SEM beam breaks per hour, adjusted for bodyweight.

704 Macroscopic assessment of cartilage damage in ipsilateral knee joints revealed
705 extensive widespread cartilage damage in MIA-injected rats of both strains, with little
706 or no damage observed in saline-injected animals (**H**). Data represent the summed

scores for each of the 5 individual joint compartments (0-5, max score 25), individual data points are shown, with bars representing the median value and error bars the IQR. #### p<0.001 versus Wistar saline, **** p<0.001 versus WKY saline.

Figure 2 - Enhanced spinal excitability and nociceptive responses in the WKY-MIA model

In vivo electrophysiological recordings of WDR neurons in the deep DH revealed enhanced wind up in the WKY strain in response to noxious electrical stimulation at 3 x C-fibre threshold (**A**). The number of action potentials recorded in the 90-1000ms post-stimulus (C-fibre to post-discharge latency range) was significantly higher in saline-injected WKY rats (stimuli 5 & 6) and MIA-injected WKY rats (stimuli 3-6) when compared to their Wistar counterparts. Data represent mean ± SEM number of action potentials. * p<0.05 versus WKY saline, + p<0.05 versus Wistar MIA, 2 way ANOVA with Tukey multiple comparisons *post-hoc* testing.

There were no significant differences in the frequency of WDR firing recorded in response to mechanical stimulation across a range of vFHs between strains or treatments (**B**). Data represent the frequency of firing in response to a 10s stimulus with each vFH. Values are means ± SEM.

Figure 3 - Reduced behavioural & spinal response to systemic morphine in the WKY/MIA model of high anxiety & OA-like pain

Systemic administration of morphine produced dose-related inhibitions in the MIA model in Wistar rats. By contrast, inhibitory effects of the low dose of morphine were

730 significantly attenuated in the WKY strain for weightbearing asymmetry (**A**) and
731 ipsilateral PWTs (**B**). Morphine did not significantly alter contralateral PWTs in MIA-
732 treated Wistar rats. However, 6 mg.kg^{-1} morphine reversed lowered contralateral
733 PWTs in MIA-treated WKY rats (**C**). Data represent % analgesia to 3 cumulative
734 doses of systemic morphine, with abolition of weightbearing asymmetry representing
735 100% analgesia in **A**, and a return to PWTs to pre-model basal values representing
736 100% analgesia in **B** & **C**. * p<0.05, ** P<0.01, ***p<0.001, ****p<0.0001, Wilcoxon
737 Signed Ranks test with a hypothetical value of 100.

738 Effects of systemic morphine on mechanically-evoked responses of spinal WDR
739 neurones was also blunted in MIA treated WKY rats. There was a blunted effect of
740 cumulative doses of 2.5 and and 6 mg.kg^{-1} morphine on 8g- (**D**), 10g- (**E**), and 26g-
741 evoked (**F**) vFH evoked responses of WDR neurones in MIA-treated WKY rats,
742 which was confirmed by AUC analysis (**G**-**I**). Data represent median ± IQR, * p<0.05,
743 ** p<0.01 Mann-Whitney *U* tests.

744

745 **Figure 4 - Altered endogenous opioid function in the WKY/MIA model**

746 Assessment of circulating plasma levels of β-endorphin in terminal blood samples
747 from naïve Wistar (*n* = 7) and WKY (*n* = 8) rats revealed a 20% increase in the WKY
748 strain (**A**). Bars represent the median value, error bars indicate IQR. * p = 0.0361,
749 one-tailed Mann-Whitney *U* test.

750 Western blotting depicting expresssion of total MOR, and MOR phosphorylated at
751 the master phosphorylation site (P-ser375) in whole lumbar spinal cord
752 homogenates (**B**). Densitometry quantification revealed no change in total MOR
753 levels (**C**) across strains and treatments (*n* = 4/group), and no significant change in

754 phosphorylation in MIA-treated Wistar rats (**D**). However, a significant increase in the
755 proportion of P-ser375-MOR was evident in MIA-treated WKY rats when compared
756 to saline-treated controls. Lines at median values, error bars represent IQR, * p =
757 0.0143, one-tailed Mann-Whitnety *U* test.

