

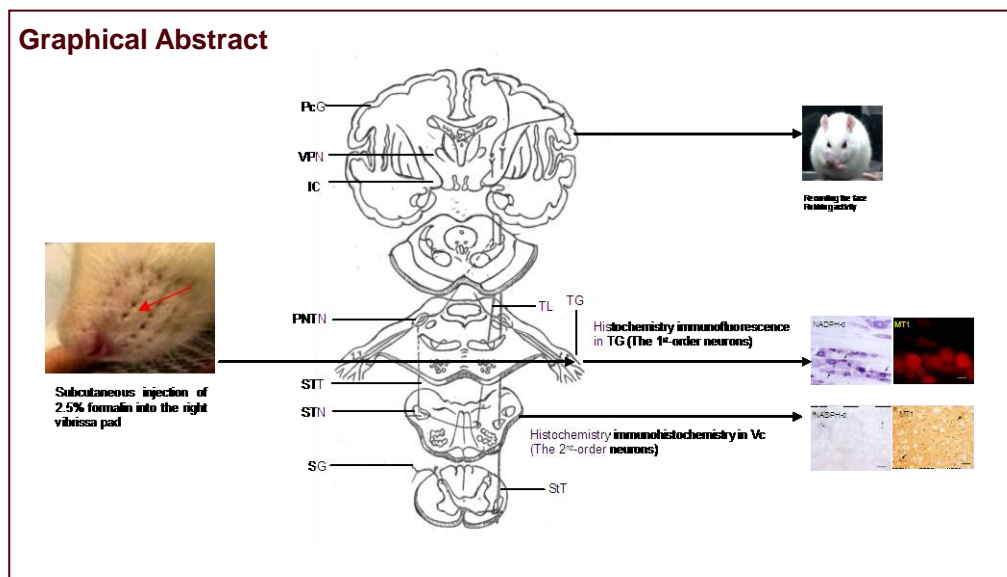
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Orofacial inflammatory pain affects the expression of MT1 and NADPH-d in rat caudal spinal trigeminal nucleus and trigeminal ganglion

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Abstract

Very little is known about the role of melatonin in the trigeminal system, including the function of melatonin receptor 1. In the present study, adult rats were injected with formaldehyde into the right vibrissae pad to establish a model of orofacial inflammatory pain. The distribution of melatonin receptor 1 and nicotinamide adenine dinucleotide phosphate diaphorase in the caudal spinal nucleus and trigeminal ganglion was determined with immunohistochemistry and mistry. The results show that there are significant differences in melatonin receptor 1 expression and nicotinamide adenine dinucleotide phosphate diaphorase expression in the trigeminal ganglia and caudal spinal nucleus during the early stage of orofacial inflammatory pain. Our findings suggest that when melatonin receptor 1 expression in the caudal spinal nucleus is significantly reduced, melatonin's regulatory effect on pain is attenuated.

Key Words

neural regeneration; pain; melatonin; nitric oxide; maxillofacial pain; caudal spinal trigeminal nucleus; trigeminal ganglia; mesencephalic trigeminal nucleus; melatonin receptor 1; nicotinamide adenine dinucleotide phosphate diaphorase; grants-supported paper; neuroregeneration

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INTRODUCTION

Melatonin is an indole neuroendocrine hormone, and has a wide range of physiological functions and effects, including regulating circadian rhythms, regulating sleep, enhancing immune function, scavenging free radicals and anti-oxidant effects^[1-2]. Accumulating research indicates that melatonin has a major role in pain transmission and has an ultra-sensitizing effect^[3-11]. However, the effects of melatonin vary greatly in different inflammatory and neuropathic pain models. Most studies suggest that melatonin reduces pain transmission or pain sensitization, showing analgesic effects^[3, 5, 8]. Conversely, other studies found that melatonin promotes pain or induces pain hypersensitivity^[9-11], and clinical findings show that pain is more intense at night, when melatonin secretion peaks^[12]. Consequently, the role of the melatonin system in pain is unclear.

Melatonin acts on cells through membrane receptors and nuclear receptors, and it can also directly enter cells to affect organelles and regulate cell function^[13-14]. The effect of melatonin in cell regulation may differ based on variations in signaling pathways, and different effects are also observed in the pain transmission process. However, most studies on the melatonin regulation of pain have focused on peripheral dorsal root ganglia and central spinal cord pathways, which are closely related to body and limb pain. However, the orofacial pain-associated trigeminal sensory pathways are rarely studied, and only a few reports have investigated the expression of membrane melatonin receptors^[15-20]. Melatonin can regulate pain through melatonin receptor 2 (MT2)^[21]; however, the function of melatonin receptor 1 (MT1) remains unclear.

Nitric oxide is a bioactive gas molecule involved in many physiological processes, such as cell signaling, nerve repair and immune regulation. Growing evidence shows that inflammatory and other types of pain are associated with the increased production of nitric oxide^[22-27]. Therefore, measuring nitric

oxide content in tissue can assist the determination of pain severity. Previous studies on body and limb pain showed that melatonin mediates pain *via* the N-methyl-D-aspartic acid (NMDA) and nitric oxide pathways through MT1/MT2^[28-31]. Pain activates α -amino-3-hydroxy-5-methyl-4-isoxazole propionic acid or NMDA receptors and increases nitric oxide or peroxynitrite free radical production, while melatonin can reverse these changes^[32]. Orofacial pain is mainly pain in the oral cavity, head and face. The first-order and second-order afferent neurons in orofacial pain transmission are located in the trigeminal ganglion and spinal trigeminal nucleus, respectively. It is known that melatonin can reduce the number of nitric oxide synthase-positive cells in the caudal spinal trigeminal nucleus, and inhibit trigeminal nerve nociception induced by cortical spreading depression^[33]. Therefore, we speculated that melatonin may play a regulatory role with nitric oxide in the orofacial pain transmission process.

Nicotinamide adenine dinucleotide phosphate diaphorase (NADPH-d) is the histochemical marker of nitric oxide synthase. Measurement of nitric oxide synthase or NADPH-d can indirectly reflect nitric oxide production rate and content^[34-35]. In this study, we examined changes in MT1 and NADPH-d expression in the spinal trigeminal nucleus, mesencephalic trigeminal nucleus and trigeminal ganglion in the orofacial inflammatory pain model using histochemistry, immunohistochemistry and immunofluorescence staining.

