Sexually Dimorphic Modulation of GABA_A Receptor Currents by Melatonin in Rat Gonadotropin-Releasing Hormone Neurons

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Abstract: Gonadotropin-releasing hormone (GnRH) neurons represent the final output neurons in the central control of reproduction. γ -Amino butyric acid (GABA), one of the major regulators of GnRH neurons, depolarizes GnRH neurons isolated from adult rats via GABA_A receptors. The presence of GABA_A receptors in GnRH neurons has also been demonstrated morphologically. Furthermore, the pineal hormone melatonin is involved in the regulation of reproductive function, including the timing of the luteinizing hormone surge. The suprachiasmatic nucleus and the GABAergic system in the medial preoptic area are considered as possible sites of the action of melatonin. Until now, however, a direct action of melatonin on GnRH neurons has not been reported. Therefore we examined the effect of melatonin on GABA_A receptor currents in GnRH neurons isolated from

GnRH-EGFP transgenic rats by means of perforated patch-clamp experiments. The GABA_A receptor currents were modulated by melatonin in a sex-specific manner. In GnRH neurons from adult males, melatonin augmented these currents in 67% of the neurons examined, but attenuated the currents in only 19% of them. These modulations were blocked by the melatonin receptor antagonist luzindole, suggesting an involvement of melatonin receptors. The modulation by melatonin was not observed in GnRH neurons isolated from infantile rats. These findings indicate that GABA affects the excitability of GnRH neurons in adult rats through GABA_A receptors, and that melatonin modifies this excitability via melatonin receptors in a sex-specific manner.

Key words: GnRH neuron, LHRH, GABA_A, melatonin, patch-clamp.

Gonadotropin-releasing hormone (GnRH) neurons constitute the final common pathway in the neuroendocrine control of reproduction. γ -Amino butyric acid (GABA), a major regulator of GnRH neurons, depolarizes GnRH neurons from adult mice [1] and rats [2] via GABA_A receptors, though Han *et al.* [3] reported that GABA hyperpolarizes GnRH neurons from adult mice. The presence of GABA_A receptors in GnRH neurons has also been demonstrated morphologically [4]. It is also suggested that the GABAergic system plays a role in the luteinizing hormone (LH) surge [5, 6], though its precise mechanism has not been clarified. Furthermore, pineal hormone melatonin is involved in the regulation of reproductive functions, including the timing of the LH surge [7–9] and the modulation of circadian rhythms in mammals [10–13]. There are several possible sites of melatonin action. The suprachiasmatic nucleus (SCN) expresses melatonin receptors [10, 14] and has a direct neuronal pathway to GnRH neurons [15]. Melatonin has been reported to affect the GABAergic neurotransmission in the rat medial preoptic

area (mPOA) [16], which contains most of the GnRH neurons [17, 18]. We find it interesting that GABA_A receptor currents are modulated by melatonin in neurons in the rat suprachiasmatic nucleus and hippocampus [19], chick spinal cord [20], and carp retina [21]. Two types of melatonin receptors are involved in these modulations in mammals: MT1 and MT2 [10, 22–24]. Their presence has been demonstrated in the brain, including in the mPOA [25, 26]. Moreover, a murine clonal cell line of GnRH neurons, GT1-7, expresses melatonin receptors [27]. These previous findings led us to speculate that melatonin modulates GABA_A receptor currents in GnRH neurons, thereby modifying the function of GnRH neurons as well as the above-mentioned mechanisms. To test this possibility, we examined the effects of GABA and melatonin in GnRH neurons isolated from GnRH-EGFP transgenic rats by means of perforated patch-clamp experiments. We revealed that GABA activated GABA_A receptor currents in the cell body of rat GnRH neurons, and these were modulated by melatonin in a sex-specific manner.

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MATERIALS AND METHODS

All experiments were performed with the approval of the Nippon Medical School Animal Care Committee. Rats expressing enhanced green fluorescent protein (EGFP) under the control of the GnRH promoter [28] were used in the present studies. In these rats, EGFP is exclusively expressed in GnRH neurons identified by immunostaining with anti-GnRH antibody, and 60%–70% of the immunoreactive GnRH neurons express EGFP [28]. The rats had free access to water and rat chow and were kept under a 14-h light, 10-h dark cycle. Eight rats (4 males and 4 females) 14 days old and 60 rats 2–3 months old were used.

