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Involvement of nNOS in the antinociceptive activity of melatonin in inflammatory pain at the level of sensory neurons

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Abstract. – OBJECTIVE: The efficacy of melatonin as an analgesic agent has been well documented in animals and humans. However, the underlying mechanisms by which melatonin exerts antinociceptive effects on inflammatory pain are poorly understood. Here, we investigated the potential of melatonin to ameliorate inflammatory pain.

MATERIALS AND METHODS: *In vitro*, ND7/23 neurons were treated with capsaicin. We used PCR and Western blot analyses to detect the expression of neuronal nitric oxide synthase (nNOS) in response to melatonin. Orofacial inflammatory pain was induced by 4% formalin administration on the right whisker pad of Sprague Dawley (SD) rats. The analgesic effect of melatonin was evaluated using mechanical threshold analyses. The expression level of nNOS in the trigeminal ganglion (TG) and trigeminal nucleus caudalis (Vc) neurons was assessed by RNAscope and immunohistochemistry.

RESULTS: *In vitro*, capsaicin upregulated the expression of nNOS, which was dose-dependently reversed by melatonin pretreatment ($p < 0.001$). In a rat model of orofacial inflammatory pain, melatonin pretreatment significantly attenuated mechanical allodynia in both the acute and chronic phases ($p < 0.05$). Furthermore, melatonin decreased the formalin-evoked elevated nNOS mRNA and protein levels in the TG and Vc neurons in the acute and chronic phases ($p < 0.05$).

CONCLUSIONS: Taken together, these results suggest that nNOS may play an active role in both peripheral and central processing of nociceptive information following orofacial inflammatory pain induction. The regulatory effect of melatonin on nNOS in inflammatory pain may have potential implications for the development of novel analgesic strategies.

Key Words:
Melatonin, Orofacial inflammatory pain, Neuronal nitric oxide synthase (nNOS), Trigeminal ganglion (TG), Trigeminal nucleus caudalis (Vc).

Introduction

Melatonin (N-acetyl-5-methoxytryptamine), a close derivative of serotonin, is a pleiotropic hormone mainly secreted by the pineal gland in vertebrates. This hormone is known for its regulatory role in the circadian rhythm[1-3], oxidative stress[4], and the immune system[5]. Growing evidence[6-9] indicates that melatonin plays a role in pain modulation.

Nitric oxide (NO) is a physiological gas molecule that was discovered as an endogenous endothelium-derived relaxing factor in blood vessels in 1987[10]. NO is synthesized with L-arginine as a substrate under the catalysis of nitric oxide synthase (NOS). NOS exists as a family of three distinct isoforms: neuronal NOS (nNOS), which is found in neuronal tissues, inducible NOS (iNOS), and endothelial NOS (eNOS)[11]. nNOS is constitutively active, and its activation is dependent on intracellular calcium ions (Ca^{2+})[12]. Considerable evidence has demonstrated that nNOS plays an important role in neural signal transmission[13] and the induction and maintenance of nociception[14-17].

Extensive evidence has shown that melatonin plays an important role in nNOS modulation[18]. In a postherpetic neuralgia model, melatonin decreased NO levels in brain and spinal cord tissues, which may be the mechanism of its analgesic effects[19].

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nNOS overexpression and elevated NO levels are observed in various inflammatory or neuropathic pain models[20,21]. However, to our knowledge, little information is available regarding whether melatonin regulates nociception by modulating nNOS in not only the peripheral but also the central nervous system.

In this study, we established an orofacial inflammatory pain model to investigate the regulation of melatonin on nNOS signaling pathways in peripheral and central sensitization. *In vitro*, we verified the role of melatonin in the regulation of nNOS in neurons pretreated with capsaicin.

Materials and Methods

Cell Culture

ND7/23 cell lines were purchased from the European Collection of Authenticated Cell Culture (London, England, UK). Cells were grown at 5% CO_2 and 37°C and maintained in complete Dulbecco's Modified Eagle's Medium (DMEM, Gibco, Grand Island, NY, USA) supplemented with 1% GlutaMAX™ (Gibco, Grand Island, NY, USA) and 10% fetal bovine serum (FBS, Gibco, Grand Island, NY, USA). Cells were split in a subconfluent culture at a 1:5 ratio and seeded at $2*10^4$ cells/cm^2. Cells were digested with TrypLE™ (Thermo Fisher Scientific, Waltham, MA, USA) for 2 min and seeded into 2 wells of six-well plates, which were divided into the following three groups[22,23]: 1) the total control group: the cells received no intervention; 2) the capsaicin (MCE, Monmouth Junction, NJ, USA) treatment group: the cells were treated with 100 nM capsaicin for 30 min[24,25]; and 3) the melatonin (Sigma-Aldrich, St. Louis, MO, USA) plus capsaicin group: the cells were treated with different concentrations of melatonin (0.25 mM, 0.5 mM, 1 mM, and 2 mM) for 6 h[26] and then cotreated with 100 nM capsaicin for 30 min.

Cell Viability Assay

The cell viability of ND7/23 neurons was assessed by Cell Counting Kit-8 (CCK-8; Dojindo, Molecular Technologies, Kumamoto, Japan) according to the manufacturer's instructions.

NOS Activity Measurement

The relative activity of intracellular NOS determined NO production. The relative activity of intracellular NOS was detected using a nitric oxide synthase assay kit (Beyotime, Songjiang, Shanghai, China) according to the manufacturer's instructions.

Real-Time PCR

Total RNA in ND7/23 neurons was extracted according to the manufacturer's instructions for the RNA-Quick Purification Kit (Yishan, Baoshan, Shanghai, China). The concentration and purity of RNA were detected with a NanoDrop™ One/One^C system (Thermo Fisher Scientific, Waltham, MA, USA). Then, an adequate amount of RNA was reverse transcribed into cDNA via Prime Script™ RT Master Mix (TaKaRa, Otsu, Shiga, Japan). Real-time PCR was performed in a Roche LightCycler® 96 (Roche, Basel, Kanton Basel, Switzerland). The gene-specific primers used in this study are listed in Table I. The relative quantification of target genes in each sample was analyzed via LightCycler® 96 SW 1.1 software. Ct values were processed using the $2^{-\Delta\Delta CT}$ method. The relative expression level of each gene was normalized to the internal control GAPDH.