RESULTS

Quantitative analysis of experimental animals

Thirteen Sprague-Dawley rats were randomly divided into control group ($n = 6$) and experimental group ($n = 7$), receiving subcutaneous injection of saline or formaldehyde, respectively, into the right vibrissae pad. One rat in the experimental group was eliminated owing to formaldehyde leakage, and the remaining rats were included in the analyses.

Nociceptive responses of rats with formaldehyde-induced orofacial inflammatory pain

Following formaldehyde injection, all the rats exhibited the following typical dual-phase nociceptive behavioral responses: initially, standing or remaining stationary for 10–30 seconds, and then quickly scratching the ipsilateral injection site using claws, accompanied by 0–3 minutes of standing or exploration (stage I); 3–12 minutes after injection, a quiet phase; subsequently, more apparent and longer scratching responses for about 33 minutes (stage II); finally, 1 hour after injection, rats gradually calm down, with occasional face-scraping, standing and exploring responses (remission phase). The rats in the control group remained behaviorally unchanged before and after the injection (Figure 1). As shown in Figure 2, the number of rats scratching the injection site at stage I and II in the experimental group was significantly higher than in the control group ($P < 0.05$), and there was no significant difference between the two groups at the quiet and regressive phases ($P > 0.05$).

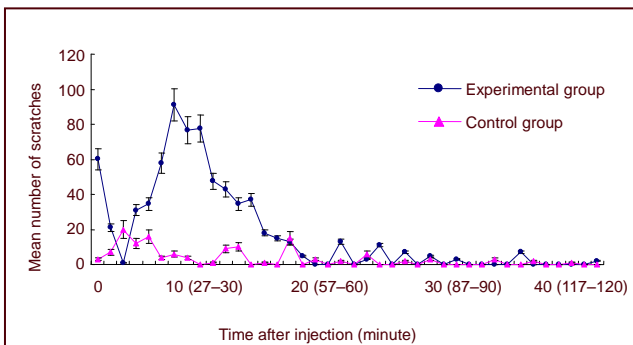


Figure 1 Time course of face scratching activity in rats after formaldehyde injection.

The mean number of scratches is plotted for each 3-minute intervals over a 2-hour post-injection observation period. The x-axis represents each 3-minute interval and the number in brackets represents the real time point. A greater number of scratches indicates a more severe nociceptive response. All data are expressed as mean \pm SD. There were six rats per group. The differences between the two groups were compared using paired *t*-test.

MT1 expression decreased in caudal spinal trigeminal nucleus neurons in rats with orofacial inflammatory pain

In the control group, MT1 was expressed in bilateral caudal spinal trigeminal nucleus neurons in normal rats, and MT1-positive neurons were located in laminae I–IV of the caudal trigeminal spinal nucleus. In laminae I and II, there were mainly medium-sized (diameter 30–40 μm) and small-sized neurons (diameter $< 30 \mu\text{m}$). In laminae III and IV, large-sized (diameter $> 40 \mu\text{m}$) and medium-sized neurons were dominant. The number of neurons in laminae III and IV was higher than in laminae

I and II, and there was no significant difference in the distribution of neurons between the two sides (Figure 3).

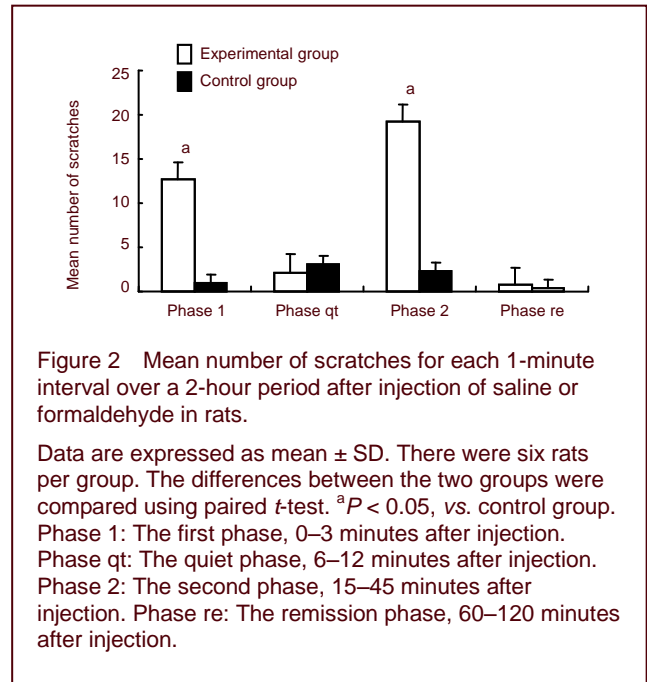


Figure 2 Mean number of scratches for each 1-minute interval over a 2-hour period after injection of saline or formaldehyde in rats.

Data are expressed as mean \pm SD. There were six rats per group. The differences between the two groups were compared using paired *t*-test. ^a $P < 0.05$, vs. control group. Phase 1: The first phase, 0–3 minutes after injection. Phase qt: The quiet phase, 6–12 minutes after injection. Phase 2: The second phase, 15–45 minutes after injection. Phase re: The remission phase, 60–120 minutes after injection.

