The occurrence and physiological functions of melatonin in the most primitive eumetazoans, the planarians

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Abstract. Asexual planarians of the species *Dugesia dorotocephala* exhibit a distinct circadian rhythm of fissioning (asexual reproduction) under the influence of normal photoperiod; fissioning occurs only at night. This rhythm is broken down by continuous illumination, continuous darkness or by decapitation. The fissioning rate increases when planarians are exposed to light for less than 1 hour/day or when they are decapitated. Fissioning of decapitated planarians is suppressed by continuous treatment with melatonin, whereas fissioning resumes when these are returned to normal culture water. Interestingly, fissioning occurs at night when decapitates are treated with melatonin in the daytime, while it is observed in the daytime with night-time melatonin treatment. Endogenous melatonin was detected by HPLC and RIA. The endogenous melatonin level is always higher in those heads collected in the scotophase than in those collected in the photophase. A type of neurosecretory cell, which may synthesize melatonin, is found in the assembly of photoreceptor cells.

Key words. Melatonin; circadian rhythm; asexual reproduction; planaria.

Planarians can reproduce either sexually or asexually[11]. However, it is still not clear whether these sexual and asexual modes of reproduction are seasonally alternated. When planarians of the species *Dugesia dorotocephala* collected from nature are maintained in the laboratory, they usually reproduce by the asexual mode, with some exceptions. This change from nature may indicate that regulatory mechanisms for these reproductive systems are altered in an artificial environment. However, sexual planarians of the species *Dugesia dorotocephala* originally collected from Buckhorn Springs, Oklahoma by Dr. Marie Jenkins have continued to reproduce in the sexual mode, although they have been maintained in the same aged tap water and fed the same diet in the laboratory as the asexual strain. When asexual planarians undergo transition into the sexual mode, they develop gonads, forego fissioning, grow larger, copulate and lay cocoons of eggs[11]. This transition is under cephalic, probably brain, control. Kenk[12] demonstrated that the heads of sexual planarians grafted onto bodies of a strain of permanently asexual planarians caused differentiation of gonads in the latter. This has also been achieved by feeding sexual planarians to asexual ones[7,22]. Seasonal factors may be involved. Ude[23] has shown that neurosecretory activity in *Dendrocoelum lacteum* is maximal in August, and that it goes through a daily cycle with a maximum at 6 p.m. Vowinckel and Marsden[24,25] found influences of day length and temperature on reproduction of *Dugesia tigrina.* It seems likely that such sexualizing effects are mediated by neurosecretory cells that have been described in the brain of planarians[13-17]. There is some experimental evidence for neurosecretory control of fissioning (asexual reproduction) and regeneration[8,15,16] stimulation of gonads and development of genital apparatus in sexual planarians[6,9,10,13], as well as sexual maturation of asexual planarians[8]. However, there is no biochemical data regarding the identity of these hormones involved in the regulation of reproduction in planarians.

In asexual reproduction, the anterior and posterior portions of the planarian tear apart at the postpharyngeal region of the body; the head side regenerates a new tail, and the tail regenerates a new head, to yield two complete, but smaller, planarians. That is, the planarian asexual reproduction consists of fissioning and regeneration. Since fissioning occurs at night, it is difficult to observe the process of fissioning. However, the fissioning process can be observed in the photoperiod if planarians are maintained in a special photocycle such as 1 h of light per day. Initially, a body constriction appears at the site where fissioning may occur; the rostral portion of the body contracts and then pulls away from the caudal portion which adheres to the surface of the glass container. The constricted region becomes narrower as it stretches. This process continues until the stretched area ruptures. Finally, the rupture closes up when the circular body wall muscles contract. The entire process of fissioning takes only about 3–4 min after appearance of the constriction. After fissioning is completed, regeneration takes place to yield two complete, but smaller, planarians. These observations suggest that fissioning primarily involves neuromuscular events which are regulated by neurotransmitters or neuromodulators.

Best et al.[1] demonstrated an interesting mechanism for control of fissioning in asexual planarians of the species *Dugesia dorotocephala*. Fissioning was suppressed in grouped planarians and released in isolated ones. This effect was not mediated by pheromones or metabolites when added to the culture water[2]. Instead, cohorts were sensed by direct contact chemoreception. Pigon et al.[21] found that clumps of neural cilia act as sensors, and have direct neural connections to the brain, which are distributed in the cephalic margins. The head, probably the brain, mediated this negative feedback effect of population density. Graduated increase in population density gave a graduated suppression of fissioning incidence[2]. When planarians were decapitated just behind the auricles, they had a high incidence of fissioning, irrespective of population density[1]. Child[3] also noted this high rate of fissioning in decapitated planarians, as well as in anesthetized planarians and suggested that removal of the brain resulted in the removal of an inhibitory influence on asexual reproduction.

In order to understand the regulatory mechanisms of fissioning, the following experiments were performed in our laboratory. Healthy planarians of the species *Dugesia dorotocephala* with similar lengths (approximately 18–20 mm) were adapted for 5 d to a given photoperiod prior to experiments. During this adaptation period, they were maintained in cylindrical glass bowls, with 70 planarians and 200 ml of aged tap water per bowl at room temperature (approx. 21 °C). This population density is adequate to inhibit fissioning during the 5 day acclimatization period[1,2]. At zero time, the adapted planarians were segregated into groups of 10 planarians with 50 ml of aged tap water per bowl. The other planarians were decapitated before segregation. The fissioning incidence was examined for 7 d, while maintaining the same photoperiod schedule and temperature used during the adaptation period. Since planarian fissioning was somehow affected by the moon cycle, all experiments were started 3 d before the full moon or the new moon.

