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Selective melatonin MT2 receptor ligands relieve neuropathic pain through modulation of brainstem descending antinociceptive pathways.

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ABSTRACT

Neuropathic pain is a major public health problem, which results in personal suffering, reduced productivity and substantial costs for health system. Only few treatments are available for this condition causing several side effects. Here, we report that the two novel MT2 melatonin (MLT) receptor partial agonists (UCM765 and UCM924) decrease allodynia in both rat spinal L5-L6 nerve ligation and spared nerve injury models. These effects are 1) dose-dependent (effective doses 20 and 40 mg/kg) and blocked by the selective MT2 receptor antagonist 4P-PDOT, 2) superior to high doses of MLT (150 mg/kg) and comparable to gabapentin (GBP, 100 mg/kg), but 3) without noticeable motor-sedative side effects in the rotarod test. Using immunohistochemistry,microscopy, we found that MT2 receptors are expressed by glutamatergic neurons in the rostral ventrolateral periaqueductal gray (VLPAG) . Using *in vivo* electrophysiologyy combined to tail flick, we observed that microinjection of UCM765 and UCM924 in the VLPAG decreases tail flick responses, depressing the firing activity of ON cells and activating the firing of OFF cells, an effect MT2 receptor-dependent. Altogether, these data demonstrate, for the first time, that MT2 receptor ligands have analgesic properties through activation of OFF cells and inhibition of the ON cells and indicate that MT2 receptors may be a novel target in the treatment of neuropathic pain.

INTRODUCTION

Neuropathic pain, characterized by chronic neurological pain, is a major health problem afflicting a significant number of patients and leading to personal suffering, reduced productivity and substantial health care costs [27]. Therapeutics for chronic pain is scant and the need for novel targets is considerable, knowing that currently available drugs show serious adverse effects.

Melatonin (N-acetyl-5-methoxytryptamine, MLT) is a neurohormone produced mainly in the pineal gland, but also in the retina and other peripherical tissues [25; 42; 44]. Most of MLT physiological effects in the brain and body result mainly from the activation of high-affinity G-protein coupled receptors, namely MT1 and MT2, which are widely expressed in the mammalian brain [11]. Interestingly, recent randomized clinical trials in humans have demonstrated that MLT has analgesic properties in chronic pain conditions such as fibromyalgia [21], irritable bowel syndrome [23; 38; 40] and migraine [34]. Similarly, cumulative evidence from animal studies has demonstrated that MLT have antinociceptive effects in animal models of acute [2; 13; 32] and chronic neuropathic pain [1; 4; 43; 47]. In neuropathic pain models, the analgesic effects of MLT are prevented by injection of the MT2 antagonist 4P-PDOT, suggesting that this effect could be mediated by MT2 melatonin receptors [4; 8]. Recently, an involvement of MT1 and MT2 receptors has been also pointed out in a rodent model of neuropathic pain and insomnia [29].

The central mechanism of neuropathic pain is far to be completely elucidated [5]. However, it is known that he brainstem modulates the spinal cord nociceptive system through important links between the periacqueductal grey (PAG) and the rostral ventromedial medulla (RVM). This network is involved in chronic pain states as well as opioid-induced analgesia [30; 46]. The RVM noci-modulatory circuit is composed of two classes of neurons defined by changes in activity associated with nocifensive reflexes such as the tail flick or paw withdrawal evoked by noxious

stimuli. ON-CELLS increase firing just before the occurrence of such reflexes, and these neurons play a pro-nociceptive role. By contrast, OFF- CELLS pause just prior to a nocifensive reflex, and selective activation of off-cells may produce antinociception.

Altogether these data impelled us to test whether the selective MT2 receptor partial agonist UCM765 [36] and its derivate UCM924 [37] possess analgesic properties following the L5-L6 spinal nerves ligation and the spared nerve injury (SNI) tests, which are both well-characterized animal models of neuropathic pain, and weather MT2 receptor is expressed in PAG and weather MT2 partial agonists modulate ON and OFF neurons, similarly to other classes of analgesic drugs [17].

MATERIAL AND METHODS

Animals

Male Wistar rats weighing 140-160 g at the beginning of the experiments were used in the two models of neuropathic pain. Male Wistar rats weighting 200-250 g were used for electrophysiological recordings coupled to tail flick. All animals were housed in standardize animal facilities under a 12 h light/dark cycle (lights on at 7 AM) with ad libitum access to food and water. All surgeries and experimental procedures were performed during the light cycle. Experimental protocols were approved by the Animal Ethics Committee of local institutional committee for animal use and care (McGill University, Qc, Canada; CINVESTAV, Mexico; Second University of Naples, Italy). These protocols follow ethical guidelines for investigation of experimental pain in conscious animals of the IASP [48], the Canadian Institute of Health Research guidelines for animal care and scientific use, and are in compliance with Italian (D.L.116/92) and E.C. (O.F. of E.C. L358/1 18/12/86) regulations on the protection of laboratory animals.

Drugs.

N-acetamide (UCM765) and *N*-acetamide (UCM924) were synthesized by the University of Urbino, Italy and by BioQuadrant Inc (Montreal, Qc, Canada). Melatonin (N-acetyl-5-methoxytryptamine, MLT) and Gabapentin (GBP, 2-[1-(aminomethyl)cyclohexyl]acetic acid) were purchased from Sigma (St. Louis, MO, USA). 4P-PDOT (4-phenyl-2-propionamidotetralin) was purchased from Tocris Bioscience. For subcutaneous administrations, MLT, UCM765, UCM924, and Gabapentin were dissolved in a vehicle (VEH) of 70% dimethylsulfoxide (DMSO) and 30% saline solution (0.5 ml volume). 4P-PDOT was dissolved in 80% DMSO and 20% saline. All treatments were administered in a single subcutaneous (s.c.) injection with a volume of 0.5 ml 30 min before the tests, unless otherwise is indicated. For intra–VLPAG administration, rats receive a single microinjection of the drugs dissolved in 0.05% DMSO in artificial cerebrospinal fluid (ACSF, composition in mM: KCl 2.5; NaCl 125; MgCl2 1.18; CaCl2 1.26) in a volume of 200 nl.

L5-L6 sciatic nerves ligation and measurement of allodynia with Von Frey filaments.

This surgery was done according to Kim and Chung [22]. Briefly, animals were anesthetized with a mixture of ketamine/xylazine/acepromazine (i.p.). Following surgical preparation and exposure of the dorsal vertebral column, left L5 and L6 spinal nerves were exposed and tightly ligated, distal to the dorsal root ganglion, using 3.0 chromic catgut. For sham operated rats, nerves were exposed but not ligated. The incisions were closed using 3.0 vicryl sutures and animals were allowed to recover for 13 days. Rats exhibiting motor deficiency (such as paw dragging) in the days following surgery were excluded from the study.

On day 14 after surgery, rats were placed one at the time in a test chamber (a cage with a metal-mesh floor) and tactile allodynia was determined by measuring paw withdrawal thresholds in response to mid-plantar hind paw stimuli with Von Frey filaments. These filaments are of a logarithmically incremental stiffness corresponding to an applied force ranging from 0.4 to 15 g.

