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Electrophysiological effects of melatonin on rat trigeminal ganglion neurons that participate in nociception *in vitro*

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Abstract. – OBJECTIVE: Melatonin (MT) is a hormone mainly produced by the pineal gland. It may be involved in the regulation of nociception, the mechanisms of which remain unclear. In the present study, electrophysiological effects of MT on neurons were studied.

MATERIALS AND METHODS: The cultured neurons were isolated from Sprague-Dawley rats trigeminal ganglia (TG). The neuron was voltage clamped using the whole cell patch clamp technique. We recorded resting membrane potential, action potential threshold and number, action potential duration and GABA-activated inward currents in the presence of 0.01 μM, 10 μM MT, and in the absence of MT.

RESULTS: In the presence of high concentration of MT, the spontaneous action potential disappeared and action potential threshold was significantly increased. GABA-activated inward currents were recorded and blocked by GABAA receptor antagonist, bicuculline, in the majority of TG neurons (91% 40/44). Continuous perfusion of MT could cause a decrease of GABA-activated currents. The inhibiting effect was dose-dependent and irreversible.

CONCLUSIONS: The results suggest that MT has several electrophysiological effects on TG neurons, which may be involved in the peripheral mechanisms of orofacial pain.

Key Words
Melatonin, Electrophysiological effects, Gamma-aminobutyric acid, Trigeminal ganglion neurons.

Introduction

Melatonin (N-acetyl-5-methoxytriptamine; MT) is a hormone mainly produced by the pineal gland in all vertebrates and has a wide range of physiological functions such as control of circadian rhythms, regulation of sleep processes, protection against cancer biology, neuroprotective effect, promoting osteoblast differentiation and so on[1-5]. Recently, accumulating evidence has shown that MT plays an important role in pain regulation[6-8] and has been shown anti-nociceptive action, the mechanisms of which are largely unknown yet.

Sensory ganglia are the first station where sensations, including pain, are generated. The orofacial region is principally innervated by the trigeminal nerve. The trigeminal ganglion (TG), which is analogous to the dorsal root ganglia (DRG) of the spinal somatosensory system, is the location of the cell bodies of most trigeminal primary afferents[9]. Gamma-aminobutyric acid (GABA) is a major inhibitory neurotransmitter in the central nervous system (CNS) and involves in afferent transmission of nociceptive information. It has been reported that GABA and GABA receptors are localized in TG neurons[10]. MT potentiates the GABA-activated current (I_{GABA}) in cultured chick spinal cord neurons[11]. However, there is no information available on the effects of MT on TG neurons involved in I_{GABA}.

The aim of this study was to examine the *in vitro* response of TG neurons to MT. The electrophysiological effects induced by MT were evaluated using the whole-cell patch-clamp technique. The current work was undertaken to advance the understanding of the peripheral mechanisms of pain regulation by MT.

Materials and Methods

Acute Dissociation of TG Neurons

Acute dissociation of TG neurons was modified from the previous study[12]. Briefly, TG neurons were isolated from Sprague-Dawley rats (3-week old, either sex) anesthetized with so-

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dium pentobarbital (50 mg/kg, intraperitoneal injection). The vascular membrane was separated from the TG in cold-ice D-Hanks' balanced salt solution under a dissecting microscope. Then, the dissected TG were minced into small pieces and incubated in DMEM/F12 solution containing type I collagenase and 0.125% trypsin for 20 min at 37°C. After appropriate digestion, the cells were washed with DMEM/F12 culture medium supplemented with 10% fetal bovine serum (FBS). Thereafter, the cells were dissociated mechanically with fire-polished Pasteur pipettes and plated on poly-L-lysine-coated glass coverslips placed in 35 mm dishes. The cells were maintained in 5% CO_2 at 37°C and used for recordings between 2 and 8 h after plating.

