Melatonin limits paclitaxel-induced mitochondrial dysfunction in vitro and protects against paclitaxel-induced neuropathic pain in the rat

Galley, Helen F.; McCormick, Barry; Wilson, Kirsten L.; Lowes, Damon A.; Colvin, Lesley; Torsney, Carole

Published in:
Journal of Pineal Research

DOI:
10.1111/jpi.12444

Publication date:
2017

Document Version
Publisher's PDF, also known as Version of record

Received: 22 June 2017 | Accepted: 18 August 2017

DOI: 10.1111/jpi.12444

ORIGINAL ARTICLE

WILEY Journal of Pineal Research
Molecular, Biological, Physiological and Clinical Aspects of Melatonin

Melatonin limits paclitaxel-induced mitochondrial dysfunction in vitro and protects against paclitaxel-induced neuropathic pain in the rat

Helen F. Galley[1] | **Barry McCormick**[1,2] | **Kirsten L. Wilson**[2] | **Damon A. Lowes**[1] | **Lesley Colvin**[3] | **Carole Torsney**[2]

[1]Institute of Medical Sciences, University of Aberdeen, Aberdeen, UK

[2]Centre for Integrative Physiology, Edinburgh Medical School: Biomedical Sciences, University of Edinburgh, Edinburgh, UK

[3]Department of Anaesthesia, Critical Care and Pain Medicine, University of Edinburgh, Edinburgh, UK

Correspondence
Helen F. Galley, Institute of Medical Sciences, University of Aberdeen, Aberdeen, UK.
Email: h.f.galley@abdn.ac.uk
and
Carole Torsney, Centre for Integrative Physiology, Edinburgh Medical School: Biomedical Sciences, University of Edinburgh, Edinburgh, UK.
Email: carole.torsney@ed.ac.uk

Funding information
The study was funded by the Association of Anaesthetists of Great Britain and Ireland, the British Journal of Anaesthesia/Royal College of Anaesthetists and the Melville Trust

Abstract

Chemotherapy-induced neuropathic pain is a debilitating and common side effect of cancer treatment. Mitochondrial dysfunction associated with oxidative stress in peripheral nerves has been implicated in the underlying mechanism. We investigated the potential of melatonin, a potent antioxidant that preferentially acts within mitochondria, to reduce mitochondrial damage and neuropathic pain resulting from the chemotherapeutic drug paclitaxel. In vitro, paclitaxel caused a 50% reduction in mitochondrial membrane potential and metabolic rate, independent of concentration (20-100 μmol/L). Mitochondrial volume was increased dose-dependently by paclitaxel (200% increase at 100 μmol/L). These effects were prevented by co-treatment with 1 μmol/L melatonin. Paclitaxel cytotoxicity against cancer cells was not affected by co-exposure to 1 μmol/L melatonin of either the breast cancer cell line MCF-7 or the ovarian carcinoma cell line A2780. In a rat model of paclitaxel-induced painful peripheral neuropathy, pretreatment with oral melatonin (5/10/50 mg/kg), given as a daily bolus dose, was protective, dose-dependently limiting development of mechanical hypersensitivity (19/43/47% difference from paclitaxel control, respectively). Melatonin (10 mg/kg/day) was similarly effective when administered continuously in drinking water (39% difference). Melatonin also reduced paclitaxel-induced elevated 8-isoprostane $F_2\alpha$ levels in peripheral nerves (by 22% in sciatic; 41% in saphenous) and limited paclitaxel-induced reduction in C-fibre activity-dependent slowing (by 64%). Notably, melatonin limited the development of mechanical hypersensitivity in both male and female animals (by 50/41%, respectively), and an additive effect was found when melatonin was given with the current treatment, duloxetine (75/62% difference, respectively). Melatonin is therefore a potential treatment to limit the development of painful neuropathy resulting from chemotherapy treatment.

KEYWORDS
antioxidant, chemotherapy, melatonin, mitochondria, neuropathic pain, oxidative stress, Paclitaxel

Helen F. Galley and Barry McCormick are equally contributed to this study.

1 | INTRODUCTION

A common side effect of cancer treatment is chemotherapy-induced neuropathic pain (CINP), which can be severe enough to require dose reduction or treatment cessation, with consequent effects on survival and the quality of life of patients with cancer.[1,2] This predominantly sensory neuropathy affects up to 68% of patients in the first month after finishing chemotherapy, with around 30% of patients still being symptomatic more than 6 months later[1]. CINP may not resolve and in some cases can worsen with time. Current treatment options are based mainly on evidence from other types of neuropathic pain;[3] duloxetine, a serotonin-norepinephrine re-uptake inhibitor, is one of the few agents with direct evidence of efficacy for CINP.[4] All of the currently used agents have significant limitations both in terms of efficacy and side effects. There is an urgent need for a treatment, which addresses the neuropathic mechanisms, and can prevent or alleviate CINP.

Paclitaxel is a commonly used chemotherapeutic agent with a high incidence of CINP.[1] It has been shown to cause mitochondrial dysfunction in vitro[5] and in peripheral nerves and dorsal root ganglia in vivo,[6,7] associated with oxidative stress. Administration of mitochondrial poisons in animals exacerbated paclitaxel-induced neuropathic pain,[8] whilst global radical scavengers (spin traps)[9], mitochondrial electron transport chain modulators[10] and reduction in mitochondrial damage with a small-molecule P53 inhibitor[11] or acetyl carnitine[12] were associated with decreases in neuropathic pain behaviours. These studies support the notion that CINP induced by paclitaxel is mediated by oxidative damage to mitochondria, and suggest that targeting treatments specifically at mitochondria may be beneficial. Our previous work showed that the mitochondria targeted antioxidant MitoVitE was able to reduce mechanical hypersensitivity in rats treated with paclitaxel.[13] However, MitoVitE has not been through Phase I studies, and its use cannot be rapidly translated into the clinical arena.

We have previously reported that MitoVitE and melatonin are equally effective at reducing mitochondrial dysfunction and markers of inflammation in other disease models both in vitro and in animals.[14] Melatonin, like MitoVitE, is able to accumulate inside mitochondria and is a potent antioxidant; its metabolites and reaction products are also effective.[15,16] Melatonin has been given safely to humans with no evidence of toxicity even at very high doses.[17] We therefore hypothesized that melatonin would reduce mitochondrial damage induced by paclitaxel in neuronal cells in vitro and would alleviate, and/or limit the development of, mechanical hypersensitivity and altered peripheral nerve function in a preclinical rat model of paclitaxel-induced painful neuropathy.

2 | MATERIALS AND METHODS

2.1 | In vitro studies

The 50B11 immortalized dorsal root ganglion (DRG) neuronal stem cell line was kindly donated by Professor Ahmet Hoke, from Johns Hopkins School of Medicine, Baltimore, MA, USA. These cells have nociceptive properties: after differentiation, they extend neurites and generate action potentials when depolarized, express key nociceptive markers and respond to capsaicin.[18] Culture and maintenance of these cells is described in our previous study.[13] They were differentiated into DRGs by exposure to 75 μmol/L forskolin for 24 hours, with outgrowth of neurites starting after about 10 hours. After differentiation, a range of concentrations of paclitaxel was added with and without 1 μmol/L melatonin or relevant solvent control for 24 hours.

2.1.1 | Mitochondrial function

Mitochondrial function was determined in intact cells after 24 hours treatment, by measurement of the mitochondrial membrane potential using the fluorescent probe JC-1 (5,5,6,6 -tetrachloro-1,1,3,3-tetraethylbenzimidazolcarbocyanine iodide, Invitrogen, Paisley, UK), and by measurement of metabolic activity using the rate of reduction of AlamarBlue™ (Invitrogen).[13,15] Mitotracker Green FM™ (Invitrogen) was used to determine mitochondrial volume.[19-21] Cell viability after treatments was measured as acid phosphatase activity as we have described previously and measures of mitochondrial function were corrected for viable cell number.[22]

2.1.2 | Cancer cell cytotoxicity

As melatonin might affect the cytotoxic capacity of paclitaxel against cancer cells, we measured paclitaxel-induced killing of the breast adenocarcinoma-like oestrogen-sensitive cell line, MCF-7, and the ovarian carcinoma cell line, A2780, in the presence of melatonin. Cancer cells were cultured for 24 hours with 0-100 μmol/L paclitaxel, with and without 1 μmol/L melatonin or solvent control as we have described for MitoVitE previously.[13] Acid phosphatase activity was used to assess cell viability as for DRG cells.

2.2 | Animal model

The animal work was approved by the UK Home Office and carried out in accordance with Animals (Scientific Procedures) Act 1986, following applicable aspects of the ARRIVE Guidelines.[23] Animal health and welfare was paramount throughout all studies, and animals were checked daily for any signs of distress. Weight gain and cage behaviour were continually recorded. All animals were handled as

gently as possible, and cage bedding was ample, to provide a soft environment.

Male and female Sprague Dawley rats weighing approximately 300 g were housed with a maximum of 6 (single sex) per cage in standard conditions, at 19-22°C, on a 12-hour light/dark cycle from 7 am to 7 pm. Food and drinking water were provided ad libitum. The rats were kept in the experimental area for a minimum of 3 days before baseline testing was started. Details of the model have been published previously[13,24] and are described only briefly here.

As rats were group housed, treatment group allocation was undertaken by cage rather than individual animal to avoid contamination by coprophagia. Rats received either 4 × doses of 2 mg/kg paclitaxel or cremophor/EL (polyethoxylated castor oil, the vehicle control for paclitaxel) diluted in saline, by intraperitoneal (i.p.) injection every second day.

