5-HT7-like Receptors Mediate Serotonergic Modulation of Photo-responsiveness of the Medulla Bilateral Neurons in the Cricket, Gryllus bimaculatus

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Source: Zoological Science, 20(3) : 303-309

Published By: Zoological Society of Japan

URL: https://doi.org/10.2108/zsj.20.303

ZOOLOGICAL SCIENCE **20**: 303–309 (2003)

5-HT$_7$-like Receptors Mediate Serotonergic Modulation of Photo-responsiveness of the Medulla Bilateral Neurons in the Cricket, *Gryllus bimaculatus*

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ABSTRACT—Serotonin (5-HT) suppresses the photo-responsiveness of medulla bilateral neurons (MBNs) that are involved in the coupling mechanism of the bilaterally paired optic lobe circadian pacemakers in the cricket, *Gryllus bimaculatus*. We found that forskolin, a highly specific activator of adenylate cyclase, mimicked the effects of serotonin on the MBNs. This fact suggests the involvement of cyclic 3', 5'-adenosine monophosphate (cAMP) in mediating the action of serotonin. We therefore tested the effects of various 5-HT receptor agonists and antagonists that are coupled to adenylate cyclase to specify the receptor involved. Application of 8-OH-DPAT that has affinity for both 5-HT$_{1A}$ and 5-HT$_7$ receptors suppressed the photo-responsiveness, like forskolin. The inhibitory effect of 8-OH-DPAT was effectively blocked by clozapine, a high affinity 5-HT$_7$ receptor antagonists with a very low affinity for 5-HT$_2$. Ketanserin, a selective 5-HT$_2$ antagonist, and NAN-190, a 5-HT$_{1A}$ antagonist, did not block it. These results suggest that serotonergic suppression of the photo-responsiveness of the MBNs is mediated by 5-HT$_7$-like receptor subtypes.

Key words: *Gryllus bimaculatus*, forskolin, 8-OH-DPAT, 5-HT$_7$, visual interneurons

INTRODUCTION

Serotonin (5-hydroxytryptamine, 5-HT), a neurotransmitter in vertebrates (Peroutka, 1990) is also widely distributed in the nervous system of a variety of insect species (Nässel, 1987). In crickets, serotonergic neurons are distributed almost through out the entire area of the optic lobes, and serotonin content in the optic lobe shows daily fluctuation in synchrony with the circadian changes in the sensitivity of the visual interneurons (Tomioka *et al*., 1993). We recently reported that serotonin is released during the day in the cricket's optic lobe and sets the day state of the medulla bilateral neurons (MBNs) (Saifullah and Tomioka, 2002). The MBNs mediate the coupling between the bilaterally paired optic lobe circadian pacemakers by encoding coupling signals as spontaneous and light-induced responses that are both greater during the night (Tomioka *et al*., 1994; Yukizane and Tomioka, 1995; Yukizane *et al*., 2002). Serotonin reduces their photo-responsiveness, but the regulatory pathway through which serotonin acts on the MBNs remains to be uncovered.

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The pathways through which serotonin acts have been explianed in many invertebrates. In *Aplysia californica*, 8-benzylthio-cAMP, an analogue of cyclic AMP, as well as forskolin, an activator of adenylate cyclase, mimicked the effects of serotonin on the circadian neural firing rhythm (Eskin *et al*., 1982; Eskin and Takahashi, 1983). Serotonin elevates the basal cAMP levels in homogenates of the mandibular closer muscles of the cricket, *Gryllus domesticus* (Baines and Downer, 1991). Thus, it is apparent that cAMP is an important component of the pathway that mediates different physiological effects of serotonin.