Western Blot

The total protein of ND7/23 neurons was extracted using radio immunoprecipitation assay (RIPA) lysis buffer supplemented with phenylmethanesulfonyl fluoride (PMSF). The protein concentration was quantified according to the manufacturer's instructions for the bicinchoninic acid assay (BCA) Protein Assay Kit (Beyotime, Shanghai, China). Denatured protein was used for 4-20% Bis-Tris sodium dodecyl sulfate polyacrylamide gel electrophoresis (Sure-PAGE, GenScript, Piscataway, NJ, USA). Then, the proteins were electrotransferred onto polyvinylidene difluoride membranes (PVDF, Millipore, Billerica, MA, USA). The membranes were blocked at room temperature and incubated overnight at 4°C with the following primary antibodies: anti-nNOS (1:1000, Cell Signaling Technology, Boston, MA, USA) and anti-β-actin (1:1000, Beyotime).

Table I. Quantitative real-time reverse transcription polymerase chain reaction primers.

Gene	Primers	Sequences (5'-3')	Length
nNOS	Forward	CGACCAATACTACTCATCCA	76 bp
	Reverse	CTCCTTGTTCACCTCCTC	
GAPDH	Forward	AACCTGCCAAGTATGATGA	119 bp
	Reverse	GGAGTTGCTGTTGAAGTC	

The membranes were then incubated with horseradish peroxidase (HRP)-conjugated anti-rabbit or anti-mouse secondary antibody (1:1000, Beyotime, Shanghai, China). Protein bands were detected by reaction with the Immobilon Western Chemiluminescent HRP Substrate (Invitrogen, Carlsbad, CA, USA). The intensities of the protein bands were quantified by the ImageJ software program (NIH, Bethesda, MD, USA). The amounts of proteins were normalized to that of β-actin, whose intensity ratio was set as 100%.

Animals and Ethics Statement

The experiment was performed on 39 male Sprague-Dawley (SD) rats weighing 250-300 g each provided by the Animal Care Committee for the Care and Use of Laboratory Animals of Sun Yat-sen University, which were maintained under a standard light-dark cycle from 7:00 am to 7:00 pm in a controlled environment (22±2°C). Food and drinking water were freely available. The animals were randomized into three different groups: 1) the control group, which received an intraperitoneal injection of saline and then a subcutaneous injection of saline 0 d, 1 d, 3 d, 7 d or 14 d later, followed by sample collection; 2) the formalin group, which received an intraperitoneal injection of saline and then a subcutaneous injection of 4% formalin 1 d, 3 d, 7 d or 14 d later, followed by sample collection; and 3) the melatonin group, which received an intraperitoneal injection of melatonin and then a subcutaneous injection of 4% formalin 1 d, 3 d, 7 d or 14 d later, followed by sample collection. The experimental protocols were approved by the Ethics Committee of Sun Yat-sen University in China.

Melatonin Administration

Melatonin (10 mg/kg body weight)[27] purchased from Sigma-Aldrich (St. Louis, MO, USA) was diluted in saline and 1% dimethylsulfoxide (DMSO). Rats in the melatonin group were injected intraperitoneally at 9:00 am 30 min before receiving 4% formalin and given a daily dose until sample collection. Rats in the control group were injected with equal amounts of saline as a control.

Orofacial Inflammatory Pain Model

Orofacial inflammatory pain was induced by subcutaneous administration of 4% formalin (formalin; 50 µl, diluted in saline, Sigma-Aldrich, St. Louis, MO, USA) into the right whisker pad. The control rats were subcutaneously injected with 50 µl of physiological saline into the right whisker pad.

Von Frey Tests

The mechanical nociceptive threshold of the whisker pad was determined using the von Frey filament test. Rats were placed in plastic chambers with a wire mesh roof for 1 h to habituate before testing. Filaments ranging from 0.008 to 60 g were applied to the whisker pad surface in ascending order, and positive indications were observed when the filaments bent and the rats produced a withdrawal response. The mechanical threshold was assessed at baseline and 1 d, 3 d, 7 d, and 14 d after administration of formalin and melatonin. The rats were euthanized by pentobarbital sodium, and trigeminal ganglion (TG) and spinal trigeminal nucleus (STN) samples were collected for subsequent RNAscope and immunohistochemistry analyses.

RNAscope In Situ Hybridization

In situ detection of nNOS mRNA in formalin-fixed and paraffin-embedded specimens (TG and STN) was performed using RNAscope® Probe Nos1 (nNOS) and RNAscope® 2.5 HD Reagent Kit-Red (ACDBio, San Francisco, CA, USA) according to the manufacturer's protocols and with standard sample pretreatment (15 min with Target Retrieval and 30 min with Protease Plus), and then the specimens were washed with distilled water. Probes were then added for 2 h at 40°C in a humidity-controlled oven. Signal amplification and detection were then performed by AMP1-6. The specimens were counterstained with Gill's hematoxylin I for 30 s at room temperature and rinsed with distilled water. Then, the slides were dried in an oven at 60°C for 20 min. Mounting media (Vector Labs, San Francisco, CA, USA) were added to the slides, and a coverslip was carefully placed on each tissue section. The slides were observed with the Aperio Digital Pathology system (Leica Biosystems, Heidelberg, Baden-Wurttemberg, Germany).