Every filament was applied during 10 seconds in the hind paw to measure the withdrawal threshold. The 2.0 g force filament was applied first, in presence of a response; the next smaller filament was applied. In absence of response, the next higher filament was applied. The test continued until six responses were collected after the first change in response, and the paw withdrawal threshold was then converted to the cutaneous nociceptive threshold by using the "up–down" method [7]. The stimulus intensity (filament stiffness) required to produce a response in 50% of the applications for each animal was defined as 50% withdrawal threshold (expressed in g). All nerve-ligated rats were verified to be allodynic, responding to a stimulus of less than 4 g. Rats without allodynia were excluded. Following establishment of basal responses, tactile allodynia was assessed again at 0.25, 1, 2, 3, 4, 5, 6, 7 and 8 h post-administration of the treatments described below. The area under the curve (AUC) of the paw withdrawal threshold during the 8 h of testing was also analyzed and compared between treatments.

Treatments in L5-L6 sciatic nerves ligation model.

Rats were randomly assigned to receive a single s.c. injection of UCM765 or UCM924 at 10, 20 or 40 mg/kg. The effects of both drugs were compared with those produced by MLT (150 mg/kg), GBP (150 mg/kg) and VEH administration. To investigate the participation of the MT2 receptors in the analgesic effects of UCM765 and UCM924, some animals received a single dose of the MT2 antagonist 4P-PDOT (10 mg/kg) 10 min before an effective dose (20 mg/kg) of UCM765 or UCM924.

Surgical preparation of electrophysiology experiments.

For electrophysiological experiments combined with the tail flick, rats were anaesthetised with pentobarbital (50 mg/kg, i.p.) 14 days after the spinal nerve ligation. Under anaesthesia, a 26-gauge, 12 mm long stainless steel guide cannula was stereotaxically lowered until its tip was 1.5 mm above the left VLPAG by applying the following coordinates from the Atlas of Paxinos and Watson [33] : A: -7.8 mm and L: 0.5 mm from bregma and V: 4.5 mm below the dura The

cannula was anchored in the skull with dental cement to a stainless steel screw. We used a David Kopf stereotaxic apparatus (David Kopf Instruments, Tujunga, CA, USA) with the animal positioned on a homeothermic temperature control blanket (Harvard Apparatus Limited, Edenbridge, Kent). The surgery for cannula implantation was carried out the same day as the electrophysiology coupled with tail flick experiments.

Intra-VL PAG microinjections.

Direct intra-VLPAG drug administration,was conducted using a stainless steel cannula connected to a SGE 1-microlitre syringe by a polyethylene tube, inserted through the guide cannula and extended 1.5 mm beyond the tip of the guide cannula to reach the VLPAG. Vehicle or drug solutions were administered into the VLPAG in a final volume of 200 nl. Microinjection was performed over a period of 60 sec and the injection cannula was gently removed 2 min later. At the end of the experiment, a volume of 200 nl of neutral red (0.1%) was also injected into the VLPAG 30-40 min before killing the rat. Rats were then perfused intracardially with 20 ml phosphate buffer solution (PBS) followed by 200 ml 10% formalin solution in PBS. The brains were removed and immersed in a saturated formalin solution for 2 days. The injection sites were ascertained by using 2 consecutive sections (40 μm), one stained with neutral red to identify nuclei and the other unstained in order to determine dye spreading. Only rats whose microinjected site was located within the VLPAG were used for data computation. Cannulae were also intentionally implanted 1 mm outside from VLPAG for microinjection site controls.

RVM extracellular recordings.

After implantation of the guide cannula into the VLPAG, a glass insulated tungsten filament electrode (3-5 MΩ) (Frederick Haer & Co., ME, USA) was stereotaxically lowered, through a small craniotomy, into the RVM (2.8 - 3.3 mm caudal and 0.4 - 0.9 mm lateral to lambda and 8.9 -10.7 mm depth from the surface of the brain) using the coordinates from the atlas of Paxinos and Watson [33]. The jugular vein was cannulated to allow intravenous anaesthetic

administration (propofol, 8-10 mg/kg/h, i.v.). The RVM ON cells were identified by a burst of activity that begins just before nocifensor reflex to the tail flick while OFF cells were identified by the fact that they cease firing at that time. Anaesthesia was adjusted so that tail flicks were elicited with a constant latency < 6 sec. The thermal stimulus was elicited by a radiant heat source of a tail flick unit (Ugo Basile, Varese, Italy) focused on the rat tail approximately 4-5 cm from its tip. The tail was placed over the surface of a slightly projecting window receiving the I.R. energy. The I.R. intensity of the tail flick unit in our experiments has been set to 50 mW corresponding to 50 mJ per sec. Tail flick latency in seconds was determined by a timer connected to a photoelectric cell which stopped the timer (and switched off the lamp) at the movement of the tail which was withdrawn. Tail flicks were elicited every 5 min for at least 15 min prior to microinjecting drugs, or the respective vehicle, 0.05 % dimethyl sulfoxide (DMSO) in ACSF into the VL PAG.

The recorded signals were amplified and displayed on an analog and digital storage oscilloscope to ensure that the unit under study was unambiguously discriminated throughout the experiment. Signals were also processed by an interface (CED 1401) (Cambridge Electronic Design Ltd., UK) connected to a personal computer. Spike2 software (CED, version 4) was used to create peristimulus rate histograms on-line and to store and analyse digital records of single-unit activity off-line. Configuration, shape and height of the recorded action potentials were monitored and recorded continuously, using a window discriminator and Spike2 software for on-line and off-line analysis. Once an RVM cell was identified from its background activity, we optimised spike size before all treatments including only neurons whose spike configuration remained constant and could clearly be discriminated from activity in the background throughout the experiment. The recording site was marked with a 20 μA DC current for 20 sec. After fixation by immersion in 10% formalin, the recording sites were identified. In each rat, only one neuron was recorded before and after vehicle or drug administration. The neuronal responses, before and after intra-VLPAG vehicle or drug microinjections were measured and expressed as

spikes/sec (Hz). At the end of the experiment, each animal was killed with a lethal dose of urethane, the microinjection site was marked with 0.2 µl of a Cresyl Violet solution, and the recording site marked with a 20 µA DC current for 20 s. After fixation by immersion in 10% formalin, the microinjection and recording sites were identified.

RVM background cell activity was expressed as means ± SEM of the spikes/s obtained by averaging the ongoing cell firing recorded in 50 s prior to tail flick trials (which were carried out every 5 min). ON cells with spontaneous activity were the only cells investigated and included in the data analysis to better characterize the activity of this ON cell subgroup and to consider post drug changes in their spontaneous activity. Tail flick–related ON cell burst was calculated as means ± SEM of the number of spikes in the 10 sec interval starting from the beginning of the increase in cell frequency (which was at least the double of its spontaneous activity). Furthermore, the onset of the ON cell burst was calculated as means ± SEM (sec) of time elapsing between the application of the noxious radiant heat and the beginning of the tail flick–related increase in cell frequency. The onset of the OFF cell pause was calculated as means ± SEM of the time (sec) elapsing between the onset of the application of thermal stimulus and the last spike. Finally, the duration of the cell pause was expressed as means ± S.E.M. of the time (sec) elapsing between the pause onset and the 1st spike after the tail flick.

Intra-VL PAG microinjection doses.

Spinal nerves ligated rats received a single intra-VLPAG administration of 200 nl vehicle (DMSO/ACSF, 0.05 %, v/v) or drug solutions 14 days after surgery. The doses used for intra-VL PAG microinjections were the following: UCM765, a MT2 selective partial agonist, (5 and 10 µg), UCM924, a MT2 selective partial agonist, (5 and 10 µg), 4P-PDOT, a selective MT2 receptor antagonist (100 µg) and MLT (100 µg). Rats received 10 µg of UCM765 or UCM924 when used in combination with 4P-PDOT (100 µg). Since only one neuron was recorded in each rat, experimental groups consisted of 14-16 rats in order to record at least 7-8 ON and OFF

cells for each treatment. When 4P-PDOT was used in combination with UCM765 or UCM924, these latter was administered 10 minutes after the antagonist.