Whole-Cell Patch-Clamp Recordings

All electrical measurements were conducted using whole-cell patch recordings and a patch-clamp amplifier with different concentrations of melatonin. The external solution contained 150 mM NaCl, 5 mM KCl, 2.5 mM $CaCl_2$, 2 mM $MgCl_2$, 10 mM HEPES, 10 mM D-glucose (PH=7.4). The internal solution contained 140 mM K-gluconate, 1 mM $CaCl_2$, 2.5 mM $MgCl_2$, 11 mM EGTA, 10 mM HEPES, 5 mM MgATP (PH=7.2). The recordings were performed at room temperature of 22-25°C. For the control groups, there was no MT exposure to the cells. For the experimental groups, the concentrations of the MT were 0.01 μM and 10 μM, respectively. The diameter of the neurons selected for whole-cell patch recordings ranged from 15 μm to 40 μm. Holding potential was -60 mV. Membrane currents were filtered at 2 kHz, digitized at 10 kHz. Both the capacitance and series resistance were well compensated. Membrane currents and membrane potential were analyzed by Clampfit 10.2 (Molecular Devices, San Jose, CA, USA) and Fitmaster 2.6 (HEKA Elektronik, Lambrecht/Pfalz, Germany).

After incubation, the coverslips were transferred to the recording chamber in a standard external solution containing 150 mM NaCl, 5 mM KCl, 2.5 mM $CaCl_2$, 2 mM $MgCl_2$, 10 mM HEPES, 10 mM D-glucose (PH=7.4). The recording chamber was mounted onto an inverted microscope equipped with phase-contrast filters, a video camera, and two micromanipulators. Under the inverted microscope, small and medium-diameter cells (15-40 μm) were selected. Fire-polished patch-pipettes were filled with external solution. In the current-clamp mode, the resting membrane potentials were recorded and action potentials were elicited by depolarizing current pulses (0-1000pA, 20pA steps, 30 ms). Initially, we determined the action potential thresholds, defined as the currents required for eliciting a single spike. Action-potential firing rates were assessed by counting the number of action potentials elicited by depolarizing pulses (twice the threshold currents). Spike duration was determined as the duration of the first spike at the half-amplitude level, as well as the duration at repolarization and duration at depolarization (Figure 1).

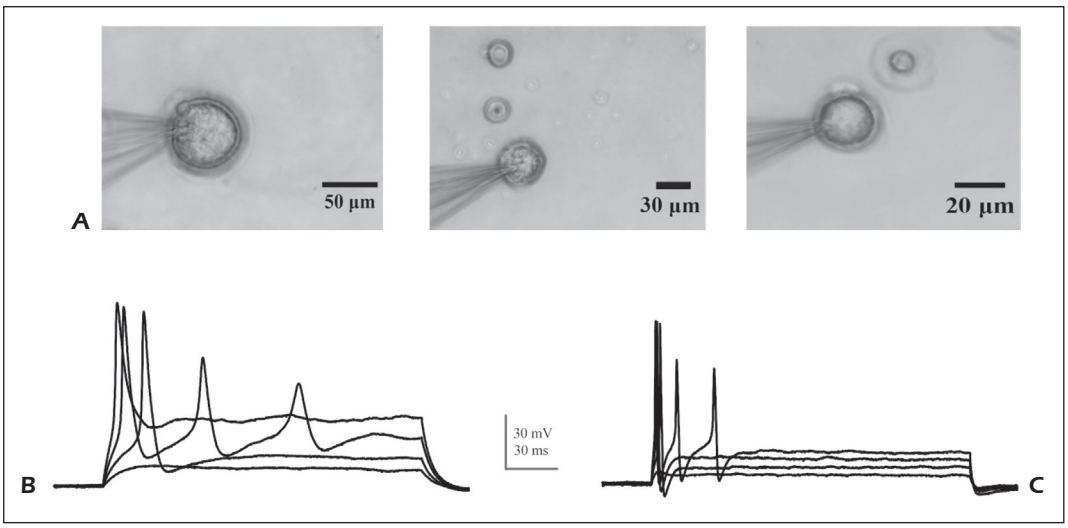

Figure 1. Diagram of whole-cell patch clamp recording. **A**, forming whole cell patch in the large-, medium-, and small-size neurons. **B**, Recording action potential in the small-size neurons (diameter < 30 μm). **C**, Recording action potential in the medium-size neurons (30-40 μm).

Drug Administration

Melatonin added to the external solution was continuously perfused during recording, while GABA (1000 μM) was ejected by DAD system (ALA, Westbury, NY, USA). GABA was typically applied for 2-3 s, at intervals of 4 min. During intervals, the cells were perfused continuously with an external solution.

