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Melatonin promotes regeneration of injured motor axons via MT_1 receptors

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Abstract

Melatonin is an ancient multi-tasking molecule produced by the pineal gland and by several extrapineal tissues. A variety of activities has been ascribed to this hormone in different physiological and pathological contexts, but little is known about its role in peripheral neuroregeneration. Here, we have exploited two different types of injury to test the capability of melatonin to stimulate regeneration of motor axons: (a) the acute and reversible presynaptic degeneration induced by the spider neurotoxin α-Latrotoxin and (b) the compression/transection of the sciatic nerve. We found that in both cases melatonin administration accelerates the process of nerve repair. This pro-regenerative action is MT_1-mediated, and at least in part due to a sustained activation of the ERK1/2 pathway. These findings reveal a receptor-mediated, pro-regenerative action of melatonin in vivo that holds important clinical implications, as it posits melatonin as a safe candidate molecule for the treatment of a number of peripheral neurodegenerative conditions.

KEYWORDS

melatonin, MT_1 and MT_2 receptors, neuromuscular junction, peripheral nerve regeneration, Schwann cells, sciatic nerve injury, α-Latrotoxin

Marco Stazi and Samuele Negro are Joint First authors.

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1 | INTRODUCTION

Melatonin is a hormone produced by the pineal gland in a photoperiod controlled mode and released in the blood flow and the cerebrospinal fluid, thus reaching many organs and tissues in the body.[1,2] It is a major regulator of the sleep/wake cycle via its binding to plasma membrane G-protein-coupled receptors dubbed MT_1 and MT_2.[3,4] Beside the pineal gland, many other body tissues produce and accumulate melatonin.[1,5] Once in the body fluids, melatonin displays a broad range of actions: circadian rhythm regulator, free radical scavenger, antioxidant, anti-inflammatory, and immunoregulating molecule, and oncostatic agent.[1,6,7] A strong neuroprotective activity of melatonin has been described in a variety of neuronal models,[8] but comparatively less attention has been dedicated to its possible contribution to nerve re-growth and neuroregeneration.[9]

To dissect the role of melatonin in peripheral nerve regeneration, we first exploited an innovative experimental model recently set up, based on the neurotoxic action of the spider toxin α-latrotoxin (α-LTx).[10] This presynaptic neurotoxin causes the rapid and selective degeneration of motor axon terminals (MATs) without inflammation, with complete recovery within a week in mice, thus providing an ideal model to investigate the molecular determinants of nerve regeneration. Indeed, using this model system, we recently identified hydrogen peroxide (H_2O_2) and the signaling axis CXCR4-CXCL12α as important contributors of the rescue of function of the injured neuromuscular junction (NMJ).[10-13] We have also tested the activity of melatonin in well-established forms of prolonged damage (compression and transection of the sciatic nerve). In both models, melatonin promotes motor axon re-growth and peripheral neuroregeneration in a receptor-mediated fashion.

2 | MATERIALS AND METHODS

2.1 | Antibodies and reagents

Primary and secondary antibodies employed in the study, and the correspondent sources and dilutions, and additional reagents for immunofluorescence, are listed in Table S1.

Agomelatine (Melatonin receptors (MTR) agonist, cat. A1362), luzindole (nonselective MTR antagonist, cat. L2407), ramelteon (MTR agonist, cat. SML2262), tasimelteon (MTR agonist, cat. SML2030), 4P-PDOT (MT_2 selective antagonist, cat. SML1189), and melatonin (cat. M5250) were purchased from Sigma-Aldrich.

Purified α-LTx was obtained by Alomone (cat. LSP-130). The purity of the toxin was checked by SDS-PAGE, and its neurotoxicity by e*x vivo* mouse nerve-hemidiaphragm preparations as previously described.[14] Unless otherwise stated, all other reagents were from Sigma.

2.2 | Animals

C57BL/6 mice expressing cytosolic GFP under the *plp* promoter,[15] kindly provided by Dr WB Macklin (Aurora, Colorado) via Dr T. Misgeld, were used in immunofluorescence experiments. CD1 mice were employed for electrophysiological recordings. Wistar IGS rats were employed for the preparation of primary cultures of Schwann cells (SC).

2.3 | Ethical statement

Mice were maintained under a 12-hour light/12-hour dark cycle in the animal facility of the Department and kept under constant temperature. Water and food were available ad libitum, and mice were fed with regular chow. All procedures were performed under general anesthesia and analgesia. Paralysis was restricted to one hind limb and did not impair food or water intake. Tissue sampling was carried out with animals sacrificed under deep anesthesia.

All experimental procedures involving animals and their care were carried out in accordance with National laws and policies (DL n. 26, March 14, 2014), with the guidelines established by the European Community Council Directive (2010/63/EU) and were approved by the local authority veterinary services and by the Italian Ministry of Health.

2.4 | Crush/cut of the sciatic nerve

Six- to eight-week-old CD1 mice weighting around 20 gr were anesthetized with a cocktail of xilazine (48 mg/Kg) and zoletil (16 mg/Kg) via i.p. injections. The sciatic nerve was exposed without damaging the gluteal musculature, and the crush performed using haemostatic forceps, predipped in powdered charcoal, to mark the crush site. The nerve was pinched 0.5 cm from the hip insertion for 20 seconds at the 3rd click. Transection of the sciatic nerve was performed using surgical scissor, leaving the edge juxtaposed.[16] The gluteal musculature was re-opposed, and the skin sutured using 6-0 braided silk, nonabsorbable sutures (ETHLCON2 biological instruments, 8697). Melatonin/luzindole i.p. injections were performed in 40 μL physiological saline plus 0.2% gelatine 30 minutes after surgery, then daily (same dosage, at 11.00 AM) for 1 week. In all in vivo experiments melatonin was i.p. injected at 30 mg/kg.