Spared nerve injury (SNI) of the sciatic nerve and measurement of allodynia with the dynamic plantar aesthesiometer.

SNI was performed according to the method of Decosterd and Woolf [10]. Rats were anaesthetized with sodium pentobarbital (50 mg/kg i.p.). The sciatic nerve was exposed at mid-thigh level distal to the trifurcation and freed of connective tissue; the three peripheral branches (sural, common peroneal, and tibial nerves) of the sciatic nerve were exposed without stretching nerve structures. Both tibial and common peroneal nerves were ligated and transected together. The sham procedure consisted of the same surgery without ligation and transection of the nerves. Fourteen days after SNI, mechanical allodynia was measured using the dynamic plantar aesthesiometer (Ugo Basile, Varese, Italy). Each rat was placed and allowed to move freely in one of the two compartments of the enclosure positioned on a metal grid surface. A mechanical stimulus was delivered to the plantar surface of the rat's hind paw through the metal grid by a steel filament (Von Frey-type), connected to a movable touch-stimulator unit exerting an increasing force of 3 g per second. The force inducing paw withdrawal was recorded to the nearest 0.1 g. Nociceptive responses for mechanical sensitivity (mechanical withdrawal threshold) were measured in grams before and after vehicle or drug administration by an experimenter blind to the treatments. A single trial at each time point was performed on the ipsilateral hind paw to SNI surgery for each rat. Nociceptive responses for mechanical sensitivity were expressed as mean ± SEM in grams. Groups of 6-7 rats per treatment were used, with each animal being used for one treatment only. As previously described for the L5-L6 model, SNI rats were randomly assigned to receive a single s.c. injection of UCM765 or UCM924 at the doses of 10, 20 or 40 mg/kg and the effects of both drugs were compared with those produced by MLT (150 mg/kg), GBP (100 mg/kg) and VEH administration.

Motor Co-ordination test (Rotarod test).

The effect of UCM924 and UCM925 administration on motor coordination was tested in an independent group of rats using an automated 4-line Rotarod apparatus (PanLab, Barcelona, Spain). The test consists on placing the animals in a cylinder (7 cm diameter) rotating at a constant speed of 10 rpm during 5 min, counting the number times that the rats fall. Rats were trained to walk on the cylinder for three consecutive sessions without any treatment. After this, treatments were s.c. administered and rats were tested in the Rotarod at 0.5, 1, 2, 3 and 5 h post-administration. UCM765 and UCM924 were tested at the doses of 10, 20 and 40 mg/kg and were compared MLT (150 mg/kg), GBP (100 mg/kg) and VEH administration.

Anatomical localization of MT2 receptors.

Single MT2 immunohistochemistry. Polyclonal rabbit anti-MT2 antibodies used for light microscopic immunohistochemistry were produced and affinity purified as described previously [3]. In previous anatomical study on rat brain tissue, selective neuronal labeling was observed with these primary antibodies [28]. The secondary antibodies were biotinylated goat anti-rabbit IgGs (Jackson Immunoresearch, West Grove, PA) revealed with the 3,3′-diaminobenzidine (DAB) substrate kit from Vector Labs (Burlingame, CA, USA). Briefly, three adult male Wistar rats (225-250 g) were deeply anesthetized with sodium pentobarbital (80 mg/kg, i.p.) and perfused through the heart with 250 ml of a fixative solution, followed by 500 ml of 4% PFA in 0.1 mM PB. The fixative solution consisted in 0.5% acrolein in 4% paraformaldehyde (PFA) prepared in 0.1 mM phosphate buffer (PB, pH 7.4). The brain was removed, postfixed in 4% PFA (60 min, 4 ºC), and washed in phosphate-buffered saline (PBS: 0.9% NaCl in 50 mM PB, pH 7.4). 50 µm-thick sections of the mesencephalon were cut with a vibratome and processed free-floating as follows. Sections were immersed (20 min) in 3% hydrogen peroxide (H_2O_2), rinsed in PBS, immersed (20 min) in 0.1% sodium borohydride in PBS, rinsed in PBS, and preincubated (2 h) in a blocking solution (BS) of PBS containing 10% normal goat serum

(Vector), 0.5% gelatine and 0.25% Triton X-100. Sections were then washed in PBS, incubated overnight at RT with anti-MT2 (1:250) antibody, rinsed in PBS, incubated (2 hr) with biotinylated goat anti-rabbit IgGs (1:1000), rinsed in PBS and incubated (1 h) in a 1:1000 dilution of horseradish peroxidase-conjugated streptavidin (Jackson Immunoresearch). After washes in PBS, labeling was revealed (2-5 min) with the DAB reagent kit. This reaction was stopped in distilled water, the sections rinsed in PB, air-dried on gelatine-coated slides, dehydrated in ethanol, cleared in toluene and mounted with DPX (Fluka, Oakville, ON, Canada). Photomicrographs were taken at low (1.6 X objective), and high (6.3 X objective) magnifications with a Leitz Diaplan optical microscope coupled to an Olympus DP21 colour digital camera and software (Olympus Corporation, Tokyo, Japan). Images were adjusted for brightness and contrast with the Adobe Photoshop software.

Double immunohistochemistry. Rats were anaesthetized with pentobarbital (50 mg/kg, i.p.) and transcardially perfused with saline solution followed by 4% paraformaldehyde in 0.1 M phosphate buffer. The brain was removed, post-fixed for 3 hours in the perfusion fixative, cryoprotected for 72 hours in 10, 20 and 30% sucrose in 0.1 M phosphate buffer and frozen in O.C.T. embedding compound. Transverse sections (15 µm) were cut using a cryostat and those containing the whole PAG were thaw-mounted onto glass slides. Sections were subsequently incubated for 1 day at room temperature in a humid chamber with the respective polyclonal antibodies (all diluted in specific block solution). All sections were processed for polyclonal rabbit anti-MT2 together with polyclonal guinea pig anti-vesicular glutamate transporter-1 (VGLUT-1, Millipore, Billerica MA, USA) or polyclonal goat anti-vesicular GABA transporter (VGAT, Santa Cruz biotechnology, Dallas, Texas). Following primary incubations, sections were washed and incubated for 3 hours with secondary antibodies (Alexa Fluor^{TM} 488 and 568-conjugated donkey anti-goat or anti-guinea pig and donkey anti-rabbit IgGs; 1:1000; Molecular Probes, USA). Slides were washed, cover-slipped with Vectashield mounting medium (Vector

Laboratories, USA) and visualized under a Leica fluorescence microscope. Negative control by using secondary antibodies alone did not reveal any positive staining.

Statistical Analysis.

Data analysis was done using SigmaPlot statistical software (Systat Software, Inc.).Two-way ANOVA for repeated measures was used to analyze data from L5-L6 and SNI models and the rotarod test using treatments (between) and testing time (within) as factors. One-way ANOVA was used to analyze AUC in the L5-L6 model. *Post-hoc* analyses were performed using Bonferroni *t*-test comparisons. All data are expressed as mean ± Standard Error of the Mean (SEM). $P < 0.05$ was considered significant.

RESULTS

UCM765 and UCM924 increase paw withdrawal threshold in rats following L5-L6 nerves ligature.