2.2.1 | Behavioural assessment of mechanical sensitivity

Pseudo-blinding of treatment allocation was achieved by mixing animals from all treatment groups immediately before testing; group allocation was only confirmed by tail number when testing was complete. Prior to mechanical sensitivity testing, animals were acclimatized in 2 × 20 minute sessions on separate days and further habituated for 20 minutes immediately before testing on any given day. Hind paw plantar withdrawal thresholds to von Frey filaments were determined every 2-6 days throughout the model, using the up-down method,[25] as we have described previously.[13]

2.2.2 | Effect of bolus doses of melatonin

To assess the effect of pretreatment with bolus doses of melatonin, male rats (n = 5-6 per group) were given 1 of 3 bolus doses of melatonin (5, 10 or 50 mg/kg, 2 µl/g body weight) or 10% ethanol vehicle control by daily oral gavage (between 10:00 and 11:00 hours) starting 3 days prior to paclitaxel or control treatment. Mechanical sensitivity was measured 1, 6 and 24 hours after gavage every second or third day. In a separate study, male rats were given a bolus dose of 10 mg/kg melatonin by oral gavage, then blood samples were obtained by cardiac puncture from groups of rats (n = 3 per group) at 1, 2, 6, 12, 24 and 48 hours after dosing, or untreated controls, to determine the pharmacokinetics of melatonin.

2.2.3 | Effect of melatonin given in drinking water

We also administered melatonin (10 mg/kg/day) or vehicle control (0.1% v/v ethanol) in drinking water starting 3 days before paclitaxel treatment, to groups of male rats (n = 5-6 per group). A control group received saline (i.p.) plus melatonin in drinking water to determine whether melatonin impacted upon baseline mechanical sensitivity. To maintain 10 mg/kg/day melatonin dose, the concentration of melatonin was adjusted to account for mean water consumption over the previous 2 days, and average current weight of rats in a given cage. Opaque bottles were used and changed every 2 days. Levels of melatonin in drinking water were measured as described for serum below to confirm that melatonin levels were stable for at least 48 hours under these conditions. The actual levels of water consumption were consistently similar to that of water with vehicle control. After experimentation, blood was collected by cardiac puncture and serum melatonin levels were determined.

The effect of discontinuing or starting melatonin treatment after mechanical hypersensitivity had developed was also assessed. Groups of rats (n = 6) received either melatonin or vehicle control in drinking water continuously starting 3 days before paclitaxel as above; melatonin starting 3 days before paclitaxel then stopping (reverting to vehicle control) at day 18 once the hypersensitivity was apparent; or vehicle starting 3 days before paclitaxel then commencing melatonin treatment at day 20 once the hypersensitivity was fully established.

The combination of melatonin with the current CINP treatment, duloxetine, was also assessed. By targeting multiple mechanisms,[26] we hypothesized that there would be an additive effect on paclitaxel-induced mechanical hypersensitivity. Given increasing awareness of sex differences in pain and analgesic sensitivity,[27,28] this was investigated in both sexes. Male and female rats (n = 6 per group) were given melatonin or vehicle control in drinking water starting 3 days before paclitaxel as above, plus daily i.p. injections of duloxetine (10 mg/kg/day; 1 µl/g body weight) or vehicle control (20% ethanol in saline), starting when the mechanical hypersensitivity had established.

2.2.4 | Sedation testing

Prior to behavioural testing, rats were assessed on a 5-point scale for righting reflexes: 0, the rat struggles when placed on its side, followed by rapid forceful righting; 1, moderate resistance when the rat is placed on its side, with rapid but not forceful righting; 2, no resistance to the rat being placed on its side, with effortful but ultimately successful righting; 3, unsuccessful righting; and 4, no movements.[29]

2.2.5 | Serum melatonin levels

Rats were anesthetized by brief inhalation of isoflurane followed by overdose of 20% w/v pentobarbitone (~1 g/kg) given by i.p. injection. The chest cavity was rapidly opened, and the right atrium was punctured to collect blood for serum samples. Melatonin levels were determined in serum and

TABLE 1 Comparison of the C-fibre electrophysiological properties in dorsal roots obtained from the different treatment groups

	Threshold (μA)	Amplitude (mV/mm)	Average CV (m/s)	Initial response width (ms/mm)
Vehicle (n = 12)	121 ± 9.65	0.12 ± 0.02	0.23 ± 0.02	4.97 ± 0.2
Paclitaxel (n = 10)	100 ± 0	0.12 ± 0.03	0.22 ± 0.2	5.57 ± 0.2
Paclitaxel and Melatonin (n = 12)	104 ± 4.17	0.13 ± 0.02	0.21 ± 0.01	5.10 ± 0.3

One-way ANOVA reveals that treatment group does not affect threshold stimulus intensity ($P = .08$), amplitude ($P = .93$), average conduction velocity ($P = .75$) or the initial C-fibre response width ($P = .23$).

drinking water using a Thermo Surveyor-TSQ Quantum liquid chromatography tandem-mass spectrometry (LC-MS/MS) system (Thermo Scientific, Hemel Hempstead, UK). This assay has a lower limit of quantitation of 0.5 ng/ml and high inter- and intra-assay precision, as we have previously described in detail.[17]

2.2.6 | 8-isoprostane $F_2\alpha$ levels

F_2-isoprostanes, which are one of the most reliable measures of oxidative stress status in vivo,[30] were measured ex vivo in peripheral nerve tissue 19 days after paclitaxel/cremophor treatment and included experimental animals from the bolus dosing experiments receiving melatonin at 10 mg/kg or vehicle treatment. Rats were decapitated under isoflurane anaesthesia and sciatic and saphenous nerve tissue were removed, placed in ice cold 0.1 mol/L phosphate buffer and homogenized, then centrifuged and frozen at −80°C. Tissue was collected throughout the day with animals from different treatment groups interleaved to ensure all treatment groups were collected over comparable time windows. $F_2\alpha$ levels were assessed using a commercially available 8-isoprostane $F_2\alpha$ ELISA kit (Enzo Lifesciences, Exeter, UK). The lower limit of detection is 40 mg/ml with median intra- and interassay coefficients of variation of 5.7% and 5.8%, respectively.

2.2.7 | C-fibre activity-dependent slowing

C-fibre function was assayed by quantifying C-fibre activity-dependent slowing (ADS) in peripheral nerve tissue from male rats receiving cremophor or paclitaxel with and without 10 mg/kg melatonin in drinking water 14-18 days following the start of paclitaxel or vehicle treatment. Rats were decapitated under isoflurane anaesthesia, at the same time in the morning, and lumbar (L4/5) dorsal roots, minus dorsal root ganglia, were isolated and incubated at 36°-37°C in oxygenated recovery solution for 1 hour. Following incubation, tissue was transferred to the recording bath of an upright microscope (Zeiss, Oberkochen, Germany) and continuously perfused with oxygenated Krebs' solution (1-2 ml/min) at room temperature. The 95% O_2/5% CO_2 saturated Krebs' solution contained (in mmol/L) the following: 125 NaCl, 2.5 KCl, 1.25 NaH_2PO_4, 26 $NaHCO_3$ 25 glucose, 1 $MgCl_2$, 2 $CaCl_2$ pH7.4. Recovery solution was identical to Krebs' solution apart from 6 mmol/L $MgCl_2$, 1.5 mmol/L $CaCl_2$.

Compound action potential recordings were carried out using 2 glass suction electrodes, one for electrical stimulation and the second for field potential recording, as we have described previously.[31,32] Dorsal roots were stimulated with an Iso-flex stimulus isolator (A.M.P.I. Jerusalem, Israel), and data were acquired and recorded using a Cygnus ER-1 differential amplifier (Cygnus Technologies Inc. Delaware, PA, USA) and pClamp 10 software (Molecular Devices, Sunnyvale, CA, USA). Data were filtered at 10 kHz and sampled at 50 kHz.

The characteristic triphasic (positive-negative-positive) C-fibre component of the compound action potential was identified, based on activation threshold and conduction velocity, which were not altered by treatment (Table 1).

To assess the frequency-dependent phenomenon of C-fibre ADS,[33,34] dorsal roots were stimulated ×40 (500 μA intensity, 0.1 ms duration) at frequencies of 1 Hz and 2 Hz. For each stimulus, the response width (first to last positive peak), indicative of the range of conduction velocities within the C-fibre population, was measured and the change in width from stimulus 1 calculated. Width change was normalized to the length of root stimulated, measured as the distance between the recording and stimulating electrodes.

2.2.8 | Statistical analysis

For in vitro studies, 6 separate experiments with 4 technical replicates were undertaken (n = 6). Data are presented as median, interquartile and full range, and statistical analysis was undertaken using Analyse-it Add-in for Microsoft Excel (Analyse-it Software Ltd., Leeds, UK). Kruskal-Wallis analysis of variance was used for each in vitro treatment, with Mann-Whitney *post hoc* testing and correction for multiple comparisons as appropriate.

For in vivo studies, area under the curve (AUC) of mechanical thresholds or C-fibre ADS was first calculated; then, all data were analysed by one-way or two-way ANOVA with appropriate *post hoc* testing as detailed (GraphPad Prism 7 Software Inc., La Jolla, CA, USA). Behavioural and C-fibre

also ADS data are shown as mean and SD; 8-isoprostane $F_2\alpha$ data are presented as median, interquartile and full range.