Immunohistochemistry and Immunofluorescence Procedure

In situ detection of nNOS protein in formalin-fixed and paraffin-embedded TG and STN specimens was performed using the Polink-2 plus® Polymer HRP Detection system (Bioss, Tongzhou, Beijing, China) according to the manufacturer's protocols. Heat antigen retrieval was performed with citrate buffer (0.01 M, pH=6.0) at 98°C for 10 min followed by cooling at room temperature for 2 h. Slides were incubated with 3% H_2O_2 at room temperature for 20 min. Bo-

vine serum albumin (BSA, 5%) in Tris-HCl buffer solution and Tween (TBST) was added to the slides at room temperature for 1 h. After removal of BSA (5%), nNOS primary antibody was added to the slides at 37°C for 2.5 h. Reagent 1 was added to the slides at 37°C for 20 min. Then, the slides were incubated with reagent 2 at 37°C for 20 min. Counterstaining was carried out with diaminobenzidine (DAB) and hematoxylin. The slides were dehydrated and made transparent by incubation at 60°C for 15 min and soaking in xylene for 10 min. Mounting media were added to the slides, and a coverslip was carefully placed on each tissue section. Finally, the slides were observed with the Aperio Digital Pathology system.

ND7/23 neurons were digested with TrypLE™ for 2 min, seeded into confocal dishes and allowed to adhere overnight. ND7/23 neurons were fixed with 4% paraformaldehyde for 20 min. Then, 0.5% Triton X-100 was added to the dish for 20 min to permeate the cells. The following steps were similar to those described above. Counterstaining was carried out with 4,6-diamidino-2-phenylindole (DAPI), and images were captured using a laser scanning confocal microscope (Zeiss, Oberkochen, Baden-Wurttemberg, Germany).

Statistical Analysis

RNAscope and immunohistochemistry micrographs were analyzed by calculating the score of every cell to obtain the weighted scores of the corresponding image. Data are presented as the mean ± standard error of the mean (SEM). One-way ANOVA was used to compare means between multiple groups, and least significant difference (LSD) tests were used for post-hoc analysis. The data were considered significant at $p < 0.05$. Statistical analysis was performed using SPSS 20.0 (IBM Corp, Armonk, NY, USA).

Results

Melatonin Pretreatment Decreased NOS Activity in Capsaicin-Treated ND7/23 Cells

A representative image of ND7/23 cells was obtained using neuron-specific enolase (NSE), and greater than 95% purity of NSE-positive neurons was calculated (Figure 1A). Cell cytotoxicity tests revealed that 100 nM capsaicin and a range of concentrations of melatonin had little impact on ND7/23 cell viability (Figure 1B). Treatment of ND7/23 cells with capsaicin without melatonin resulted in significantly increased NOS activity as shown by a ~20% increase in the relative fluorescence units (Figure 1C, $p < 0.001$). When cells were co-exposed to capsaicin plus melatonin, NOS activity decreased, resulting in a reduction in NO production ($p < 0.001$).

Melatonin Treatment Downregulated nNOS Expression in Capsaicin-Treated ND7/23 Cells

Western blot analysis showed that capsaicin-induced inflammation significantly increased nNOS levels at 30 min after capsaicin administration (Figure 2A and B, $p < 0.001$). Preincubation with melatonin induced a significant decrease in the nNOS level, which was lower than that observed in response to capsaicin stimulation ($p < 0.01$). In addition, high-dose melatonin (2 mM) caused substantially decreased expression of nNOS protein compared with low-dose melatonin (0.25 mM, $p < 0.01$). These Western blot data suggested that melatonin inhibited the stimulatory effect of capsaicin on nNOS in a dose-dependent manner.

Similar to the Western blot results, capsaicin treatment increased nNOS mRNA levels, while the cultures pretreated with melatonin showed lower levels than the unstimulated control cultures (Figure 2C, $p < 0.01$). With increasing melatonin concentrations, melatonin dose-dependently attenuated the nNOS mRNA levels in response to capsaicin ($p < 0.01$).

Melatonin Alleviated Mechanical Allodynia in Formalin-Treated Rats

In vivo, we first measured the mechanical withdrawal thresholds of the saline- and formalin-treated rats to verify whether the inflammatory pain model was established. Mechanical thresholds were assessed at baseline (0 d, prior to formalin injection) and at 1 d, 3 d, 7 d, and 14 d after formalin administration. Formalin administration caused a reduction in the mechanical withdrawal threshold values that developed progressively over 1 d (Figure 3, $p < 0.05$). The rats that received formalin and melatonin by the subcutaneous and intraperitoneal routes, respectively, had less mechanical hypersensitivity than the rats given formalin plus saline (Figure 3, $p < 0.05$). LSD analysis of mechanical withdrawal thresholds revealed that melatonin limited mechanical hypersensitivity at all time points ($p < 0.05$).