Serotonin acts through multiple receptors to mediate a large variety of functions in both vertebrates and invertebrates. So far, 14 structurally distinct mammalian 5-HT receptors have been identified and they are classified into seven families according to their gene structure, the second messenger systems to which they are coupled, and their functional characteristics (Hoyer *et al*., 1994). The 5-HT$_{1, 5}$ and 5-HT$_{4, 6, 7}$ subtypes inhibit and activate adenylate cyclase, respectively, whereas the 5-HT$_2$ subtype stimulates phospholipase C, and the 5-HT$_3$ is an ionotropic receptor (Peroutka, 1995). In invertebrates, the 5-HT receptor was first cloned from *Drosophila*, and subsequently 11 additional receptor genes have been cloned from *Drosophil*a, molluscs (*Lymnaea* and *Aplysia*) and nematodes (*Caenorhabditis* and

Ascaris), and classified according to mammalian category (Tierney, 2001). Although the pharmacological profiles of some invertebrate 5-HT receptors differ somewhat from those of vertebrate receptors, homologous relationships exist in some vertebrate and invertebrate 5-HT receptor types due to the ancient origin of the 5-HT signaling system (Tierney, 2001). In the present study, we investigated the 5-HT receptor subtypes that are involved in the serotonergic regulation of photo-responsiveness of the MBNs and possible involvement of cAMP in it using techniques combining electrophysiology and pharmacology in the cricket, *Gryllus bimaculatus*.

MATERIALS AND METHODS

Experimental animals

Adult male crickets, *Gryllus bimaculatus*, were used for all experiments. They were obtained from laboratory colonies maintained at a constant temperature of 25±0.5°C and a 12h light: 12h dark cycle (light: 0600 to 1800; Japanese standard time) with a continuous supply of food (laboratory chow, Nihon Clea) and water.

Electrophysiology

For extracellular recording of the neural activity of the MBNs, an adult male cricket was fixed on a specially designed plastic platform. A small square piece of head cuticle was removed to expose the optic stalk. The optic stalk was then subdivided into fine nerve filaments using a fine needle and severed near the medulla. Since the MBNs run along the ventral side of the optic stalk, the cut end of a nerve filament from the ventral side of the optic stalk was sucked firmly into a plastic capillary filled with Ringer's solution (Fielden, 1960). A silver reference electrode was placed near the tip of the suction electrode. After electrode implantation, the animals were kept in the dark at a constant temperature of 24–25°C.

Electrical signals from the suction electrode were amplified by a biophysical amplifier (Nihon Kohden, AVB-9), displayed on an oscilloscope (Nihon Kohden, VC-9), then fed into a computer (IBM, 300GL) via a 1401 Plus A/D converter (Cambridge Electronic Design Limited) and analyzed by Spike-2 software (Cambridge Electronic Design Limited).

A slide projector (ELMO, CSII) equipped with a 150W lamp (Philips, KP-8) was used as a light source to obtain light-induced response of MBNs. Light from the slide projector was focused on one end of the plastic light guide (3.5 mm in diameter) and the opposite end was placed close to the compound eye. Neutral density filters (Shonan Kogaku Co.) ranging from 10% to 50% were placed between the shutter and the light source to attenuate light intensity. Light pulses of 1000 ms with various intensities were given to the contralateral compound eye by an electric shutter controlled by an electronic stimulator (Nihon Kohden, SEN-3210). The maximum light intensity (log $I = 0$) on the surface of the compound eye was 0.4mW/cm^2 as measured with an optical powermeter (UDT instruments, Model 371).

Chemicals used

The compounds used were forskolin (Sigma), (±)-8-hydroxy-2-(DL-N-propylamino) tetralin hydrobromide (8-OH-DPAT, RBI), NAN-190 hydrobromide (NAN-190, RBI), Ketanserin tartrate (Tocris) and Clozapine (Tocris). They were dissolved in Ringer's solution, except forskolin and clozapine, which were dissolved in a small volume of DMSO before final dilution. Final DMSO concentration was less than 0.001%. DMSO at this concentration did not affect the photo-responsiveness of the MBNs.