The time curse effect of the different doses of UCM765 (5-40 mg/kg) is shown in Fig. 1A; two-way ANOVA analysis revealed a significant interaction between treatment and time of testing ($F_{(36,225)}$=7.929, p<0.001). *Post-hoc* analysis revealed that compared with basal paw withdrawal threshold (0h), doses of 20 mg/kg (p<001) and 40 mg/kg (p=0.016) of UCM765 induce a significant increase of withdrawal threshold starting at 1 and 0.5h post-administration, respectively (Fig. 1A). The highest increase in withdrawal threshold was observed at 3h post-administration for the dose of 20 mg/kg (419% from basal) and at 2h post-administration for the dose of 40 mg/kg (395% from basal), and lasted for up to 6h and 5h post-administration, respectively. A significant interaction among treatments and time of test was also detected when the effects of UCM765 (20mg/kg) were compared with MLT and GBP treated rats and with rats receiving the MT2 antagonist 4P-PDOT (10 mg/kg) prior to UCM 765 (20mg/kg) ($F_{(36,225)}$ = 5.115, p<0.001; Fig. 1B). Remarkably, post-hoc analysis of area under the curve (AUC, Fig. 1C) also shows that UCM765 (20mg and 40 mg) is superior to MLT (150mg) (p>0.05).

In particular, AUC's post-hoc analyses across 8 h indicates that, compared to VEH-treated rats (measured as mean±SEM, weight (g) of mechanical allodynia, 36.24 ± 1.8 g), UCM765 treatment induced a significant dose-dependent anti-allodynia effect (UCM765, 5 mg/kg: 81.11 ± 4.8 g; 10 mg/kg: 70.24 ± 4.23 g; 20 mg/kg: 219.12 ± 24.13 g; 40 mg/kg: 220.13 ± 13.65 g; $F_{(7,40)}$=35.723, p<0.001, Fig. 1C). The effect of both drugs at the doses of 20 and 40 mg/kg were comparable with the AUC produced by administration of a 100 mg/kg dose of GBP (249.33 ± 22.1 g) and were higher than the effects produced by MLT administration (150 mg/kg = 131.75 ± 7.9, p<0.01). Pre-treatment with 4P-PDOT (10 mg/kg) blocked the analgesic effects of UCM765 (98.22 ± 9.54 g) and UCM924 (89.69 ± 7.16 g) as indicated in Fig. 1C (p>0.05 when compared to vehicle).

The time curse of the effects of UCM924 is shown in Fig 2A; two-way ANOVA analysis revealed a significant interaction between treatment and time of testing ($F_{(36,225)}$=5.886, p<0.001). Compared with basal withdrawal threshold (0h), doses of 10 mg/kg (p=0.009), 20 mg/kg (p<0.001) and 40 mg/kg (p=0.003) of UCM924 increased paw withdrawal threshold starting at 1h post-administration. The effect of the 10mg/kg dose lasted up to 3h, but the effects of the doses of 20 and 40 mg/kg remained stable for up to 6h post-administration with a maximum increase of threshold at 5h (20 mg/kg: 394% from basal; 40 mg/kg: 427.65% from basal). Compared with VEH-treated rats, UCM924 at 10 mg/kg dose increased paw withdrawal threshold at 1, 2, 3, 4 h post-administration, whereas the dose of 20 and 40 mg/kg increased it from 1h to 6h post-administration (see Fig. 2A).

Similarly, a significant interaction between treatment and time of test was also detected when the effects of UCM924 (20mg/kg) were compared with MLT and GBP treated rats and with rats receiving the MT2 antagonist 4P-PDOT (10 mg/kg) prior UCM 765 (20mg/kg) ($F_{(36,255)}$ = 4.967, p<0.001; Fig. 2B).

Effects of UCM765 and UCM924 were similar to those observed in rats treated with GBP at 100 mg/kg (Fig 1B and Fig 2B, respectively). GBP treatment increased paw withdrawal threshold from 1 h to 6 h compared with basal withdrawal threshold (0 h) and from 1 h to 7 h compared with VEH treated rats. The maximum withdrawal threshold increase induced by GBP was observed at 4 h (420% from basal). MLT treated rats, showed an increased withdrawal threshold from 1 h to 3 h compared with their basal threshold and with VEH group, with a maximum increase in threshold at 2 h post-treatment (335% from basal). Pre-treatment with 4P-PDOT blocked the effects of both UCM765 and UCM924 during the 8 h of testing (Fig. 1B and Fig. 2B, respectively).

Analysis of the AUC during the 8 h of testing indicates that, compared to VEH-treated rats (measured as mean±SEM, weight (g) of mechanical allodynia 36.24 ± 1.8 g), UCM924 treatments induced a significant dose-dependent anti-allodynia effect (UCM924, 5 mg/kg: 89.03 ± 6.33 g; 10 mg/kg: 127.75 ± 12.08 g; 20 mg/kg: 245.9 ± 19.9 g; 40 mg/kg: 230.33 ± 17.69 g; $F_{(7,40)}$=35.416, p<0.001, Fig. 2C). The effect of UCM924 at the doses of 20 and 40 mg/kg were comparable with the AUC produced by administration of a 100 mg/kg dose of GBP (249.33 ± 22.1 g) and were higher than the effects produced by MLT administration (150 mg/kg = 131.75 ± 7.9, p<0.01). Pre-treatment with 4P-PDOT (10 mg/kg) blocked the analgesic effects of UCM924 (89.69 ± 7.16 g) as indicated in Fig. 2C, (p>0.05 when compared to vehicle).

UCM765 and UCM924 increased paw withdrawal threshold in SNI rats.

SNI of the sciatic nerve resulted in a significant decrease in mechanical withdrawal threshold in the ipsilateral side of SNI rats (13.5 ± 0.6 g), though not on the contralateral sides 7 days after surgery. Administration of vehicle did not change MWT in the SNI rats (Fig. 3). In SNI rats, a reduction of the mechanical allodynia was caused by the administration of UCM765 (20 mg/Kg: $F_{(7,49)}$=12.612, p<0.001; and 40 mg/kg: $F_{(7,49)}$=9.762, p<0.001, Fig. 3A) but not by UCM765 10mg/Kg and UCM924 (20 mg/Kg: $F_{(7,49)}$=24, p<0.001; and 40 mg/kg: $F_{(8,54)}$=24.9, p<0.001, Fig. 3B) but not by UCM924 10mg/Kg. The effect of UCM765 at all the doses tested lasted no longer than 6 h after administration, and reduced mechanical allodynia up to 18.7 ± 0.2 g at the higher dose (40 mg/kg) (Fig. 3A). UCM924 (40 mg/kg) was effective up to 8 h after administration and reversed almost completely mechanical allodynia (24.9 ± 0.7 g) (Fig. 3B).

The subcutaneous administration of MLT (150 mg/kg) decreased mechanical allodynia (18.7 ± 0.2 g; $F_{(8,54)}$=12.256, p<0.001) an effect apparent up to 4 h after administration (Fig. 3C). Subcutaneous administration of GBP (100 mg/kg) decreased mechanical allodynia (25.7 ± 0.4 g; $F_{(8,54)}$=14.831, p=0.003), an effect apparent up to 6 h after administration (Fig. 3C).

Post-hoc-analysis shows a difference between UCM924 and MLT (P<0.01, Fig 3C).

UCM765 and UCM924 do not affect motor coordination in rats.