2.5 | Cell cultures

Rat primary cerebellar granular neurons (CGN) and SC were isolated and cultured as described in.[10] For phospho-ERK detection, SC plated onto 35-mm dishes (20.000 cells/well)

were exposed to either 10 nmol/L melatonin, ramelteon (10-100 nmol/L), agomelatine (10-100 nmol/L), or 100 nmol/L luzindole (added 30 minutes before melatonin).

2.6 | Electrophysiological recordings

Electrophysiological measurements were performed in 6- to 8-week-old CD1 mice weighting around 20 gr, anesthetized with a cocktail of xylazine (48 mg/Kg) and zoletil (16 mg/Kg) via i.p. injections.

2.6.1 | Evoked junctional potentials (EJPs)

Mice were locally injected with α-LTx in the hind limb (5 μg/Kg in 15 μL physiological saline plus 0.2% gelatine), w/o daily i.p. injections of 30 mg/Kg melatonin (in 40 μL physiological saline plus 0.2% gelatine), and electrophysiological recordings were performed on soleus muscles 72 hours later. In experiments with the MTR agonists ramelteon or tasimelteon (2 mg/Kg in 40 μL physiological saline plus 0.2% gelatine, daily local injections), soleus muscles were collected 72 hours after-intoxication, while with the nonselective MTR antagonist luzindole (0.2 mg/Kg in 40 μL physiological saline plus 0.2% gelatine, daily local injections), two different treatment protocols were employed: (a) to test the contribution of endogenous melatonin, soleus muscles were collected 96 hours after intoxication; (b) to test the receptor-mediated contribution of exogenously administered melatonin, drug injection was performed before i.p. administered melatonin, and muscles were collected 72 hours after injury. In the case of the MT_2 selective antagonist 4P-PDOT (4 mg/Kg in 40 μL physiological saline plus 0.2% gelatine, daily local injections), drug injection was performed before i.p. administered melatonin, and muscles were collected 72 hours after injury. All drugs were administered at 11 AM. Recordings were performed in oxygenated Krebs-Ringer solution on soleus muscles using intracellular glass microelectrodes (WPI) filled with one part of 3 M KCl and two parts of 3 M CH3COOK. Evoked neurotransmitter release was recorded in current-clamp mode, and resting membrane potential was adjusted with current injection to −70 mV. EJPs were elicited by supramaximal nerve stimulation at 0.5 Hz using a suction microelectrode connected to a S88 stimulator (Grass). To prevent muscle contraction after dissection, samples were incubated for 10 minutes with 1 μmol/L μ-Conotoxin GIIIB (Alomone). Signals were amplified with intracellular bridge mode amplifier (BA-01X, NPI), sampled using a digital interface (NI PCI-6221, National Instruments). Peak amplitude was measured with Clampfit software.

2.6.2 | Compound muscle action potential (CMAP)

Following general anesthesia, the sciatic nerve was exposed without damaging the gluteal musculature, then a small piece of parafilm was put under the nerve, which was kept wet by a drop of PBS. The stimulation electrode was placed upstream the lesion, and the recording one in the gastrocnemius muscle. To reach supramaximal stimuli (5-15 V for controls, up to 50 V after nerve damage) the sciatic nerve was stimulated with increasing stimuli until CMAP value ceases to increase. The rate of stimulation was kept at 0.5 Hz, with a constant duration of 0.4 ms. Experimental groups: (a) control crushed mice (daily i.p. injected with 40 μL physiological saline plus 0.2% gelatine; (b) mice daily i.p. injected with melatonin (30 mg/kg in 40 μL physiological saline plus 0.2% gelatine) for 1 week after crush; (c) and (d) mice daily i.p. injected with luzindole (0.2 mg/Kg in 40 μL physiological saline plus 0.2%) w/o melatonin for 1 week after injury. For groups 1-2, CMAPs were recorded 7, 18, 26, and 36 days after injury, for groups 3-4 measurements were performed 18 days after crush. Traces were recorded with WinEDR V3.4.6 software and analyzed using pCLAMP 10.3 software for CMAP area measurement.

2.7 | Immunohistochemistry

Anesthetized mice were locally injected with α-LTx close to soleus or to *Levator auris longus* (LAL) muscles. Muscles were dissected at different time points, fixed in 4% PFA in PBS for 30 minutes at RT, and quenched in 0.24% NH_4Cl PBS for 20 minutes. After permeabilization and 2 hours saturation in blocking solution (15% goat serum, 2% BSA, 0.25% gelatine, 0.20% glycine, 0.5% Triton X-100 in PBS), samples were incubated with primary antibodies for 72 hours in blocking solution at 4°C. Muscles were then washed and incubated with secondary antibodies. Images were collected with a Leica SP5 Confocal microscope equipped with a 40× HCX PL APO NA 1.4 oil immersion objective. Laser excitation line, power intensity, and emission range were chosen accordingly to each fluorophore in different samples to minimize bleed-through. Orthogonal projection analysis was performed with ImageJ software (*Orthogonal views* command in *Image-Stacks* section). This method is used with a stack to display the XZ and YZ planes at a given point in the 3D image. For phospho-ERK detection at the NMJ, 5 animals for each experimental condition were employed, and an amplification step was performed (Tyramide Signal Amplification Kit, Thermo Fisher Scientific, cat. B40923) following manufacturer's instructions.