Statistical Analysis

The indexes included resting membrane potential, action potential threshold, action potential threshold and number, action potential duration, I_{GABA}.

The effects of melatonin on I_{GABA} of TG medium or small neurons were measured by inhibition ratio. The inhibition ratio of various concentrations was calculated using the following equation.

$$\text{Inhibition ratio} = (I_{GABA\text{-}C} - I_{GABA\text{-}E})/I_{GABA\text{-}C} * 100\%$$

Where inhibition ration represents the effect of MT on I_{GABA} of TG medium or small neurons, $I_{GABA\text{-}C}$ represents the I_{GABA} of the control group and $I_{GABA\text{-}E}$ represents the I_{GABA} of the experimental groups. Numerical values are provided as the mean±SEM. Significant differences in the mean amplitude were tested using the *t*-test or paired *t*-test. Values of $p<0.05$ were considered significantly different. All statistical analyses were performed using SPSS16.0 (SPSS Inc., Chicago, IL, USA) and Visio 2010 Microsoftware.

Results

Effect of MT on the Neuronal Excitability of TG Neurons

To determine whether MT is related to the excitability of TG neurons, we first examined the resting membrane potential of small and medium-diameter TG neurons, in the presence of different concentrations of MT. The resting membrane potential recorded in the whole-cell patch clamp mode were showed in Figures 2A-B. MT perfusion caused change at the average resting membrane potential, but no significance

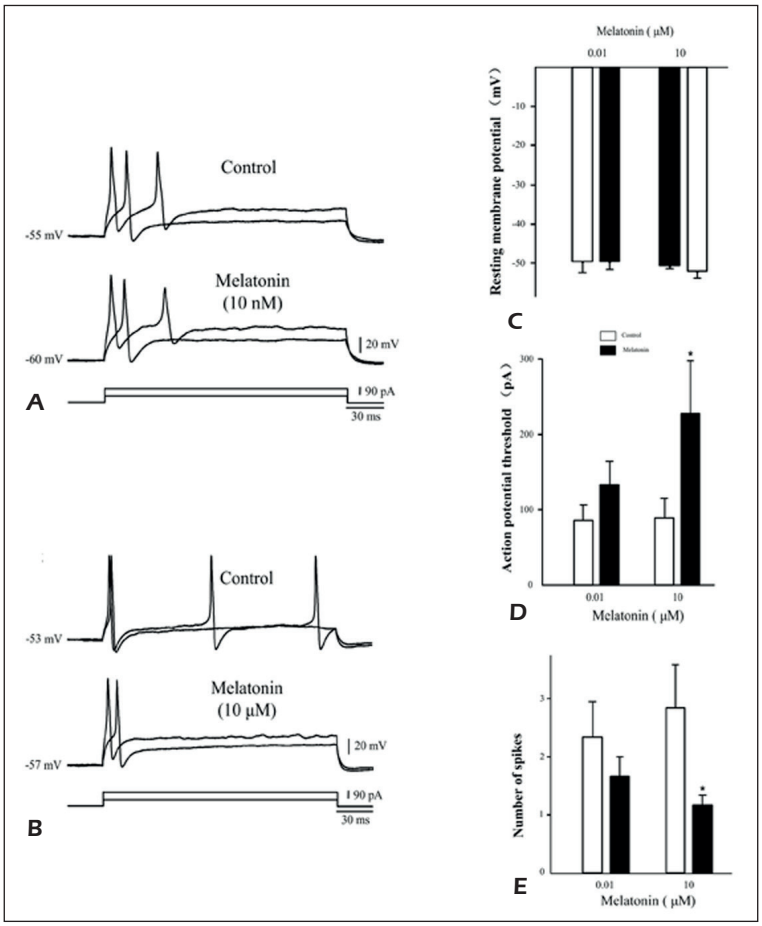

Figure 2. Effect of MT on action potential threshold and number in rat TG neurons. **A**, 0.01 μM group. **B**, 10 μM group. **C**, Showing statistic results of resting membrane potential. **D**, showing statistic results of action potential threshold. **E**, Showing statistic results of action potential threshold number. *significantly different at $p<0.05$ vs. control. (n = 8, 8 respectively).