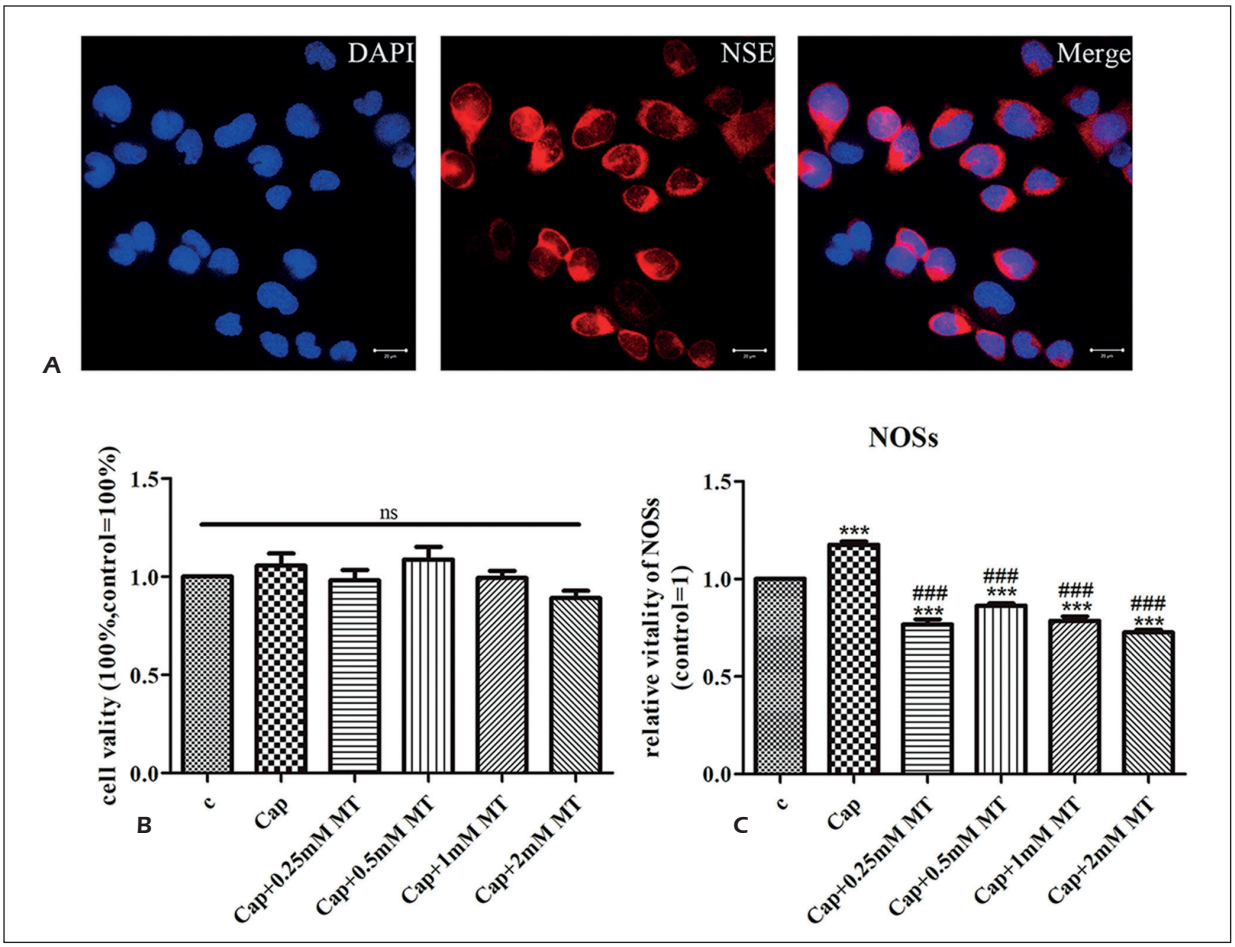

Figure 1. The morphology of ND7/23 cells and the effect of melatonin on the proliferation and NOS activity of ND7/23 cells. **A**, Representative immunofluorescence image of ND7/23 cells (NSE). High-purity NSE (> 95%) was calculated as the number of NSE-positive neurons (red) divided by the total number of neurons (blue). Scale bar =20 μm. **B**, The cell cytotoxicity test in various groups of ND7/23 neurons. **C**, The relative activity of intracellular NOS under various treatments in ND7/23 neurons. The ND7/23 cells were treated with different reagents as follows: the C group (without any intervention), Cap group (treated with 100 nM capsaicin), Cap + 0.25 mM MT group (treated with 100 nM capsaicin plus 0.25 mM melatonin), Cap + 0.5 mM MT group (treated with 100 nM capsaicin plus 0.5 mM melatonin), Cap + 1 mM MT group (treated with 100 nM capsaicin plus 1 mM melatonin), and Cap + 2 mM MT group (treated with 100 nM capsaicin plus 2 mM melatonin), $^{***}p < 0.001$ *vs.* C; $^{\#\#\#}p < 0.001$ *vs.* Cap; ns: no statistical significance. n=4 for each group. Error bars represent the mean±SEM.

Melatonin Treatment Downregulated nNOS mRNA and Protein Expression in the TG Neurons of Formalin-Treated Rats

In the ipsilateral TG of the saline-treated rats, few nNOS-positive neurons were detected (Figure 4E and F). Significant expression of nNOS mRNA and protein was detected in TG neurons 1 d after formalin administration (Figure 4A, C, E and F, $p < 0.05$). Moreover, nNOS immunoreactivity decreased in a time-dependent manner and reached a plateau at 7 d after formalin administration (Figure 4E and F, $p < 0.05$). Furthermore, the optical densities of nNOS mRNA and protein and nNOS-positive neurons were increased in the ipsilateral TG at 14 d after formalin injection in rats compared with rats treated with saline (Figure 4E and F, $p < 0.05$). Melatonin treatment resulted in significant downregulation of nNOS protein and mRNA expression and nNOS-positive neurons in the TG of the formalin-treated rats not only in the acute phase but also in the chronic phase (Figure 4B, D, E and F, $p < 0.05$).

Melatonin Treatment Downregulated nNOS mRNA and Protein Expression in the Trigeminal Nucleus Caudalis (Vc) of Formalin-Treated Rats

The Vc is considered an important brain stem transmitter of orofacial nociceptive information[28] and is located on the caudal side of the STN. Melatonin has previously been shown to downreg-