Sciatic nerves were isolated, fixed in 4% PFA in PBS for 30 minutes, sucrose cryoprotected o.n., and embedded in OCT. Samples were slowly frozen in isopentane vapors and cryo-sliced in 20 µm thick sections using Leica CM1520 cryostat. Slices were processed for immunostaining as described above. In some cases, *whole-mount* staining of the nerve was performed as described in.[16] Five animals for each experimental condition were employed. To quantify p-ERK signal in cross sections, 4 fields were captured at the crush site, and for each field five different regions of interest were analyzed. The average intensity of the fluorescent signal was measured with ImageJ software and normalized to controls.

For proliferation assay, sciatic nerves were longitudinally cryo-sliced and stained with DAPI and the anti-Ki-67 primary antibody. The ratio between Ki-67 positive (ie, proliferating cells) and DAPI positive (total nuclei) was determined in a 785 × 785 µm region of interest. Four fields were captured at the crush site (middle region in controls) and analyzed for each condition.

For immunohistochemistry on brain slices, mice were sacrificed and perfused with 4% PFA. Brains were isolated and incubated over night in 4% PFA plus 15% sucrose in PBS, followed by 48 hours in 30% sucrose in PBS. Samples were then included in 4% agarose and sagittaly sliced (30 µm thick) by a vibratome Leica VT 1200 S. Immunostaining was performed as described above.

2.8 | Immunofluorescence

Cells were fixed for 15 minutes in 4% PFA in PBS, quenched (0.24% NH_4Cl in PBS), and permeabilized with 0.3% Triton X-100 in PBS for 5 minutes at RT. After saturation with 3% goat serum in PBS for 1 hour, samples were incubated with primary antibodies, diluted in 3% goat serum in PBS overnight at 4°C, washed, then incubated with the corresponding secondary antibodies Alexa-conjugated for 1 hour at RT. Coverslips were mounted in Mowiol and examined by confocal (Leica SP5) microscopy.

2.9 | Western blotting

Cells were lysed in Lysis Buffer (Hepes 10 mmol/L, NaCl 150 mmol/L, SDS 1%, EDTA 4 mmol/L, protease and phosphatase inhibitors). Protein concentration was quantified using the BCA assay (Protein Assay Kit, Pierce).

Seven to 10 µg of total SC lysates were loaded into NuPage 4-12% Bis-Tris gels and separated by electrophoresis in MES buffer (Life Technologies, B0002). For Western blotting, proteins were transferred onto Protran nitrocellulose membranes and saturated for 1 hour in PBS-T (PBS, 0.1% Tween 20) supplemented with 5% BSA (Sigma, A4503-100G). Incubation with primary antibodies was performed overnight at 4°C. Thereafter, membranes were washed and incubated with specific HRP-conjugated secondary antibodies for 1 hour. After additional washings signals were revealed with Luminata™ using an Uvitec gel doc system (Uvitec Cambridge). For densitometric quantification, the bands of interest were normalized to the housekeeping protein Hsp90.

2.10 | Statistical analysis

Sample sizes were determined by analysis based on data collected by our laboratory in published studies. We used at least N = 4 mice/group for electrophysiological analysis. We ensured blinded conduct during electrophysiological recordings, and the subsequent data analysis. For cell cultures studies, at least 3 independent replicates were performed. For imaging analysis, the quantitation was conducted by an observer who was blind to the experimental groups. No samples or animals were excluded from the analysis. Data displayed as histograms are expressed as means ± SEM or SD. GraphPad Prism software was used for all statistical analyses. Statistical significance was evaluated by unpaired Student's t-test or by an one-way analysis of variance (ANOVA) with Tukey's post-test when more than 2 experimental conditions were compared each other. Data were considered statistically different when $*P < .05$, $**P < .01$, $***P < .001$, $****P < .0001$.

3 | RESULTS

3.1 | Melatonin accelerates peripheral nerve regeneration

To test whether melatonin promotes regeneration of the injured MAT, we recorded the evoked junctional potentials (EJPs) of soleus muscles 72 hours after α-LTx injection in the mice hind limb, with/without daily i.p. injections of the hormone, using controlateral muscles as controls. α-LTx causes the rapid degeneration of the presynaptic nerve terminals that recover in a few days, as indicated by the progressive reappearance of the EJPs, and of presynaptic markers. A faster neurotransmission rescue takes place in muscles that received melatonin (Figure 1A), a result confirmed by the lower number of denervated NMJs in melatonin-treated animals 72 hours after intoxication (Figure 1B,C). The extent of MAT degeneration caused by α-LTx, shown by the progressive loss of staining of the presynaptic marker syntaxin, and of neurofilaments (NF) in the MAT, is not affected by melatonin administration (Figure S1), thus arguing against a neuroprotective action of the hormone.