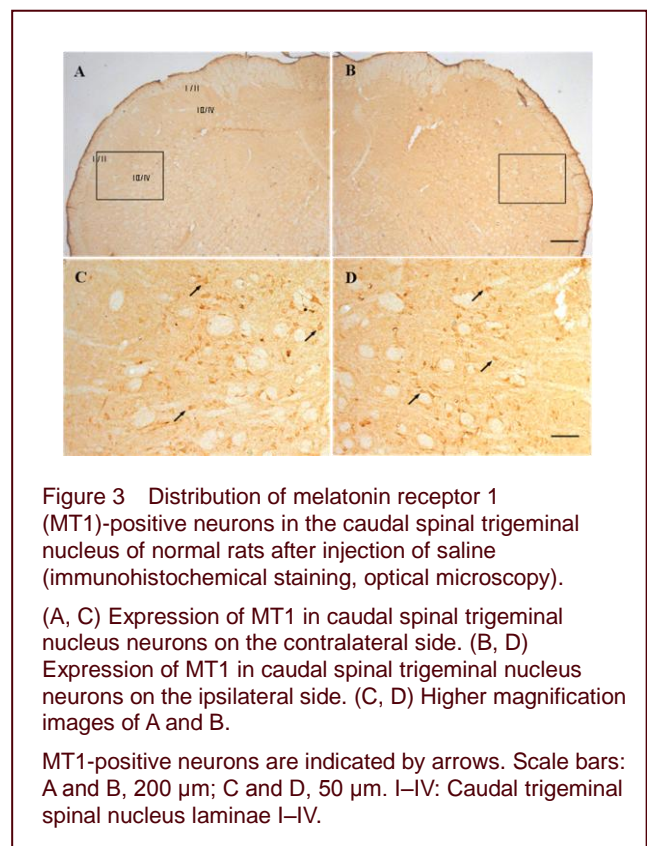


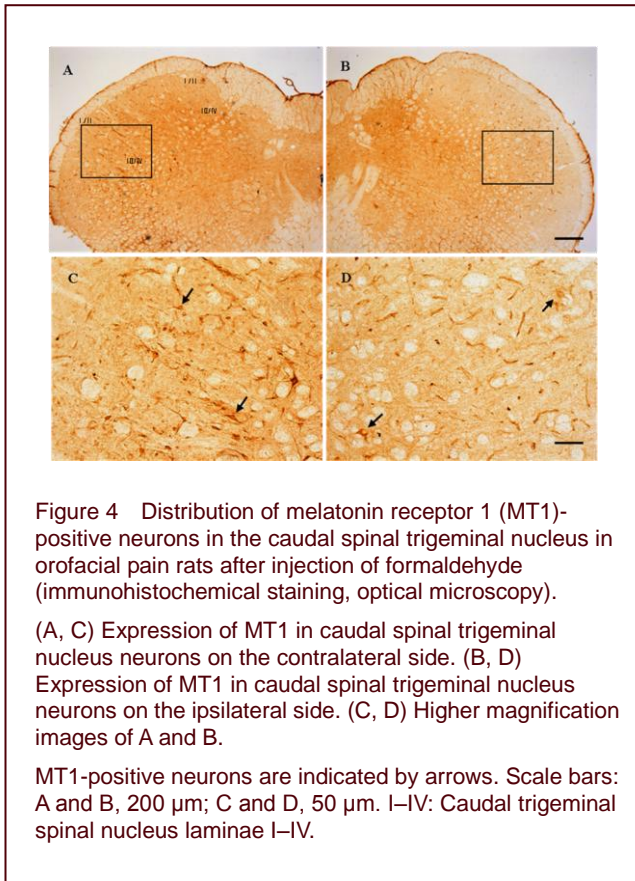
Figure 3 Distribution of melatonin receptor 1 (MT1)-positive neurons in the caudal spinal trigeminal nucleus of normal rats after injection of saline (immunohistochemical staining, optical microscopy).

(A, C) Expression of MT1 in caudal spinal trigeminal nucleus neurons on the contralateral side. (B, D) Expression of MT1 in caudal spinal trigeminal nucleus neurons on the ipsilateral side. (C, D) Higher magnification images of A and B.

MT1-positive neurons are indicated by arrows. Scale bars: A and B, 200 μm ; C and D, 50 μm . I–IV: Caudal trigeminal spinal nucleus laminae I–IV.

In the experimental group, MT1 expression was observed in the bilateral caudal spinal trigeminal nucleus neurons of rats with orofacial pain, and MT1-positive neurons were located in laminae I–IV. In laminae I and II, there were mainly medium-sized and small-sized neurons. In laminae III and IV, large-sized and medium-sized

neurons were dominant. The neuronal distribution was similar to the control group. In addition, the number of MT1-positive neurons in the ipsilateral caudal trigeminal spinal nucleus was significantly reduced compared with the contralateral side, and was also significantly lower than in the control group. This result shows that the distribution of MT1-positive neurons in the caudal spinal trigeminal nucleus is reduced, especially in laminae I and II (Figure 4).

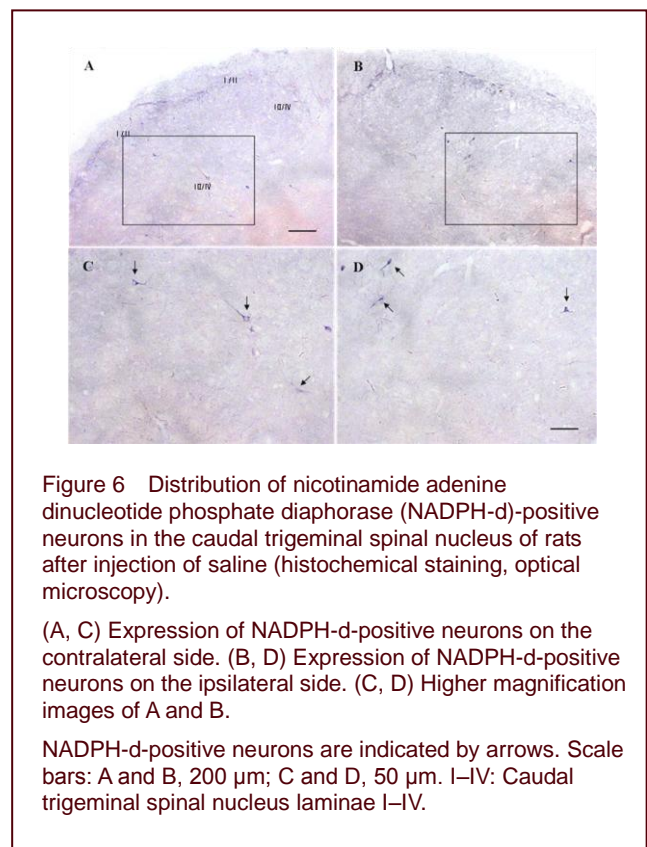
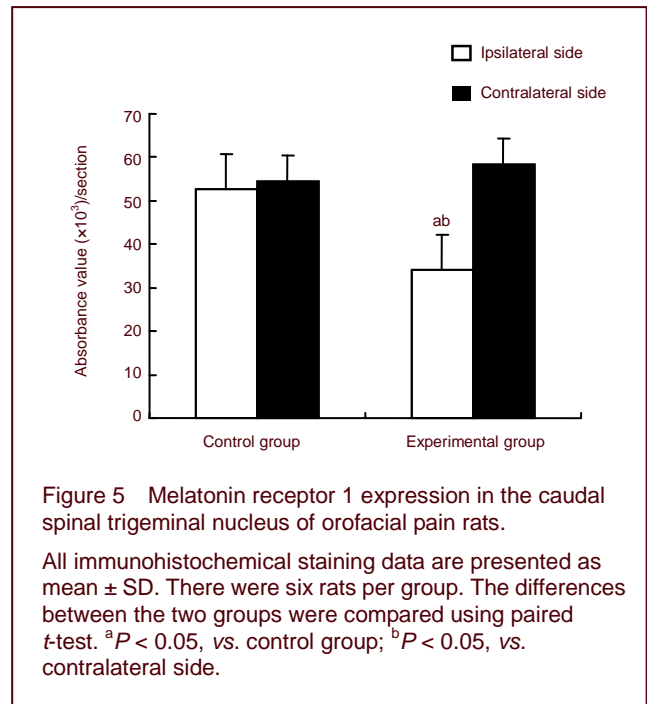