The drugs used in this study are well characterized. Forskolin is a highly specific activator of adenylate cyclase (Seamon and Daly, 1981; Eskin and Takahashi, 1983). 8-OH-DPAT is a 5-HT receptor agonist with high affinity for the 5-HT$_{1A}$ receptor subtype and moderate affinity for the 5-HT$_7$ receptor (Lovenberg *et al*., 1993; Hoyer *et al*., 1994). NAN-190 is a high affinity 5-HT$_{1A}$ antagonist (Williams and Dourish, 1992). Clozapine is a putative 5-HT$_7$ antagonist (Ying and Rusak, 1997; Yu *et al*., 2001) and shows moderate affinity for 5-HT$_2$ receptor subtypes (Canton *et al*., 1994). Ketanserin is an antagonist for 5-HT$_2$ receptor subtypes (Van Nueten *et al*., 1981).

Drugs were injected into the medulla area of the optic lobe using a glass micropipette equipped with a nanoliter injector (WPI, A203XVY) mounted on a micromanipulator (Narishige, M-3333). The injected volume ranged from 18–28, averaging 22.2±2.1 nl (mean ± SD). A similar amount of Ringer's solution was injected as control. In order to ensure successful injections, tips of the micropipettes were checked before and after injection.

Data collection and analysis

The light-evoked response was estimated by subtracting the number of spikes occurring during the 1000 ms period just before the light pulse from those during the 1000 ms light pulse. The effects of treatments on the MBNs' light-induced response were calculated by the following equation: Response Modulation Index (RMI) = 100 × (A-B)/B, where A and B represent the total number of spikes induced by a series of light pulses with intensities of log I = –8 to 0 before (B) and after (A) injection, respectively. In all figures each data point is presented as mean ± S.E.M, and n represents the number of animals used for each test. Statistical comparisons were made using Student's t-test. The difference was considered significant when $P<0.05$.

RESULTS

Effects of forskolin on MBNs' photo-responsiveness

Extracellular recordings of brain efferents were made from the proximal optic stalk of dark-adapted animals using a suction electrode. Light stimuli of 1000 msec given to the contralateral compound eye induced a burst discharge in the brain efferents, which was almost entirely from the MBNs (Tomioka *et al*., 1994). Consistently with the previous report (Saifullah and Tomioka, 2002), the light-induced response of MBNs was intensity dependent and significantly greater during the night than that of the day at all light intensities (Fig. 1B). Injection of forskolin (22 nl of 10^{-5} M) into the contralateral optic lobe reduced the photo-responsiveness of the MBNs (Fig. 1A). The suppression occurred within a few minutes of the injection and persisted for more than 30 minutes. Figure 1B shows the average intensity-response curves of the MBNs' light-induced response before and after the injection of 10^{-5} M forskolin during the day ($n = 5$) and during the night ($n = 5$). The entire intensity-response curve shifted downward in response to forskolin in both the day and night, indicating a clear suppression of the photo-responsiveness. The effect of forskolin was greater during the night than during the day. No significant change in electrical responses of the MBNs was observed when the same amount of vehicle was injected into the optic lobe either in the day or in the night (Fig. 2, control).