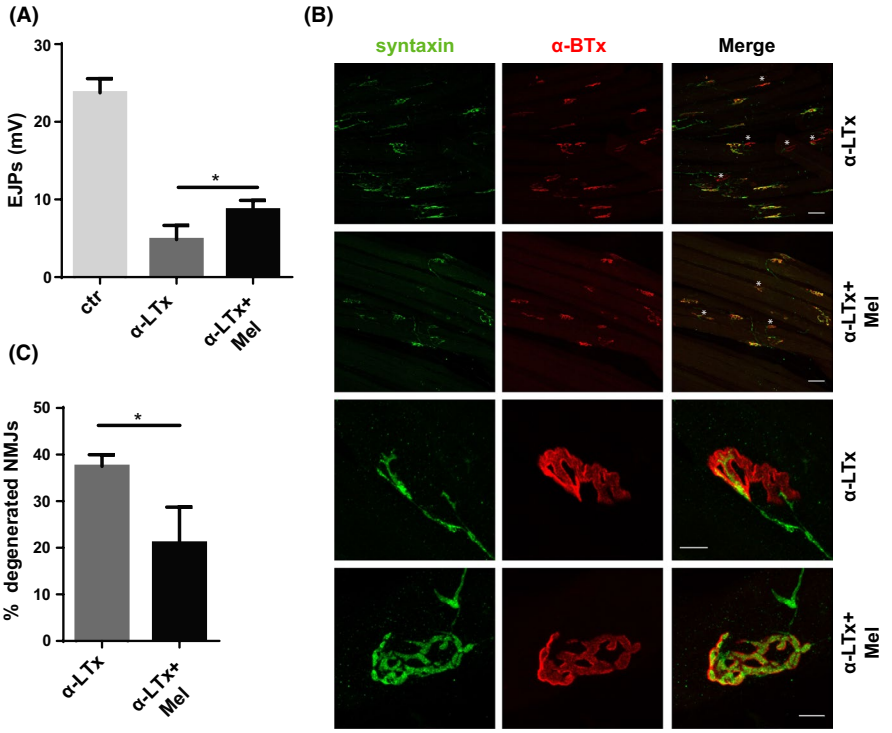

FIGURE 1 Melatonin promotes nerve terminal regeneration. A, Evoked junctional potentials (EJPs) amplitude of soleus muscles 72 h after α-LTx injection in the hind limb, w/o melatonin i.p. injections. Each bar represents mean ± SEM from N = 4, number of analyzed fibers: 10, *$P < .05$. B, Representative immunostaining and (C) quantitation performed on the same muscles in A. *$P < .05$. MAT is identified by syntaxin immunostaining (*green*), postsynaptic AChRs by fluorescent α-BTx (*red*). White asterisks indicate still degenerated NMJs. Lower panels show NMJs at higher magnification. Scale bars: 20 μm upper panels, 10 μm lower panels [Colour figure can be viewed at wileyonlinelibrary.com]

To extend the investigation to more severe and prolonged forms of damage, we tested the possible pro-regenerative effect of melatonin upon compression (crush) and transection (cut) of sciatic nerves. While the crush interrupts all axons preserving both SC and the basal lamina (BL), thus facilitating regeneration, the complete transection of the nerve cuts axons, connective sheaths and BL, making regeneration less efficient, and functional recovery incomplete.[17,18] CMAP recordings performed in gastrocnemius muscles show a significant improvement in neurotransmission recovery in melatonin-treated animals 18 and 26 days after crush, compared with untreated crushed controls. Twenty-six days after crush neurotransmission recovery is complete in animals that received melatonin, whereas it is still ongoing in untreated crushed mice (Figure 2A). Figure 2B reports representative CMAP traces of the same muscles of panel A at different time points after injury. Eighteen days after crush the amplitude of the main peak is higher in melatonin-treated animals with respect to untreated crushed controls. The multiple peaks beside the main biphasic trace indicate the presence of damaged fibers: at 18 and 36 days in melatonin-treated mice these peaks are fewer and smaller in amplitude compared with untreated crushed animals, indicative of a more synchronous neurotransmission.

To better evaluate the effect of melatonin on axonal regeneration, we performed the transection of the nerve, an injury that damages all motor axons, to easily monitor axon re-growth, and we compared the immunostaining of NF in controls and in melatonin-treated mice (12 days after injury) expressing a cytoplasmic GFP in SC (*plp*-GFP) (Figure 2C): we observed a striking difference between controls and mice that received melatonin, the latter displaying increased axon length.

It is long known that axonal regeneration is sustained and guided by SC, which proliferate upon nerve injury and elongate, thus paving the way for axonal re-growth.[19,20] Accordingly, we found an increase in the global proliferation rate at the injured sciatic nerve (mainly of SC, which can be easily identified by GFP expression), which was further stimulated by melatonin (Figure S2).

3.2 | Melatonin receptor MT_1 is expressed at the NMJ and along the sciatic nerve and mediates melatonin pro-regenerative action

Next, we tested the possibility that melatonin pro-regenerative action was receptor-mediated. Mammals express two melatonin receptors (MTRs) , both belonging to the G-protein-coupled (GPCR) superfamily.[3,4] MTRs are engaged during MAT regeneration after α-LTx treatment promoted by both endogenous and exogenously administered melatonin, as local injection of luzindole, a nonselective MTR antagonist, reduces the extent of NMJ functional recovery induced by melatonin (Figure 3A). Accordingly, local injections of the non-selective MTR agonists ramelteon and tasimelteon increase the regeneration rate after injury (Figure 3B). Noticeably, treatment with the MT_2 selective antagonist 4P-PDOT[21] does not affect the rate of NMJ neurotransmission rescue induced by exogenous melatonin, indicating the MT_1 engagement in melatonin-triggered MAT regeneration.