3 | RESULTS

3.1 | In vitro studies

3.1.1 | Cancer cell cytotoxicity

Paclitaxel caused loss of DRG cell viability of cancer cells such that median [range] viability in A2780 cells was 60.0 [52.1-68.6]% in the presence of 100 µmol/L paclitaxel without melatonin and 56.6 [48.0-64.3]% with 100 µmol/L paclitaxel plus 1 µmol/L melatonin. Viability of MCF7 cells was 39.2 [15.8-41.3]% with paclitaxel without melatonin and 36.3 [18.9-55.4]% with melatonin, indicating that cell killing by paclitaxel was not reduced by co-exposure of cells to melatonin in either of the cancer cell lines.

3.1.2 | Mitochondrial function

Treatment of DRG cells with paclitaxel without melatonin resulted in significantly reduced mitochondrial membrane potential as shown by a ~50% reduction in JC-1 red/green fluorescence ratio, regardless of dose ($P < .0001$, Figure 1A). When cells were co-exposed to paclitaxel plus melatonin, membrane potential actually increased except at the highest concentration of paclitaxel (Figure 1A). Mitochondrial metabolic activity was also significantly reduced when DRG cells were treated with paclitaxel, independently of dose ($P < .0001$, Figure 1B); no such reduction was seen when cells were co-treated with melatonin (Figure 1B). Paclitaxel exposure of DRG cells caused a marked dose-dependent increase in mitochondrial volume (Figure 1C, $P < .0001$), and in cells co-treated with melatonin, this effect was not seen (Figure 1C).

3.2 | In vivo studies

Melatonin administration did not produce sedative effects; all rats, irrespective of treatment, scored zero on the 5-point scale for righting reflexes (data not shown). Melatonin also did not impact upon weight gain in any experimental group tested (Fig. S1).

3.2.1 | Bolus dosing with melatonin

Paclitaxel administration caused a reduction in mechanical withdrawal threshold values that developed progressively over 2 weeks, as we have shown previously[13] (Figure 2A).

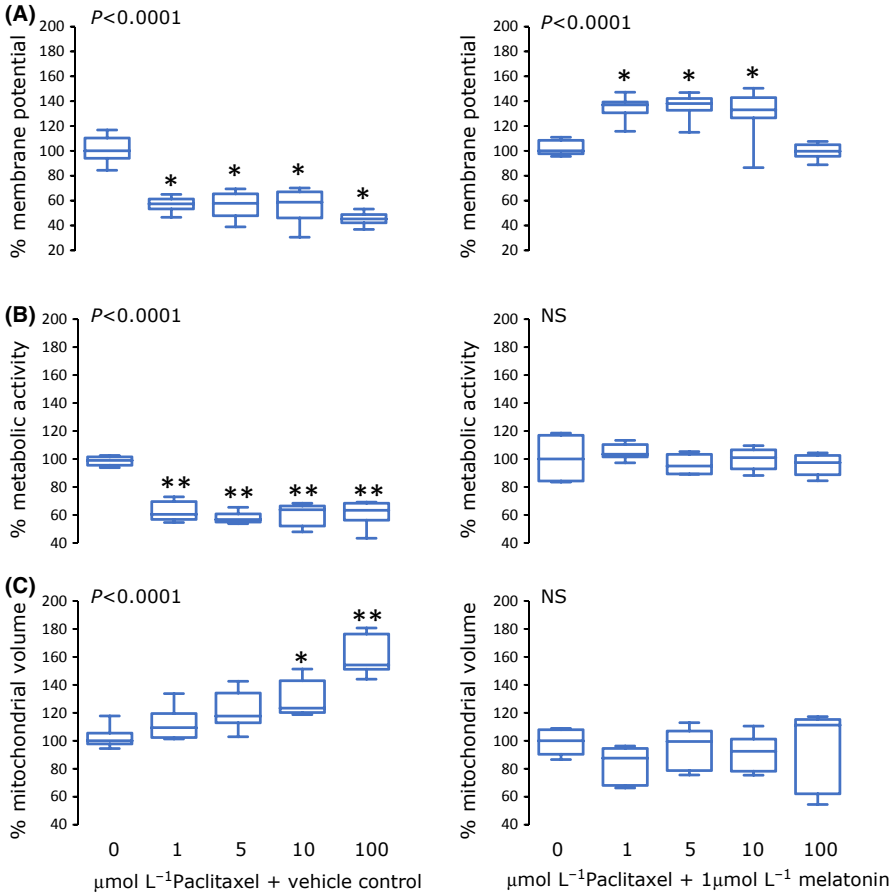

FIGURE 1 Effect of a range of concentrations of paclitaxel plus vehicle control (left) or plus 1 µmol/L melatonin (right) on (A) mitochondrial membrane potential, (B) mitochondrial metabolic activity and (C) mitochondrial volume, in a dorsal root ganglion neuronal cell line. Results are presented as percentage of data at baseline, that is vehicle control-treated cells without paclitaxel but with melatonin treatment. Data are shown as box-and-whisker plots showing median, interquartile and full range (n = 6). *P* value is Kruskal-Wallis. Asterisks = significantly different to without paclitaxel (*$P < .05$, **$P < .01$)

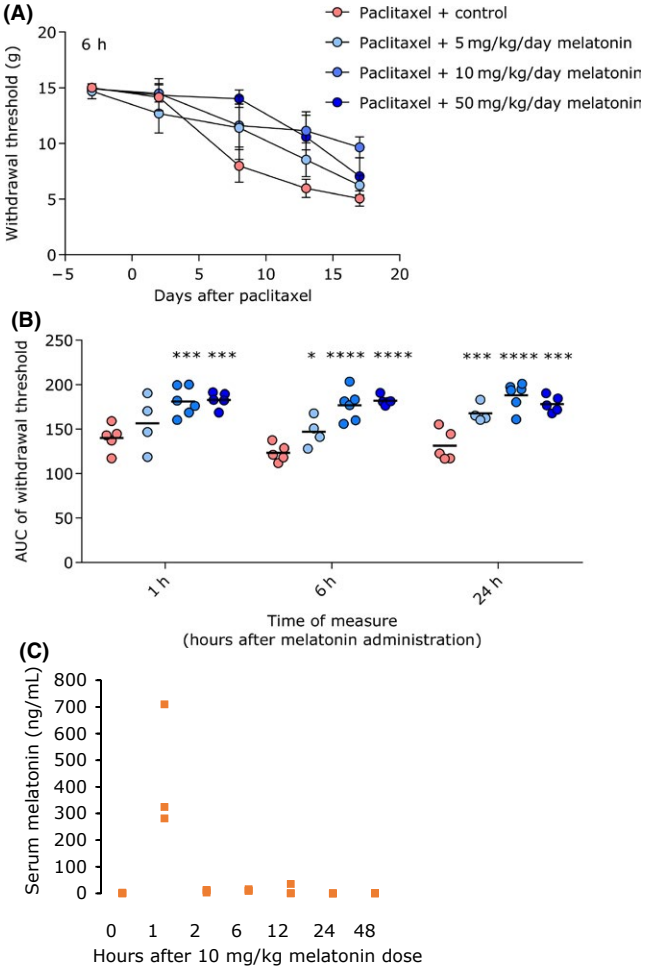

FIGURE 2 Mechanical hind paw withdrawal thresholds (A) of male rats receiving paclitaxel with vehicle control, paclitaxel with 5 mg/kg melatonin, paclitaxel with 10 mg/kg melatonin and paclitaxel with 50 mg/kg melatonin measured 6 hours after oral gavage melatonin/vehicle administration. AUC analysis of withdrawal thresholds (B) 2-17 days following paclitaxel treatment, measured at 1, 6 and 24 hours after oral gavage. Behavioural data are shown as mean (SD), n = 5-6 per treatment group. Two-way RM ANOVA (melatonin treatment $P < .0001$) followed by Dunnett's multiple comparisons test used to compare all groups to paclitaxel with vehicle control. *$P < .05$; ***=$P < .001$; ****$P < .0001$. There was no significant effect of time of measure. (C) Serum melatonin levels from paclitaxel-treated male rats given 10 mg oral melatonin by gavage. Individual raw data points are shown (n = 3)

Rats receiving paclitaxel plus melatonin by oral gavage had less mechanical hypersensitivity compared with rats given paclitaxel plus vehicle (Figure 2A and B, $P < .0001$). AUC analysis of mechanical withdrawal thresholds revealed that melatonin limited mechanical hypersensitivity at all 3 doses given. However, there was no additional effect of 50 mg/kg melatonin compared to 10 mg/kg. Notably, the reduction in hypersensitivity was independent of when sensitivity testing was performed in relation to dosing, as similar effects were observed when testing was performed at 1, 6 or 24 hours after the bolus dose was given (Figure 2B). In contrast, there was a marked increase (~400-fold) in serum melatonin levels at 1 h after dosing, which had returned to baseline values by 24 hours (Figure 2C). Six rats were allocated to each treatment group at the start of the study; however, data from 3 rats (×1 vehicle, ×1 5 mg/kg melatonin, ×1 50 mg/kg melatonin treated) were excluded as they were euthanized before the end of the study due to complications with repeated oral gavage.

