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GABA modulates day–night variation in melatonin levels in the cerebral ganglia of the damselfly *Ischnura graellsii* and the grasshopper *Oedipoda caerulescens*

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Abstract

The relationship between daily rhythms in GABA content and melatonin (MEL) content, as well as the effect of GABA treatment during either the day time and night time phases on MEL levels and *N*-acetyltransferase (NAT) activity, were studied in the brains of two insect species, the grasshopper *Oedipoda caerulescens* and the damselfly *Ischnura graellsii.* In *O. caerulescens*, levels of GABA in the optic lobes showed significant daily variation, with a marked increase during the light-to-dark transition period. In contrast, in the brain of *I. graellssi*, two daily peaks in GABA levels were observed, during the light-to-dark and the dark-to-light transition periods. In both insects the maximal levels of GABA occurred 4–6 h in advance of the nocturnal MEL peak, which was associated with a reduction in GABA levels. In both insects, treatment with GABA (1 μg/μl, intracranial injection) during the night was followed by a significant reduction in melatonin levels and NAT activity. In contrast, GABA administered during the day time increased brain MEL levels and synthesis. These data suggest that GABA acts as a modulator of light/dark-dependent melatonin synthesis in the insect brain.
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Circadian rhythmicity is an endogenous timing mechanism of vital importance in all animals living in natural conditions. It is well known that insects also 'time' their lives in a very precise way to the 24-h daily solar cycle, and many species exhibit adaptive rhythmic behaviours in response to seasonal changes in daylength [29]. Insects, like other invertebrates, use the photoperiod as a temporal cue for initiating postembryonic events such as molting, eclosion, and diapause [5,12,22,28]. Melatonin is a highly conserved molecule that is involved in photoperiodic time measurement in both invertebrates and vertebrates; it can be considered as an endocrine signal of the endogenous clock that serves to synchronize physiological responses. As in vertebrates, the synthesis of melatonin in insects is a circadian rhythmic process, which is entrained by the environmental light/dark cycle and shows a marked peak during the scotophase [9,10,12,15,26,33,34].

Melatonin synthesis in both the retina and pineal gland of mammals is partially modulated by neural GABAergic activity. GABA has been shown to inhibit the noradrenaline-induced increase in melatonin production in bovine [7] and rat [27] pineal explants, and recently it has been suggested to act as an important signal mediating the light-induced inhibition of melatonin synthesis in the rat pineal gland in vivo [18,23,24]. By contrast, in golden hamster retina in vitro GABA dose-dependently increased the melatonin content [16]. In insects, melatonin synthesis is likely to occur in photic structures and cerebral ganglia [1], which also contain neural mechanisms involved in generating circadian oscillations [8,29]. The region of the optic lobes is strongly influenced by changes in the light/dark cycle, and also contains a

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population of GABAergic neurons innervating the neuropiles [11,13,14,20]. Furthermore, in the cockroach, which has a circadian pacemaker located in the accessory medulla (a neuropil at the base of the optic lobe), GABA has been suggested to act as a neurotransmitter involved in the entrainment of the circadian clock [25]. However, little is known concerning the role of GABA in the photic regulation of clock-containing neural areas and in the modulation of melatonin production in insects. In the present work, we investigated daily variations in GABA levels in the cerebral ganglia of two insect species, the damselfly *Ischnura graellsii* (Odonata) and the grasshopper *Oedipoda caerulescens* (Orthoptera). We also studied interactions between GABA administration and melatonin production in these two insect species.

Male adult insects (7–10 days old) were used in the experiments. Specimens of *I. graellsii* were raised in the colony maintained by Dr. A. Cordero and kindly donated to our laboratory where they were housed under a 15 h light/9 h dark cycle (lights on at 07:00 a.m.) with controlled temperature (20 ± 1 °C) and humidity (60–80%). Specimens of *O. caerulescens* were collected from a controlled field population in our locality and maintained under a 12 h light/12 h dark cycle (lights on at 8:00 a.m.) with controlled temperature (25 ± 1 °C) and humidity (60%). Food was available ad libitum. Brain tissues were dissected out from cold anaesthetized animals [31]. When insects were sacrificed at night, a dim red light source was used.

In the first experiment, daily changes in brain GABA levels in *O. caerulescens* were studied separately in the optic lobes and the midbrain (i.e., the supraesophageal ganglia minus the optic lobes), whereas in *I. graellsii* the whole supraesophageal ganglia (referred to as 'brain' in the text) was included due to the difficulty of separating the optic stalks. In the second and third experiments, daily changes in melatonin levels and the effect of GABA on melatonin synthesis were studied in the brains of both insects.