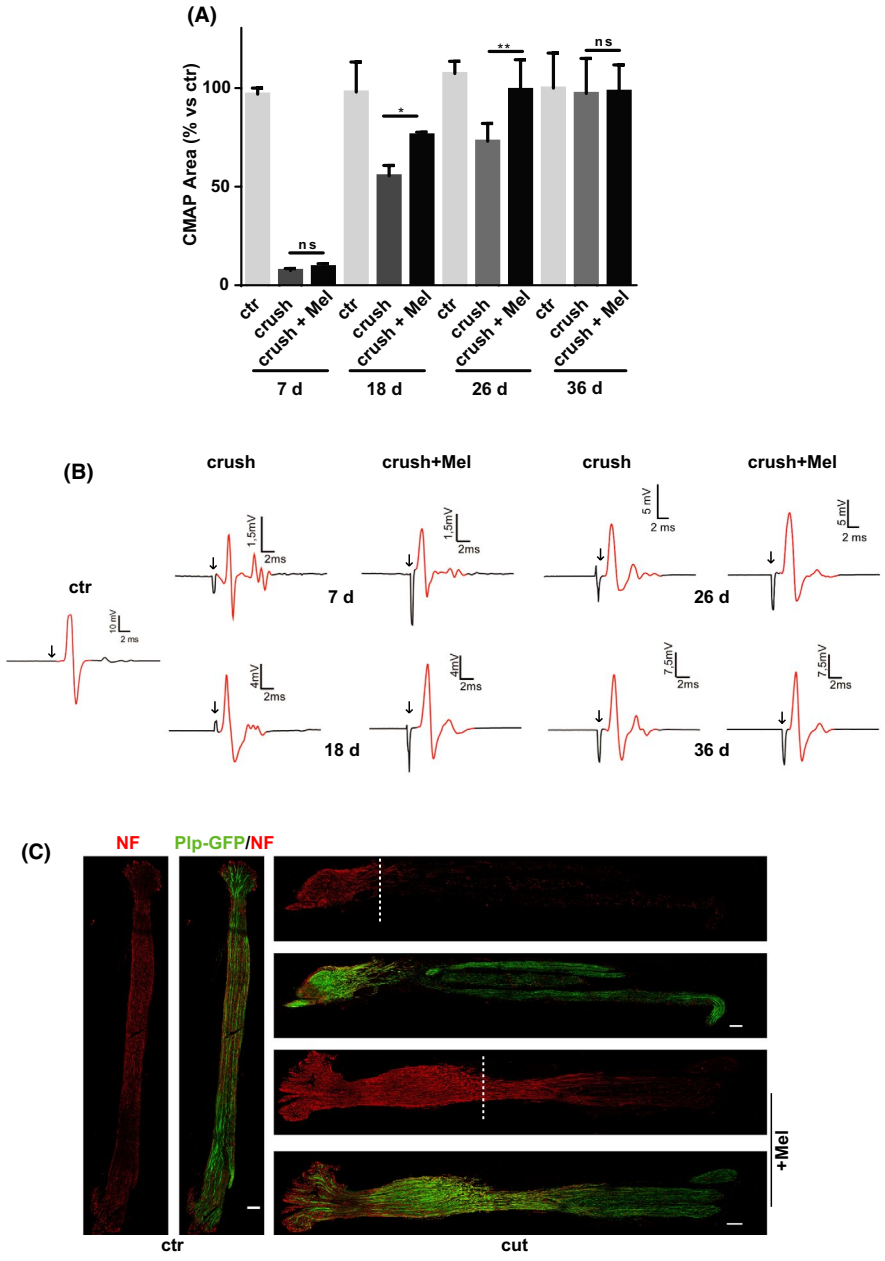

FIGURE 2 Melatonin promotes sciatic nerve regeneration upon a mechanical trauma. A, CMAP recordings on gastrocnemius muscles 7, 18, 26, and 36 d after compression of the sciatic nerve, w/o melatonin i.p. administration. Data are expressed as CMAP area (% vs ctr). $*P < .05$, $**P < .01$, ns: not significant. F-value (DFn, DFd) = 47.81 (7,24); *P*-value < .0001. B, Representative CMAP traces of gastrocnemius muscles before and 7, 18, 26, and 36 d after sciatic nerve crush (w/o melatonin i.p. treatment). Black arrows point to the stimulation peak. Red traces have been analyzed, and values are reported in panel A. C, *Whole-mount* control and transected sciatic nerves from *plp*-GFP mice (12 d after cut; the lesion site is indicated by dashed lines) immunostained for NF (*red*). SC are GFP-positive (*green*). Scale bars: 200 μm. [Colour figure can be viewed at wileyonlinelibrary.com]

These results are further supported by the analysis of MTR expression at the NMJ. Only MT_1 is expressed at *Levator auris longus* (LAL) (Figure 3C) and soleus (Figure S3A) NMJs, with a strong expression in controls mainly in the MAT (upper panels). MT_1 signal is lost from presynaptic nerve terminals 24 hours after α-LTx injection, in line with the ongoing fragmentation of the MAT (shown by disappearance of NF staining), although, intriguingly, the receptor becomes expressed by terminal or perisynaptic SC (PSC) (Figure 3C, lower panels and orthogonal projection). Validation of recently generated monoclonal anti-MT_1 and anti-MT_2 antibodies[22] was performed in brain slices, where neurons expressing MT_1 and MT_2 are detectable in the hypothalamus and in the cerebral cortex (Figure S3 panels B and C, respectively).[23] No background signal was obtained by incubation with secondary antibodies only (Figure S3D).

In line with results obtained at the NMJ, the pro-regenerative role of melatonin administered upon sciatic nerve crush occurs via MTR, as luzindole abolishes it (Figure 3D). In control sciatic nerves MT_1 localizes along the axon (NF-positive), while 3 days upon nerve injury the receptor becomes expressed mainly in SC, identified by GFP fluorescence (Figure 3E). Conversely, MT_2 was not detectable by antibody labeling (data not shown), whose specificity was shown in Figure S3B-D. Hence, as: (a) MT_1, and not MT_2, is engaged during MAT rescue, (b) MTR are involved in regeneration after crush, and (c) MT_2 is not expressed either at the NMJ or in the sciatic nerve, we can infer that the pro-regenerative action of melatonin is mediated by MT_1.

