Full Paper

Melatonin Modulates the GABAergic Response in Cultured Rat Hippocampal Neurons

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Abstract. In the present study, we investigated the effect of melatonin on the GABA-induced current (I_{GABA}) and GABAergic miniature inhibitory postsynaptic currents (mIPSCs) in cultured rat hippocampal neurons using the whole-cell patch-clamp technique. We found that melatonin rapidly and reversibly enhanced I_{GABA} in a dose-dependent manner, with an EC_{50} of 949 μM . Melatonin markedly enhanced the peak amplitude of a subsaturating I_{GABA} but not that of a saturating I_{GABA} . Interestingly, melatonin was effective only when GABA and melatonin were applied together. Furthermore, the effect of melatonin on I_{GABA} was voltage-independent and did not change the ion selectivity of the GABA_A receptor. The melatonin enhancement on I_{GABA} can not be blocked by luzindole, a melatonin receptor antagonist, indicating that melatonin-induced I_{GABA} enhancement was not via activation of its own membrane receptors. However, this enhancement may be mediated via high-affinity benzodiazepine sites as it was inhibited by the classical benzodiazepine antagonist flumazenil, suggesting an allosteric modulation of melatonin by binding to the sites of GABA_A receptors. In addition, melatonin increased both amplitude and frequency of GABAergic mIPSCs, indicating that melatonin enhances GABAergic inhibitory transmission. Hence, our observation that melatonin has an enhancing effect on the GABAergic system may implicate a potential pathway for the neuroprotective effects of melatonin.

Keywords: melatonin, GABA_A receptor,
GABAergic miniature inhibitory postsynaptic current (mIPSC),
whole-cell patch-clamp technique, hippocampal neuron

Introduction

Melatonin, the main secretory product of the pineal gland, regulates many biological functions including circadian rhythm, sex maturation, immune responses, etc. In addition, as an intracellular antioxidant, melatonin exerts significant neuroprotection in different models of neuronal degeneration (1 – 8). Our previous studies have reported a dramatic decrease in cerebrospinal fluid (CSF) melatonin levels with increasing age, and even more so in Alzheimer's disease (AD) (9, 10).

The GABA type A receptor ($\text{GABA}_\text{A}\text{R}$) is one of the major inhibitory receptors in the adult central nervous system (CNS) (11 – 14). GABAergic neurotransmission

play a crucial role in regulating the balance between excitation and inhibition in CNS neural network activity. Occurrence of many nervous system diseases is related to the change of GABAergic inhibition, directly or indirectly, such as epilepsy, depression, and anxiety (15 – 19). Recently, much of the accumulated data indicate that the $\text{GABA}_\text{A}\text{R}$ on neurons is modulated by melatonin. Melatonin potentiates the GABA_A receptor-mediated current in cultured chick spinal cord neurons (20). On the other hand, it oppositely modulates the function of GABA_A receptors via differentially expressed melatonin receptors $\text{Mel}_{1\text{a}}$ and $\text{Mel}_{1\text{b}}$ in the rat suprachiasmatic nucleus and hippocampus (21). It is well known that the hippocampus is essential for memory formation and cognition (22), and highly vulnerable to many pathological insults (23, 24). Meanwhile, melatonin was found to modulate the hippocampal neuronal excitability and plasticity (25 – 27). However, whether melatonin has any direct

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effect on the GABA-induced current in the hippocampus is still unknown. Thus, in the present study, we investigated the effects of melatonin on GABA-induced currents (I_{GABA}) and GABAergic miniature inhibitory postsynaptic currents (mIPSCs) in cultured rat hippocampal neurons by using the whole-cell patch-clamp technique.