The effects of forskolin on the light-induced responses

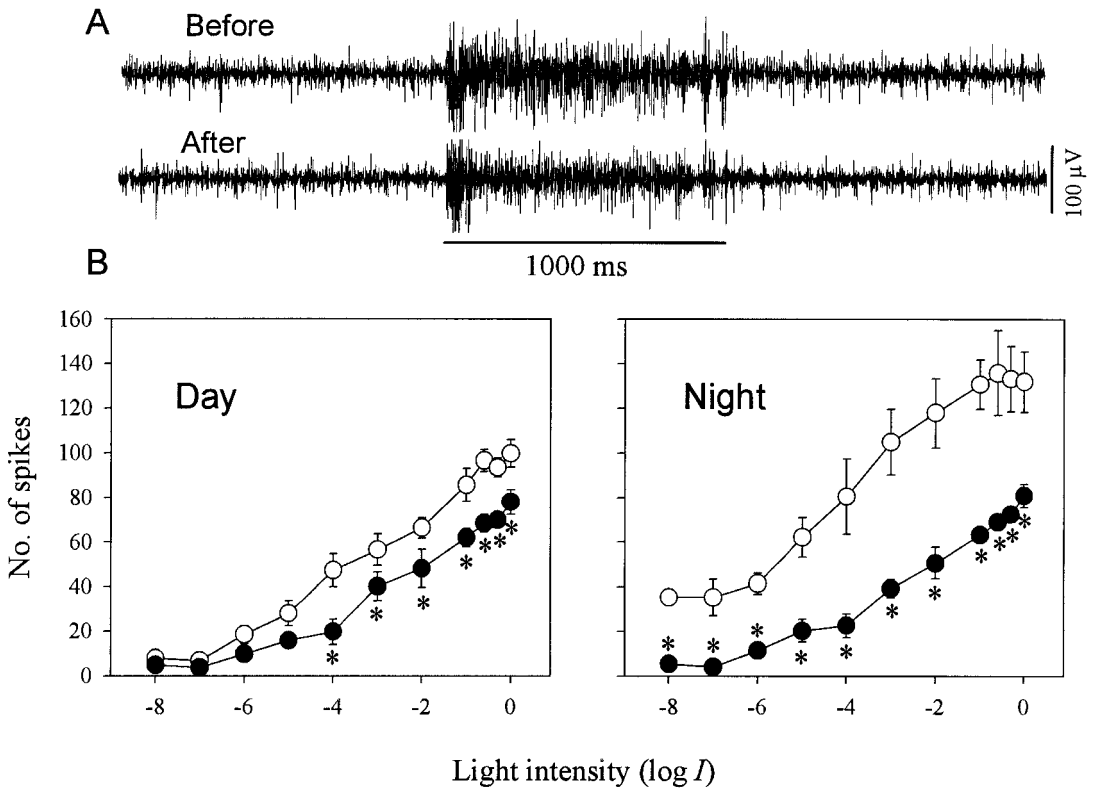

Fig. 1. Effects of forskolin on the photo-responsiveness of the MBNs in the cricket, *G. bimaculatus*. (A) Extracellular recordings from optic stalk brain efferents at log $I = 0$ before and after application of forskolin (22 nl of 10^{-5} M). The light induced response of MBNs was reduced by forskolin. Bar at the bottom indicates light pulse. (B) Average intensity response curves before (open circles) and after (filled circles) forskolin injection during the day ($n = 5$) and night ($n = 5$). Vertical bars indicate S.E.M. Forskolin suppressed the photo-responsiveness in both the day and night with greater suppression during the night. Asterisks indicate significant difference between the values before and after injection (t-test, $P<0.05$).

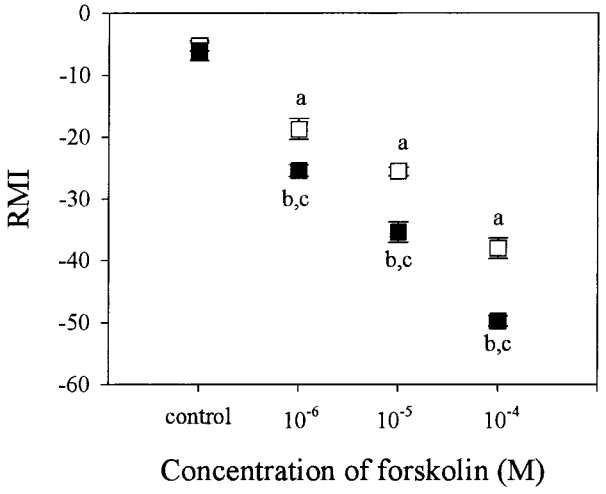

Fig. 2. Dose-response curves of the effect of forskolin on MBNs' photo-responsiveness during the day (open squares) and night (closed squares). The forskolin-induced suppression was always significantly greater during the night than during the day (c, $P<0.01$). Values are means ± S.E.M. of 4–6 preparations. Letters a and b indicate a significant difference ($P<0.01$) compared with Ringer-injected control values for day and night, respectively.