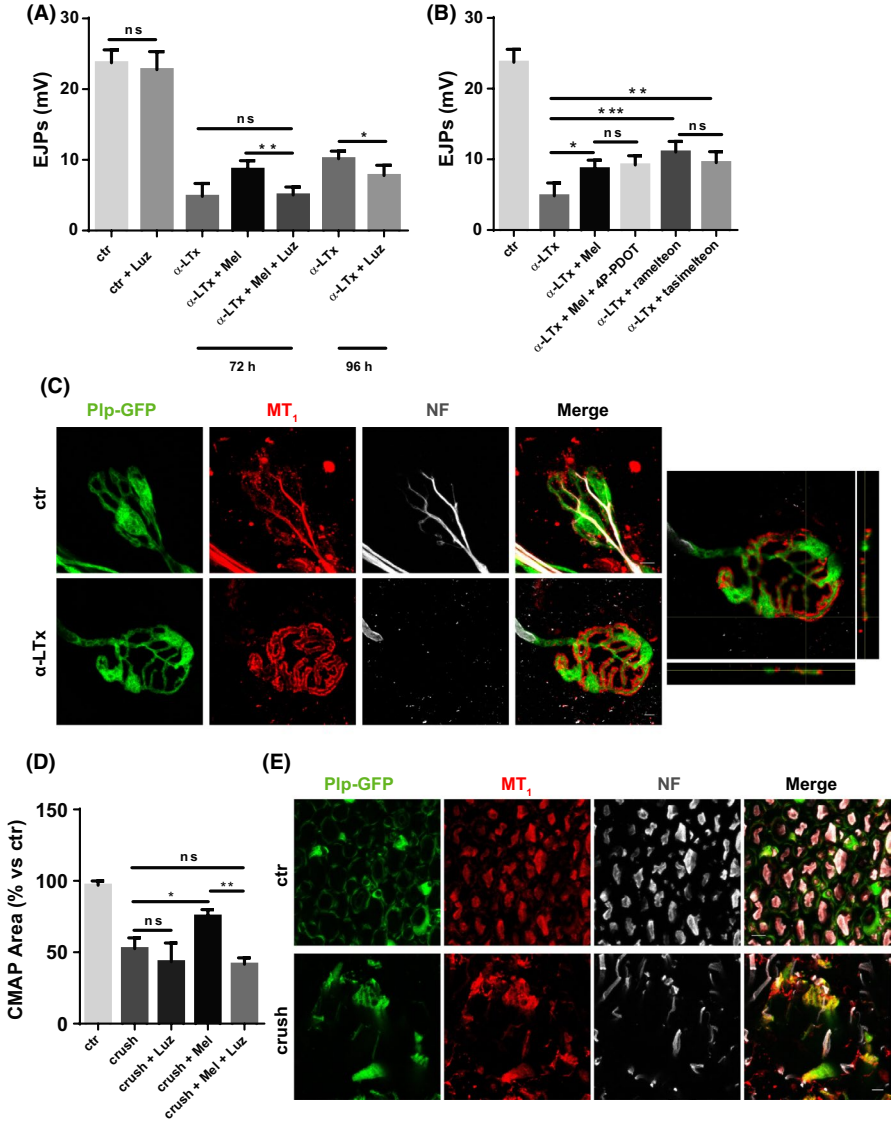

FIGURE 3 MT_1 receptors are expressed at the NMJ and along the sciatic nerve and mediate the pro-regenerative action of melatonin. A, EJPs amplitude of soleus muscles: (a) in control conditions (± luzindole); (b) +α-LTx ± i.p. daily treatment with melatonin ± luzindole daily local injections (time point: 72 h after injury); (c) +α-LTx ± luzindole daily local injections (time point: 96 h after injury). Each bar represents mean ± SEM from N = 4 (number of analyzed fibers: 10). *$P < .05$, **$P < .01$, ns = not significant. *F*-value (DFn, DFd) = 13.65 (4,19); *P*-value < .0001. B, EJPs amplitude of soleus muscles: (a) in control conditions; (b) + α-LTx ± i.p. daily melatonin; (c) +α-LTx + i.p. daily melatonin +4P-PDOT daily local injections; (d) +α-LTx +ramelteon or tasimelteon daily local injections. For all conditions: time point = 72 h after injury. Each bar represents mean ± SEM from N = 4 (number of analyzed fibers: 10). *$P < .05$, **$P < .01$, ***$P < .001$, ns = not significant. *F*-value (DFn, DFd) = 9.459 (4,15); *P*-value = .0005. C, MT_1 staining (mAb-A06, *red*) at LAL NMJs in controls (upper panels) and 24 h after α-LTx injection (lower panels). PSCs are GFP-positive (*green*), the axon terminal is identified by NF staining (*white*). Scale bars: 5 μm. Right panel: the orthogonal projection of one α-LTx poisoned NMJ shows MT_1 spots along PSC membrane. D, CMAP recordings in gastrocnemius muscles before and 18 d after crush (± luzindole daily i.p. administration, or ±i.p. melatonin, or +luzindole and melatonin). *$P < .05$, **$P < .01$, ns = not significant. *F*-value (DFn, DFd)=10.78 (3,8); *P*-value = .0035. E, MT_1 expression (AMR031, *red*) in cross sections of sciatic nerves before (upper panels) and 3 d after crush (lower panels). PSCs are in *green* (GFP-positive), axons in *white* (NF-positive). Scale bars: 10 μm [Colour figure can be viewed at wileyonlinelibrary.com]