Materials and Methods

Cell culture

The use and care of animals in the present study followed the guidelines and protocols approved by the Institutional Animal Care and Use Committee of the University of Science and Technology of China. All efforts were made to minimize the number of animals used. The neurons used for cell culture were dissociated from the hippocampus of Wistar rats (postnatal day 0, 24 rats). The neonatal rat was deeply anesthetized with isoflurane, transferred to the chilled Hank's buffered salt solution, and then decapitated. The whole brain was removed and was placed in the iced Hank's buffered salt solution. The hippocampus was collected under a dissection microscope and neurons were isolated by a standard enzyme treatment protocol. Briefly, the tissues were incubated in saline with 0.25% trypsin (Sigma, St. Louis, MO, USA) for 15 min at 37°C and mechanically dissociated by trituration with a pipette tip in Dulbecco's modified Eagle's medium (DMEM) (Invitrogen/Gibco, Grand Island, NY, USA). The tissue was then plated (1.5×10^6 cell/ml) on a poly-L-lysine (Sigma)-coated cover glasses. The isolated neurons were grown in DMEM with L-glutamine plus 10% fetal bovine serum (FBS), 10% F-12 nutrient mixture, 100 U/ml penicillin, and 100 µg/ml streptomycin (Invitrogen/Gibco) for 24 h. Then, neuron-basal medium (1.5 ml) with 2% B₂₇ (Invitrogen/Gibco) was replaced every 3–4 days. The cultures were treated with 5-fluoro-5'-deoxyuridine (20 µg/ml, Sigma) on the fourth day after plating to block cell division of non-neuronal cells, which helped to stabilize the cell population. The cultures were maintained at 37°C in a 5% CO₂ humidified atmosphere. Cells were used for electrophysiological recordings 7–15 days after plating.

Solutions and drugs

The standard external solution contained 150 mM NaCl, 5 mM KCl, 1 mM MgCl₂, 2 mM CaCl₂, 10 mM glucose, and 10 mM Hepes. The pH was adjusted to 7.4 with Tris base. The osmolarity of the solutions was adjusted to 310–320 mOsm/l with sucrose. The pipette solution for whole-cell patch-clamp technique contained 120 mM KCl, 30 mM NaCl, 1 mM MgCl₂, 0.5 mM CaCl₂, 5 mM EGTA, 2 mM Mg-ATP, and 10 mM Hepes. The pH of the internal solution was adjusted to 7.2 with

Tris base. When the relationship between the effect of melatonin on the I_{GABA} and the membrane potential was examined, voltage-activated Na⁺, Ca²⁺, and K⁺ channels were blocked by adding 0.3 µM tetrodotoxin and 0.2 mM CdCl₂ to the standard external solution and by replacing K⁺ with Cs⁺ in the pipette solution. Drugs used in the present experiments were purchased from Sigma. Melatonin was initially dissolved as concentrated stocks solutions in dimethylsulphoxide (DMSO, Sigma) and subsequently diluted to the desired concentration in the standard external solution. In the vehicle controls, the highest final concentration of DMSO employed (0.1%) had no detectable effect on the GABA response in our experiments. Other drugs were first dissolved in ion-free water and then diluted to the final concentrations in the standard external solution just before use. Drugs were applied using a rapid application technique which is termed the 'Y-tube' method (28) throughout the experiments. This system allows a complete exchange of external solution surrounding a neuron within 20 ms.

Electrophysiological recordings

The electrophysiological recordings were performed in conventional whole-cell patch-recording configuration under the voltage-clamp mode. Patch pipettes were pulled from glass capillaries with an outer diameter of 1.5 mm on a two-stage puller (PP-830; Narishige, Tokyo). The resistance between the recording electrode filled with pipette solution and the reference electrode was 4–6 MΩ. Membrane currents were measured using a patch-clamp amplifier (Axon 200B; Axon Instruments, Union City, CA, USA), sampled using a Digidata 1320A interface connected to a personal computer, and analyzed with Clampex and Clampfit software (Version 8.1, Axon Instruments). The average input resistance was 507.4 ± 18.8 MΩ ($n = 24$), the average C_m was 12 ± 3.1 pF ($n = 24$), and the average series resistance was 10 ± 2.3 MΩ ($n = 24$). If the series resistance changed by > 20%, the data were discarded. The membrane potential was held at -50 mV throughout the experiment. All the experiments were carried out at room temperature (22°C–25°C).