758

759 **Figure 5 - Altered endogenous opioid tone in rats with an anxiety-like**
760 **phenotype**

761 Systemic administration of the MOR antagonist naloxone (0.1-1 $mg.kg^{-1}$, s.c.)
762 significantly reduced ipsilateral PWTs in MIA-treated, but not saline-treated, Wistar
763 rats (**A**), suggesting elevated opioidergic tone in the model of OA-like pain. In WKY
764 rats, naloxone produced a significant, dose-dependent, and bilateral reduction in
765 PWTs in both saline- and MIA-treated WKY rats (**A**&**B**), suggesting a more
766 generalised elevation of opioidergic tone in this strain. Data represent mean ± SEM
767 for baseline PWTs, and MMI values for each cumulative dose of naloxone. # p<0.05,
768 ## p<0.01, ### p<0.001, #### p<0.0001 versus Wistar saline; *p<0.05, ****
769 p<0.0001 versus WKY saline; + p<0.05, ++p<0.01, +++ p<0.001, ++++ p<0.0001
770 versus Wistar MIA, 2 way ANOVA with Tukey multiple comparison *post-hoc* testing.

771 AUC analysis of dose response curves revealed a significantly greater effect of
772 naloxone on ipsilateral (**C**) and contralateral (**D**) PWTs in WKY rats when compared
773 to Wistar rats. Individual data points are shown with bars representing median values
774 and error bars depicting IQR. ## p<0.01, ### p<0.001 versus Wistar saline;
775 ++p<0.01 versus Wistar/MIA, Kruskal-Wallis test with Dunn's multiple comparison
776 *post-hoc* testing.

	Wistar/Saline	**Wistar/MIA**	**WKY/Saline**	**WKY/MIA**
Total No.	**41**	**48**	**41**	**45**
Cohort 1 (M)	8	10	8	10
Cohort 2 (N)	12	12	10	11
Cohort 3 (E)	17	22	19	20
Bodyweight (Cohort 3, g, mean ± SD)				
Basal	**183±15**	**184±14**	**157±14** ^^^^	**160±16** ^^^^
Day 21	**340±23**	**337±28**	**251±15** ^^^^	**247±12** ^^^^
Pain				
Basal WB %	**50.16**	**49.75**	**50.42**	**50.13**
Cohort 1 (M)	49.69	49.67	50.74	50.54
Cohort 2 (N)	50.39	48.74	50.94	50.04
Cohort 3 (E)	50.23	50.37	49.98	49.95
Day21 WB %	**48.89**	**36.84** ####	**50.11**	**43.12** ****, +
Cohort 1 (M)	50.08	38.99	50.99	43.97
Cohort 2 (N)	48.36	38.24	49.59	44.71
Cohort 3 (E)	48.64	34.90	49.96	41.85
Basal ipsilateral PWT (g)	**22.12±5.35**	**23.35±5.21**	**20.39±6.42**	**19.78±6.12**
Cohort 1 (M)	21.88±5.69	23.30±5.81	19.13±5.69	18.90±6.30
Cohort 2 (N)	23.00±5.14	26.00±0	18.00±8.55	20.50±5.88
Cohort 3 (E)	21.60±5.58	22.26±5.75	22.12±5.42	19.94±6.41
D21 ipsilateral	**19.47**	**12.71**	**12.69**	**7.17**