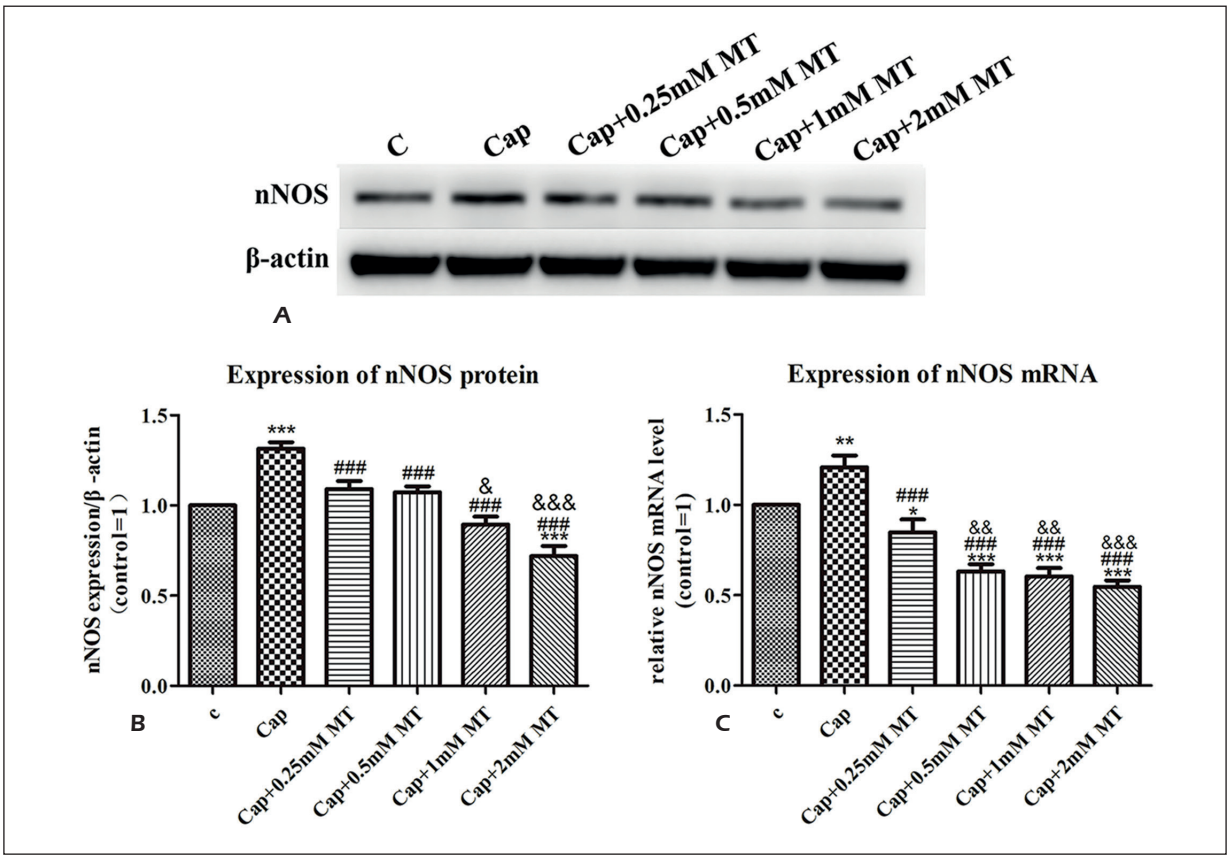

Figure 2. The effect of melatonin on the regulation of nNOS mRNA and protein in ND7/23 neurons in vitro. **A-B**, nNOS protein expression under different treatments in ND7/23 cells. C, nNOS gene expression under different treatments in ND7/23 cells. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$ *vs.* C; $^{\#\#\#}p < 0.001$ *vs.* Cap; $^{\&\&}p < 0.01$, $^{\&\&\&}p < 0.001$ *vs.* Cap+0.25 mM MT; n=4 for each group. Error bars represent the mean±SEM.

ulate nNOS expression in response to formalin stimulation in TG neurons. To determine whether melatonin regulation of nociception involves modulation of nNOS expression in the superior nerve center, we collected Vc samples and then stained the samples with an antibody and an RNA probe directed against nNOS. In the Vc of the formalin-treated rats, the densities of nNOS mRNA and protein and nNOS-positive neurons were markedly increased at 1 d after formalin injection (Figure 5A, C, E and F, $p < 0.05$). However, the expression of nNOS decreased in a time-dependent manner. No significant change in the number of nNOS-positive neurons was observed between 7 d and 14 d after formalin administration (Figure 5E and F, $p > 0.05$). Treatment with melatonin reduced the number of neurons with detectable levels of nNOS and resulted in significant downregulation of nNOS mRNA and protein expression in response to formalin (Figure 5B, D, E and F, $p < 0.05$).

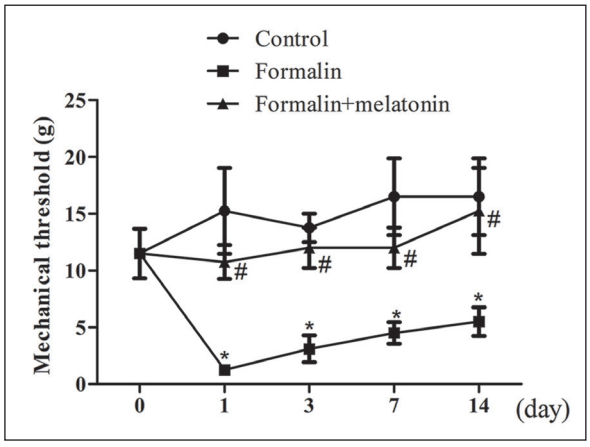

Figure 3. The effect of melatonin on the mechanical withdrawal threshold of rats. The withdrawal threshold (g) of the right whisker pad was measured by the von Frey filament test. Abscissa: the day after formalin injection. The SD rats were treated with different reagents as follows: the C group (treated with saline); F group (treated with formalin) and MT group (treated with melatonin plus formalin). *$p < 0.05$ *vs.* C; #$p < 0.05$ *vs.* F. n=4 for each group. Error bars represent the mean±SEM.