GABA content was measured by HPLC with electrochemical detection, as described previously [3] with slight modifications. To measure the levels of GABA in optic lobes and midbrain, tissue samples were transferred to Eppendorf microtubes and sonicated in a solution of 500 μl of cold 0.1N perchloric acid containing norleucine as an internal standard. The homogenate was centrifuged ($17{,}700 \times g$) at 4 °C for 15 min; a volume of the supernatant was neutralized with an equal volume of 0.2 N K_2CO_3 before derivatization with *o*-phthaldehyde and β-mercaptoethanol, and then injected into HPLC-EC system. This included a BAS LC-4B/17A amperometric detector (BAS, West Lafayette, IN) coupled with TL-5A glassy carbon electrode set at +700 mV (versus Ag/AgCl). The injection valve was a Rheodyne M7125 (Berkeley, CA) with a 20 μl sample loop. Reverse-phase separation was done at room temperature using a guard column (50 mm × 4.6 mm, 5 μm) and a Spherisorb C_{18} ODS2 (150 × 4.6 mm i.d., 5 μm) analytical column (Phase Separations, Deeside, UK). Mobile-phase conditions and other chromatographic details were as described previously [3].

To quantify MEL content, brain tissue samples were sonicated in Eppendorf microtubes containing 100 μl of cold 0.1N perchloric acid. The homogenate was centrifuged ($40{,}000 \times g$) at 4 °C for 15 min, and the supernatant was filtered through microspin membranes (0.22 μm). Aliquots of filtered supernatant (10–50 μl) were injected into the chromatographic system comprising a Coulochem 5100A detector (ESA, Bedford, MA, USA) equipped with a conditioning M5021 cell set at +100 mV and an analytical M5011 cell set at +350 mV (first electrode) and +500 mV (second electrode). The injection valve was equipped with a 50-μl sample loop. Chromatographic separation was done at room temperature using a guard column (Spherisorb C_8, 10 mm × 4.6 mm, 5 μm) and a Spherisorb C_8 analytical column (100 mm × 4.6 mm, 3 μm; Tracer Analítica, Barcelona, Spain). Mobile-phase conditions and other chromatographic details were as described previously [32].

The effects of GABA on the activity of the enzyme arylalkylamine *N*-acetyltransferase (NAT) and on melatonin production were evaluated by administering GABA during either the day or the night. An injection (1 μl) of a solution containing 1.0 μM GABA or the corresponding vehicle saline was administered into the occipital region of randomly sampled individuals. Day time injections were administered at 10:00 h, while night time injections were administered 1 h before the MEL peak, at 23:00 h in *O. caerulescens* and at 03:00 h in *I. graellsii*. All animals were sacrificed 1 h after treatment.

NAT activity in the brain was evaluated by the method described by Martin and Downer [19]. Tissue samples were homogenized in 100 μl of a cold solution containing 0.25 M KCl and 1.0 mM dithiothreitol (DTT), and then centrifuged (4 °C) at $40{,}000 \times g$ for 15 min. Aliquots of the supernatant (10 μl) were incubated in a water bath (30 °C) for 10 min in a solution containing 200 mM KCl, 20 mM sodium phosphate buffer (pH 7.0), 0.40 mM DTT, 1.0 mM 5-HT and 2.5 mM acetyl-CoA. The reaction was stopped by adding 100 μl of cold 0.1 M perchloric acid, and a 20-μl aliquot was used for determination of the levels of *N*-acetylserotonin (NAS) by HPLC-EC [31]. Protein content was determined in a volume of tissue supernatant by the method of Bradford [2]. NAT activity was expressed as nmol NAS formed/min/mg protein.

The existence of significant daily variations was evaluated by a Kruskal–Wallis range test followed by a post hoc multiple comparisons test (Tukey test). To evaluate differences between GABA-treated and non-treated insects a non-parametric Mann–Whitney rank sum test was used (Sigma Stat 2.0 statistical software).

No changes were observed in the levels of GABA in the midbrain of *O. caerulescens* throughout the day–night cycle (Fig. 1C). However, significant daily variation was observed in the optic lobes ($p < 0.0001$) (Fig. 1B). In this region the concentration of GABA started to increase at the end of the light phase and reached maximal values at the onset of darkness (38.2 ± 3.1 nmol/tissue), then decreased abruptly (by 60%) during the first part of the night. After

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