PWT (g)				
Cohort 1 (M)	22.38±6.97	15.70±5.89	13.63±3.89	8.00±6.46
Cohort 2 (N)	20.09±7.01	14.44±8.76	17.14±9.35	10.25±3.24
Cohort 3 (E)	17.47±7.60	10.32±5.15	10.41±4.15	5.33±3.82
Mean Δ ipsilateral PWT (vFH)	**-0.44**	**-1.68** #	**-1.44** #	**-3.22** ####, *, ++
Cohort 1 (M)	-0.13±0.99	-0.88±0.99	-0.88±1.73	-2.90±1.29
Cohort 2 (N)	-0.36±1.03	-1.78±1.48	-0.57±1.81	-1.76±1.16
Cohort 3 (E)	-0.67±1.18	-2.11±1.45	-2.06±1.34	-4.06±2.15
Basal contralateral PWT (g)	**22.29**	**24.31**	**21.33**	**20.95**
Cohort 1 (M)	21.25±6.73	26.00±0.00	21.88±5.69	21.11±5.80
Cohort 2 (N)	23.00±5.14	26.00±0.00	19.75±8.65	21.88±5.69
Cohort 3 (E)	22.33±5.37	22.53±5.25	21.82±5.94	20.50±5.64
Day 21 contralateral PWT (g)	**23.41**	**24.08**	**13.18**	**9.68**
Cohort 1 (M)	24.63±3.89	26.00±0.00	15.75±5.25	8.56±2.70
Cohort 2 (N)	23.00±5.14	23.00±5.14	14.63±8.18	10.50±6.48
Cohort 3 (E)	23.07±5.04	23.68±4.61	11.29±3.02	9.85±4.89
Mean Δ	**0.12**	**-0.03**	**-1.21**	**-2.06**

contralateral PWT (vFH)		####	####, ++++	####, ++++
Cohort 1 (M)	0.38±0.92	0.00±0.00	-1.00±1.41	-2.20±1.03
Cohort 2 (N)	0.00±0.77	-0.30±0.48	-0.88±1.13	-1.75±1.16
Cohort 3 (E)	0.07±0.70	0.11±0.46	-1.47±0.80	-2.11±1.18
Basal Anxiety (open arm AUC)				
Cohort 3 (E)	33.60 (19.20-58.87)		17.40 (4.92-31.68) ^^	
Anxiety Post-Model Induction (open arm AUC)				
Cohort 3 (E)	16.56 (4.40-24.84)	32.16 (15.46-50.61)	1.38 (0.03-3.96) ##, +++	2.58 (0.12-14.40) ++

Lillywhite & Woodhams et al, 2020 - Table 1 – Summary of animal numbers and behavioural data