In the rotarod test, a significant interaction between the treatment and testing time was observed in the number of falls when effects of UCM765 ($F_{(20,84)}$=2.908, p<0.001) and UCM924 ($F_{(20,84)}$=3.256, p<0.001) where compared against GBP (100 mg/kg) and MLT (150 mg/kg). In both cases, the differences detected with two-way ANOVA were due to GBP administration. As shown in Table 1 the different doses of UCM765 for UCM924, (10, 20 and 40 mg/kg) did not significantly increased the number of falls in the 5 h period of the testing. GBP administration increased the number of falls at 2 h (p<0.02) and 3 h (p<0.001) post-administration in comparison with UCM765-, UCM924-, MLT- and VEH-treated rats (Table 1).

MT2 receptors are expressed by glutamatergic neurons in the VLPAG.

Our immunohistochemical study indicates that MT2 receptors were found in the rostral third of the periaqueductal gray (PAG) (Fig. 4). MT2 immunoreactivity appeared moderate and seemed to be restricted to the ventrolateral aspect of the PAG across the different antero-posterior levels of this area (Fig 4A). MT2 immunostaining was absent from the dorsomedial (DMPAG), dorsolateral (DLPAG), and lateral (LPAG) PAG, but MT2-positive neurons could be observed more densely in the ventrolateral subdivision (VLPAG). Moreover, double immunofluorescence revealed that MT2 receptors in this area are preferentially expressed by neurons expressing the vesicular glutamate transporter-2 (vGlut-2) rather than by axon terminals expressing the vesicular GABA transporter vGAT (Fig. 4B).

Effect of intra-VL PAG UCM765 and UCM924 alone or in combination with 4P-PDOT on the ongoing activity of RVM ON and OFF cells in SNL rats.

The results are based on RVM neurons (one cell recorded from each animal per treatment) at a depth of 9,900-10,955 mcm from the surface of the brain. All recorded neurons identified as ON cells, by a burst of activity immediately prior to tail flick responses, were spontaneously active in 29.2% of cases and inactive in the remaining ones. Only ON cells showing spontaneous activity

were chosen and included in the analysis of the data so as to characterize the activity of this ON cell subgroup more accurately and to consider post-drug changes in their spontaneous activity. In L5-L6 spinal nerve ligated rats the population of ON cells had a mean frequency of spontaneous activity of 14.3 ± 1.4 spikes/s. Microinjection of vehicle (0.05% DMSO in ACSF) did not change the spontaneous activity of ON cells (14.7 ± 1.2 spikes/s) (Fig. 6A and B). A decrease in the spontaneous firing activity of the ON cells was observed after the microinjection intra-VL PAG of 5 µg UCM765 (10.2 ± 1.1 spike/s, $F_{(7,49)}$=34.015, p=0.003) and 10 µg UCM765 (9.3 ± 0.9 spike/s, $F_{(7,49)}$=32.623, p<0.001, Fig 6A). A reduction of ON cell firing was also observed with 5 µg UCM924 (8.7 ± 0.48 spike/s, $F_{(7,49)}$=33.847, p=0.004, Fig 6A), 10 µg UCM924 (2.8 ± 1 spike/s, $F_{(7,49)}$=28.227, p<0.001, Fig 6A) and 100 µg MLT (8.5 ± 0.5 spike/s, $F_{(7,49)}$=35.431, p=0.003, Fig 6B). The population of OFF cells had a mean frequency of spontaneous activity of 5.2 ± 1 spikes/s in SNL rats. Microinjection of vehicle did not change the spontaneous activity of OFF cells (4.3 ± 1.2 spikes/s) (Fig. 6C and D). An increase in the spontaneous firing activity of the OFF cells compared to the vehicle injection was caused by the microinjections intra-VL PAG of 5 µg UCM765 (10.7 ± 1.6 spike/s, $F_{(7,49)}$=36.415, p=0.002), 10 µg UCM765 (22.6 ± 1.3 spike/s, $F_{(7,49)}$=33.741, p<0.001, Fig 6C), 5 µg UCM924 (9.4 ± 1.2 spike/s, $F_{(7,49)}$=30.213, p=0.003) and 10 µg UCM924 (17.8 ± 1.0 spike/s, $F_{(7,49)}$=29.114, p<0.001, Fig 6C) and 100 µg MLT (16 ± 1.6 spike/s, $F_{(7,49)}$=30.201, p=0.002, Fig 6D).

The effects of both UCM765 and UCM924 (10 µg) on ongoing activity of the ON cells ($F_{(7,49)}$=8.401, p<0.001, and $F_{(7,49)}$=4.252, p<0.001, respectively) and OFF cells ($F_{(7,49)}$=11.417, p<0.001, and $F_{(7,49)}$=8.503, p<0.001, respectively) were completely abolished by a 10 min pre-treatment with 4P-PDOT (100 µg) which was inactive per se at the same dose (Fig 6B and D).

Effect of intra-VL PAG MLT, UCM765 and UCM924 alone or in combination with 4P-PDOT on tail flick-related ON and OFF cell activity in SNL rats.

In L5-L6 spinal nerve ligation rats the population of ON cells had a tail flick-induced burst of firing of 21.7 ± 1.5 spikes/s, the population of OFF cells had a pause of 14.67 ± 1.25 s. Microinjections of VEH did not change the tail flick-induced ON cell burst and OFF cell pause (22.6 ± 2.0 spikes/s, n=7 and 15.0 ± 1.0 s, n=7, respectively) (Fig. 7A and B and Fig. 8A and B). Intra-VL PAG microinjections of MLT (100 µg) decrease significantly the ON cell burst (7.8 ± 1 spikes/s, $F_{(7,49)}$=25.224, p=0.004; Fig. 7B) and the OFF cell pause (7 ± 0.9 s, $F_{(7,49)}$=29.332, p<0.001) (Fig. 8B). Intra-VL PAG microinjections of UCM765 (10 µg) decrease significantly the ON cell burst (6.5 ± 1.8 spikes/s, $F_{(7,49)}$=20.253, p<0.001) (Fig. 7A) and the OFF cell pause (5 ± 1.3 s; $F_{(7,49)}$=30.151, p= 0.003) (Fig. 8A). The lower dose of UCM765 (5 µg) did not change the tail flick-related ON and OFF cell activity (n=8 for both cells) (Fig. 7A and 8A). Intra-VL PAG microinjections and $F_{(7,49)}$=7.258, p<0.001, respectively) and OFF cell activity ($F_{(7,49)}$=10.015, p<0.001, and $F_{(7,49)}$=6.403, p<0.001, respectively). 4P-PDOT was inactive per se at the same dose (Fig. 7B and 8B).of UCM924 significantly decrease the ON cell burst at 5 µg (17.1 ± 0.9 spikes/s, $F_{(7,49)}$=25.254, p=0.003) and 10 µg (0.6 ± 1.1 spikes/s, $F_{(7,49)}$=24.662, p<0.001) (Fig. 7A). UCM924 changed also the OFF cell pause at 5 µg (10.26 ± 1.4 s; $F_{(7,49)}$=28.475, p=0.002) and 10 µg (7.56 ± 1.5 s, $F_{(7,49)}$=26.448, p< 0.001) (Fig. 8A). 4P-PDOT (100 µg) completely prevented the effects induced by the highest dose of UCM765 and UCM924 on tail flick-related ON ($F_{(7,49)}$=9.205, p<0.001).

Effect of intra-VL PAG MLT, UCM765 and UCM924 alone or in combination with 4P-PDOT on the onset of the ON cell burst and the OFF cell pause in SNL rats.