Immunohistochemical staining showed that the accumulated absorbance value of MT1-positive cells in the ipsilateral caudal spinal trigeminal nucleus in orofacial pain rats was significantly reduced compared with that on the contralateral side ($P < 0.05$), and was also significantly lower than in the control group ($P < 0.05$; Figure 5).

Effect of orofacial inflammatory pain on NADPH-d expression in caudal spinal trigeminal nucleus neurons

Small numbers of NADPH-d-positive cells and fibers were visible in the caudal trigeminal spinal nucleus in laminae I-IV, in both normal and orofacial pain rats, and the positive cells were scattered in both the ipsilateral and contralateral sides. There was no significant difference in the number of NADPH-d positive cells between the two groups ($P > 0.05$), or between the two sides in

the experimental group ($P > 0.05$; Figures 6-8).



Effect of orofacial inflammatory pain on MT1 expression in trigeminal ganglion neurons

MT1-positive neurons of varying sizes were observed in both orofacial pain rats and normal rats. The medium and small neurons, which are related to pain^[36], were more numerous than large neurons (Figure 9).

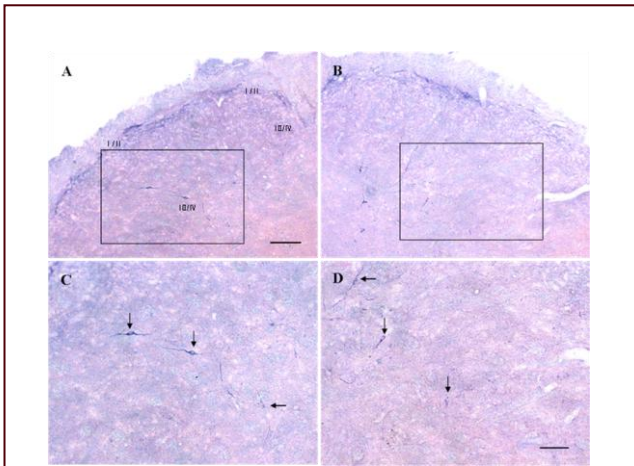


Figure 7 Distribution of nicotinamide adenine dinucleotide phosphate diaphorase (NADPH-d)-positive neurons in the caudal trigeminal spinal nucleus of rats after injection of formaldehyde (histochemical staining, optical microscopy).

(A, C) Expression of NADPH-d-positive neurons on the contralateral side. (B, D) Expression of NADPH-d-positive neurons on the ipsilateral side. (C, D) Higher magnification images of A and B.

NADPH-d-positive neurons are indicated by arrows. Scale bars: A and B, 200 μm ; C and D, 50 μm . I–IV: Caudal trigeminal spinal nucleus laminae I–IV.

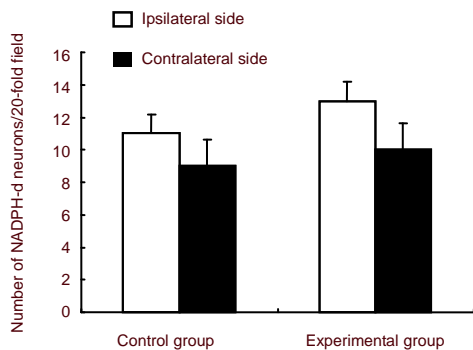


Figure 8 Number of nicotinamide adenine dinucleotide phosphate diaphorase (NADPH-d)-positive neurons in the caudal trigeminal spinal nucleus of rats after injection of formaldehyde.

The number of NADPH-d-positive neurons in the caudal trigeminal spinal nucleus was counted in laminae I–IV. There is no difference in the number of NADPH-d neurons between the two groups or between the bilateral sides in each group. All histochemical staining data are presented as mean \pm SD. There were six rats per group. The differences between the two groups were compared using paired *t*-test.

In the control group, the large, medium and small MT1-positive neurons accounted for 25.9%, 29.9% and 44.2%, respectively, of the total number of neurons. In the experimental group, the large, medium and small MT1-positive neurons accounted for 26.8%, 29.4% and

43.8%, respectively, of the total number of neurons. There was no significant difference in the number of large, medium or small MT1-positive neurons between the two groups ($P > 0.05$). The mean absorbance of MT1-positive neurons in the trigeminal ganglia of rats in the experimental group was similar to that in the control group ($P > 0.05$; Figure 10).

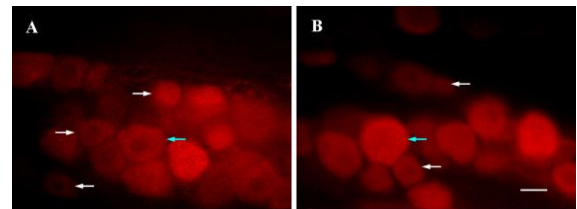


Figure 9 Expression of melatonin receptor 1 (MT1) in trigeminal ganglion neurons after injection of saline or formaldehyde (immunofluorescence staining, optical microscopy).

(A) MT1-positive neurons in the control group. (B) MT1-positive neurons in the experimental group.