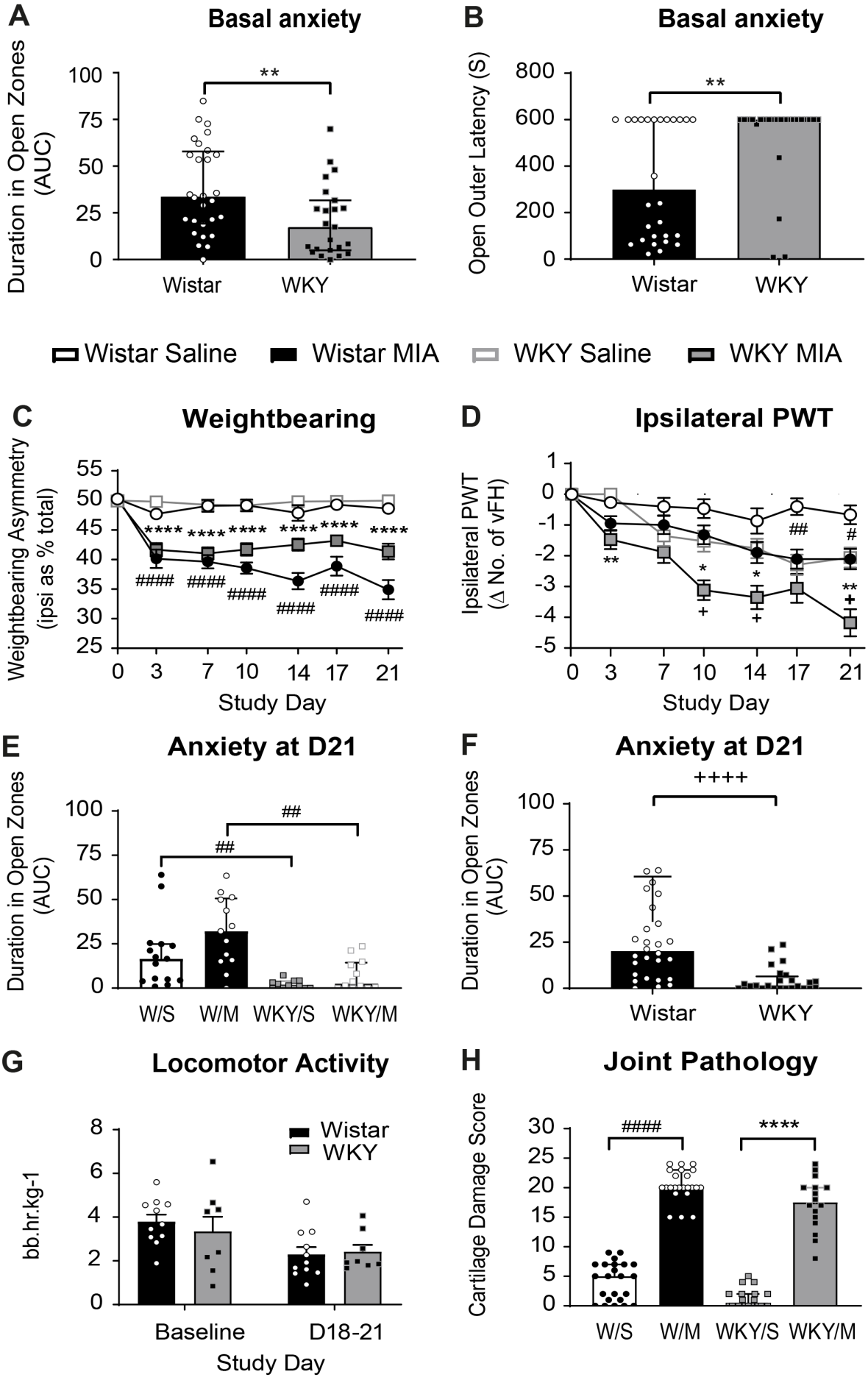

Figure 1 - Lillywhite & Woodhams et al, 2020

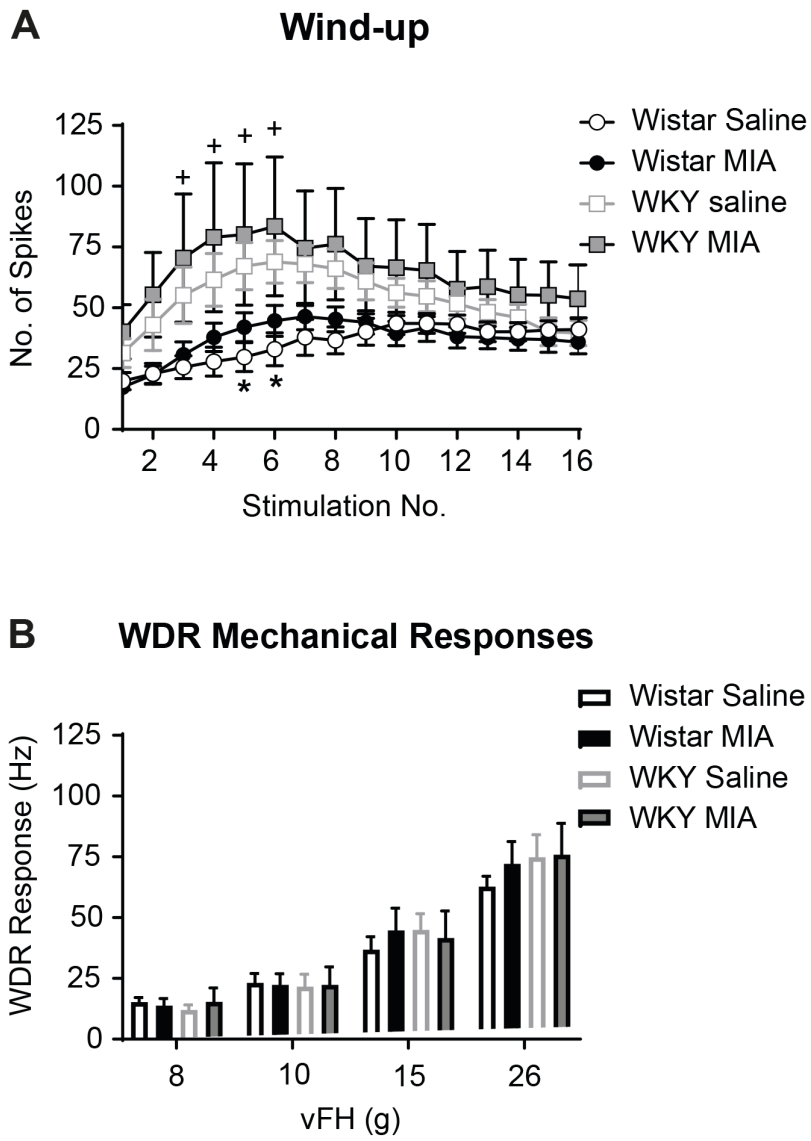