In L5-L6 spinal nerve ligation rats the onset of ON cells burst was 415 ± 64 ms and the onset of the OFF cell pause was 500 ± 60 ms. Microinjections of vehicle did not change the onset of the ON cell burst and the OFF cell pause (465 ± 60 ms, n=7 and 470 ± 40 ms, n=7, respectively) (Fig. 7C and D and 8C and D). Intra-VL PAG microinjections of MLT (100 µg) increase significantly the onset of the ON cell burst (830 ± 27 ms, $F_{(7,49)}$=21.223, p=0.002) (Fig 7D) and

the onset of the OFF cell pause (760 ± 38 ms; $F_{(7,49)}$=24.331, p=0.003) (Fig 8D). Intra-VL PAG microinjections of UCM765 (10 µg) increase significantly the onset of the ON cell burst (840 ± 30 ms, $F_{(7,49)}$=31.825, p<0.001 (Fig 7C) and the onset of the OFF cell pause (780 ± 50 ms; $F_{(7,49)}$=29.521, p<0.001 (Fig 8C). The lower dose of UCM765 (5 µg) did not change the onset of the ON cell burst and the OFF cell pause (n=8 for both cells) (Fig. 7C and 8C). Intra-VL PAG microinjections of UCM924 increase significantly the onset of the ON cell burst at (5 µg) (690 ± 54 ms, $F_{(7,49)}$=26.802, p=0.002) and at (10 µg) (820 ± 33 ms, $F_{(7,49)}$=22.364, p<0.001) (Fig 7C) and the onset of the OFF cell pause at 5 µg (680 ± 33 ms, $F_{(7,49)}$=29.607, p=0.003) and 10 µg (870 ± **10** ms, $F_{(7,49)}$=26.581, p<0.001, Fig 8C) 4P-PDOT (100 µg) completely prevented the effects of the highest dose of UCM765 and UCM924 on the onset of the ON cell burst ($F_{(7,49)}$=11.203, p<0.001 and $F_{(7,49)}$=9.741, p<0.001, respectively) and the OFF cell pause ($F_{(7,49)}$=13.207, p<0.001, $F_{(7,49)}$=5.584, p<0.001, respectively) (Fig 7D and 8D). 4P-PDOT (100 µg) which was inactive per se at the same dose (Fig. 7D and 8D). UCM924 seems more potent than UCM765 in inhibiting ongoing and tail flick-related ON cell activity.

Effect of intra-VLPAG UCM765 and UCM924 alone or in combination with 4P-PDOT on tail flick latency in SNL rats.

Tail flicks were elicited every 5 min for 15 min prior to microinjecting drugs or respective vehicle into the VL PAG. In neuropathic rats basal tail flick latencies were related to pre-treatment intervals (3 ± 0.5 s, n=14). Intra-VL PAG microinjection of vehicle did not change tail flick latency in ligated rats (3.3 ± 0.5 s, n=16) (Fig. 9A and B) compared with basal values. Intra-VL PAG microinjections of MLT (100 µg) caused an increase in the tail flick latency at 15 min after microinjection (7 ± 0.7 s, $F_{(7,49)}$=18.117, p<0.001, Fig. 9B). An increase in the tail flick latency visible already at 15 min after microinjection was observed after intra-VLPAG microinjections of both 5 µg UCM765 (4.92 ± 0.3 s, $F_{(7,49)}$=24.362, p=0.003), 10 µg UCM765 (6.14± 0.12 s,

$F_{(7,49)}$=20.314, p<0.001, Fig. 9A), 5 μg UCM924 (8.56 ± 0.60 s, $F_{(7,49)}$=12.365, p=0.003) and 10 μg UCM924 (9.3 ± 0.2 s, $F_{(7,49)}$=10.405, p<0.001, Fig. 9A).

The antinociceptive effect induced by the highest dose of UCM765 and UCM924 (10 μg) was completely prevented by 10 min pre-treatment with 4P-PDOT (100 μg), which was inactive per se at the same dose ($F_{(7,49)}$=14.061, p<0.001, and $F_{(7,49)}$=12.647, p<0.001, respectively, Fig. 9B).

DISCUSSION

The present study is the first demonstration that systemic administration of selective MT2 partial agonists, UCM765 and UCM924, induces an antiallodynia effect in two models of chronic neurophatic pain in rats. These effects are mediated by melatonin MT2 receptor since are blocked by the selective MT2 antagonist 4P-PDOT and are comparable to the effects produced by gabapentine, without producing motor coordination impairments. We also demonstrated that MT2 receptors are expressed in the glutamateric neurons of the VL PAG and when UCM765 or UCM924 are microinjected in this area induce a decrease in firing activity of ON cells and an increase in firing of OFF cells in the RVM.

Several studies have shown that MLT is active in models of inflammatory pain. MLT inhibits the nociceptive responses, hyperalgesia and oedema induced by carrageenan [2] and in the formalin test attenuates the licking mostly in the second phase of the test, corresponding to the inflammatory mediated response [32]. Most importantly, previous studies have demonstrated that MLT reduces neuropathic pain in different animal models: partial ligation of sciatic nerve [43], L5-L6 ligation [1], chronic constriction nerve injury [47] and also in the streptozotocin-induced hyperalgesia and allodynia, that is a model of diabetic neuropathic pain [4].

In neuropathic pain models the analgesic effects of MLT were prevented by the injection of the MT2 antagonist 4P-PDOT or by naltrexone, suggesting that this effect could be mediated by the MT2 receptor and partially by opioids [4]. In our study the activation of MT2 receptor was sufficient to induce a decrease in allodynia and the effects were blocked by the antagonist 4P-PDOT in both pain models and electrophysiological experiments, however a co-activation of opioid system in this mechanism cannot be rule-out. Remarkably, we have also demonstrated that MT2 selective agonists, in particular UCM924, was more potent than MLT itself in decreasing allodynia in L5-L6 models and in the activation of both ON/OFF cells; this effects

can be linked to the higher affinity of UCM924 (pK_i = 10.2) for the MT2 receptor compared to melatonin itself (pK_i = 9.6) confirming the specific role of MT2 receptor in the physiopathology of neuropathic pain.

Other studies have also identified melatonin receptors in areas related with pain. MT1 and MT2 receptor mRNA expression has been observed in hippocampus as well as in the cerebral and cerebellar cortex, in the habenulaand thalamus [11] and hypothalamus [26; 39]. MT2 receptors are also detectable in the CA3 and CA4 pyramidal neurons, which receive glutamatergic excitatory inputs from the entorhinal cortex [12] and our recent studies have found the expression of MT2 receptors in thalamus, ventral pallidum, CA2, dentate gyrus, and dorsal raphe (unpublished results), which are also indirectly involved in the pain status. More importantly, this study using MT2 polyclonal antibodies and microscopy have confirmed the presence of MT2 receptors at the level of PAG, and the capacity to activate the firing of OFF cells and decrease this of ON cells in the RVM, when MT2 ligands are directly microinjected into the PAG. PAG is an analgesic site within the pain-descending pathway, whose stimulation produces analgesia [15; 35]. PAG-induced analgesia passes through the RVM, an intermediate projection site before synapsing and inhibiting spinal cord dorsal horn neurons [6; 16], PAG receives also major inputs from the frontal neocortex, the hypothalamus and amygdala. The PAG-RVM network can enhance or suppress nociceptive processing, and it plays a role in facilitated pain states as well as opioid analgesia [30; 46] and also maintains nerve-injury induced sensitization in neuropathic pain [45]. Systemic or local administration of opioids or cannabinoids, able to inhibit nociception, inhibit ON cells whilst increase OFF cells [9; 14; 16; 19]. Changes of ON and OFF cell activity are thus predictive of pain response modulation for putative analgesic drugs.