3.3 | Melatonin sustains and prolongs injury-induced ERK phosphorylation

Peripheral regeneration is driven by several pathways, including ERK1/2 signaling.[24,25] The acute MAT degeneration by α-LTx activates ERK1/2 in PSC through phosphorylation,[10] which is clearly evident 24 hours after intoxication, when regeneration is under way, while the same pathway is turned off after 72 hours (Figure 4A). Melatonin treatment increases and prolongs ERK activation, as phospho-ERK signal is still detectable 72 hours after intoxication (Figure 4A). Whole-mount preparations and cross sections of sciatic nerves

3 days after crush show that p-ERK signal: (a) increases close to the compression site, (b) is stronger in mice that received melatonin, and (c) localizes in SC and other cell types (Figure 4B,C).

In primary SC (S100 positive), melatonin administration induces a rapid ERK phosphorylation (Figure S4). In these cells, which express MT_1 (Figure S5A,B), ERK activation is MTR-mediated, as pre-incubation with luzindole abolishes it (panels C-D), while the exposure to the MTR agonists agomelatine and ramelteon closely mimicks the time-course of phospho-ERK increase in primary SC induced by melatonin (panels E-H).

4 | DISCUSSION

Here, we have investigated the role of melatonin in peripheral nerve regeneration, showing that it accelerates the recovery of neurotransmission after different types of axonal damage. First, we evaluated the ability of melatonin to promote neuroregeneration following the acute and reversible damage to the MAT caused by α-LTx. In this highly reproducible model system, the selective damage to the MAT does not trigger inflammation, and a complete regeneration is achieved in about a week in mice.[10] The toxin binds specifically to the presynaptic membrane, where it makes pores through which Ca^{2+} enters, triggering the complete degeneration of the MAT,[26,27] similarly to the initial stages of dying back motor neuron neuropathies such as amyotrophic lateral sclerosis.[28,29] Using this experimental model, we recently identified H_2O_2 and the signaling axis CXCR4-CXCL12α as important contributors of the rescue of function of the injured neuromuscular junction (NMJ).[10-13] We also employed the crush and cut models of peripheral nerve injury, that well approximate some traumatic lesions affecting human patients. In both models, melatonin displayed a defined pro-regenerative action.

Melatonin exerts many different biological activities via binding to G-protein-coupled receptors, MT_1 and MT_2.[3,4]