Figure 2 - Lillywhite & Woodhams et al, 2020

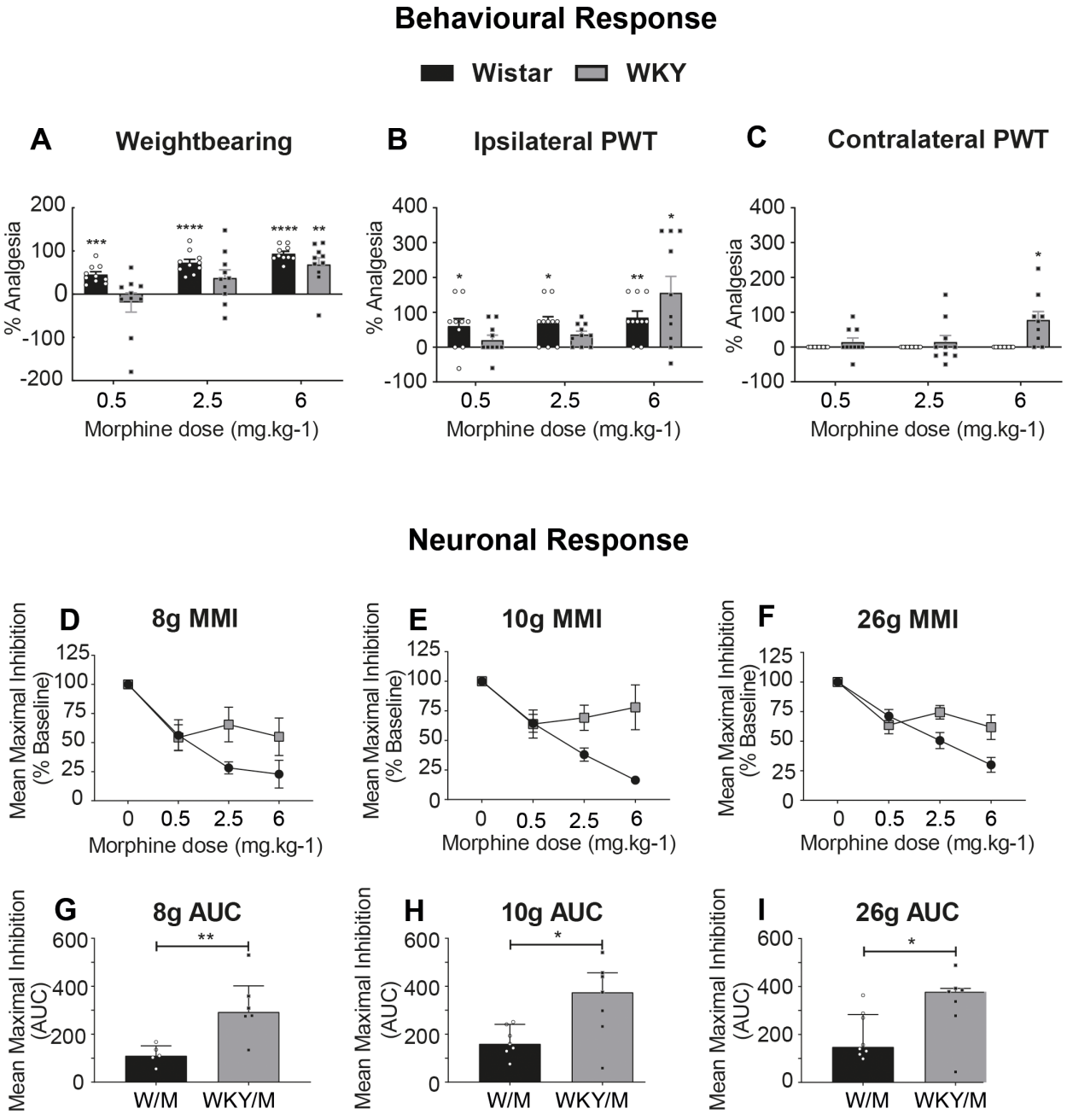