3.2.2 | Melatonin in drinking water

Administration of melatonin in drinking water facilitated longer-term monitoring of the effect of melatonin upon paclitaxel-induced mechanical hypersensitivity that progressively develops over 2 weeks before then plateauing at peak hypersensitivity for around 2 weeks (Figure 3A). Melatonin alone did not affect mechanical sensitivity in naïve subjects as assessed using AUC analysis of mechanical withdrawal thresholds (Figure 3A and B). Melatonin therefore does not appear to produce sedative effects that would confound assessment of its effect upon mechanical hypersensitivity in neuropathic animals. Rats given paclitaxel plus 10 mg/kg melatonin in drinking water had less mechanical hypersensitivity than rats given paclitaxel plus vehicle throughout the time course of the model (Figure 3A-C, $P < .0001$). Comparison of the effect of 10 mg/kg/day melatonin given as oral gavage or drinking water, using AUC analysis of data from day 2-17 after paclitaxel administration started, revealed that the melatonin effect was not dependent on the administration route (Figure 3C, melatonin $P < .0001$; interaction $P = .49$). Serum melatonin levels on day 42 in animals given melatonin were significantly higher than those which received vehicle (Figure 3D, $P = .001$). Given that administration of melatonin in drinking water was as effective, but less problematic than the oral gavage route, melatonin was administered in drinking water for the remainder of the behavioural studies.

When melatonin was given as an intervention to rats with established paclitaxel-induced mechanical hypersensitivity, there was no difference between AUC withdrawal threshold (day 20-30) values of groups receiving melatonin or vehicle control (Figure 4). Additionally, both were significantly lower than AUC values of the group receiving paclitaxel and melatonin throughout ($P < .01$), suggesting melatonin was not an effective intervention to the established phenotype. However, when melatonin treatment was started before paclitaxel and then stopped once the hypersensitivity phenotype was apparent, AUC (day 20-30) analysis indicated that withdrawal thresholds remained higher than rats receiving paclitaxel without melatonin ($P < .0001$) and were indistinguishable from those given

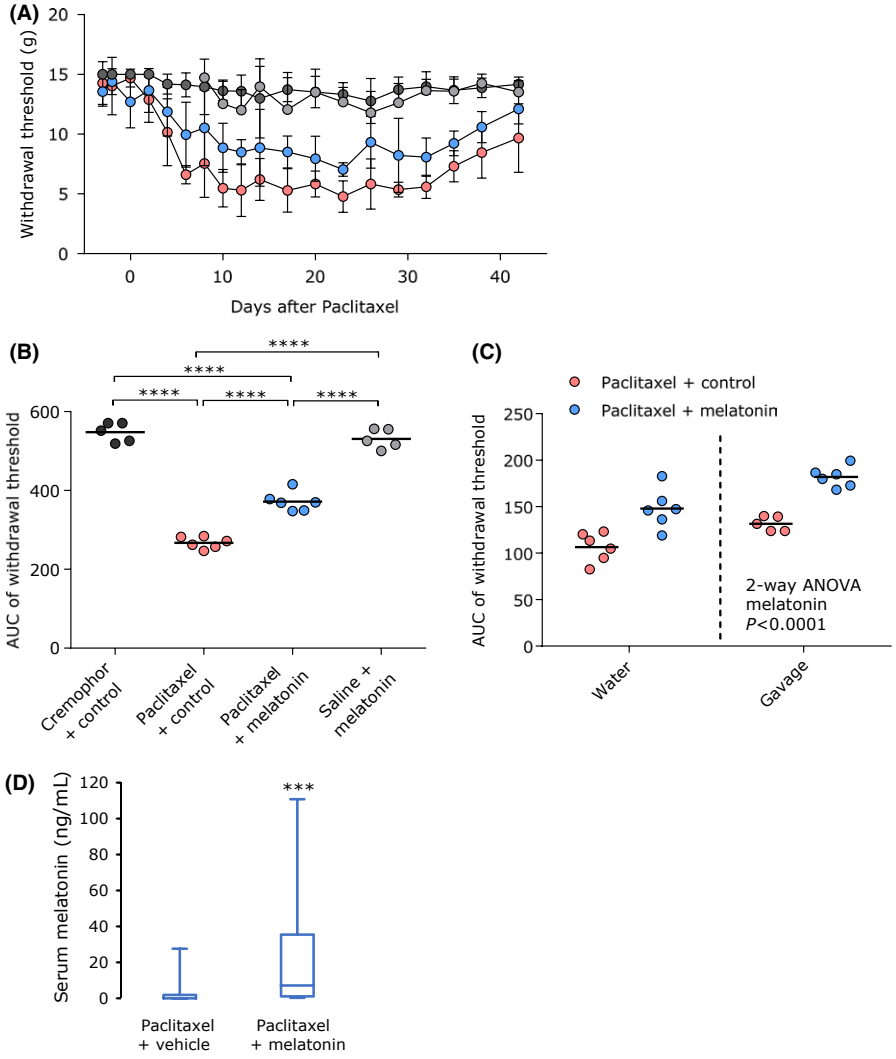

FIGURE 3 Mechanical hind paw withdrawal thresholds (A) of male rats receiving paclitaxel with vehicle control, paclitaxel with 10 mg/kg melatonin, cremophor (paclitaxel vehicle) with vehicle control and saline with 10 mg/kg melatonin with melatonin/vehicle administered in drinking water. AUC analysis of withdrawal thresholds (B) 2-42 days following paclitaxel treatment. AUC analysis of withdrawal thresholds (C) 2-17 days following paclitaxel treatment with 10 mg/kg melatonin/vehicle administered in drinking water or by oral gavage. Behavioural data are shown as mean (SD), n = 5-6 per treatment group. In (B), one-way ANOVA followed by Tukey's multiple comparisons test ****$P < .0001$. In (C), 2-way ANOVA, melatonin treatment $P < .0001$, interaction $P = .49$. (D) Serum melatonin in male rats given paclitaxel plus melatonin or vehicle in drinking water. Data are presented as box-and-whisker plots showing median, interquartile and full range (n = 15). *** = significantly higher than rats not given melatonin ($P = .001$)

melatonin continuously (Figure 4), suggesting that melatonin administration had a preventative effect that persisted beyond the cessation of treatment.

3.2.3 | Peripheral nerve 8-isoprostane $F_2\alpha$ levels

8-isoprostane $F_2\alpha$ levels were measured ex vivo in sciatic and saphenous nerve tissue collected at the peak of the model and normalized to sample protein content. Increased 8-isoprostane $F_2\alpha$ levels were found in peripheral nerve tissue from paclitaxel-treated rats (Figure 5A, $P = .003$). Melatonin treatment was found to significantly reduce the elevated 8-isoprostane $F_2\alpha$ levels in paclitaxel-treated rats (Figure 5B, $P = .0015$).

3.2.4 | C-fibre activity-dependent slowing

Treatment with paclitaxel with and without melatonin had no effect on C-fibre threshold stimulus intensity, amplitude, average conduction velocity or initial response width, indicative of the initial range of conduction velocities within the C-fibre population (Table 1).

Repetitive stimulation of isolated dorsal roots produced a progressive increase in C-fibre response width (Figure 6A and B). AUC analysis confirmed that this C-fibre ADS was frequency-dependent, with stimulation at 2 Hz producing greater ADS than at 1 Hz ($P < .0001$, Figure 6C). C-fibre ADS was lower in rats treated with paclitaxel ($P < .001$), an effect that was prevented by melatonin ($P < .0001$), independent of stimulation frequency.

3.2.5 | Combination treatment with duloxetine

Paclitaxel produced mechanical hypersensitivity in male and female animals that was significantly ameliorated by melatonin administered in drinking water in both sexes (Figure 7). Duloxetine limited mechanical hypersensitivity to an extent comparable with melatonin in both sexes (Figure 7).

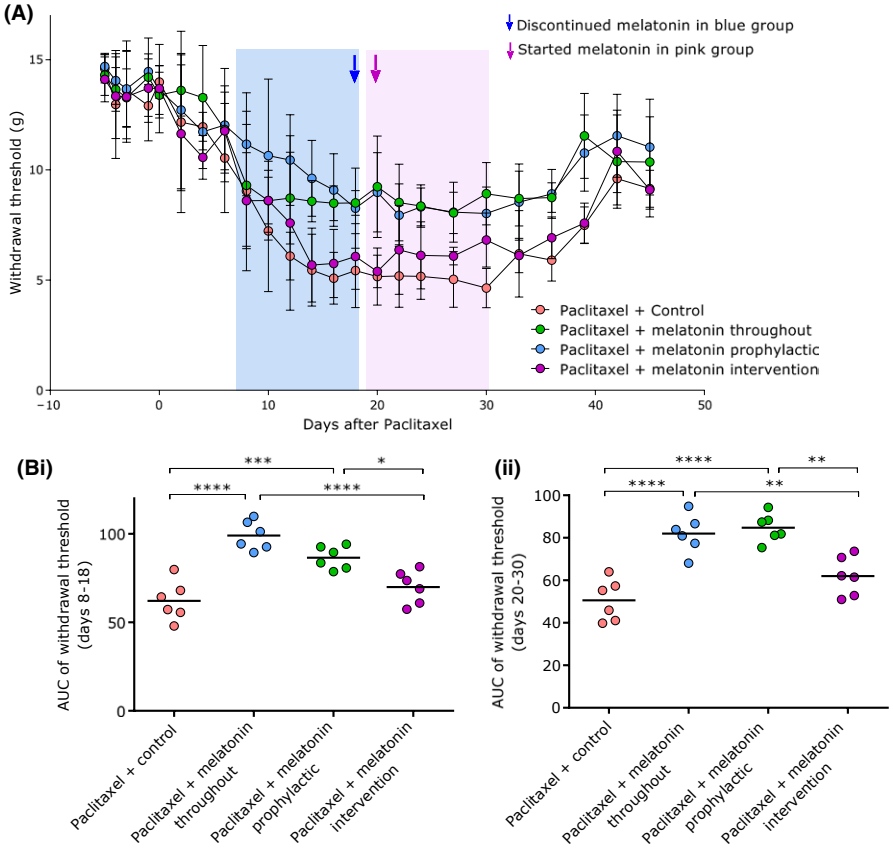

FIGURE 4 Mechanical hind paw withdrawal thresholds (A) of male rats receiving paclitaxel with vehicle control, paclitaxel with melatonin throughout, paclitaxel with melatonin discontinued at day 18, and paclitaxel with melatonin starting day 20. 10 mg/kg melatonin/vehicle administered in drinking water. AUC analysis of withdrawal thresholds (Bi) 8-18 days or (Bii) 20-30 days following paclitaxel treatment. Data are shown as mean (SD), n = 6 per treatment group. In (B), one-way ANOVA followed by Tukey's multiple comparisons test $*P < .05$; $**P < .01$; $***P < .001$;$****P < .0001$