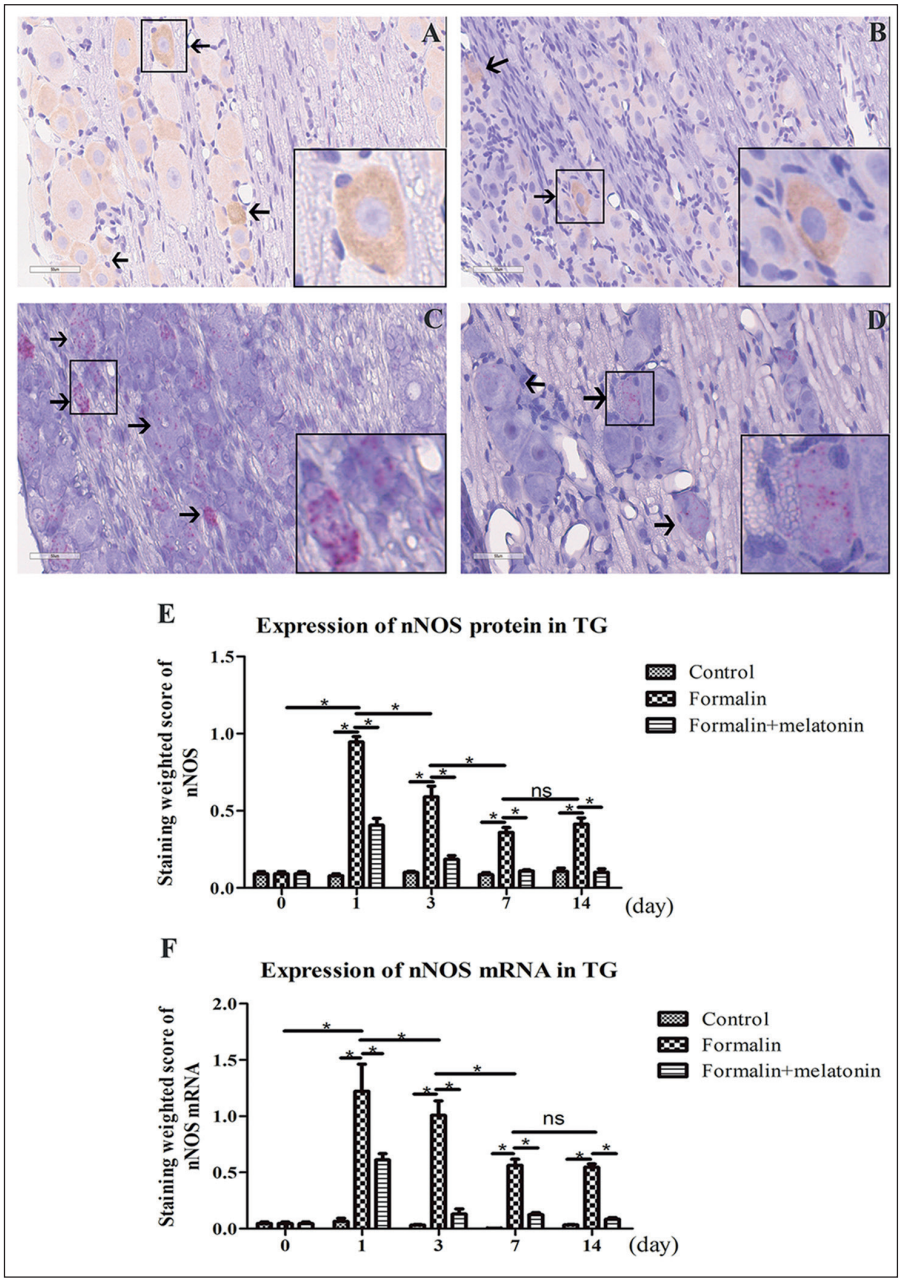

Figure 4. The effect of melatonin on the regulation of nNOS mRNA and protein in TG neurons of formalin-treated rats. **A**, A photomicrograph showing nNOS immunoreactivity in the ipsilateral TG neurons 1 d after formalin injection in rats (F-1 d group). **B**, A photomicrograph showing nNOS immunoreactivity in the ipsilateral TG neurons 1 d after melatonin pretreatment in response to formalin in rats (MT-1 d group). **C**, A photomicrograph showing the nNOS mRNA density in the ipsilateral TG neurons 1 d after formalin administration in rats (F-1 d group). **D**, A photomicrograph showing the nNOS mRNA density in the ipsilateral TG neurons 1 d after melatonin administration in response to formalin in rats (MT-1 d group). **E**, Relative nNOS protein expression was analyzed. F, Relative nNOS mRNA expression was analyzed. Representative nNOS-positive neurons *(arrows)*. Scale bar: 50 μm. **p* < 0.05. n=4 for each group. Error bars represent the mean ± SEM.

Discussion

In the present study, we tested mechanical allodynia using the Von Frey test as a behavioral indicator for subsequent nNOS RNAscope and immunohistochemical analyses. Our results indicated that melatonin attenuated nNOS mRNA and protein expression in TG and Vc neurons treated with formalin in rats. In addition, melatonin dose-dependently decreased the expression of nNOS in primary sensory neurons *in vitro*.

Animal models of chronic pain play a critical role in preclinical pain research. Formalin and capsaicin are widely used chemicals for inducing nociceptive reactions[29-31]. Orofacial inflammatory pain was induced by 4% formalin[32] administration in the right whisker pads of SD rats. *In vitro*, ND7/23 cells were treated with capsaicin[33,34]. Dussor et al[35] explored the role of choline in the modulation of neuropeptide release in buccal mucosae treated with capsaicin. *In vivo*, these researchers established an orofacial