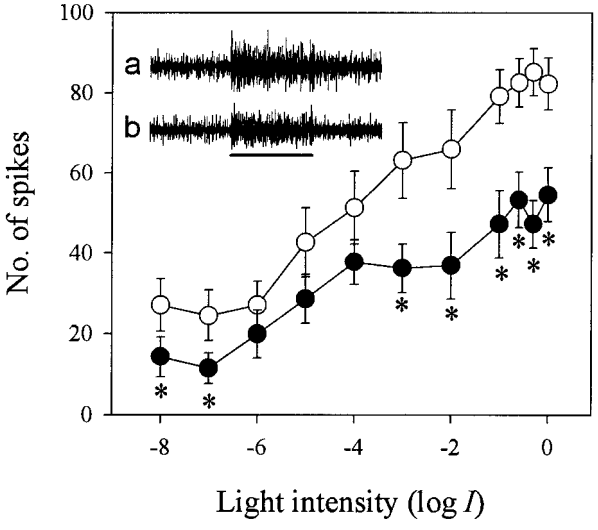

Fig. 3. Effects of 8-OH-DPAT on the photo-responsiveness of MBNs during the day. The intensity response curve shifted downward after injection of 8-OH-DPAT. Open and filled circles indicate values before and after injection of 22 nl of 10^{-3} M 8-OH-DPAT ($n = 7$), respectively. Asterisks indicate significant difference between values before and after injection. Inset shows sample waveforms at log $I = 0$ before (a) and after (b) injection of 8-OH-DPAT. Bar under the record indicates light pulse.

of the MBNs were clearly dose-dependent. Significant suppressions by forskolin were observed at 10^{-6} M in both the day and night. Higher doses induced greater suppression. The response modulation indexes (RMIs) were always greater during the night than during the day at all doses examined (Fig. 2, $P<0.01$).

Effects of 8-OH-DPAT on MBNs' photo-responsiveness

Since forskolin is a highly specific activator of adenylate cyclase and mimics the effect of serotonin on the MBNs (Saifullah and Tomioka, 2002), it is likely that the serotonergic suppression of the MBNs' photo-responsiveness occurred through 5-HT receptors that are coupled to adenylate cyclase. To determine involvement of cAMP coupled 5-HT receptors, we examined the effect of 8-OH-DPAT, an agonist of 5-HT_{1A} and 5-HT_7 receptors that are coupled to cAMP, on the light-evoked response of the MBNs. We made the experiment during the day, since preliminary experiment revealed that the chemical caused a significant change in the photo-responsiveness of the MBNs during the day of which the magnitude was almost equivalent to that caused by the same dose of serotonin during the night. Injection of 22 nl of 10^{-3} M 8-OH-DPAT significantly suppressed the light-evoked response of the MBNs within a few minutes of application. The intensity response curve was shifted downward similar to the case of forskolin injection (Fig. 3), suggesting that the suppressing effect of serotonin was due to the activation of either 5-HT_{1A} or 5-HT_7 receptors.

Pharmacological identification of the 5-HT receptor involved

To determine whether the effect of 8-OH-DPAT was due to the activation of 5-HT_{1A} receptor, 8-OH-DPAT was applied in the presence of a selective 5-HT_{1A} receptor antagonist, NAN-190 (22 nl of 10^{-3} M). The suppression of the photo-responsiveness of the MBNs by 8-OH-DPAT was not blocked by NAN-190 (Figs. 4B and 5). The magnitude of suppression was not significantly different from that observed when 10^{-3} M 8-OH-DPAT was applied alone (Figs. 4A and 5, $P>0.05$), suggesting that 8-OH-DPAT does