White arrows indicate medium and small MT1-positive neurons. Blue arrows indicate large MT1-positive neurons. Scale bar: 50 μm .

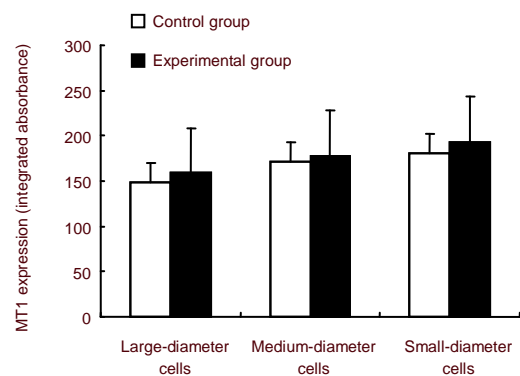


Figure 10 Expression of melatonin receptor 1 (MT1) in trigeminal ganglion neurons after injection of formaldehyde.

The diameters of small, medium and large trigeminal ganglion neurons were $< 30 \mu\text{m}$, $30\text{--}40 \mu\text{m}$ and $> 40 \mu\text{m}$, respectively. There was no significant difference between the two groups ($P > 0.05$). All immunofluorescence data are expressed as mean \pm SD. There were six rats per group. The differences between the two groups were compared using paired *t*-test.

Effect of orofacial inflammatory pain on NADPH-d expression in trigeminal ganglion neurons

NADPH-d-positive neurons of varying sizes were observed in both orofacial pain rats and normal rats. The deeply stained medium and small neurons in the experimental group were more numerous than in the control group (Figure 11).

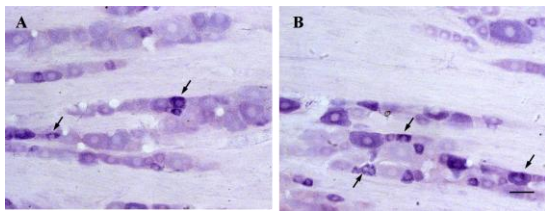


Figure 11 Expression of nicotinamide adenine dinucleotide phosphate diaphorase (NADPH-d) in ipsilateral trigeminal ganglion neurons after injection of saline or formaldehyde (histochemical staining, optical microscopy).

(A) In the control group, large NADPH-d-positive neurons are weakly stained. (B) In the experimental group, the number of densely stained medium and small neurons was increased. Arrows indicate intense staining of medium and small cells. Scale bar: 40 μ m.

In the control group, the large, medium and small NADPH-d-positive trigeminal ganglia neurons accounted for 24.4%, 28.7% and 46.9%, respectively, of the total number of neurons. In the experimental group, the large, medium and small neurons accounted for 25.4%, 28.1% and 46.5%, respectively, of the total number of neurons. There was no significant difference in the numbers of the various sizes of NADPH-d-positive neurons between the two groups ($P > 0.05$). The staining intensity (integrated absorbance) of medium and small NADPH-d-positive neurons in the trigeminal ganglia was significantly higher in the experimental group than in the control group ($P < 0.05$), but there was no significant difference in the staining intensity of large neurons between the two groups ($P > 0.05$; Figure 12).

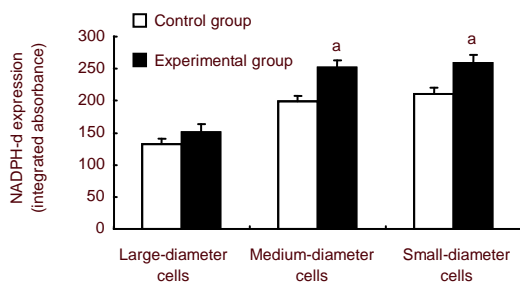


Figure 12 Expression of nicotinamide adenine dinucleotide phosphate diaphorase (NADPH-d) in the ipsilateral trigeminal ganglion of rats after injection of formaldehyde.

All data are expressed as mean \pm SD. There were six rats per group. The differences between the two groups were compared using paired *t*-test. ^a $P < 0.05$, vs. control group.

Effect of orofacial inflammatory pain on MT1 and NADPH-d expression in mesencephalic trigeminal nucleus neurons

Both MT1 and NADPH-d-positive neurons were found in

mesencephalic trigeminal nucleus neurons in normal rats and orofacial pain rats.

The majority of positive neurons were large and had a spherical or oval shape. The expression levels of MT1 and NADPH-d were the same ($P > 0.05$; Figures 13, 14, Table 1).

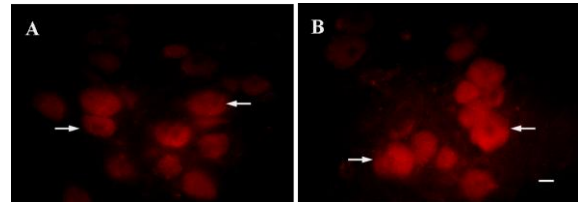


Figure 13 Expression of melatonin receptor 1 (MT1) in the ipsilateral mesencephalic trigeminal nucleus neurons after injection of saline or formaldehyde (immunofluorescence staining, optical microscopy).

(A) MT1-positive neurons in the control group. (B) MT1-positive neurons in the experimental group.

MT1 positive neurons are indicated by arrows. Scale bar: 20 μ m.

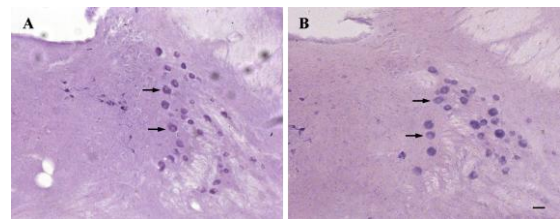


Figure 14 Expression of nicotinamide adenine dinucleotide phosphate diaphorase (NADPH-d) in ipsilateral mesencephalic trigeminal nucleus neurons after injection of saline or formaldehyde (histochemical staining, optical microscopy).

(A) NADPH-d-positive neurons in the control group. (B) NADPH-d-positive neurons in the experimental group.

NADPH-d-positive neurons are indicated by arrows, and are large cells, with a spherical or ovoid shape. Scale bar: 40 μ m.