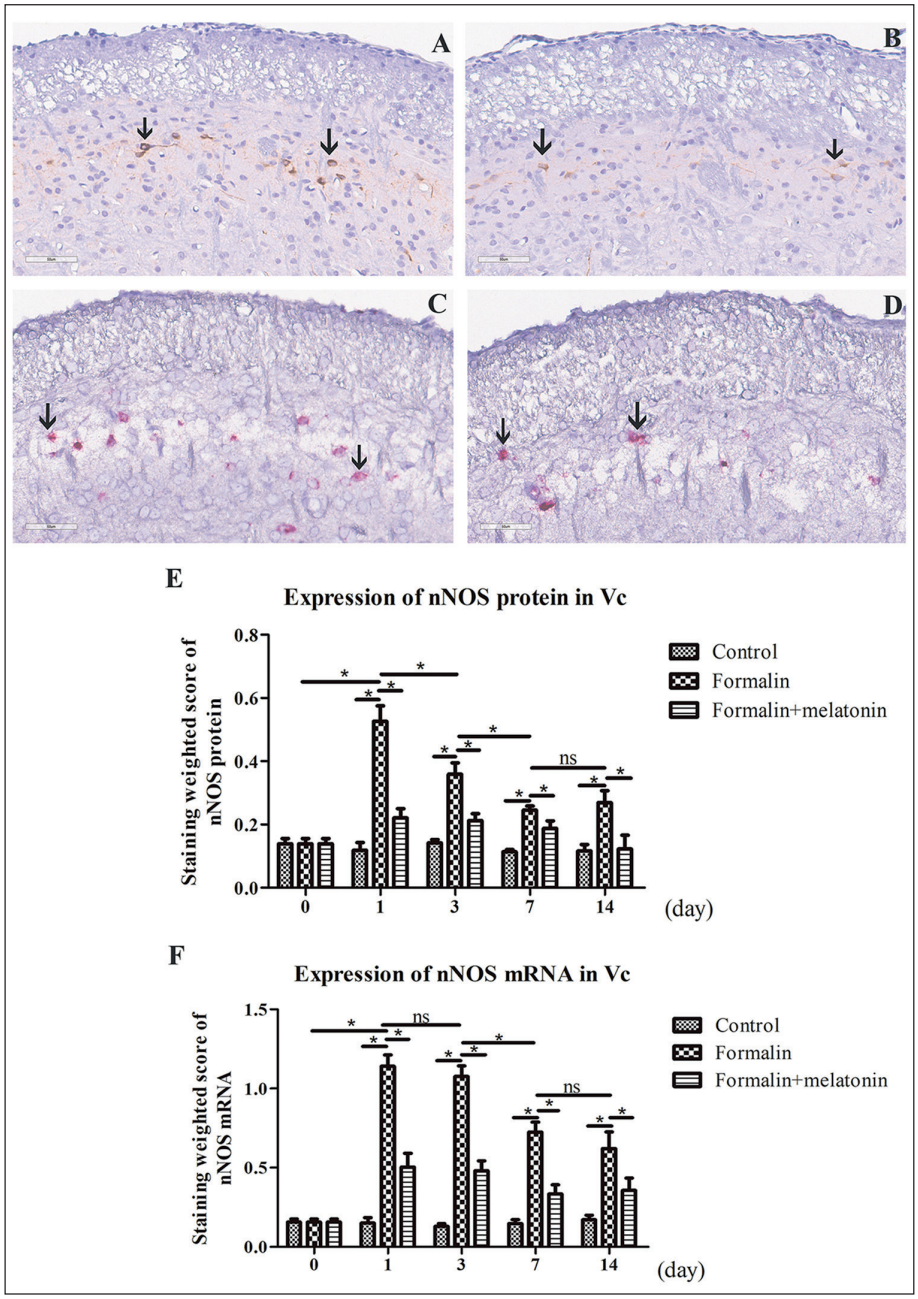

Figure 5. The effect of melatonin on the regulation of nNOS mRNA and protein in Vc neurons of formalin-treated rats. **A**, A photomicrograph showing nNOS immunoreactivity in the ipsilateral Vc neurons 1 d after formalin injection in rats (F-1 d group). **B**, A photomicrograph showing nNOS immunoreactivity in the ipsilateral Vc neurons 1 d after melatonin pretreatment in response to formalin in rats (MT-1 d group). **C**, A photomicrograph showing the nNOS mRNA density in the ipsilateral Vc neurons 1 d after formalin administration in rats (F-1 d group). **D**, A photomicrograph showing the nNOS mRNA density in the ipsilateral Vc neurons 1 d after melatonin pretreatment in response to formalin in rats (MT-1 d group). **E**, Relative nNOS protein expression was analyzed. **F,** Relative nNOS mRNA expression was analyzed. Representative nNOS-positive neurons (arrows). Scale bar: 50 μm. *$p < 0.05$. ns: no statistical significance. n=4 for each group. Error bars represent the mean±SEM.

inflammatory pain model following administration of formalin to investigate the regulation of choline on nociceptive responses at the level of primary sensory neurons.

The TG and dorsal root ganglia (DRG) are clusters of cell bodies of primary sensory neurons that are responsible for transmitting information about the environment to the central nervous system. Due to the homology of TG and DRG neurons, they are overwhelmingly similar. They possess pain-related receptors situated in the membranes of sensory neurons, such as Toll-like receptors (TLRs), opioid receptors (ORs), gamma-aminobutyric acid receptors (GABAs), and metabolic glutamate receptors[36,37]. In addition, the two types of neurons also show similarities in processing cell membrane ion channels, such as voltage-gated sodium ions (Na^+) channels, voltage-gated Ca^{2+} channels, inward rectifier potassium ions (K^+) channels, and transient receptor potential vanilloid type 1 (TRPV1)[36]. The responses of cultured TG and DRG neurons to capsaicin,

bradykinin, substance P and prostaglandin show properties similar to those of *in vivo* sensory neurons[33]. ND cell lines are created by fusing mouse N18Tg2 neuroblastoma cells with adult rat DRG sensory neurons[38]. ND cells have been used to study responses to substance P, bradykinin, capsaicin, and opioids[39]. In summary, in this study, we used ND7/23 cells to explore the role of melatonin in the regulation of nNOS *in vitro*.

nNOS is predominantly expressed in neurons and is found in both the central and peripheral nervous systems[40]. Growing evidence demonstrates that nNOS plays a critical role in the development and/or maintenance of inflammatory pain[41,42]. nNOS expression is substantially increased in lesioned neurons during peripheral nerve injury, where prolonged levels can cause tissue damage and mechanical allodynia[20,43]. So, in mice, stimulation with complete Freund's adjuvant (CFA) was shown to cause hypersensitivity to thermal and mechanical pain and resulted in augmentation of nNOS[41]. In another study, significant upregulation of the levels of three types of NOS was observed after capsaicin-induced inflammation at the immediate time points. However, CFA-evoked inflammation did not result in marked changes in nNOS at any time point[44]. Therefore, differential expression of nNOS may be associated with the time course of inflammatory pain and may be due to distinct stimuli. In our study, we showed that increased expression of nNOS is induced by capsaicin or formalin. Our findings are consistent with data from studies on sensory neurons in the central or peripheral nervous system in which increased NOS expression is involved in pathological processes in response to peripheral nerve injury or inflammatory stimuli.