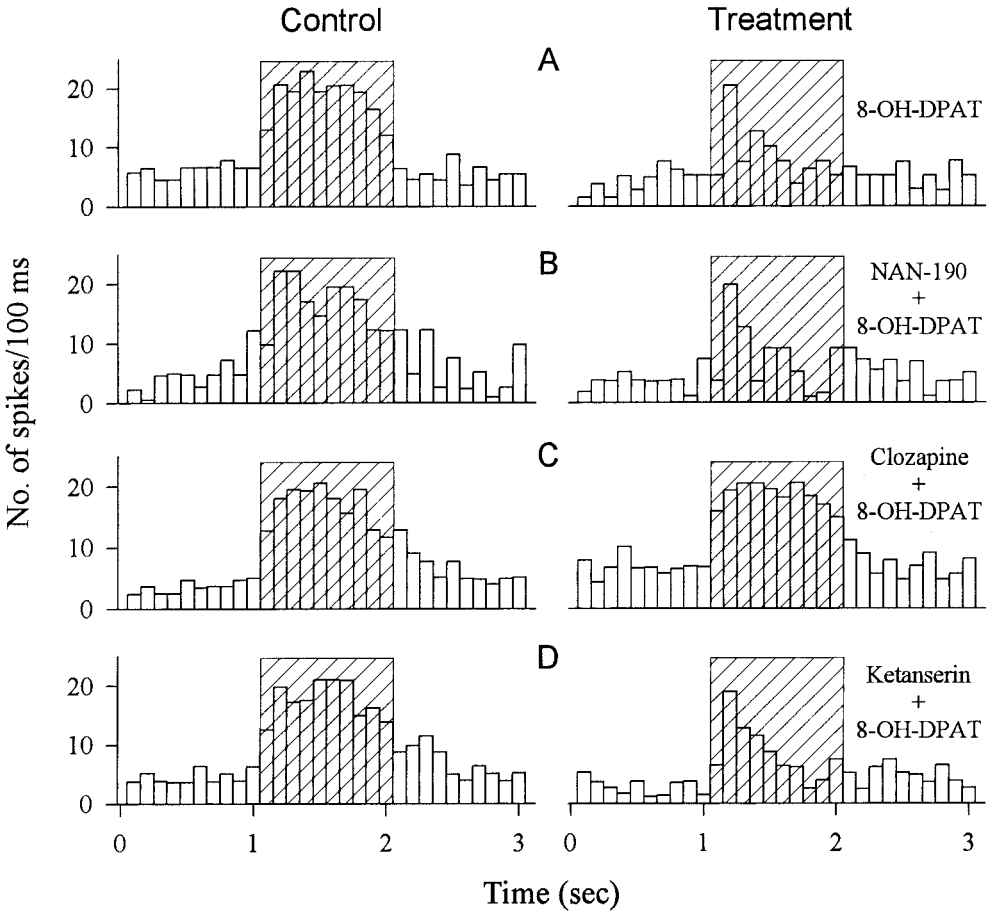

Fig. 4. Examples of histograms of firing rate (per 100 ms) of the optic stalk efferents before (control) and after (treatments) injection of various 5-HT receptor agonists and antagonists, illustrating the ability of 5-HT receptor antagonists to block the effect of 22 nl of 10^{-3} M 8-OH-DPAT. Each row of histograms was obtained from a different animal. (A) Inhibitory action of 8-OH-DPAT on the photo-responsiveness of MBNs. (B) NAN-190 (22 nl of 10^{-3} M), a 5-HT_{1A} antagonist, failed to block the effect of 8-OH-DPAT. (C) Clozapine (22 nl of 10^{-3} M) effectively antagonized the effect of 8-OH-DPAT. (D) Ketanserin (22 nl of 10^{-2} M), a 5-HT_2 antagonist, failed to block the effect of 8-OH-DPAT. Shaded areas indicate responses when a 1000 ms light pulse (0.4mW/cm^2) was given.