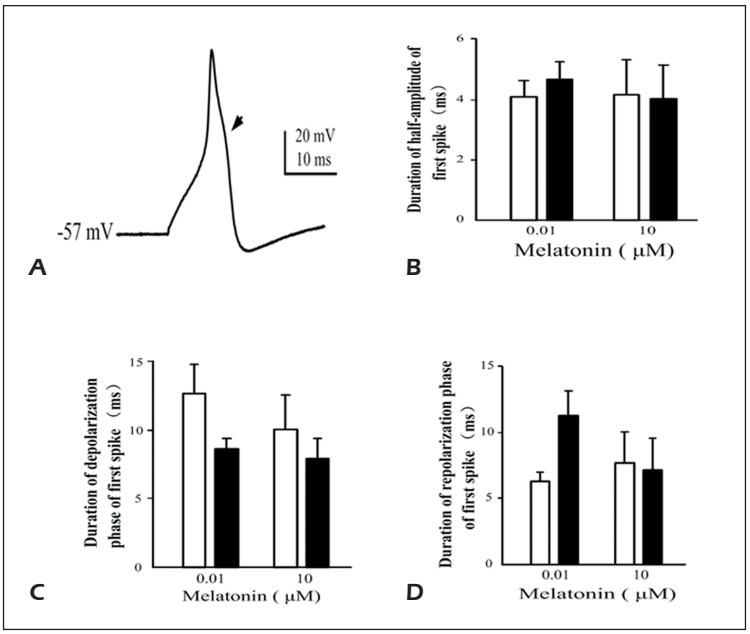

Figure 3. Effect of MT on action potential duration in rat TG neurons. **A**, Action potential of control group. **B**, Showing statistic results of action potential duration at half amplitude. **C**, Showing statistic results of action potential duration at depolarization. **D**, Showing statistic results of action potential duration at repolarization. (n = 8, 8 respectively).

between before and after MT perfusion (Figure 2C). Then, in the current-clamp mode, we recorded the action potential when the small and medium neurons were stimulated by a series of current stimulation (Figures 2A-B). In the presence of 10 μM MT, the action potential threshold was higher than that before MT perfusion ($p < 0.05$, Figure 2D). In the presence of 10 μM MT, 10 min, and stimulating by twice the threshold current, the number of spikes decreased when compared with that before MT perfusion ($p < 0.05$, Figure 2E).

We next investigated the effect of MT on action potential duration in rat TG neurons. Evoked by a depolarization current pulse, typical waveforms of action potential with a prominent shoulder on the repolarization phase were shown in Figure 3A. Action potential duration showed no significant change before and after 0.01 μM, 10 μM MT perfusion (Figures 3B-D). The first action potential at half amplitude, action potential duration at repolarization and depolarization were not significantly affected in the presence of MT compared with the control group.

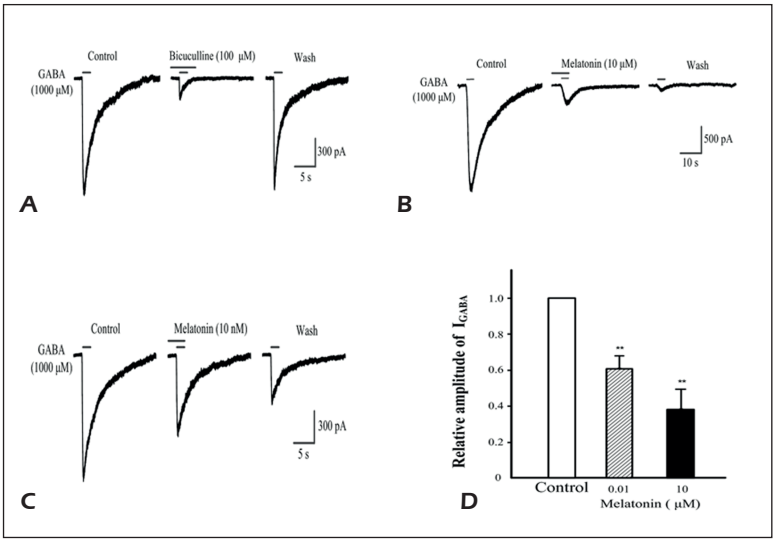

Figure 4. Inhibiting effect of MT on IGABA in rat TG neurons. **A**, IGABA could be reversibly blocked by bicuculline, an antagonist of GABAA receptor. **B**-**C**, Records of current traces show the inhibition of MT (0.01 μM, 10 μM) on IGABA. **D**, Inhibition rate of IGABA after perfusion of MT at different concentrations. **significantly different at $p<0.01$ vs. control. (n = 5, 5 respectively)