The MT2 partial agonists UCM765 and UCM924, when microinjected into the PAG, inhibited the spontaneous and tail flick-evoked activity of ON cells while enhancing that of OFF cells,

suggesting a novel and before unknown mechanism involved in the control of ON/OFF cells. Thus MT2 receptor activation within the VL PAG leads to antinociception and simultaneous ON cell inhibition and OFF cell stimulation similarly to cannabinoid type 1 (CB1) and transient receptor potential vanilloid type 1 (TRPV1) receptor stimulation [9; 31; 41], but differently from opioids that act directly on OFF cells though mu receptors, and indirectly on ON cells [17; 24]. Interestingly the UCM924, comparing to UCM765, seems having a superior effect on deactivating the ongoing and tail flick-evoked activity of the ON cells (Figs. 6A and 7A). This effect is consistent and match with the greater behavioural analgesia showed by UCM924 compared to UCM 765 (Figs 3 and 9). Indeed, the inhibition of the ON cell activity seems to be a critical event in antinociception [18]. The difference among UCM924 and UCM765 potency could be linked to their different affinity for the MT2 receptor [8].

Until now, the molecular mechanisms by which melatonin modulates or controls pain activity has not been understood yet, but have been reported that the peripheral antinociceptive mechanisms of melatonin may involve the activation of NO-cyclic GMP-PKG ATP-sensitive and Ca2+-activated K+ channels pathway [20] or the activation of the melatonin receptors may modify K+ and Ca2+ ion channel function and induce cell hyperpolarization. In fact, the binding of melatonin to MT2 receptors generates a decrease of the intracellular concentration of AMPc, GMPc, Ca2+, diacylglycerol and arachidonic in the anterior pituitary of rats [44]. Since the MT2 receptors are expressed at the level of PAG glutamatergic neurons it is likely that a decrease in AMPc, GMPc and Ca2+can occur at these levels, but further research will be necessary to understand molecular mechanism.

Finally, we also found that UCM765 and UCM924 unlike gabapentine did not produced fall at the rotorod tests, indicating that this novel class of ligands can produce analgesic effects avoiding the motor adverse effects produced by current clinically prescribed analgesic.

In a previous research we have demonstrated that the UCM765 at the dose of 40mg/kg, but not 10-20 mg/kg, reduces the latency of sleep and increase the total duration of non-rapid eyes sleep during the inactive phase in rodents through the activation of reticular thalamic nucleus [28]. From these experiments instead it is clear that lower doses (20 mg/kg) of UCM765 and UCM924 are sufficient to induce analgesic properties. This dose-dependent effect may be due to a differential activity of G-protein receptor MT2 receptors at the level of PAG (in case of analgesia) and of reticular thalamic nucleus (in case of sleep), but further research are needed to identify the intracellular pathways activated by MT2 receptor ligands in these different areas. Moreover, the involvement of MT2 receptors in both pain and sleep regulation will also help to better understand the pathophysiology of fibromyalgia and chronic fatigue syndrome, two medical conditions in which severe pain and insomnia are often associated and for which only limited treatments are available. Despite these open questions, our findings provide a preclinical validation for melatonin MT2 receptor partial agonists as potential analgesic drugs for neuropathic pain with a direct action on RVM ON/OFF cells.

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Authors' contribution. EP, LL: *in vivo* electrophysiology, analysis of data, writing assistance, immunohistochemistry; BL, LD: immunohistochemistry studies; SDL: conception of experiments and data analysis; DA: preparation of MT_2 antibodies; FF: measures of affinity and intrinsic

activity of drugs, preparation of MT_2 antibodies; GT, GS. AB: synthesis of drugs, LD: immunohistochemistry studies and writing assistance; SM, VGS: experimental planning and neuropathic pain expertise, manuscript revision; GG: conception of idea and experimental organization; supervision of experiments and data analysis; international team coordination; manuscript writing.

FIGURE LEGENDS

Figure 1. MT2 partial agonist UCM765 reduces allodynia in a MT2 selective manner, with an efficacy comparable to gabapentine in *a* L5-L6 ligation model.

(A) Time course of paw withdrawal threshold after Von Frey filaments stimulation in rats with L5-L6 spinal nerves ligation before (time 0) after (0.5-8 hours) subcutaneous administration of increasing doses of UCM765 (5, 10, 20 and 40 mg/kg), in comparison with vehicle (VEH) treated rats, (***B)*** of UCM765 (20 mg/kg), gabapentin (GBP, 100mg/kg), melatonin (MLT, 150 mg/kg), 4P-PDOT (10 mg/kg) administered 10 min prior to UCM765 (20 mg/kg).

Intermittent line in A and B represents the threshold cut-off (4 g) for allodynia, values above this line are considered antiallodynic effective.

(C) Area under the curve (AUC) of the antiallodynic effect (expressed in grams) of increasing doses of UCM765 compared to GBP (100mg/kg), MLT (100mg/kg), and 4P-PDOT (10 mg/kg) prior to UCM765 (10mg/kg). VEH. Data are expressed as mean ± SEM. * $p < 0.05$, ** $p < 0.01$ and *** $p < 0.001$ vs. vehicle.

(Animals per group=XX)

Figure 2. MT2 partial agonist UCM924 reduces allodynia ina MT2 selective manner, with an efficacy comparable to gabapentine in *a* L5-L6 ligation model.

(A) Time course of paw withdrawal threshold after Von Frey filaments stimulation in rats with L5-L6 spinal nerves ligation before (time 0) after (0.5-8 hours) subcutaneous administration of increasing doses of UCM924 (5, 10, 20 and 40 mg/kg), in comparison with vehicle (VEH) treated rats; (***B)*** of UCM924 (20 mg/kg), gabapentin (GBP, 100 mg/kg), melatonin (MLT, 150

mg/kg) and VEH. 4P-PDOT (10 mg/kg) administered 10 min before UCM765 (20 mg/kg) blocks the antiallodynic effect of UCM765 administration.

Intermittent line in A and B represents the threshold cut-off (4 g) for allodynia, values above this line are considered antiallodynic effective.

(C) Area under the curve (AUC) of the antiallodynic effect (expressed in grams) of increasing doses of UCM924 compared to to GBP (100mg/kg), MLT (100mg/kg), and 4P-PDOT (10 mg/kg) prior to UCM924 (10mg/kg). Data are expressed as mean ± SEM. * $p < 0.05$, ** $p < 0.01$ and *** $p < 0.001$ vs. vehicle.

(Animals per group=XX)

Figure 3. Antihyperalgesic effects of MT2 partial agonists in the Spared d Nerve Injury (SNI) model. ***(A)*** Effects of subcutaneous administration of UCM765 (10, 20 and 40 mg/kg), ***(B)*** UCM924 (10, 20 and 40 mg/kg), **(C)** MLT (150 mg/kg), GBP (100 mg/kg), on mechanical withdrawal threshold in SNI rats. Each point represents threshold mean ± S.E.M. (Animals per group=7-8). * $p < 0.05$, ** $p < 0.01$ and *** $p < 0.001$ indicate statistically significant difference vs. vehicle. $^{+}$ indicates statistically significant difference between different doses of UCM765 and UCM924. $^{\#}$ indicates statistically significant difference versus melatonin.