Table 1 MT1 and NADPH-d expression (integrated absorbance) in the mesencephalic trigeminal nucleus after injection of saline or formaldehyde

Group	MT1		NADPH-d	
	Ipsilateral	Contralateral	Ipsilateral	Contralateral
Control	166.8 \pm 22.8	160.6 \pm 24.1	189.1 \pm 25.6	179.5 \pm 26.1
Experimental	156.5 \pm 23.2	172.1 \pm 24.5	201.3 \pm 22.3	195.1 \pm 18.3

All data are expressed as mean \pm SD. There were six rats per group. The differences between the two groups were compared using paired *t*-test. MT1: Melatonin receptor 1; NADPH-d: nicotinamide adenine dinucleotide phosphate diaphorase.

DISCUSSION

Behavioral responses and neuronal excitability in orofacial inflammatory pain

The results of this study show that the animal model of maxillofacial inflammatory pain exhibits a dual-phase nociceptive response after subcutaneous injection of formaldehyde, consistent with previous studies^[37-41]. The typical nociceptive behavioral responses were not seen in the control group, indicating that they are not caused by the injection itself, but are a response to the formaldehyde-induced inflammatory pain.

In contrast to previous studies, our findings revealed no significant difference in behavioral responses at 1–2 hours after injection in the two groups. Previous studies on the neuronal excitability marker c-Fos have shown that nerve impulses are not diminished, but are enhanced 2 hours after formaldehyde injection, with c-Fos expression peaking 1.5–4 hours after stimulation^[40, 42-43]. Nitric oxide is closely related to c-Fos expression in the central nervous system. When peripheral inflammatory pain occurs, nitric oxide levels sharply and rapidly increase. Nitric oxide synthase is a key enzyme in the biosynthesis of nitric oxide, and NADPH-d is considered to be a histochemical marker of nitric oxide synthase^[34-35]. Thus, measurement of nitric oxide synthase or NADPH-d can indirectly reflect nitric oxide production rate and content. As previously described^[44], we observed MT1 and NADPH-d expression in the rat trigeminal sensory system 2 hours after formaldehyde injection.

Effect of maxillofacial inflammatory pain on MT1 and NADPH-d expression in the trigeminal ganglion

The trigeminal ganglion is involved in the maxillofacial sensory response to mechanical force, heat and pain. Pain sensations are mediated by medium and small neurons^[45-46]. In this study, NADPH-d activity in the medium and small trigeminal ganglion neurons was significantly enhanced 2 hours after formaldehyde injection, while large neurons did not exhibit a change. There is little data on the role of nitric oxide in maxillofacial pain. Borsani *et al*^[47] found that nitric oxide synthase expression in the trigeminal ganglia 3 hours after formaldehyde-induced maxillofacial pain was significantly higher than in the control group, mainly in small cells on the ipsilateral side. At 24 hours, expression was similar to that in the control group. Purinergic receptor antagonists were found to reduce formaldehyde-induced grasping behavior and downregulate nitric oxide synthase ex-

pression in the trigeminal ganglia and reduce c-Fos expression in the trigeminal spinal nucleus. The observation time point in this study was earlier than in previous studies; 2 hours after inflammation, we already observed enhanced NADPH-d activity in the trigeminal ganglia. This finding demonstrates the early involvement of nitric oxide in the modulation of formaldehyde-induced inflammatory pain, and a potential role for the molecule in regulating nociceptive pathways in the central nervous system.

In addition, our findings revealed no significant difference in the percentage of the various sizes of MT1-positive neurons in the ipsilateral trigeminal ganglia or in the absorbance values 2 hours after injection. This suggests that pain impulses have no impact on MT1 expression in the trigeminal ganglion at the early stage of inflammatory pain. However, exogenous melatonin has a strong antinociceptive effect in different inflammatory and neuropathic pain models^[3, 5, 8]. Melatonin significantly decreases nitric oxide and malondialdehyde levels, and reduces edema^[48-49]. Melatonin also reverses endotoxin-induced hyperalgesia and inhibits tumor necrosis factor production^[50]. Some researchers have shown a pain-promoting effect of endogenous melatonin in the mouse spinal cord^[9-12]. Thus, the role of melatonin in maxillofacial pain is unclear. Preliminary work by our research group demonstrates that 84.7% of MT1-positive neurons in the trigeminal ganglia co-express NADPH-d (data not shown). In contrast to pain at the spinal cord level, the trigeminal ganglia do not show synchronous changes among melatonin receptors and NADPH-d, *i.e.*, melatonin receptors in trigeminal ganglion neurons are not closely related to nitric oxide in maxillofacial inflammatory pain. This contrasting finding may be due to tissue differences.

Effect of maxillofacial inflammatory pain on MT1 expression in the caudal spinal trigeminal nucleus

Autoradiography^[15-17] and *in situ* hybridization^[19] have shown expression of MT1 in the spinal dorsal horn, brainstem spinal trigeminal nerve and spinal trigeminal nucleus, but tissue and cellular distribution remained unclear. We applied the immunohistochemical method to examine MT1 expression in the caudal trigeminal spinal nucleus laminae I–IV. We found a small amount of nitric oxide synthase expression; more importantly, we observed that formaldehyde-induced maxillofacial inflammatory pain significantly reduced MT1 expression in the ipsilateral caudal trigeminal spinal nucleus, while the control group was unaffected. Formaldehyde-induced inflammatory pain robustly reduces the number of

MT1-positive neurons in the caudal spinal trigeminal nucleus by downregulating MT1 expression.

The function and number of melatonin receptors are influenced by many factors and physiological stimuli, such as melatonin levels, light-dark cycles and the biological clock. Melatonin acts through receptors to effectuate its biological functions, which include regulating sensory transmission in the spinal cord^[51]. Perissin *et al*^[9] showed that pain stimulation results in the reduction of melatonin binding sites; the number of 2-[¹²⁵I]-Mel binding sites in the bilateral spinal dorsal horn was significantly reduced at night, when pain is more apparent. This indicates that melatonin receptor activity is attenuated, but the regions presenting the reduced expression remained unclear.