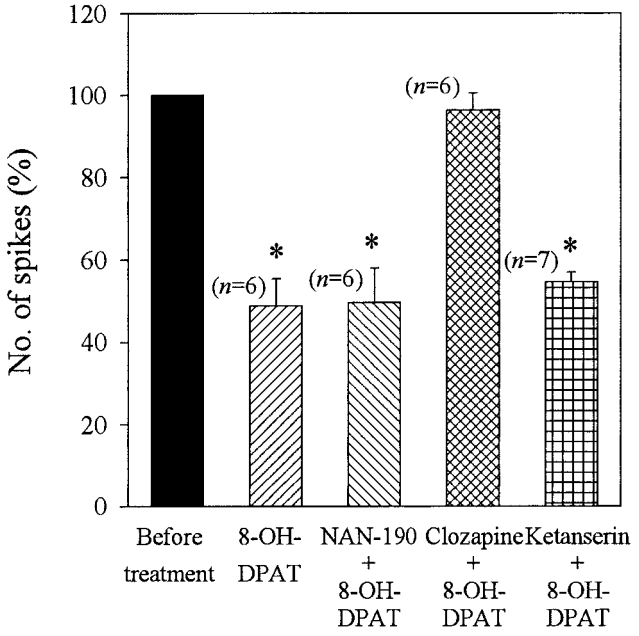

Fig. 5. Comparison of the effects of 8-OH-DPAT (22 nl of 10^{-3} M) on MBNs' photo-responsiveness in presence of different 5-HT receptor antagonists, including the results shown in Fig. 4. Values are the number of spikes (mean ± S.E.M.) induced by a light pulse (log I = 0) and are presented in percentage of those of untreated control. The inhibitory effect of 8-OH-DPAT was effectively antagonized by clozapine (22 nl of 10^{-3} M), but not by NAN-190 (22 nl of 10^{-3} M) or by ketanserin (22 nl of 10^{-3} M). Numbers in parenthesis indicate the number of preparations used. Asterisks indicate significant suppression compared to the values before treatment.

not act through 5-HT$_{1A}$ receptors.

Since 8-OH-DPAT has a moderate affinity for 5-HT$_7$ receptor subtypes (Lovenberg *et al.*, 1993), we examined whether clozapine, a putative 5-HT$_7$ receptor antagonist, blocked the effect of 8-OH-DPAT. We first applied clozapine (22 nl of 10^{-3} M), and then injected 8-OH-DPAT (22 nl of 10^{-3} M) into the optic lobe. Clozapine effectively antagonized the suppressing effect of 8-OH-DPAT on the MBNs' photo-responsiveness (Figs. 4C and 5). Since clozapine also shows some affinity for 5-HT$_2$ receptors (Canton *et al.*, 1994), the effect of ketanserin, a selective 5-HT$_2$ antagonist, was then tested to examine whether the antagonizing action of clozapine occurred through 5-HT$_2$ receptors. Ketanserin (22 nl of 10^{-2} M) failed to block the effect of 8-OH-DPAT on the MBNs' photo-responsiveness when injected into the optic lobe (Figs. 4D and 5), indicating that the 5-HT$_2$ receptors are not involved in the serotonergic suppression of the MBNs' photo-responsiveness.

DISCUSSION

The results of the present study indicate that injection of forskolin into the optic lobe suppressed the photo-responsiveness of the MBNs with greater suppression during the night and that the suppression occurred in a dose-dependent manner. The intensity response curves after forskolin injection and the dose-response curves for forskolin can be compared with those obtained for serotonin described elsewhere (Saifullah and Tomioka, 2002). Since forskolin is an activator of adenylate cyclase and mimics the effect of serotonin, it is likely that serotonin suppresses the photo-responsiveness of the MBNs by stimulating adenylate cyclase, hence by increasing cyclic AMP. The involvement of cAMP in the intracellular pathway through which serotonin acts has been proven for circadian systems in both vertebrates and invertebrates. In the ocular circadian clock of *Aplysia*, 5-HT and cAMP analogs such as 8-benzylthio-cAMP induce phase shifts in a similar way and 5-HT increases the cAMP level (Eskin *et al.*, 1982). Similarly, the circadian clock of the mammalian SCN phase shifts in response to serotonin and to analogs of cAMP (Prosser *et al.*, 1994). Taken together with the results of the present study, the cAMP seems to be a common constituent of the pathway through which serotonin acts in the circadian system in a variety of animals including mammals, molluscs, and insects.