Data analyses

Clampfit software (Version 8.1) and Origin (Version 7.5; OriginLab Corp., Northampton, MA, USA) were used for data analysis. All the data are represented as the mean ± S.E.M. Statistical significances between two groups were determined with Student's *t*-test. One way analysis of variance (ANOVA) was employed for statistical significance between multiple groups. The Mini Analysis Program (version 6.0; Synaptosoft, Inc., Decatur, GA, USA) was used to analyze mIPSCs. Cumu-

lative curves were compared using the Kolmogorov-Smirnov test for significant differences. Statistically significant differences were assumed as $P < 0.05$; P and n represent the value of significance and the number of neurons, respectively.

Results

Melatonin enhances the whole-cell current induced by GABA

Under the present experimental conditions, extracellular application of $10\ \mu\text{M}$ GABA evoked an inward current in all tested neurons at a holding potential (V_H) of $-50\ \text{mV}$. The I_{GABA} could be completely abolished by $10\ \mu\text{M}$ bicuculline, a selective GABA_A receptor antagonist, while the GABA_B agonist baclofen ($10\ \mu\text{M}$) failed to evoke any currents, suggesting that the currents were mediated by GABA_A receptors in the present preparation (Fig. 1A). Melatonin did not induce any detectable currents in these neurons when the drug was applied alone. However, melatonin rapidly and reversibly enhanced I_{GABA} in a concentration-dependent manner when it was co-applied with GABA (Fig. 1B). The averaged EC_{50} for melatonin enhancement of I_{GABA} was $949\ \mu\text{M}$ ($n = 5$, Fig. 1C).

Modulation of melatonin on I_{GABA} at different GABA concentrations

To investigate the mechanism of melatonin action on the GABA_A receptors, we examined the enhancement of I_{GABA} by $1\ \text{mM}$ melatonin at three different GABA concentrations. As shown in Fig. 2A, the enhancement of I_{GABA} by melatonin was dependent on the GABA concentration. At $10\ \mu\text{M}$ GABA, the enhancement was 1.58 ± 0.06 -fold, whereas at a saturating concentration ($1\ \text{mM}$), little effect was observed (Fig. 2B). Melatonin shifted the GABA dose-response curve to the left, decreasing the mean EC_{50} at GABA_A (Fig. 2C).

Comparison of melatonin on I_{GABA} with different drug application protocols

To further explore the mechanism by which melatonin enhanced I_{GABA} , we applied three different drug application protocols on the same neurons. In the pretreatment protocol (protocol b), neurons were pretreated with melatonin for $15\ \text{s}$ and then applied with melatonin and GABA together; in the sequential application protocol (protocol c), neurons were applied with GABA alone immediately after $15\ \text{s}$ perfusion of melatonin; in the co-application protocol (protocol a), neurons were co-applied with melatonin and GABA. As shown in Fig. 3A, the enhancement efficacy varied in the three drug application protocols. These enhancements were statistically significant when melatonin and GABA were applied to-

Fig. 1. Effect of melatonin at various concentrations on the I_{GABA} in cultured rat hippocampal neurons. A) Identification of bicuculline-sensitive, baclofen-insensitive GABA_A -receptor currents. B) Representative current traces induced by $10\ \mu\text{M}$ GABA in the presence or absence of melatonin at various concentrations. The enhancement of the peak amplitude of I_{GABA} depended on the concentration of melatonin. All the currents were recorded from one cell. Mel stands for melatonin. C) Concentration-response relationship for melatonin-induced enhancement of the I_{GABA} ($n = 5$). The holding potential (V_H) was $-50\ \text{mV}$. In this figure and the following figures, data are shown as the mean $\pm$ S.E.M.

gether (protocol a and b, $P < 0.01$ in each case; Fig. 3B), but the enhancement in I_{GABA} was not statistically significant with sequential application of melatonin and GABA (protocol c, $P > 0.05$; Fig. 3B).