Figure 3 - Lillywhite & Woodhams et al, 2020

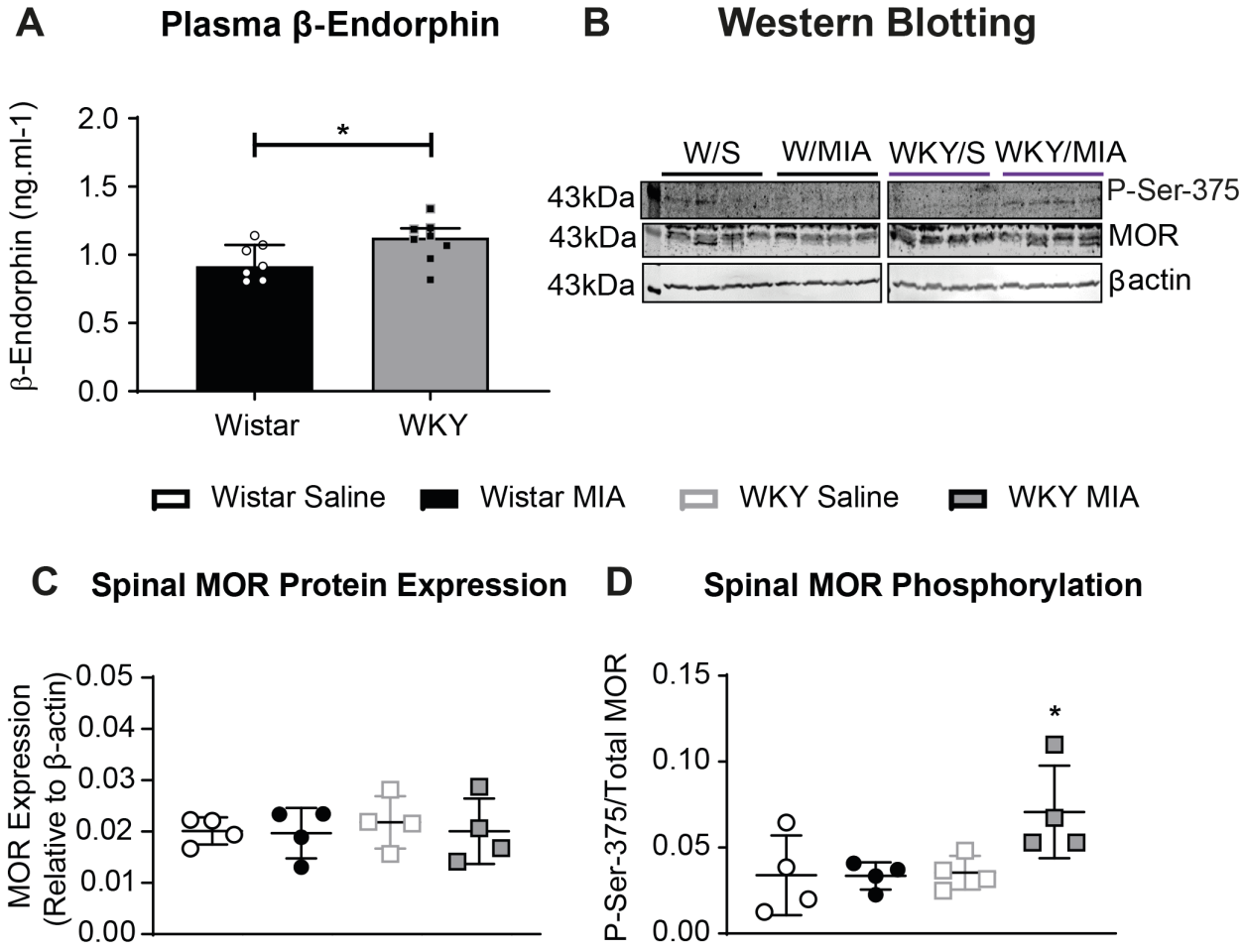

Figure 4 - Lillywhite & Woodhams et al, 2020

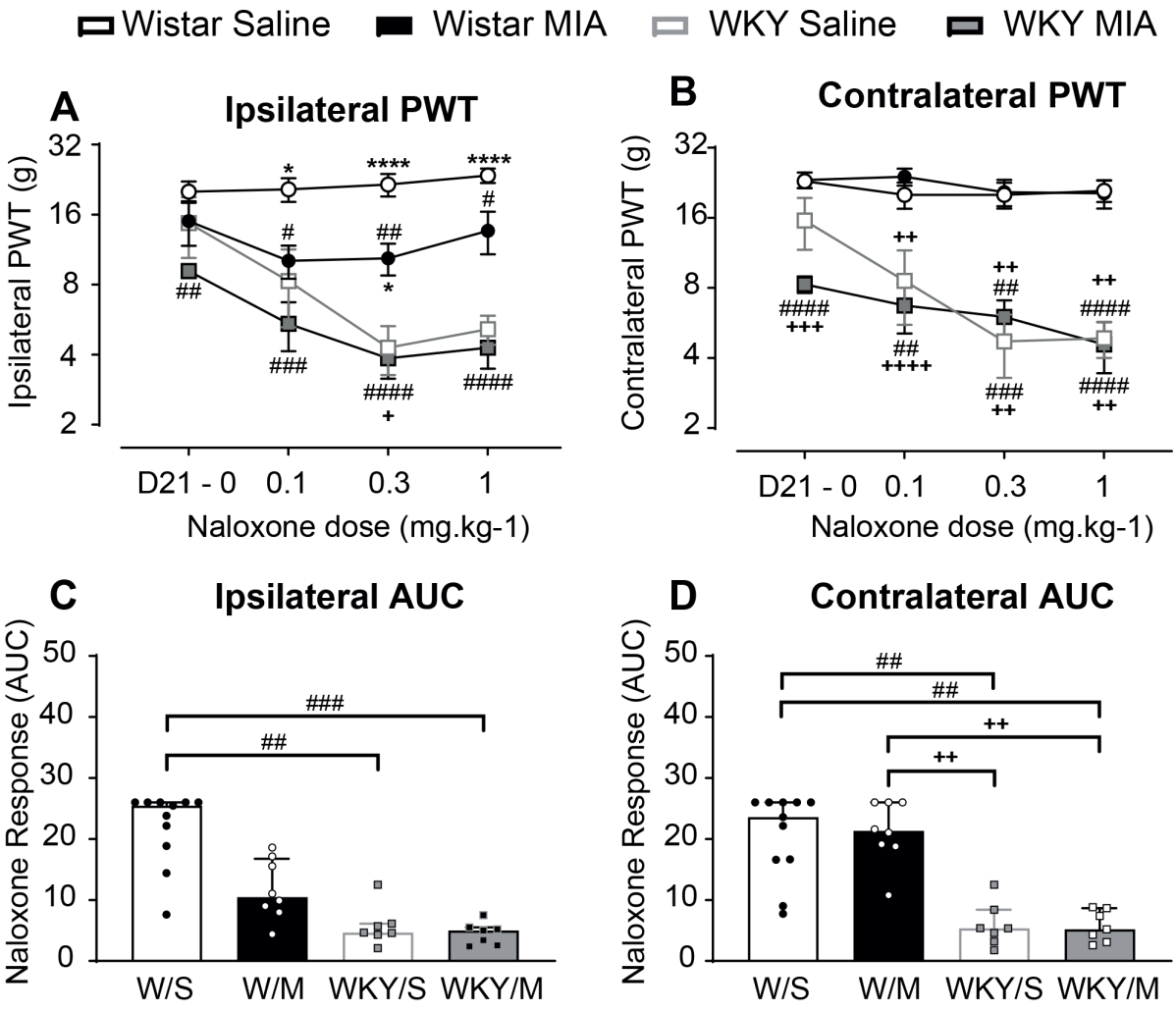

Figure 5 - Lillywhite & Woodhams et al, 2020