In the present study, we showed cAMP is involved in mediating the serotonergic action on the MBNs, but how cAMP regulates the photo-responsiveness of the MBNs remains to be clarified. The role of cAMP is usually activation of protein kinases, which regulate the activity of cellular proteins by phosphorylation. For example, serotonergic phase advances of the mammalian circadian clock are induced by activation of protein kinase A (Prosser *et al.*, 1994). In molluscs, protein synthesis is also required in the serotonergic phase shifts (Eskin *et al.*, 1984), suggesting some factors regulating transcription or translation such as cAMP response element (Kaang *et al.*, 1993) may be activated through cAMP pathway. Future investigation of the cAMP dependent kinases will reveal the mechanism through which serotonin suppresses the photo-responsiveness of the MBNs.

Our results demonstrated that 8-OH-DPAT, a 5-HT$_{1A/7}$ receptor agonist, significantly suppressed the photo-responsiveness of the MBNs and that the suppression was not blocked by a 5-HT$_{1A}$ receptor antagonist, NAN-190, suggesting that the effect of serotonin on the MBNs is not mediated through 5-HT$_{1A}$ subtypes. The fact that the effects of 8-OH-DPAT were effectively blocked by clozapine, a 5-HT$_7$ receptor antagonist, but not by ketanserin, a 5-HT$_2$ antagonist, suggests that 5-HT$_7$ receptor subtypes mediate the suppressing effects of 5-HT on the MBNs. In addition to the 5-HT$_7$ receptor, one may argue that 5-HT$_4$ and 5-HT$_6$ receptor subtypes might contribute in mediating the action of serotonin, since they are also positively coupled to adenylate cyclase (Bockaert *et al.*, 1990; Sebben *et al.*, 1994). But this is highly unlikely, since there is no evidence that 8-OH-DPAT shows affinity for 5-HT$_4$ or 5-HT$_6$ receptor subtypes. Our previous study revealed that 5-HT phase shifts the circadian clock in the cricket optic lobe in a phase-dependent manner and that the shifts are mediated by 5-HT$_{1A}$-like receptors during the subjective night (Tomioka, 1999). It is thus suggested that at least two types of 5-HT receptors are

present in the cricket optic lobe and that different receptor types are responsible for the phase regulation of the circadian clock and for the modulation of the photo-responsiveness of the coupling pathway between the two clocks, respectively.

Some invertebrate 5-HT receptors have close relationship with mammalian 5-HT receptors in both pharmacological and structural aspects (Tierney, 2001). The *Drosophila* 5-HT_7 receptor (5-HT_{7Dro}) shares the property of increasing adenylate cyclase activity (Witz *et al*., 1990) with mammalian 5-HT_7 receptors (Bard *et al*., 1993). It has high homology with rat (56%) and human (57%) 5-HT_7 receptors within transmembrane domains (Tierney, 2001). Likewise, the *Aplysia* 5-HT_{1AP} receptor and *Caenorhabditis elegans* 5-HT_{1Ce} receptor also share the physiological and structural properties with mammalian 5-HT_1 receptors (Olde and McCombie, 1997; Angers *et al*., 1998). In the present study, we suggested that serotonergic suppression of the photo-responsiveness of the MBNs is mediated by 5-HT_7-like receptors because cricket's 5-HT_7 receptors are pharmacologically similar to mammalian 5-HT_7 receptors. However, the primary amino acid sequence of the cricket receptor will have to be elucidated before full comparisons can be made.

ACKNOWLEDGEMENTS

This work was supported in part by a grant-in-aid for Scientific Research from the Ministry of Education, Science, Sports, Culture and Technology of Japan.

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(Received October 21, 2002 / Accepted November 25, 2002)