Co-treatment of paclitaxel-treated animals with melatonin in drinking water plus duloxetine injections revealed a clear additive effect, with higher withdrawal thresholds in co-treated animals compared to those given either melatonin (males $P < .05$) or duloxetine alone (males $P < .0001$; females $P < .05$).

4 | DISCUSSION

We have shown that in vitro, paclitaxel treatment caused mitochondrial dysfunction in DRG cells, with reduced membrane potential and metabolic activity, and evidence of mitochondrial swelling. However, when the cells were treated with paclitaxel plus melatonin, mitochondrial damage was attenuated. Importantly, co-treatment of breast or ovarian cancer cells with melatonin had no impact on the cytotoxicity of paclitaxel. In a rat model of paclitaxel-induced neuropathic pain, we found that oral melatonin pretreatment was protective, whether given as a bolus or in drinking water. Furthermore, melatonin reduced paclitaxel-elevated peripheral nerve 8-isoprostane $F_2\alpha$ levels and prevented paclitaxel reduced C-fibre ADS. When given in a prophylactic manner, melatonin significantly attenuated paclitaxel-evoked mechanical hypersensitivity in both male and female animals, and there was an additive affect when melatonin was given along with duloxetine. Melatonin treatment was well tolerated with no effects on animals' weight gain, general well-being and sedation levels. This work suggests that melatonin may be a useful preventive treatment for chemotherapy-induced painful neuropathy in patients.

Melatonin is known to be an effective antioxidant, and many of its reaction products and metabolites (e.g. 6-hydroxymelatonin) also possess antioxidant activity.[15,16] Melatonin is able to cross cell membranes and is reported to concentrate particularly inside mitochondria,[35] where it may potentially interact with mitochondrial MT1 receptors to modulate mitochondrial function.[36,37] Mitochondrial damage caused by paclitaxel has been reported previously in several cell types[5,38-40] including peripheral nerve cells.[6] Our in vitro data show that paclitaxel caused a loss of mitochondrial membrane potential and metabolic activity in DRG cells, as previously reported[13] and that melatonin ameliorated this damage. MitoTracker™ Green is a marker which fluoresces inside mitochondria, regardless of membrane potential and is an indicator of mitochondrial volume, reacting with free thiol groups in cysteine residues of mitochondrial proteins.[19] The increase in observed mitochondrial volume could reflect increased number of mitochondria, or mitochondrial swelling, the latter of which has been documented in peripheral nerve axons from paclitaxel-treated rats.[6] Again melatonin prevented this. Moreover, these in vitro

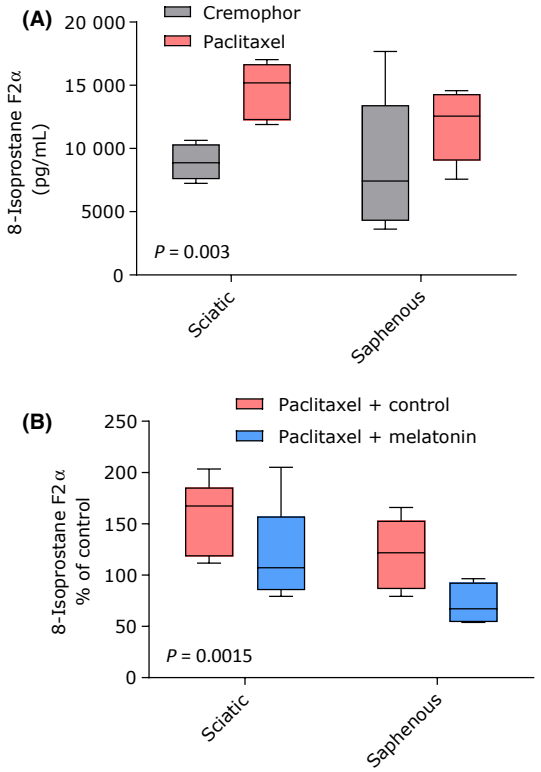

FIGURE 5 8-Isoprostane $F_2\alpha$ levels in sciatic and saphenous nerve tissue isolated at day 19 of model from (A) cremophor- (n = 6) or paclitaxel (n = 6)-treated male rats or (B) paclitaxel-treated male rats administered 10 mg/kg melatonin by oral gavage (n = 6) or vehicle/no treatment (n = 11). Data expressed as % change from average control cremophor values (n = 12). Data are shown as box-and-whisker plots showing median, interquartile and full range. Two-way ANOVA with *P* values indicating treatment significance

effects were in keeping with findings in vivo, where levels of 8-isoprostane $F_2\alpha$, an end product of reactions promoted in conditions of oxidative stress,[30] were increased in peripheral nerve tissue isolated from paclitaxel-treated animals, an effect diminished in paclitaxel-treated animals co-treated with melatonin.

Altered peripheral nerve function in the rat model of paclitaxel-induced neuropathy has been demonstrated with in vivo electrophysiological recordings revealing that ~30% of C-fibres display spontaneous activity that is reduced by prophylactic treatment with acetyl-L-carnitine[41], which is known to limit mitochondrial dysfunction.[42] Altered C-fibre function can also be characterized, in both preclinical pain models[43,44] and chronic pain patients,[45-48] by measuring the phenomenon of C-fibre ADS, which is a progressive slowing of nociceptive C-fibre conduction velocity in response to repetitive stimulation.[33,34] However, to date, this had not been addressed for CINP. Here, we demonstrate that in peripheral nerve tissue from paclitaxel-treated rats, C-fibres had significantly lower ADS, an effect that was prevented in those rats co-treated with melatonin. We have very recently reported that C-fibre ADS alters the temporal relay of pain input to the spinal cord and that a reduction in ADS, as we have observed in the paclitaxel model, facilitates central pain processing, and likely contributes to pain hypersensitivity.[49]

The rat model of paclitaxel-induced neuropathy employed has been well characterized[6,13,24] and features mechanical hypersensitivity as indicated by the reduced mechanical threshold of the flexion withdrawal reflex in the present study. Melatonin given by oral gavage was both time-consuming and stressful for the animal exemplified by the loss of 3 animals after multiple oral gavages. Attempts to give melatonin to individual rats in jelly cubes were unsuccessful, and so we administered the melatonin in drinking water as described previously.[50,51] There was no effect of melatonin on water consumption, weight gain and no apparent sedative effect given there was no change in righting reflex activity, food or water intake, nor indeed altered mechanical sensitivity in animals given melatonin alone. Furthermore, when melatonin was discontinued, the reduction in mechanical hypersensitivity persisted and when given as an intervention in animals when CINP was established, melatonin did not limit mechanical hypersensitivity, effects inconsistent with a melatonin "sedative" effect accounting for the attenuation of CINP. Significantly higher serum melatonin levels at the end of the study were seen in animals given melatonin compared to those which did not and the magnitude of the melatonin protective effect was similar for rats given melatonin by bolus doses or in drinking water. We therefore conclude that oral melatonin pretreatment, either as a bolus dose or in drinking water was effective at reducing paclitaxel-induced mechanical hypersensitivity. During these experiments, a melatonin dose of 10 mg/kg per day was used. This dose has been demonstrated to be effective in reducing oxidative stress and symptom severity in preclinical models of a number of diseases, including epilepsy, diabetes, ethanol-induced neurotoxicity and oxidative lung toxicity.[52-55]

The capacity of melatonin to protect against the development of paclitaxel-induced mechanical hypersensitivity is most likely due to its antioxidant activity. Oxidative stress occurs in peripheral nerves in the paclitaxel rat model[56], and other antioxidant strategies, including spin trap agents[9], mitochondrial electron transport chain modulators[10] and the antioxidant MitoVitE[13] reduce symptoms in this model. In support, we demonstrate that melatonin limits paclitaxel-induced elevation of 8-isoprostane $F_2\alpha$ levels in peripheral nerves in vivo. Therefore, although melatonin may influence pain processing via its MT_1/MT_2 membrane receptors and effects on neurotransmitter systems[57], the protective effect we observe may be more likely to be due to its antioxidant action, perhaps via mitochondrial MT1 receptors[36,37] as firstly melatonin prevented but did not reverse established mechanical hypersensitivity; secondly, the reduction in mechanical hypersensitivity produced by daily bolus administration was