Voltage-dependence of modulation of melatonin on I_{GABA}

To learn the voltage dependence of the effect of melatonin on I_{GABA} , we examined the effect of melatonin on the I_{GABA} by using a ramp protocol (Fig. 4A). The effect

Fig. 2. Effect of melatonin on I_{GABA} at different GABA concentrations. A) Actual currents induced by GABA at various concentrations in the absence or presence of 1 mM melatonin. Mel stands for melatonin. B) Statistical results illustrating effects of 1 mM melatonin on amplitude of I_{GABA} . The ordinate represents the ratio of I_{GABA} amplitudes to the control. * $P < 0.05$, ** $P < 0.01$, significantly different from the control (Student's paired t -test). C) The concentration-response relationships of the I_{GABA} in the presence or absence of 1 mM melatonin. These data were fitted with the Michaelis-Menten equation. For every neuron recorded, the currents were normalized to the peak amplitude of the I_{GABA} induced by 1 mM GABA and each point represents the averaged value of 4 neurons.

Fig. 3. Modulation of melatonin on I_{GABA} with different drug application protocols. A) Sample recordings demonstrating the modulation of 1 mM melatonin on 10 μ M I_{GABA} with three different drug application protocols (a, b, and c). Mel stands for melatonin. B) Statistical results of the ratio of I_{GABA} amplitudes to the control ($n = 5$) in the three application protocols. ** $P < 0.01$, significantly different from the control (Student's paired t -test).

of melatonin showed no voltage dependence over a range of membrane potential from -80 to $+80$ mV. The reversal potential of I_{GABA} in both the absence and the presence of melatonin were close to the theoretic chloride equilibrium potential, indicating that melatonin did not change the ion selectivity of GABA_{A} R.

Failure of luzindole to block melatonin-induced enhancement on I_{GABA}

To investigate whether the melatonin receptors are involved in the melatonin-induced enhancement on I_{GABA} , we observed the effects of luzindole, a melatonin-receptor antagonist, on the melatonin-induced I_{GABA} enhancement. Figure 5A shows typical effects of luzindole on the enhancement of melatonin. It shows that luzindole (100 μ M) did not affect I_{GABA} and the enhancement of melatonin. Figure 5B summarizes the results in five recorded neurons, showing that the I_{GABA} enhancement of melatonin (1 mM) were $152\% \pm 2.9\%$ and $157\% \pm 1.4\%$ in the absence and presence of luzindole, respectively ($P > 0.05$).

Fig. 4. Voltage-dependence of modulation of melatonin on I_{GABA} . A) Voltage-ramp protocol was applied for 10 μ M I_{GABA} with and without application of 1 mM melatonin, respectively. B) Current-voltage relationships derived from panel A. Melatonin did not alter the reversal potential of I_{GABA} . Mel stands for melatonin.

Flumazenil inhibits the enhancement of melatonin on I_{GABA}

To investigate whether melatonin acts through the benzodiazepine sites to enhance the GABA response, we observed the effects of flumazenil, the classical benzodiazepine antagonist, on the melatonin-induced I_{GABA} enhancement. Flumazenil inhibits the enhancement of melatonin on I_{GABA} (Fig. 6A). Figure 6B summarizes the results in four recorded neurons, showing that the I_{GABA} enhancement of melatonin (1 mM) was $152\% \pm 5.5\%$ and $102\% \pm 1.7\%$ in the absence and presence of flumazenil, respectively ($P < 0.05$).

Melatonin modulation of GABAergic mIPSCs

GABAergic spontaneous mIPSCs were recorded at a V_H of -50 mV in cultured rat hippocampal neurons, while superfusing the tested neurons with 10 μ M DL-2-amino-5-phosphovaleric acid (APV), 3 μ M 6-cyano-7-nitroquinoxaline-2,3-dione (CNQX), 0.3 μ M strychnine, and 0.3

Fig. 5. Failure of luzindole to block melatonin-induced I_{GABA} enhancement. A) Representative effects of 100 μ M luzindole on 1 mM melatonin-induced I_{GABA} enhancement. B) Statistical results demonstrating that luzindole could not block melatonin's effect. Mel stands for melatonin. L stands for luzindole. ** $P < 0.01$, significantly different from the control (Student's paired t -test).