Short-term dissociated culture. The brains were excised from the rats under ether anesthesia. The medial septum, diagonal band of Broca, organum vasculosum of the lamina terminalis, and medial preoptic area were cut out using razor and surgical blades. The sections were minced and treated with papain (21 U/ml; Funakoshi, Tokyo, Japan) for 45 min at 30°C with gentle agitation. The tissues were triturated with a 5 ml plastic pipette after several washes with DMEM (Sigma, St. Louis, MO). The cell suspension was subjected to discontinuous Percoll (Amersham Biosciences, Uppsala, Sweden) density gradient centrifugation composed of 1.0, 1.023, and 1.078 g/ml layers. The cells obtained from the middle layer were plated on polylysine-coated cover slips and then incubated overnight in Neurobasal-A medium (Invitrogen, Carlsbad, CA) supplemented with 0.5 mM L-glutamine and B-27 (Invitrogen) at 37°C. Most of the GnRH neurons were either round or spindle shaped, and some possessed short processes.

Electrophysiology. A List EPC-9 patch-clamp system (HEKA Elektronik, Lambrecht/Pfalz, Germany) was used for recordings and data analyses. The GnRH neurons were identified as EGFP-positive ones under inverted fluorescence microscope (IX70; Olympus, Tokyo, Japan). Whole-cell currents were measured by means of the perforated patch-clamp technique with amphotericin B (Seikagaku Corp., Tokyo, Japan) [28] at room temperature (25°C). The final concentration of amphotericin B in the pipette solution was 0.05 mg/ml. The pipette solution consisted of (mM) 95 cesium aspartate, 47.5 CsCl, 1.0 MgCl₂, 0.1 EGTA, and 10 HEPES (pH 7.2), the osmolality being adjusted to 270 mOsm. The extracellular solution consisted of (mM) 137.5 NaCl, 5 KCl, 2.5 CaCl₂, 0.8 MgCl₂, 0.6 NaHCO₃, 10 glucose, and 20 HEPES (pH 7.4), the osmolality being adjusted to 300 mOsm. Pipettes were fabricated from borosilicate glass capillaries and had a resistance of 6–8 MΩ. A pipette was targeted to a GnRH neuron. After the cell had been touched, slight negative pressure was applied to the pipette, which led to a seal resistance of ~5 GΩ. Perforation with amphotericin B was achieved within 3–7 min after giga-seal formation. The currents were filtered at 2.3 kHz, digitized at 10 kHz, and

Fig. 1. GABA-induced currents in GnRH neurons. **A:** GABA was applied with a 600 ms puff, as indicated with the horizontal bar above current traces, at a holding potential of -80 mV. Current traces activated by various concentrations of GABA are shown. The number for each trace indicates the micromolar concentration of GABA. **B:** Concentration–response relationship of GABA. Peak currents activated by various concentrations of GABA are plotted. Each filled circle with an error bar indicates the mean of 103 cells with 30 μM GABA, and of 3–5 cells with other concentrations. **C:** Example traces for determining the reversal potential of GABA using the amphotericin B perforated patch-clamp technique in which the $[Cl^-]_i$ was nearly equalized with the $[Cl^-]$ in the pipette solution. We applied 30 μM GABA at holding potentials ranging from -80 mV to 0 mV. In this cell, the current was reversed between -40 and -30 mV. **D:** Reversal potential of GABA-induced currents. The peak currents are plotted against the holding potentials. Each filled circle with an error bar indicates the mean of 5 cells. The interpolated estimation of the reversal potential was -28.8 mV, which is identical to the equilibrium potential of Cl⁻ under the present experimental condition ($[Cl^-]_o$, 149.1 mM; $[Cl^-]$ in the pipette solution, 49.5 mM). The straight line is drawn by the least-square method.

then stored. The series resistance was 70% electronically compensated. Data were taken when the series resistance was stable and less than 30 M Ω . The cell capacitance was 11.3 ± 2.2 pF (mean $\pm$ SD, $n = 103$). Leak currents were not subtracted.

GABA was applied with a 600 ms puff (41 kPa) through a glass pipette with an inner tip diameter of ~ 6 μ m. The pipette was connected to a Pressure System IIe (Toohey, Fairfield, NJ), and the tip was positioned 30–40 μ m from the cell. Other drugs were bath applied through perfusion with a flow rate of 1 ml/min. Data were taken only when the GABA-induced peak current at -80 mV was more negative than -200 pA, except the experiment of concentration–response relationship.

Chemicals. GABA was obtained from Wako Junyaku (Kyoto). Melatonin, luzindole, and bicuculline were from Sigma. All drugs were prepared on the day of use.