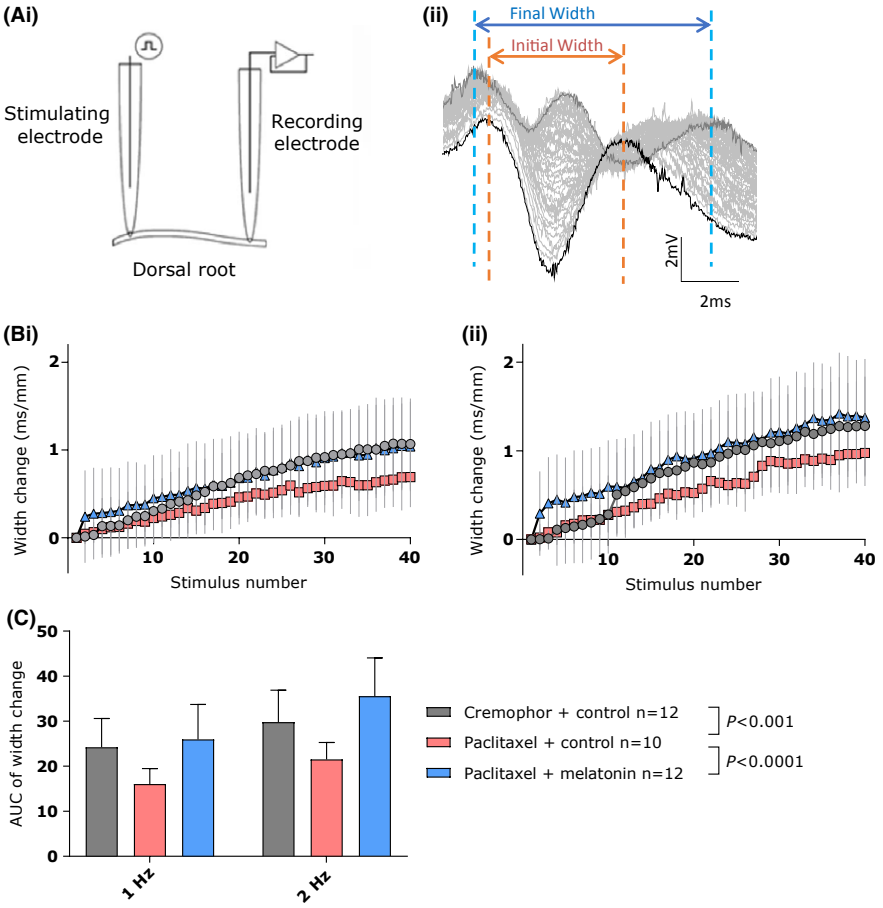

FIGURE 6 C-fibre activity-dependent slowing (ADS) recorded using (Ai) 2 suction electrodes to stimulate and record compound action potentials from L4/L5 dorsal roots from male rats. (Aii) Representative compound action potentials illustrating the slow C-fibre conducting component. The ×40 traces recorded in response to 2 Hz dorsal root stimulation are shown (trace 1 black; traces 2-39 light grey; trace 40 dark grey). Initial width (orange dashed lines) and last width (blue dashed lines) denoted. Repetitive stimulation of dorsal roots at 1 Hz (Bi) and 2 Hz (Bii) results in a progressive increase in response width. AUC analysis of width change (C) reveals that the frequency-dependent progressive width change (2-way ANOVA $P < .0001$) is reduced by paclitaxel (2-way ANOVA, Tukey's multiple comparisons test $P < .001$), an effect prevented with 10 mg/kg/day melatonin treatment in drinking water (2-way ANOVA, Tukey's multiple comparisons test $P < .0001$). Data are shown as mean (SD)

stable over 24 hours yet serum melatonin levels peak at 1 h and return to baseline by 24 hours. Interestingly, this antioxidant protective effect fits well with the demonstration that reactive oxygen species levels peak in the DRG during the onset rather than the peak of the paclitaxel model,[56] although it has been demonstrated that antioxidant strategies can reduce established paclitaxel-induced hypersensitivity.[9,10] Furthermore, the recent demonstration that melatonin limits oxaliplatin-induced mitochondrial dysfunction and peripheral neuropathy[58] suggests that melatonin is a potential disease-modifying treatment for CINP. However, it could also be that melatonin antioxidant capacity combines with its receptor-mediated effects upon pain processing to collectively provide the reduction in hypersensitivity observed. Of interest, given the observed additive effect of melatonin and duloxetine, agomelatine, a new class of antidepressant and a melatonergic and serotonergic receptor agonist, with evidence of antioxidant activity[59,60], has very recently been shown to be effective against chemotherapy-induced neuropathy.[61]

We also found that melatonin did not inhibit the cytotoxic action of paclitaxel in 2 relevant cancer cell lines, in agreement with studies using other antioxidants.[13,62] Treatment of cancer patients with melatonin in several small studies did not impact on the effectiveness of chemotherapy[63-65], and a meta-analysis of 10 randomized controlled trials of over 600 patients with advanced solid tumours reported that melatonin treatment reduced the risk of death at 1 year.[66] Another recent meta-analysis of 8 trials and 700 patients similarly reported that adjuvant melatonin treatment in patients with cancer resulted in improved 1-year survival.[67] A more recent small trial showed no difference in survival of patients with non-small-cell lung cancer who received 10 or 20 mg melatonin daily for 6 months, although after 22 months, only patients given melatonin had survived, with more patients surviving who received 20 mg than had received 10 mg.[68] Other studies report mechanisms

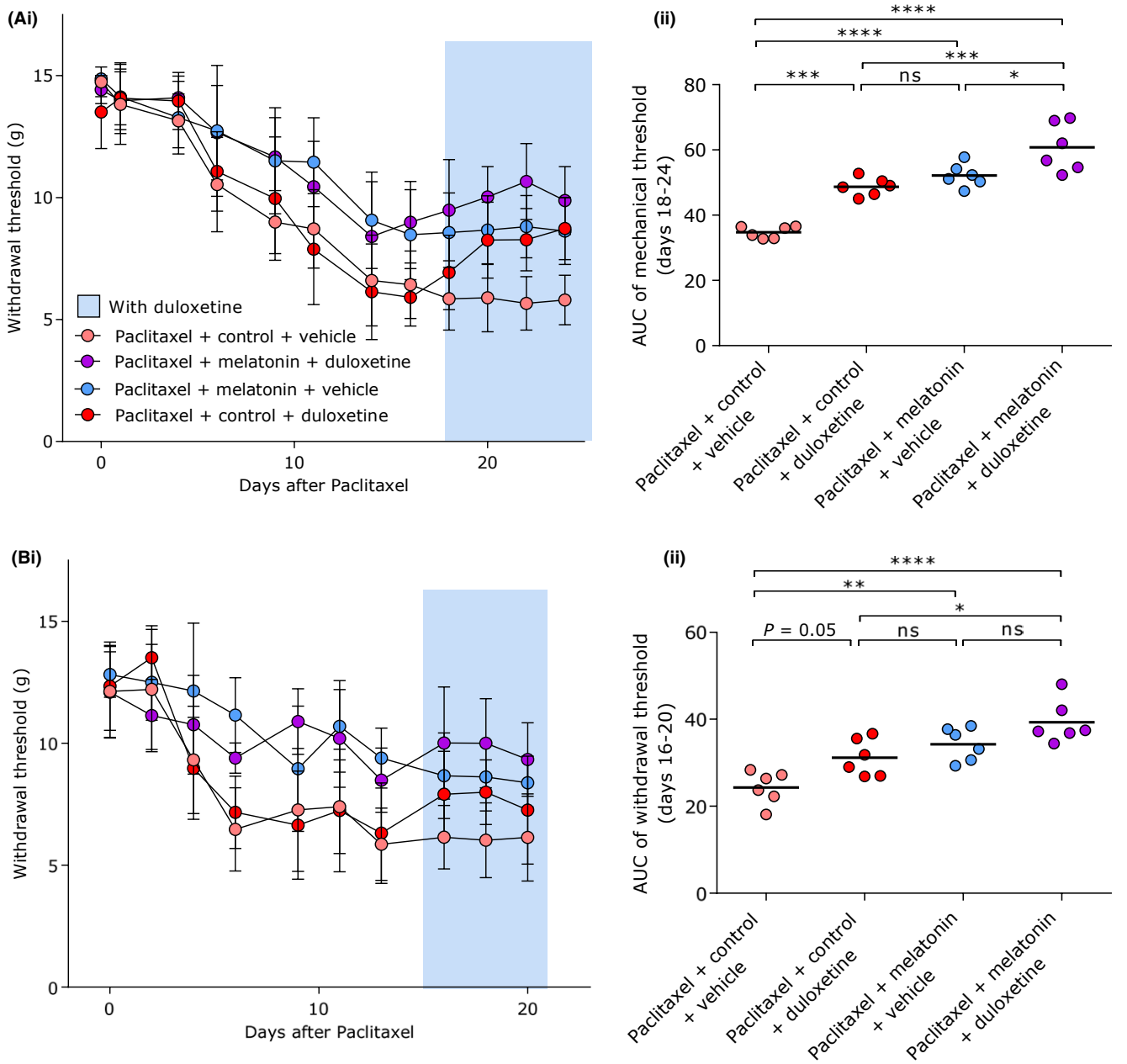

FIGURE 7 Mechanical hind paw withdrawal thresholds of (Ai) male or (Bi) female rats receiving paclitaxel, paclitaxel with melatonin, paclitaxel with duloxetine intervention treatment or paclitaxel with melatonin and duloxetine intervention treatment. 10 mg/kg/day melatonin/control administered in drinking water; 10 mg/kg/day duloxetine-/vehicle-injected i.p. AUC analysis of withdrawal thresholds in (Aii) males or (Bii) females during duloxetine intervention. Data are shown as mean (SD), n = 6 per treatment group. In (A/Bii), one-way ANOVA followed by Tukey's multiple comparisons test *$P < .05$; **$P < .01$; ***$P < .001$; ****$P < .0001$

of how melatonin may potentiate chemotherapy, reduce metastatic progression and prevent resistance to chemotherapy.[69-72] Moreover, the ability of gliomas to synthesize melatonin negatively correlates with tumour malignancy.[73] Melatonin appears to be without side effects, other than mild drowsiness,[17,74] and has been administered to thousands of patients without toxic effects, even at high doses, for a variety of conditions.