μ M tetrodotoxin (TTX), which fully blocked the action potential generation in our preparation. As expected, these events disappeared after addition of 10 μ M bicuculline. In order to investigate the effects of melatonin on GABAergic neurotransmission, the activity was recorded from each neuron in the control condition, during melatonin application, and after washing for 5–8 min. As illustrated in Fig. 7B, application of 1 mM melatonin increased both the amplitude and the frequency of GABAergic mIPSCs. These effects were quantitatively demonstrated by comparison of the cumulative curve: melatonin induced both a shift to the right of the cumulative curve of peak amplitude and a shift to the left of the cumulative of inter-event intervals (Fig. 7D). Similar results were obtained in the other four experiments performed under identical conditions. As shown in Fig. 7E, melatonin increased the amplitude, frequency, and decay time to $173\% \pm 34.8\%$ ($P < 0.05$), $183\% \pm 44.4\%$ ($P < 0.05$), and $148\% \pm 17.2\%$ ($P < 0.05$), respectively, whereas the rise time was not significantly affected ($94.5\% \pm 2.6\%$, $P > 0.05$).

Fig. 6. Flumazenil inhibits the enhancement of melatonin on I_{GABA} . A) Representative effects of 10 μM flumazenil on 1 mM melatonin-induced I_{GABA} enhancement. B) Statistical results demonstrating that flumazenil can block melatonin's effect. Mel stands for melatonin. F stands for flumazenil. ** $P < 0.01$, significantly different from the control (Student's paired t -test).

Discussion

In the present research, we used the whole-cell patch-clamp technique to study the effects of melatonin on the GABAergic response in cultured rat hippocampal neurons. In our study, although melatonin did not activate currents by itself, it could significantly enhance I_{GABA} . The time course of melatonin enhancement of GABA $_A$ Rs was very rapid, and the response was completely reversed after washout. These effects are compatible with the fact that melatonin acts directly on the GABA $_A$ Rs. The observed enhancement in I_{GABA} caused by melatonin depended on concentrations of both melatonin and GABA (Figs. 1 and 2). Melatonin's EC_{50} for GABA $_A$ Rs was about 949 μM (Fig. 1C). Melatonin was more potent in enhancing I_{GABA} induced by low GABA concentration (Fig. 2). These results suggest that the melatonin effects may induce an altered potency of GABA $_A$ R rather than a change in receptor efficacy (20, 29).

In our experiment, the potency of melatonin enhancement on I_{GABA} is dependent on the sequence of drug application (Fig. 3). The enhancement of I_{GABA} was only seen when GABA and melatonin were applied together. Sequential application of melatonin and GABA did not result in a significant enhancement of I_{GABA} . Because GABA was applied immediately after melatonin pretreatment, it is unlikely that the effect of melatonin on I_{GABA} is attributed to a metabolic process associated with second messenger production (30). Besides, the reversal potential was close to the theoretic chloride equilibrium potential during melatonin application (Fig. 4), indicating that melatonin enhanced I_{GABA} without changing the ion selectivity of GABA $_A$ Rs. In addition, the effect of melatonin on I_{GABA} showed no voltage dependence, and no rebound current was observed in all experiments after the washout of melatonin and GABA. These results excluded the possibility that melatonin acts as an open-channel blocker.

Previous study has suggested that melatonin (1 nM) oppositely modulates the function of GABA $_A$ Rs via differentially expressed melatonin receptors Mel $_{1a}$ and Mel $_{1b}$ in the rat suprachiasmatic nucleus and hippocampus (21). It seems unlikely that the modulatory effects of melatonin on the GABA $_A$ Rs reported here are mediated by melatonin receptors. Firstly, luzindole, a specific melatonin-receptor antagonist (31), could not block the melatonin's effects. In addition, it was previously demonstrated that the melatonin concentration needed to activate melatonin receptors is of the micromolar order (20, 21, 32). It was found in the present work, however, that the modulatory effects of melatonin were not observed at concentrations lower than 100 μM . In addition to melatonin, the GABA $_A$ receptors are also positively modulated by benzodiazepines and steroids (33). It was shown in cultured chick spinal cord neurons that melatonin enhanced the GABA-induced current in a dose-dependent manner, with an EC_{50} of 716 μM , which was thought to be caused by melatonin acting on a site different from that for steroid (20). The current study showed that melatonin enhancement on I_{GABA} was inhibited by the classical benzodiazepine antagonist flumazenil, indicating that this enhancement may be mediated via high-affinity benzodiazepine sites (34, 35). On the basis of the above observations, we suggest that melatonin most probably exerts positive allosteric modulation.