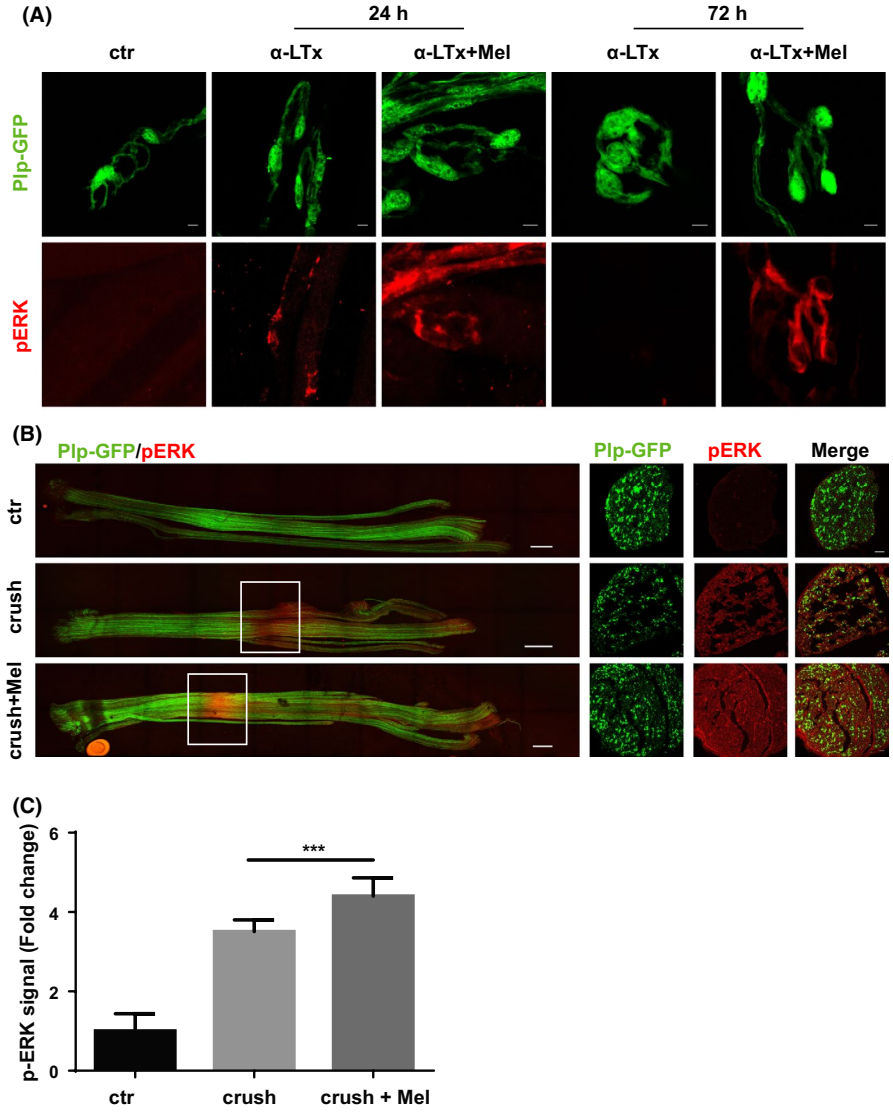

FIGURE 4 Melatonin administration prolongs injury-induced ERK phosphorylation. A, Phospho-ERK 1/2 signal (*red*) at LAL NMJs in controls and 24/72 h after α-LTx injection, w/o melatonin i.p. treatment. PSC are *green* (GFP-positive). Scale bars: 5 μm. B, Phospho-ERK 1/2 signal (*red*) in *whole-mount* (left) and cross sections (right) of sciatic nerves before and 3 d after crush (w/o melatonin i.p. treatment). White squares indicate the crush site. PSC are in *green*. Scale bars: 500 μm (left) and 20 μm (right). C, quantification of p-ERK signal in cross sections. ***$P < .001$. F-value (DFn, DFd) = 123.1 (2,22); P-value < .0001 [Colour figure can be viewed at wileyonlinelibrary.com]

Once activated, these inhibit forskolin-stimulated cAMP, protein kinase A signaling, and CREB phosphorylation.[30-32] Both receptors also increase phosphorylation of mitogen-activated protein kinase 1/2 and extracellular signal-regulated kinase 1/2.[33] A pharmacological investigation, employing MTR agonists and antagonists, allowed us to demonstrate that melatonin pro-regenerative action is receptor-mediated; in addition, the inability of a selective MT_2 antagonist to affect melatonin pro-regenerative action, together with the lack of MT_2 expression at the NMJ and along the sciatic nerve, led us to infer that MT_1 is the MTR subtype mediating neurotransmission rescue promoted by melatonin. Although the present study does not include the use of MTR KO mice due to their present unavailability, we used a pharmacological approach that provided valuable evidence of the role of MTR in melatonin action. MT_1 is well expressed at the MAT in control NMJ, and in PSC upon injury; along the same line, in control sciatic nerves it displays an axonal localization, while upon compression it is strongly expressed by SC and other cell types. This suggests that a damage of the MAT (in the case of α-LTx) or of motor axons (crush or cut of the sciatic nerve) is sensed by SC, which respond by expressing MT_1, the engagement of which contributes to prolong ERK activation, which promotes nerve regeneration.[10,24] Following melatonin administration, we observed, together with a sustained ERK activation in myelinating SC, and a faster motor axonal re-growth, an increased SC number in vivo, previously reported for in vitro conditions by Chang et al,[34] in line with the fundamental role of SC in guiding and sustaining motor axon elongation.[19,20]

Various peripheral effects have been ascribed to melatonin: better structural preservation of myelin sheaths upon cut or crush of the sciatic nerve,[35] slower wound healing in pinealectomised rats,[36] reduced collagen accumulation in the neuroma of a suture repair site that physically prevents axon development.[37] Damage to peripheral nerves causes the accumulation of free oxygen radicals and other toxic agents around the site of injury: as melatonin is an antioxidant, capable of reaching all cell compartments, it has been generically considered neuroprotective.[38] No neuroprotective effects can be ascribed to the molecule in the present context, as melatonin administration does not affect the progression of MAT degeneration induced by α-LTx.

Atik and *colleagues* reported that upon nerve transection and coaptation only rats that received melatonin at high doses had significantly less and better-organized collagen than pinealectomized animals, while melatonin had little effect in the physiologic range.[39] Here, we show that luzindole local administration delays neurotransmission recovery at the NMJ following injury, conceivably by blocking MTR activation by endogenous melatonin. Although no information on the proficiency of CD1 mice strain (employed for electrophysiological experiments) for pineal melatonin is available, as virtually all common laboratory strains (except C3H and CBA) are pineal melatonin deficient,[40,41] we speculate that melatonin is likely produced locally and may accumulate in the synaptic cleft, where it could reach high concentrations. This goes along with the notion that, while physiological levels of endogenous melatonin in the serum (produced by the pineal gland) are in nanomolar concentrations, its levels in different tissues appear to be considerably higher.[42] By interacting with MT_1, melatonin participates in MAT regeneration, presumably thanks to the high concentrations reached in situ. Differently from the NMJ, at the sciatic nerve level, despite the change in MT_1 localization upon crush, luzindole treatment fails to delay regeneration upon crush (though it reduces the beneficial effects of melatonin administration), presumably due to insufficient levels of endogenous melatonin.

Melatonin is an evolutionarly conserved molecule that has been proposed to have arisen in photosynthetic bacteria to neutralize toxic O_2 species during photosynthesis.[7,43,44] As mitochondria and chloroplasts derive from bacteria (which produce melatonin), it is not surprising that these organelles have kept the ability to synthetize this molecule.[45] It was recently reported that mitochondria synthesize and release melatonin, and express MT_1 in their outer membrane.[46] These findings, together with the abundance of mitochondria at the NMJ, make the local synthesis of the molecule at this synapse very likely, an intriguing possibility that, however, requires a further, extensive investigation.

Overall, our study reveals a MT_1-mediated, pro-neuroregenerative action of melatonin that holds important clinical implications, as it posits this molecule for the treatment of several peripheral neurodegenerative disorders.

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AUTHORS CONTRIBUTION

MS, SN, AM, and GD performed the experiments and analyzed the results; MSo and RJ provided essential reagents; FL, CM, and MR designed and supervised the project; CM and MR wrote the manuscript, with contributions from all authors.

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SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section.

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