Statistics. Data are expressed as means $\pm$ SEM. The paired t -test, the Kruskal-Wallis test, and the Mann-Whitney U -test were used for statistical analysis. The significance level was set at $P < 0.05$.

RESULTS

Response to GABA and the effect of bicuculline

GABA elicited inward currents at a membrane potential of -80 mV in a concentration-dependent manner from 1 to 100 μ M (Fig. 1). The half-maximal concentration of GABA was 30.5 μ M ($n = 103$). There was no apparent attenuation of the current during the 600 ms application of GABA at concentrations of 100 μ M or lower. Above this concentration, the response to GABA was clearly desensitized. Therefore 30 μ M GABA was used in the following experiments. The GABA-induced current was -460 ± 29 pA ($n = 103$) at -80 mV and was reversed at -28.8 ± 2.7 mV ($n = 5$). This reversal potential is close to the equilibrium potential of Cl[−] (-27.8 mV), as calculated from the Cl[−] concentrations of the extracellular and pipette solutions (Fig. 1, C and D). The GABA_A receptor antagonist bicuculline (see [29, 30] for reviews) blocked these GABA-induced currents (Fig. 2). Bicuculline suppressed the response by $20\% \pm 2.7\%$ ($n = 5$) at 1 μ M, $64\% \pm 4.3\%$ ($n = 4$) at 10 μ M, and $96\% \pm 0.2\%$ ($n = 4$) at 100 μ M. The effect of the blocker was statistically significant ($P < 0.001$, Kruskal-Wallis test) at all concentrations examined.

Effects of melatonin

After the control current had been recorded, melatonin was introduced from 5 min prior to the application of GABA. It modulated the response to GABA in a sex-specific manner at concentrations of 10 nM or higher (Fig. 3). In female GnRH neurons, melatonin attenuated the GABA-induced response in 68% (17/25) of the neurons

Fig. 2. GABA_A receptor antagonist blocked the response. Upper panel: Example traces. Bicuculline, bath-applied from 5 min before application of GABA concentration-dependently suppressed the response to GABA. Bicuculline (100 μ M) almost completely blocked the response. The lower panel shows the concentration response relationship of bicuculline ($n = 4$ –5). IC₅₀ was 4.4 μ M. ** $P < 0.001$ vs. control by paired t -test.

examined at 10 nM, in 59% (10/17) at 100 nM, and in 56% (19/34) at 1 μ M, but augmented the response in only 16% of the neurons examined at 10 nM, in 18% at 100 nM, and in 35% at 1 μ M. In male GnRH neurons, attenuation was observed in only 23% (5/22) of the neurons examined at 10 nM, in 16% (3/19) at 100 nM, and in 14% (4/28) at 1 μ M, whereas the augmentation was observed in 64% at 10 nM, in 69% at 100 nM, and in 75% at 1 μ M. Therefore the attenuation of currents was dominant in females, and their augmentation was dominant in males. The difference between males and females was statistically significant (U -test, $P < 0.05$). Melatonin did not modulate the GABA-induced currents in GnRH neurons from infantile rats (14 days old) of either sex (Fig. 3C).

These attenuations and augmentations by melatonin were blocked by 1 μ M luzindole (melatonin receptor antagonist) [31, 32], as shown in Fig. 4, A and B. Furthermore, these effects of melatonin on the GABA-induced response were completely eliminated by overnight treatment with 100 ng/ml pertussis toxin (PTX; Fig. 4, C and D).

Fig. 3. Melatonin modulated the response to GABA in a sex-specific manner in GnRH neurons from adults, but not from infantile rats. **A:** Example responses induced by 30 μ M GABA and their modulation by 100 nM melatonin in GnRH neurons from adult rats. **B:** Frequency histogram of the melatonin action on the response to 30 μ M GABA. Abscissa, % change produced by melatonin at the concentrations indicated; –, reduction; +, augmentation. The bin was set at 5%. Open bars, data from females; closed bars, data from males. Numerals indicate the number of cells. **C:** Effect of melatonin on the response to GABA in GnRH neurons from 14-day-old (infantile) rats.

DISCUSSION

The present experiments confirmed that GABA evokes membrane currents via GABA_A receptors in rat GnRH neurons and revealed that melatonin modulated these currents. This modulation was observed with 10 nM or higher concentrations of melatonin. Melatonin is released from the pineal gland and reaches central neurons through either the cerebrospinal fluid or blood circulation. The maximum concentrations of melatonin in the cerebrospinal fluid are 400 pM in rats [33], 20 nM in ewes [34, 35], 200 pM in rhesus monkeys (see [36] for review), and 1–10 nM in humans [37, 38]. The melatonin concentration is higher in the cerebrospinal fluid than in the blood [39, 40], suggesting that melatonin acts on central neurons through the cerebrospinal fluid.