To explore the melatonin effects on the GABAergic neurotransmission, we examined the modulatory effects of melatonin on GABAergic mIPSCs. Our results showed that melatonin increased both the amplitude and the frequency of GABAergic mIPSCs (Fig. 7). The increased amplitude and frequency of mIPSCs could be recovered to normal levels by washout with standard external solu-

Fig. 7. Effect of melatonin on GABAergic mIPSCs. A) Recording of GABAergic mIPSCs at the V_H of -50 mV in the presence of $0.3 \mu\text{M}$ TTX, $0.3 \mu\text{M}$ strychnine, $3 \mu\text{M}$ CNQX, and $10 \mu\text{M}$ AP-5. However, not a single one was detectable after perfusion of $10 \mu\text{M}$ bicuculline. B) Sample recordings showing the GABAergic mIPSCs in the absence or presence of 1 mM melatonin. C) Averaged trace of 191 and 236 GABAergic mIPSCs before and after application of melatonin, respectively. They are superimposed for comparison. D) Normalized cumulative curves showing the modulatory effects of melatonin on the amplitude and frequency of mIPSCs from the sample neuron. E) Statistical analysis of the effects of melatonin on the amplitude, frequency, decay time, and rise time of GABAergic mIPSCs. Each column represents the averaged value of five neurons. * $P < 0.05$, significantly different from the control (Student's paired t -test).

tion. These results suggest that melatonin can reversibly increase the amplitude and the frequency of mIPSCs in cultured rat hippocampal neurons. The enhancement of melatonin on the amplitude of mIPSCs indicates a post-

synaptic mechanism. We have demonstrated that melatonin enhanced the function of GABA_A via allosteric regulation. So it is not strange that melatonin increased the amplitude of GABAergic mIPSCs, which reflected

the increase of postsynaptic GABA_AR function. In contrast, the enhancement of melatonin on the frequency of mIPSCs indicates a presynaptic mechanism. There are several potential mechanisms underlying the enhancement. First, melatonin might increase Ca²⁺ influxes through voltage-gated Ca²⁺ channels in the presynaptic nerve terminals, resulting in the increase of GABA exocytosis. Second, melatonin might inhibit K⁺ channels, thus depolarizing the presynaptic membrane and increasing synaptic release. Finally, melatonin might activate intracellular signal transduction pathways and increase transmitter release machinery in presynaptic terminals.

The reported plasma concentrations of endogenous melatonin in humans at night are in the nanomolar range (36), below the concentration range (> 100 μM) at which neuromodulation is observed. This may indicate that melatonin is not the best candidate for being an endogenous modulator of the GABA_AR. So the dose used in the present study is a pharmacological dose. A similar high concentration of melatonin (500 μM) was needed to protect neurons in vitro from the kainite-induced excitotoxicity, thought to be due to the capability of melatonin to scavenge free radicals (37). This might be related to the characteristics of the in vitro system. Our observation that melatonin at higher concentrations is able to enhance the GABA response may also contribute to the neuroprotective effect of melatonin.

Melatonin is a secretory product that plays not only a major role in the regulation of the circadian rhythms, but also exerts neuroprotection in different models of neuronal degeneration (10). For example, the existence of a relationship between melatonin and seizures has been reported. Various studies suggest that melatonin is an anticonvulsant substance (38–43), but the mechanisms of its anticonvulsant actions are not fully understood. A loss of hippocampal GABAergic inhibition is believed to underlie the neuronal hyperexcitability in human temporal lobe epilepsy. In fact, it is well known that complete blockade of GABA_A receptors can readily induce epileptiform activity in hippocampal slices (44). The results of the present study therefore open a new field for explaining anticonvulsant actions of melatonin. Since melatonin is safe and nontoxic, more experimental studies should be conducted to explore the synergetic actions of melatonin with other drugs that are presently applied clinically.

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