Figure 5. Immunohistochemical localization of MT2 receptors in the periaqueductal gray matter. ***(A)***, Two antero-posterior levels of the rostral PAG are illustrated. MT2 immunostaining in the rostral PAG reveals immunoreactive neurons (arrows) in the ventrolateral PAG (red squares). ***(B)*** Double labelling demonstrates the preferential expression of MT2 receptors by vGlut-2-positive neurons. Insets are magnification crops of above images.

Figure 6. Intra-VL PAG microinjections of MT2 partial agonist UCM765 and UCM924 increases spontaneous firing activity of ON cells and decrease firing activity of OFF cells of RVM.

TOP. Schematic illustration of the location of ventrolateral periaqueductal gray (VL PAG) microinjection sites *(**A**)* and RVM ON or OFF cell recording sites *(**B**).* Vehicle or drug microinjections were performed in the VL PAG (filled squares). The open circles indicate microinjections accidentally or intentionally performed outside of VL PAG, the effects of which (n=7) have been considered in the study for location specificity *(**A**).* Cell recordings were performed by lowering a tungsten electrode into the RVM. ON cells (filled triangles) or OFF cells (open triangles) recording sites are shown in ***B***. Many sites are not shown because of symbol overlapping. Distances (in mm) from the interaural line are indicated.

BOTTOM. Examples of ratemater records which illustrate the effect of intra-VL PAG microinjection of vehicle, MLT (100 μg), UCM765 and UCM924 (10 μg) alone or in combination with 4P-PDOT (100 μg) on either the ongoing or tail flick-related burst of activity of identified RVM ON cells (C, E, G, I, K, M) and ongoing or tail flick related pause of identified RVM OFF cells (D, F, H, J, L, M, N) in SNL rats. Intra-VL PAG microinjection of vehicle did not change the ongoing activity and tail flick-related activity of the ON and OFF cells (C and D). Intra-VL PAG microinjection of UCM765 and UCM924 (10 μg) decreased the ongoing activity and tail flick-related burst of the ON cells (G and I, respectively). The same treatment increased the ongoing activity and reduced the tail flick-related pause of the OFF cells (H and J, respectively). The effects induced by the highest dose of UCM765 and UCM924 on the ongoing activity and tail flick-related activity of the ON and OFF cells were completely prevented by pre-treatment with 4P-PDOT (100 μg) (K, L, M, N,). Scales bars indicate 5 min for ratemater records, while small full arrows indicate the noxious stimulation. At the right a time expanded scale illustrates pause

duration changes (scale bar = 5 sec). The grey arrows show the noxious stimuli application and the black one the tail flick reflex.

Figure 7. UCM765, UCM924 and MLT increase spontaneous firing activity of ON cells and decrease firing activity of OFF cells across time, this effect is blocked by the MT2 receptor antagonist 4P-PDOT.

Effects of intra-VL PAG microinjections of vehicle (0.05% DMSO in ACSF), UCM765 and UCM924 (5 and 10 µg) alone on the spontaneous firing (spikes/sec or Hz) of RVM ON ***(A)*** and OFF cells (***C)***. * $p < 0.05$, ** $p < 0.01$ and *** $p < 0.001$ indicate statistically significant difference vs. vehicle. $^{+}$ indicates statistically significant difference between different doses of UCM765 and UCM924. Effects of MLT (100 µg), UCM765 and UCM924 (10 µg) alone or in combination with 4P-PDOT (100 µg) on the spontaneous firing of RVM ON (B) and OFF cells (D). Vehicle or drugs were administered at the time 0, whereas 4P-PDOT (100 µg) was administered 10 min beforehand. Each point represents the mean ± S.E.M of 6-7 neurons. ** $p < 0.05$, *** $p < 0.01$ indicates statistically significant difference vs. vehicle.

Figure 8. UCM765, UCM924 and MLT decrease tail flick-evoked burst of firing and enhance the onset of the burst of the ON cells in the RVM across time, this effect is blocked by the MT2 receptor antagonist 4P-PDOT. Effects of intra-VL PAG microinjections of vehicle (0.05% DMSO in ACSF), UCM765 and UCM924 (5 and 10 µg) alone and MLT (100 ug), UCM765 (10 µg) and UCM924 (10 µg) alone or in combination with 4P-PDOT (100 µg) on tail flick-evoked burst of firing (**A** and **B**) and onset of the burst (**C** and **D**) of the ON cells in the RVM. Each point represents the mean ± S.E.M of 7-8 neurons of different treated groups of rats.). * $p < 0.05$, ** $p < 0.01$ and *** $p < 0.001$ indicate statistically significant difference vs. vehicle. $^{+}$ indicates statistically significant difference between different doses of UCM765 and UCM924.

Figure 9. UCM765, UCM924 and MLT decrease tail flick-evoked pause duration and enhance the pause onset of OFF cells in the RVM across time, this effect is blocked by the MT2 receptor antagonist 4P-PDOT. Effects of intra-VL PAG microinjections of vehicle (0.05% DMSO in ACSF), UCM765 and UCM924 (5 and 10 µg) alone, and MLT (100 µg) or UCM765 and UCM924 (10 µg) in combination with 4P-PDOT (100 µg) on OFF cell tail flick-evoked pause duration (**A** and **B**) and onset of the pause (**C** and **D**) of the OFF cells in the RVM. Each point represents the mean ± S.E.M of 7-8 neurons of different treated groups of rats). * $p < 0.05$, ** $p < 0.01$ and *** $p < 0.001$ indicate statistically significant difference vs. vehicle. $^{+}$ indicates statistically significant difference between different doses of UCM765 and UCM924.

Figure 11. Increase of tail flick latency after intra-VL PAG microinjections of UCM765, UCM924 and MLT and block by the MT2 antagonist 4P-PDOT. (A) Tail flick latencies after intra-VL PAG microinjections of vehicle (VEH, 0.05% DMSO in ACSF), UCM765 and UCM924 (5 and 10 µg) or (**B**) VEH, MLT (100 µg), UCM765 and UCM924 (10 µg) in combination with 4P-PDOT (100 µg). Each point represent the mean ± S.E.M of 14-16 rats per group. * $p < 0.05$, ** $p < 0.01$ and *** $p < 0.001$ indicate statistically significant difference vs. vehicle. $^{+}$ indicates statistically significant difference between different doses of UCM765 and UCM924.

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Table 1

Number of falls of rats tested in the Rotarod at different time points after subcutaneous administration of different doses (10, 20 and 40 mg/kg) of UCM765 and UCM924 in comparison with melatonin (MLT, 150 mg/kg), gabapentin (GBP, 100 mg/kg) and vehicle (VEH) treated rats. Bars represent mean ± SEM. *** p < 0.001 and ** p< 0.01 vs. vehicle

	Vehicle	MLT 150	GBP 100	UCM765 10	UCM765 20	UCM765 40	UCM924 10	UCM924 20	UCM92 40
0.5h	1±0.41	0.5±0.29	0.25±0.25	0.2±0.2	0.4±0.25	0.6±0.6	0.2±0.2	1.4±0.51	0.2±0.2
1 h	0	0.25±0.25	0.25±0.25	0.4±0.25	0	0.4±0.4	0.2±0.2	0.2±0.2	0
2 h	0	0	2.25±0.85**	0	0	0.4±0.4	0	0.4±0.4	0.2±0.2
3 h	0	0.25±0.25	3.5±2.021***	0.2±0.2	0	0.2±0.2	0	0.2±0.2	0.4±0.4
5 h	0	0.25±0.25	1.5±0.289	0	0.2±0.2	0	0	0.2±0.2	0