Our study clearly shows the potential of melatonin as a preventative therapeutic intervention for patients undergoing chemotherapy, to limit development of neuropathic pain, with no obvious risk to the efficacy of chemotherapy or outcome.

ACKNOWLEDGEMENTS

Thank you to Professor Ahmet Hoke (Johns Hopkins, Baltimore, USA) for the gift of DRG cells and to Professor Patrick M. Dougherty (MD Anderson Cancer Center, Texas, USA) for sharing his expertise in the rat model.

AUTHOR CONTRIBUTIONS

All authors made a substantial contribution to the conception and design, acquisition of data or analysis and interpretation of data, were involved in drafting the article or revising it critically for important intellectual content, and approved the final version. They have all agreed to be accountable for all aspects of the work in terms of accuracy and integrity. All authors contributed to and approved the final version of the manuscript. HFG conceived of and designed the study, analysed data and drafted the manuscript. BM conceived of and designed the study, conducted in vitro and in vivo experimental works and analysed in vivo data and contributed to writing the manuscript. KLW: conducted electrophysiological recordings, analysed data and contributed to writing the manuscript. CT conceived of and designed and supervised in vivo work and contributed to writing the manuscript. DL helped conduct and supervised in vitro experimental work. LC conceived of and designed study, supervised conduct and contributed to writing the manuscript.

ORCID

Carole Torsney http://orcid.org/0000-0002-2448-3364
Helen Galley https://orcid.org/0000-0002-9517-0074

REFERENCES

1. Seretny M, Currie GL, Sena ES, et al. Incidence, prevalence, and predictors of chemotherapy-induced peripheral neuropathy: a systematic review and meta-analysis. *Pain*. 2014;155:2461-2470.
2. Cavaletti G, Marmiroli P. Chemotherapy-induced peripheral neurotoxicity. *Nat Rev Neurol*. 2010;6:657-666.
3. Finnerup NB, Attal N, Haroutounian S, et al. Pharmacotherapy for neuropathic pain in adults: a systematic review and meta-analysis. *Lancet Neurol*. 2015;14:162-173.
4. Smith EM, Pang H, Cirrincione C, et al. Effect of duloxetine on pain, function, and quality of life among patients with chemotherapy-induced painful peripheral neuropathy: a randomized clinical trial. *J Am Med Assoc*. 2013;309:1359-1367.
5. Kidd JF, Pilkington MF, Schell MJ, et al. Paclitaxel affects cytosolic calcium signals by opening the mitochondrial permeability transition pore. *J Biol Chem*. 2002;277:6504-6510.
6. Flatters SJ, Bennett GJ. Studies of peripheral sensory nerves in paclitaxel-induced painful peripheral neuropathy: Evidence for mitochondrial dysfunction. *Pain*. 2006;122:245-257.
7. Duggett NA, Griffiths LA, Flatters SJL. Paclitaxel-induced painful neuropathy is associated with changes in mitochondrial bioenergetics, glycolysis, and an energy deficit in dorsal root ganglia neurons. *Pain* 2017;158:1499-1508.
8. Xiao WH, Bennett GJ. Effects of mitochondrial poisons on the neuropathic pain produced by the chemotherapeutic agents, paclitaxel and oxaliplatin. *Pain*. 2012;153:704-709.
9. Fidanboylu M, Griffiths LA, Flatters SJ. Global inhibition of reactive oxygen species (ROS) inhibits paclitaxel-induced painful peripheral neuropathy. *PLoS ONE*. 2011;6:e25212.
10. Griffiths LA, Flatters SJ. Pharmacological modulation of the mitochondrial electron transport chain in paclitaxel-induced painful peripheral neuropathy. *J Pain*. 2015;16:981-994.
11. Krukowski K, Nijboer CH, Huo X, et al. Prevention of chemotherapy-induced peripheral neuropathy by the small-molecule inhibitor pifithrin-mu. *Pain*. 2015;156:2184-2192.
12. Jin HW, Flatters SJ, Xiao WH, et al. Prevention of paclitaxel-evoked painful peripheral neuropathy by acetyl-L-carnitine: Effects on axonal mitochondria, sensory nerve fiber terminal arbors, and cutaneous Langerhans cells. *Exp Neurol*. 2008;210:229-237.
13. McCormick B, Lowes DA, Colvin L, et al. MitoVitE, a mitochondria-targeted antioxidant, limits paclitaxel-induced oxidative stress and mitochondrial damage in vitro, and paclitaxel-induced mechanical hypersensitivity in a rat pain model. *Br J Anaesth*. 2016;117:659-666.
14. Lowes DA, Webster NR, Murphy MP, et al. Antioxidants that protect mitochondria reduce interleukin-6 and oxidative stress, improve mitochondrial function, and reduce biochemical markers of organ dysfunction in a rat model of acute sepsis. *Br J Anaesth*. 2013;110:472-480.
15. Lowes DA, Almawash AM, Webster NR, et al. Melatonin and structurally similar compounds have differing effects on inflammation and mitochondrial function in endothelial cells under conditions mimicking sepsis. *Br J Anaesth*. 2011;107:193-201.
16. Reiter RJ, Mayo JC, Tan DX, et al. Melatonin as an antioxidant: under promises but over delivers. *J Pineal Res*. 2016;61:253-278.
17. Galley HF, Lowes DA, Allen L, et al. Melatonin as a potential therapy for sepsis: A phase I dose escalation study and an ex vivo whole blood model under conditions of sepsis. *J Pineal Res*. 2014;56:427-438.
18. Chen W, Mi R, Haughey N, et al. Immortalization and characterization of a nociceptive dorsal root ganglion sensory neuronal line. *J Peripher Nerv Syst*. 2007;12:121-130.
19. Presley AD, Fuller KM, Arriaga EA. MitoTracker Green labeling of mitochondrial proteins and their subsequent analysis by capillary electrophoresis with laser-induced fluorescence detection. *J Chromatogr B Analyt Technol Biomed Life Sci*. 2003;793:141-150.
20. Agnello M, Morici G, Rinaldi AM. A method for measuring mitochondrial mass and activity. *Cytotechnology*. 2008;56:145-149.
21. Cottet-Rousselle C, Ronot X, Leverve X, et al. Cytometric assessment of mitochondria using fluorescent probes. *Cytometry A*. 2011;79:405-425.
22. Yang TT, Sinai P, Kain SR. An acid phosphatase assay for quantifying the growth of adherent and nonadherent cells. *Anal Biochem*. 1996;241:103-108.
23. Kilkenny C, Browne WJ, Cuthill IC, et al. Improving bioscience research reporting: The ARRIVE guidelines for reporting animal research. *PLoS Biol*. 2010;8:e1000412.
24. Polomano RC, Mannes AJ, Clark US, et al. A painful peripheral neuropathy in the rat produced by the chemotherapeutic drug, paclitaxel. *Pain*. 2001;94:293-304.
25. Chaplan SR, Bach FW, Pogrel JW, et al. Quantitative assessment of tactile allodynia in the rat paw. *J Neurosci Methods*. 1994;53:55-63.
26. Gilron I, Jensen TS, Dickenson AH. Combination pharmacotherapy for management of chronic pain: From bench to bedside. *Lancet Neurol*. 2013;12:1084-1095.