Effect of MT on GABA-Activated Currents of TG Neurons

We chose the isolated round cells for current recording, which had a clear outline and specific diameter (15-40 μm). The majority of cells examined, which is freshly isolated from the TG, responded to GABA (1000 μM) applied externally with an inward current (I_{GABA}) (91%, 44/47). The I_{GABA} could be blocked reversibly by $GABA_A$ receptor selective antagonist, bicuculline (100 μM).

In our study, MT of different concentrations (0.01 μM, 10 μM) was not able to elicit an inward current. Continuous perfusion of 0.01 μM, 10 μM MT could cause a decrease of I_{GABA}. The inhibiting effect was dose-dependent and irreversible (Figure 4B-D). In the presence of 0.01 μM and 10 μM MT, 3-10 min, the average inhibiting rate of I_{GABA} was 39.2±7.31% ($p < 0.01$), 61.7±11.15% ($p < 0.01$) (Figure 4D). The inhibiting effect of MT on I_{GABA} appeared dose-dependent, but the average amplitude of I_{GABA} decreased gradually (Figure 4D).

Discussion

MT has been previously found to inhibit the activity of CNS neurons including suprachiasmatic nucleus, hippocampal neurons, cerebellum and striatum neurons[13-16] but no data on primary sensory neurons was available. To test the effect of MT on the activity of sensory neurons, in the present study, we used *in vitro* culture systems to determine the response of TG neurons to MT.

MT exerts a wide range of physiological functions and many pharmacological effects. The physiological concentrations of MT range from 1 pM to 10 nM, while the pharmacological concentrations range from 1 μM to 100 μM[17]. We tested the effects of melatonin at 0.01 μM and 10 μM on the activity of TG neurons using the whole-cell patch-clamp technique. The results showed that in the presence of high concentration of MT, the spontaneous action potential disappeared and action potential threshold was significantly increased. These results suggest that high concentration of MT inhibits the excitability and activity of small and medium TG neurons. The electrophysiological results are in agreement with those obtained in CNS neurons *in vitro*[13-16]. The inhibitory effects might be attributed to regulation of ion channels by MT[18-21], which might be one mechanism of the analgesic effects of MT that showed *in vivo* studies[22-24]. The mechanism underlying the inhibition of MT in TG neurons is needed to be elucidated in detail. GABA is a major inhibitory neurotransmitter in the CNS[25]. However, morphologic studies reveal that GABA and its specific receptors are expressed in neuron cell bodies in TG[10]. The peripheral GABA system within TG cells might be a unit of function involved in the processing of the orofacial sensory input. Recently, evidence has showed that MT plays an important role in orofacial pain regulation and may be a promising drug in alleviating the pain[8]. In the present work, I_{GABA} were recorded and blocked by $GABA_A$ receptor antagonist, bicuculline, in the majority of TG neurons (91% 40/44). It suggests that GABA might act through $GABA_A$ receptor in TG since $GABA_A$ receptor is expressed in the majority of neurons of TG[10]. In addition, MT decreased I_{GABA} at physiological and pharmacological concentrations. The inhibitory effect of MT was dose-dependent. The results indicate, for the first time, that MT can alter I_{GABA} in the TG neurons. In addition, the findings suggest that the effect of MT on neurons in sensory ganglion is not the same as that in the CNS since several studies[26,27] have demonstrated that MT increases the frequency of I_{GABA} in neurons of CNS. The TG is one peripheral location of $GABA/GABA_A$ receptor, the mechanisms of effect of MT on which is required to be illuminated.

Conclusions

We demonstrated that electrophysiological has effects of MT on rat TG neurons *in vitro*. Our results may contribute to further understanding the peripheral mechanisms of modulatory effects of MT. Since MT is safe and nontoxic, more studies should be conducted to explore the effects of MT with other drugs that are presently applied clinically.

Acknowledgments

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Conflict of interest

The Authors declare that they have no conflict of interest.

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