In this study, MT1 expression was downregulated in the rat caudal spinal trigeminal nucleus laminae I–IV, especially in lamina I and II and on the ipsilateral side, indicating that the decreased expression is likely a direct result of inflammatory stimuli. Nelson and colleagues^[52] found that melatonin receptor activity may be altered because of pain stimuli or the activation of preganglionic sympathetic neurons and sympathetic efferent fibers in the pineal body^[53], but the underlying mechanisms remain unclear. Roy *et al*^[54] proposed that the high levels of melatonin could be induced in the spinal cord gray matter through internal mechanisms. Under stress conditions, melatonin circulating levels are elevated and melatonin receptor levels are reduced^[55]. However, previous studies focused on the decreased activity of melatonin receptors and protein transcription levels in the spinal cord. Our findings are the first on MT1 expression in trigeminal spinal nucleus neurons, which are closely related to maxillofacial pain. In addition, we also found that MT1 expression in the caudal spinal trigeminal nucleus was reduced during maxillofacial pain. This shows that the reduction in melatonin receptors underlies the decrease in melatonin binding sites.

Effect of maxillofacial inflammatory pain on NADPH-d expression in the caudal spinal trigeminal nucleus

In this study, a number of NADPH-d positive neurons in the caudal spinal trigeminal nucleus showed no significant changes, which differs from previous studies. It is known that after hind paws or limbs are injected with a proinflammatory agent, the number of nitric oxide synthase-positive neurons and NADPH-d activity in the spinal dorsal horn are significantly increased, especially on the ipsilateral side. However, the observation period was 24 hours, or even several days, after injection.

Traub and colleagues^[56] found that the number of NADPH-d-stained neurons was slightly increased 2 hours after subcutaneous injection of carrageenan in rats, but the difference was still insignificant compared with the control group. This is consistent with our experimental results. Furthermore Traub *et al* found that the number of positive neurons is increased at 6 hours. This indicates that inflammatory stimuli affect MT1 earlier than nitric oxide. Fan and colleagues^[57] found that NADPH-d activity in the caudal trigeminal spinal nucleus begins to increase on day 7 after pulp exposure in the chronic pulpitis model, corresponding with changes in the neuronal activity marker c-Fos. Similar results were also observed in other studies^[58]. Yonehara and colleagues^[59] showed that the number of nitric oxide synthase-positive neurons is significantly increased in the ipsilateral caudal spinal trigeminal nucleus in laminae I/II after inferior alveolar nerve ligation in rats. The discrepancy may be due to early changes induced by acute inflammation. In the study by Leong and colleagues^[44], only 14% of c-Fos-positive neurons co-expressed nitric oxide synthase in the caudal trigeminal spinal nucleus 2 hours after formaldehyde injection. This is evidence that nitric oxide synthase in the caudal spinal trigeminal nucleus is not particularly involved in pain at the early stage, and that melatonin has a more apparent effect than nitric oxide in central maxillofacial pain transmission at the early stage of inflammatory pain.

In summary, in this study, we examined, for the first time, MT1 distribution in the caudal spinal trigeminal nucleus, and we show a downregulation of MT1 expression during early inflammatory pain. In addition, we found significant differences in MT1 and NADPH-d expression during early inflammatory pain. NADPH-d expression increases significantly in peripheral trigeminal ganglia, but remains unchanged in the central caudal spinal trigeminal nucleus. Furthermore, MT1 and NADPH-d expression in the mesencephalic trigeminal nucleus showed no significant difference. MT1 expression is significantly downregulated in the caudal spinal nucleus, but does not change in the trigeminal ganglia. Further study is required to determine whether the decreased MT1 expression in the central caudal trigeminal spinal nucleus can attenuate the analgesic effect of the melatonin/melatonin receptor/nitric oxide pathway.

MATERIALS AND METHODS

Design

A randomized controlled animal study.

Time and setting

Experiments were performed from August 2011 to April 2012. Animal experiments were performed in the Experimental Animal Center of Sun Yat-sen University (North Campus) in China, and histological experiments were performed in Guanghua School of Stomatology, Hospital of Stomatology, Sun Yat-sen University, Guangdong Provincial Key Laboratory of Stomatology in China.

Materials

Healthy adult female Sprague-Dawley rats, aged 6 weeks, weighing 190–220 g, and of specific pathogen free grade, were provided by the Experimental Animal Center of Sun Yat-sen University (North Campus), China with animal license number of SYXK (Yue) 2007-0081. Animals were housed in a quiet environment with good ventilation and air filtration systems, at 22–24°C and 55–65% humidity, under a 12-hour light-dark cycle, with free access to food. The feeding cages and bedding were changed daily. Experimental procedures were in accordance with the *Guidance Suggestions for the Care and Use of Laboratory Animals*, issued by the Ministry of Science and Technology of China^[60].

Methods

Establishment of maxillofacial inflammatory pain model

All animals were acclimatized to the feeding for 1 week, and were forced to exercise for 30 minutes per day 2 days prior to the experimentation. The feeding cage was a 47 cm × 25 cm × 21 cm transparent plexiglass box with the same lighting and temperature conditions. On the day of the experimentation, rats were fixed and rapidly injected with 50 µL of 1% formaldehyde (Guangdong Guanghua Chemical Factory Co., Ltd., Guangzhou, Guangdong Province, China) using a microsyringe *via* the right whisker pad (at the junction of the upper lip skin and mucous membranes). The control group received 50 µL saline injection^[61].

Pain behavioral tests

Rat behavior was recorded and evaluated immediately after injection, every 3 minutes, for a total of 2 hours. The number of times the rats scratched the facial injection site with their claws was recorded as a quantitative indicator of pain. At the same time, behavior was photographed using a high-definition digital camera (Shanghai Soughing Electronics Co., Ltd., Shanghai, China).

Harvesting specimens

All rats were anesthetized with 10% chloral hydrate (400 mg/kg) *via* intraperitoneal injection 2 hours after

injection. The heart was rinsed with 4°C 0.01 mol/L PBS and fixed in 4% paraformaldehyde, and the trigeminal ganglia and brainstem were harvested. The brainstem was labeled using a scalpel at the ventral side of brain stem opposite to the injection side, then fixed for 6–8 hours, gradient dehydrated in 10%, 20% and 30% sucrose, and preserved at 4°C. Specimens were sliced into successive frozen slices, with the trigeminal ganglion at 25-µm thickness, and the mesencephalic trigeminal nucleus and spinal trigeminal nucleus at 25-µm thickness. All slices were collected in 0.01 mol/L PBS.