27. Mogil JS. Sex differences in pain and pain inhibition: Multiple explanations of a controversial phenomenon. *Nat Rev Neurosci*. 2012;13:859-866.
28. Bartley EJ, Fillingim RB. Sex differences in pain: A brief review of clinical and experimental findings. *Br J Anaesth*. 2013;111:52-58.
29. Devor M, Zalkind V. Reversible analgesia, atonia, and loss of consciousness on bilateral intracerebral microinjection of pentobarbital. *Pain*. 2001;94:101-112.
30. Montuschi P, Barnes PJ, Roberts LJ. 2ND Isoprostanes: markers and mediators of oxidative stress. *FASEB J*. 2004;18:1791-1800.
31. Dickie AC, Torsney C. The chemerin receptor 23 agonist, chemerin, attenuates monosynaptic C-fibre input to lamina I neurokinin 1 receptor expressing rat spinal cord neurons in inflammatory pain. *Mol Pain*. 2014;10:24.
32. Torsney C. Inflammatory pain unmasks heterosynaptic facilitation in lamina I neurokinin 1 receptor-expressing neurons in rat spinal cord. *J Neurosci*. 2011;31:5158-5168.
33. Thalhammer JG, Raymond SA, Popitz-Bergez FA, et al. Modality-dependent modulation of conduction by impulse activity in functionally characterized single cutaneous afferents in the rat. *Somatosens Mot Res*. 1994;11:243-257.
34. Gee MD, Lynn B, Cotsell B. Activity-dependent slowing of conduction velocity provides a method for identifying different functional classes of C-fibre in the rat saphenous nerve. *Neuroscience*. 1996;73:667-675.
35. Venegas C, Garcia JA, Escames G, et al. Extrapineal melatonin: Analysis of its subcellular distribution and daily fluctuations. *J Pineal Res*. 2012;52:217-227.
36. Wang X, Sirianni A, Pei Z, et al. The melatonin MT1 receptor axis modulates mutant Huntingtin-mediated toxicity. *J Neurosci*. 2011;31:14496-14507.
37. Gbahou F, Cecon E, Viault G, et al. Design and validation of the first cell-impermeant melatonin receptor agonist. *Br J Pharmacol*. 2017;174:2409-2421.
38. Zhang X, Zhang S, Zhu S, et al. Identification of mitochondria-targeting anticancer compounds by an in vitro strategy. *Anal Chem*. 2014;86:5232-5237.
39. Andre N, Braguer D, Brasseur G, et al. Paclitaxel induces release of cytochrome c from mitochondria isolated from human neuroblastoma cells'. *Can Res*. 2000;60:5349-5353.
40. Selimovic D, Hassan M, Haikel Y, et al. Taxol-induced mitochondrial stress in melanoma cells is mediated by activation of c-Jun N-terminal kinase (JNK) and p38 pathways via uncoupling protein 2. *Cell Signal*. 2008;20:311-322.
41. Xiao WH, Bennett GJ. Chemotherapy-evoked neuropathic pain: Abnormal spontaneous discharge in A-fiber and C-fiber primary afferent neurons and its suppression by acetyl-L-carnitine. *Pain*. 2008;135:262-270.
42. Virmani A, Gaetani F, Binienda Z. Effects of metabolic modifiers such as carnitines, coenzyme Q10, and PUFAs against different forms of neurotoxic insults: Metabolic inhibitors, MPTP, and methamphetamine. *Ann N Y Acad Sci*. 2005;1053:183-191.
43. Wang XC, Wang S, Zhang M, et al. Alpha-Dendrotoxin-sensitive Kv1 channels contribute to conduction failure of polymodal nociceptive C-fibers from rat coccygeal nerve. *J Neurophysiol*. 2016;115:947-957.
44. Shim B, Ringkamp M, Lambrinos GL, et al. Activity-dependent slowing of conduction velocity in uninjured L4 C fibers increases after an L5 spinal nerve injury in the rat. *Pain*. 2007;128:40-51.
45. Orstavik K, Namer B, Schmidt R, et al. Abnormal function of C-fibers in patients with diabetic neuropathy. *J Neurosci*. 2006;26:11287-11294.
46. Orstavik K, Weidner C, Schmidt R, et al. Pathological C-fibres in patients with a chronic painful condition. *Brain*. 2003;126:567-578.
47. Kleggetveit IP, Namer B, Schmidt R, et al. High spontaneous activity of C-nociceptors in painful polyneuropathy. *Pain*. 2012;153:2040-2047.
48. Serra J, Collado A, Sola R, et al. Hyperexcitable C nociceptors in fibromyalgia. *Ann Neurol*. 2014;75:196-208.
49. Dickie AC, McCormick B, Lukito V, et al. Inflammatory pain reduces C fibre activity-dependent slowing in a sex dependent manner, amplifying nociceptive input to the spinal cord. *J Neurosci*. 2017;37:6488-6502.
50. di Paolo C, Cabre M, Domingo JL, et al. Melatonin does not modify the concentration of different metals in AbetaPP transgenic mice. *Food Chem Toxicol*. 2014;70:252-259.
51. Petkova Z, Tchekalarova J, Pechlivanova D, et al. Treatment with melatonin after status epilepticus attenuates seizure activity and neuronal damage but does not prevent the disturbance in diurnal rhythms and behavioral alterations in spontaneously hypertensive rats in kainate model of temporal lobe epilepsy. *Epilepsy Behav*. 2014;31:198-208.
52. Tchekalarova J, Petkova Z, Pechlivanova D, et al. Prophylactic treatment with melatonin after status epilepticus: Effects on epileptogenesis, neuronal damage, and behavioral changes in a kainate model of temporal lobe epilepsy. *Epilepsy Behav*. 2013;27:174-187.
53. Amin AH, El-Missiry MA, Othman AI. Melatonin ameliorates metabolic risk factors, modulates apoptotic proteins, and protects the rat heart against diabetes-induced apoptosis. *Eur J Pharmacol*. 2015;747:166-173.
54. Bagheri F, Goudarzi I, Lashkarbolouki T, et al. Melatonin prevents oxidative damage induced by maternal ethanol administration and reduces homocysteine in the cerebellum of rat pups. *Behav Brain Res*. 2015;287:215-225.
55. Shokrzadeh M, Chabra A, Naghshvar F, et al. Protective effects of melatonin against cyclophosphamide-induced oxidative lung toxicity in mice. *Drug Res (Stuttg)*. 2015;65:281-286.
56. Duggett NA, Griffiths LA, McKenna OE, et al. Oxidative stress in the development, maintenance and resolution of paclitaxel-induced painful neuropathy. *Neuroscience*. 2016;333:13-26.
57. Danilov A, Kurganova J. Melatonin in chronic pain syndromes. *Pain Ther*. 2016;5:1-17.
58. Areti A, Komirishetty P, Akuthota M, et al. Melatonin prevents mitochondrial dysfunction and promotes neuroprotection by inducing autophagy during oxaliplatin-evoked peripheral neuropathy. *J Pineal Res*. 2017;62:e12393.
59. Aguiar CC, Almeida AB, Araujo PV, et al. Effects of agomelatine on oxidative stress in the brain of mice after chemically induced seizures. *Cell Mol Neurobiol*. 2013;33:825-835.
60. Yigitturk G, Acara AC, Erbas O, et al. The antioxidant role of agomelatine and gallic acid on oxidative stress in STZ induced type I diabetic rat testes. *Biomed Pharmacother*. 2017;87:240-246.

61. Chenaf C, Chapuy E, Libert F, et al. Agomelatine: A new opportunity to reduce neuropathic pain-preclinical evidence. *Pain*. 2017;158:149-160.
62. Sprouse AA, Herbert BS. Resveratrol augments paclitaxel treatment in MDA-MB-231 and paclitaxel-resistant MDA-MB-231 breast cancer cells. *Anticancer Res*. 2014;34:5363-5374.
63. Lissoni P, Tancini G, Barni S, et al. Treatment of cancer chemotherapy-induced toxicity with the pineal hormone melatonin. *Support Care Cancer*. 1997;5:126-129.
64. Sarma A, Rodriguez MA, Cabanillas F, et al. A randomized trial of CHOP chemotherapy with or without melatonin in patients with favorable prognosis large B-cell lymphoma. *J Clin Oncol*. 2004;22:745s-745s.
65. Nahleh Z, Pruemer J, Lafollette J, et al. Melatonin, a promising role in taxane-related neuropathy. *Clin Med Insights Oncol*. 2010;4:35-41.
66. Mills E, Wu P, Seely D, et al. Melatonin in the treatment of cancer: A systematic review of randomized controlled trials and meta-analysis. *J Pineal Res*. 2005;39:360-366.
67. Wang YM, Jin BZ, Ai F, et al. The efficacy and safety of melatonin in concurrent chemotherapy or radiotherapy for solid tumors: A meta-analysis of randomized controlled trials. *Cancer Chemother Pharmacol*. 2012;69:1213-1220.
68. Sookprasert A, Johns NP, Phunmanee A, et al. Melatonin in patients with cancer receiving chemotherapy: A randomized, double-blind, placebo-controlled trial. *Anticancer Res*. 2014;34:7327-7337.
69. Gao Y, Xiao X, Zhang C, et al. Melatonin synergizes the chemotherapeutic effect of 5-fluorouracil in colon cancer by suppressing PI3K/AKT and NF-kappaB/iNOS signaling pathways. *J Pineal Res*. 2017;62:e12380.
70. Lopes JR, Da Silva Kavagutti M, Medeiros FA, et al. Evaluation of melatonin effect on human breast cancer stem cells using a three-dimensional growth method of mammospheres. *Anticancer Agents Med Chem.* 2017;17:961-965.
71. Nooshinfar E, Safaroghli-Azar A, Bashash D, et al. Melatonin, an inhibitory agent in breast cancer. *Breast Cancer*. 2017;24:42-51.
72. Xiang S, Dauchy RT, Hauch A, et al. Doxorubicin resistance in breast cancer is driven by light at night-induced disruption of the circadian melatonin signal. *J Pineal Res*. 2015;59:60-69.
73. Kinker GS, Oba-Shinjo SM, Carvalho-Sousa CE, et al. Melatonergic system-based two-gene index is prognostic in human gliomas. *J Pineal Res*. 2016;60:84-94.
74. Andersen LP, Gogenur I, Rosenberg J, et al. The safety of melatonin in humans. *Clin Drug Investig*. 2016;36:169-175.

SUPPORTING INFORMATION

Additional Supporting Information may be found online in the supporting information tab for this article.

How to cite this article: Galley HF, McCormick B, Wilson KL, Lowes DA, Colvin L, Torsney C. Melatonin limits paclitaxel-induced mitochondrial dysfunction in vitro and protects against paclitaxel-induced neuropathic pain in the rat. *J. Pineal Res*. 2017;63:e12444. https://doi.org/10.1111/jpi.12444