The modulation of GABA_A receptor currents by melatonin is probably mediated through G_i/G_o-coupled melatonin receptors, either MT1 or MT2 [41, 42], because the modulation was completely blocked by the melatonin receptor antagonist luzindole [31, 32] and by pretreatment with PTX [43]. Luzindole has been reported to be a selective antagonist of MT2 with a K_i of 10^{-8} M, but blocks both MT1 and MT2 at higher concentrations [44]. In the present experiments, luzindole was effective only at concentrations higher than 1 μ M, which block both subtypes of melatonin receptors. Therefore we could not determine pharmacologically whether MT1 or MT2 is involved.

It is interesting that there was sexual dimorphism in the modulation of currents by melatonin. In GnRH neurons from adult males, melatonin augmented the GABA_A receptor currents in 70% of the neurons examined, but atten-

Fig. 4. Effects of luzindole and PTX. **A, B:** The action of melatonin was blocked by the melatonin receptor antagonist luzindole. After recording the control response to melatonin, 1 μM luzindole was introduced with melatonin from 5 min before the application of GABA, reversibly blocking both the attenuation (**A**) and augmentation (**B**) induced by melatonin. **C, D:** Pertussis toxin prevented the modulation by melatonin. The cells were incubated overnight with PTX (100 ng/ml). This treatment suppressed the effect of melatonin (100 nM, 1 μM) on the GABA-induced currents. **C:** Example traces are shown. Four traces are overlapped. **D:** Frequency histogram. 1 μM melatonin had no effect.

uated them in only 18% of neurons. However, in GnRH neurons from adult females, melatonin augmented the currents in only 25% of the neurons examined, but attenuated them in 61% of neurons. Wang *et al.* [19] reported that the activation of MT1 augments the GABA_A receptor currents, and the activation of MT2 attenuates these currents as judged from gene expression and patch-clamp experiments on HEK293 cells. Taken together, these find-

ings suggest that the expression of MT1 is higher in male GnRH neurons than in female ones, and that the expression of MT2 is higher in female GnRH neurons than in male ones.

In physiological implications, the generation of action potentials is essential to release GnRH at the nerve terminal of GnRH neurons in the median eminence. A low concentration (3 μM) of GABA elicits trains of action potentials in rat GnRH neurons, whereas 20 μM GABA elicits only a single action potential by producing a persistent depolarization beyond a threshold of the voltage-gated Na⁺ channels [2]. The activation of GABA_A receptors beyond a certain level decreases a firing frequency of action potentials in GnRH neurons, even though GABA depolarizes GnRH neurons via GABA_A receptors. Present results, together with the previous findings [2], indicate that GABA acts on extrasynaptic GABA_A receptors in the cell body of GnRH neurons. In fact, only a few synaptic boutons are present in the soma of rat GnRH neurons [45]. In this case an ambient concentration of GABA may closely be involved in determining the cell excitability of GnRH neurons. Within a physiological range of ambient GABA concentration, that is, from submicromolar to micromolar [46], melatonin would increase the excitability of GnRH neurons via MT1 and decrease it via MT2. The next question is how this sort of regulation is involved in reproductive physiology. In female rats, LH surge occurs at the late afternoon of proestrus through the early morning of estrus [47], whereas melatonin level in the blood increases from 2–3 h after the onset of the dark period and remains high throughout the period [39]. This increase in melatonin level coincides with the falling phase of LH surge. The falling phase, which is caused by the decline of the activity of GnRH neurons, would be determined at least in part by the activity of GABA_A receptors. The inhibitory action of melatonin is also demonstrated by the experiment that exogenous melatonin facilitates the offset of LH surge [7]. We can therefore postulate that melatonin decreases the activity of GABA_A receptors through MT2, thereby inhibiting the neuronal excitability. More work is needed to examine the above-mentioned hypothesis and to elucidate the mechanism of how direct action of melatonin on GnRH neurons functions in reproductive physiology, both in males and females.

In conclusion, adult rat GnRH neurons express GABA_A receptors on their soma membranes, and melatonin modulates their function positively in males and negatively in females. These melatonin actions are probably mediated by either MT1 or MT2. These results suggest that melatonin regulates the cell excitability of rat GnRH neurons by modulating GABA-induced depolarization in a sex-specific manner.

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