Immunohistochemical staining for MT1 expression in the rat spinal trigeminal nucleus

The two adjacent sections of the spinal trigeminal nucleus were used for immunohistochemical staining and histochemical staining for MT1 and NADPH-d, respectively. Slices were rinsed with 0.01 mol/L PBS three times, for 5 minutes each, and incubated with 0.3% H₂O₂ at room temperature for 20 minutes to eliminate endogenous peroxidase activity. After another 0.01 mol/L PBS wash, Ultra V Block (LabVision, Kalamazoo, MI, USA) was applied to block non-specific antigens for 20 minutes at room temperature. Slices were then incubated with rabbit anti-rat MT1 polyclonal antibody (1:200; Abbiotec Company, San Diego, CA, USA) at 4°C overnight, then with biotinylated goat anti-rabbit secondary antibody (ABC immunohistochemistry kit, LabVision) at 37°C for 1 hour, and finally with SABC reagents at 37°C for 30 minutes. Between each step, slices were rinsed with 0.01 mol/L PBS. Afterwards, slices were developed with 3,3'-diaminobenzidine at room temperature. Negative controls were incubated with control antibody. All slices were mounted onto glass slides and air dried, followed by gradient ethanol dehydration, xylene clearing (twice, 10 minutes each), and neutral gum mounting.

Immunofluorescence staining for MT1 expression in rat trigeminal ganglia and mesencephalic trigeminal nucleus

The two adjacent sections of trigeminal ganglia and mesencephalic trigeminal nucleus were selected for MT1 immunofluorescence staining and NADPH-d histochemical staining. After rinsing in 0.01 mol/L PBS, three times for 5 minutes each, and blocking with 5% bovine serum albumin for 30 minutes, the sections were incubated with rabbit anti-rat MT1 polyclonal antibody (1:200; Abbiotec) at 4°C overnight and then with TMRITC-labeled donkey anti-rabbit IgG (1:200; Abcam Corporation, Cambridge, MA, USA) at 37°C in the dark for 1 hour. Between each step, sections were rinsed with 0.01 mol/L PBS three times, for 5 minutes each. Subsequently, the sections

were mounted in weak natural light with glycerol buffer (Tianjin Guangcheng Chemical Reagent Company, Tianjin, China), and stored at 4°C in the dark overnight. Negative controls were incubated without primary antibody.

Histochemical staining for NADPH-d expression in the rat spinal trigeminal nucleus, trigeminal ganglia and mesencephalic trigeminal nucleus

The sections of spinal trigeminal nucleus, trigeminal ganglia and mesencephalic trigeminal nucleus were rinsed with TBS three times for 5 minutes each and incubated with 0.1 mol/L Tris buffer (pH 8.0) containing 1 mmol/L NADPH-d (Sigma, St. Louis, MO, USA) and 0.1 mmol/L nitro blue tetrazolium (Sigma) at 37°C for 2 hours, followed by another TBS wash. Negative controls were incubated without NADPH-d or NBT at 37°C for 2 hours. Slices were mounted on glass slides, air dried, gradient ethanol dehydrated, xylene cleared (twice, 10 minutes each), and mounted with neutral gum.

Image analysis

Images were collected under an optical microscope (Axioskop 40, Zeiss, Jena, Germany) using a charge-coupled device camera (Carl Zeiss, Hallbergmoos, Jena, Germany). Images were analyzed using a photo MicroGraph digitized integration system (Carl Zeiss) and Image Pro-Plus 6.0 software (Media Cybernetics, Silver Spring, MD, USA). From each rat, three slices were randomly selected for analysis of two fields under 20 × magnification^[62]. The integrated absorbance of MT1 in the caudal spinal trigeminal nucleus, as well as of MT1 and NADPH-d in the mesencephalic trigeminal nucleus, was recorded. The number of NADPH-d-positive neurons was also measured in the caudal trigeminal spinal nucleus. The caudal spinal trigeminal neurons are divided into three sub-groups: small- (< 85 μm²), medium- (85–150 μm²), large-sized (> 150 μm²); and the trigeminal ganglion neurons are divided into three sub-groups: small (< 30 μm), medium (30–40 μm) and large (> 40 μm). The percentage of MT1- and NADPH-d-positive neurons of varying sizes in the trigeminal ganglion was calculated. Only those cells with visible nuclei were included and counted.

Statistical analysis

All data are expressed as mean ± SD, and statistically analyzed using one-way and two-way analyses of variance. When analysis of variance showed significant differences, the mean pairwise comparisons were done using Student-Newman-Keuls *post-hoc* test. Differences of the mean value between groups were compared with paired *t*-test. *P* < 0.05 was considered statistically signif-

icant. All statistical analyses were performed using Sigma Stat for Windows (version 3.1; Jandel Corporation, Las Vegas, NV, USA).

Research background: Only a few studies on the melatonin regulation of pain have investigated membrane receptors at the protein level. It has been reported that melatonin can regulate pain *via* melatonin receptor 2, but the function of melatonin receptor 1 remains unclear.

Research frontiers: Very little is known about the interactions of melatonin and melatonin receptors with nitric oxide in the orofacial pain transmission process.

Clinical significance: Our findings will further our understanding of the effect of orofacial inflammatory pain on the caudal spinal trigeminal nucleus and trigeminal ganglia, melatonin receptor 1 and nicotinamide adenine dinucleotide phosphate diaphorase expression. We investigate the mechanisms of orofacial inflammatory pain in an effort to help the development of new anti-pain strategies.

Academic terminology: Trigeminal sensory pathway: the pain, warmth and touch-pressure sensation pathways in the head and facial skin, as well as in the oral cavity and mucosa. It is composed of level 3 neurons that signal to the cerebral cortex, resulting in a feeling of consciousness.

Peer review: This is the first study to examine the distribution of melatonin receptor 1 in the caudal spinal nucleus in the formaldehyde pain model. The authors observed differences in melatonin receptor 1 expression as well as nicotinamide adenine dinucleotide phosphate diaphorase expression in the trigeminal ganglia and caudal spinal nucleus at the early stage of maxillofacial inflammatory pain. When melatonin receptor 1 expression in the caudal spinal nucleus was significantly reduced, melatonin's regulatory effect was attenuated.

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