Melatonin plays an important role in pain modulation through multiple mechanisms[45,46]. Lin et al[46] indicated that in a neuropathic pain model, melatonin administration attenuated mechanical allodynia and inhibited activation of neuroinflammation by downregulating nNOS expression. Likewise, another study revealed that during a neuropathic pain event, melatonin mediates its anti-thermal hypersensitivity effect via downregulation of nNOS expression[47]. Notably, melatonin at nanomolar concentrations has been reported to increase the expression of nNOS and the production of nitrite and nitrate[48]. Melatonin seems to play a dual role in the regulation of nNOS pathways based on its distinct concentration. Consistent with most of the above literature, according to our findings, the stimulatory effect of capsaicin or formalin on nNOS expression was reversed by melatonin treatment. nNOS has constitutively active properties, which are dependent on an increased intracellular Ca^{2+} concentration and subsequent binding to calmodulin[16,40]. Opening of voltage-gated Ca^{2+} channels increases the influx of Ca^{2+}, which binds to calmodulin and activates nNOS[49]. However, melatonin modulates the Ca^{2+} influx via desensitization of TRPV1 and transient receptor potential melastatin type 2 (TRPM2)[50]. Furthermore, melatonin may suppress the elevated calmodulin induced by nerve injury[8,51]. Therefore, melatonin exerts an antinociceptive effect by inhibiting the expression and activation of nNOS. The antinociceptive effect of melatonin against chronic constriction injury of the sciatic nerve in rats is significantly reversed by L-arginine pretreatment, suggesting the involvement of the NO pathway in the protective effect of melatonin against CCI-induced behavioral and biochemical alterations in rats[52]. The L-arginine/NO/cyclic guanosine monophosphate (cGMP)/K^+-ATP pathway plays an important role in peripheral nociception[53,54]. nNOS/NO pathways contribute to behavioral pain responses evoked by the inferior alveolar nerve[14].

In this study, the stimulatory effect of capsaicin on NOS activity appeared to be primarily inhibited by melatonin. However, no difference in NOS activity was found among the groups receiving different doses of melatonin treatment. Our findings are different from those of studies on cerebellar and hypothalamic cells in that melatonin inhibited NOS activity in a dose-dependent manner at concentrations ranging from 1 nM to 1 mM (dilution factor=100). In cerebellar and hypothalamic cells, NOS activity was measured by monitoring the conversion of L-(^{3}H) arginine to L-(^{3}H) citrulline[55,56]. In the present study, the activity of NOS was determined indirectly by measuring the fluorescence of the benzotriazole derivative using the DAF-FM probe, which depends on the amount of NO production. NO is known to have a very short half-life of seconds or less and is converted into one of several classes of more stable metabolites designated as NOx, including nitrate and nitrite[57,58]. In addition, the dilution factor of the melatonin concentration in our study was 2, which is less than that used in cerebellar and hypothalamic cells. However, the inhibitory effect of melatonin on NOS activity is strongly dependent on Ca^{2+}/calmodulin. The inconsistency between NOS activity and nNOS expression in response to melatonin may be related to the low

content of Ca^{2+} in ND7/23 neurons, the distinct melatonin concentrations, and different methods of measuring NOS activity.

iNOS is expressed in certain cell types, including macrophages, glia and neurons, and is usually expressed at low levels under physiological conditions[15]. iNOS is distinct because it is not constitutively active but is substantially increased during pathological processes[59]. The increased iNOS expression was observed in cultured TG neurons treated with artemin[60]. In mouse paws, stimulation with lipopolysaccharide (LPS) was shown to cause activation of iNOS in the acute phase[61]. Similarly, elevated iNOS expression has been found in the ipsilateral cortex 24 h after brain contusion[62]. Park et al[63] found increased iNOS expression 3 d after spinal cord injury, which then decreased over time. Taken together, these data suggest that iNOS may be involved in nociceptive activation. eNOS is found predominantly in the vascular endothelium, and eNOS expression in neurons is minimal[64,65]. However, certain findings indicate that eNOS is present in primary cortical and hippocampal neurons and is localized in dendritic spines[66]. However, whether melatonin exerts analgesic effects through regulation of iNOS and eNOS in the nervous system is controversial and requires further study.

Melatonin has been suggested to regulate pain via membrane receptors, nuclear receptors, and simple diffusion[46,67,68]. Melatonin binds to membrane receptors with high affinity in the picomolar range and/or to the nuclear receptor RZR/ROR in the nanomolar range. At even higher concentrations, melatonin can directly enter the cell and also has a free radical scavenging function[69]. The concentration of melatonin in our study ranged from 0.25 mM to 2 mM, which is higher than the concentration needed to bind to membrane receptors. Moreover, the addition of luzindole does not distinctly influence the expression of nNOS, suggesting that the antinociceptive effect of melatonin in this pathway is not mediated by melatonin receptors[46]. Melatonin is considered to attenuate morphine-induced hypersensitivity and tolerance by suppressing N-methyl-D-aspartate (NMDA) receptor subtype 1 activities in the spinal cord[45]. Inhibition of NO production leads to a decrease in the protein kinase C-dependent NMDA receptor GluN1 subunit and ultimately contributes to improving mechanical allodynia following peripheral nerve injury[70].

Previous studies related to the potential analgesic activity of melatonin through nNOS regulation merely emphasized the detection of nNOS mRNA expression by collecting tissue mRNA instead of *in situ* hybridization[71,72]. In our study, we detected the expression and localization of nNOS mRNA in the TG and Vc by RNAscope analyses (a reliable technique of RNA in situ hybridization), which provided direct morphological evidence.

Conclusions

This research demonstrates that nNOS was upregulated in TG and Vc cells following inflammation *in vivo* and in ND7/23 cells in response to capsaicin *in vitro*. These findings suggest that nNOS may play an active role in both peripheral and central processing of nociceptive information following orofacial inflammatory pain induction. The increased response of nNOS was inhibited by melatonin. In addition, melatonin significantly alleviated the mechanical allodynia induced by formalin. The regulatory role of melatonin on nNOS in inflammatory pain may have potential implications for the development of novel analgesic strategies.

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Conflict of Interests

The authors declared there is no conflict of interest.

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