Under the normal photoperiod (8–12 h of light per day), 65.9 ± 3.3% of intact planarians and 82.4 ± 2.4% of the decapitates fissioned within 7 d following segregation[18]. Decapitated planarians showed a higher rate of fissioning than intact planarians. These results were consistent with those reported previously by Best[1] and Child[3]. Interestingly, we found that almost all of the intact planarians fissioned during the dark period. Decapitated planarians, on the other hand, fissioned indiscriminately in both light and dark periods during the first 3 d after decapitation and segregation. During the 4th to 7th days, fissioning occurred only at night similar to the fissioning pattern of intact planarians. This is probably due to the emergent functional recovery associated with the newly regenerating brain and eyes. These results indicate that intact planarians fission with a distinct day-night rhythm under the influence of a normal photoperiod. This distinct rhythm of fissioning is broken down by continuous illumination, continuous darkness, or by decapitation. The fissioning rate increases when planarians are exposed to light for less than 1 h per day, or when they are decapitated. Since the activity of the inhibitory factor released from the brain seems to have a distinct day-night rhythm, various neurotransmitters and neuromodulators as well as inhibitors such as serotonin, melatonin, norepinephrine and reserpine, which are related to a circadian rhythm in their activity, were tested on the groups of decapitated planarians. Interestingly, we found that only melatonin inhibited fissioning. In addition, we found that fissioning of decapitated planarians was suppressed by continuous treatment with melatonin in their culture water, while fissioning resumed when these were returned to normal culture water. When decapitated planarians were treated with 18.75 ppm melatonin in the daytime and returned to normal aged tap water at the end of daytime, fissioning occurred only at night similar to the intact planarians. When decapitated planarians were treated with 18.75 ppm melatonin at night, fissioning occurred only in the daytime. These results suggest that the planarian head, probably brain, releases melatonin that inhibits fissioning, and that the synthesis and release of melatonin are closely related to the environmental photoperiod[18].

In order to confirm the existence of endogenous melatonin, the heads of 300 normal planarians were collected in the middle of the photophase, while the heads of another 300 planarians were collected in the middle of the scotophase. The heads of another 300 planarians that had been adapted to the reversed-phase schedule were collected during the mid-photophase. Another 300 heads of such reversed-phase planarians were collected during the mid-scotophase. In addition, the heads of 150 sexual planarians were harvested during photophase, and the heads of another 150 sexual planarians were collected during scotophase. Each group of heads were prepared for HPLC. The results of these melatonin analyses indicate that heads of both sexual and asexual planarians contain measurable amounts of melatonin[20]. No matter what photoperiod they were adapted to, melatonin content (pg/head) was an average of 14.2 in the midphotophase and 21.1 in the midscotophase in asexual planarians, while it was an average of 13.2 in the midphotophase and 25.8 in the midscotophase in sexual planarians. That is, the melatonin level in the head was consistently higher during scotophase than during photophase. These results are consistent with the hypothesis that melatonin is synthesized in and released from the planarian head, and that it mediates fissioning under the influence of the environmental photoperiod. The chemical composition of the endogenous melatonin detected by HPLC was further confirmed by the radioimmunoassay technique[20]. Extracts of head material were purified by HPLC chromatographic fraction and divided into two groups in order to determine amounts of melatonin by HPLC and RIA. Both assay methods, HPLC and RIA, agreed over the range of melatonin concentration. Recently, endogenous melatonin was also detected in the planarian *Dugesia japonica*, particularly in the head region, by Yoshizawa et al.[26], using radioimmunoassay.

The results described above suggest that the melatonin-synthesizing cells located in the planarian head, are possibly in close association with the photoreceptor system. In other experiments, we found a type of neurosecretory cell in the apical portion of the photoreceptor cell assembly near the eye and we examined whether or not the activity of these neurosecretory cells changed during the photoperiods[19]. Planarian heads were collected by decapitation just behind the auricles at midphotophase and midscotophase 2 d after experimental segregation and prepared for electron microscopy. As postulated, the neurosecretory cells in the specimens collected at midscotophase contained significantly more secretory granules than those collected at midphotophase. In addition, electron-dense material similar to that contained in secretory granules was observed in the cisternae of Golgi complexes and Golgi vesicles of the neurosecretory cells collected at midscotophase. On the other hand, the neurosecretory cells collected at midphotophase exhibited fewer secretory granules and no electron-dense material in the cisternae of Golgi complexes. These observations suggest that the secretory material of this neurosecretory cell is actively synthesized and condensed in the secretory granules during scotophase and is released during the photophase, and that the optic neurosecretory cells observed here may receive synaptic input directly or indirectly from the photoreceptor cells under the influence of photoperiods. Although additional techniques, such as immunocytochemistry or high resolution autoradiography, would be required to identify the exact nature of the secretory material, it is possible that the neurosecretory cells located in the photoreceptor cell assembly are synthesizing melatonin.

Csaba and Bierbauer[5] and Csaba et al.[4] demonstrated that exogenous melatonin inhibits formation of pigments in regenerating specimens of *Dugesia lugubris*. More recently, Yoshizawa et al.[26] reported that when decapitated planarians were maintained in a 1 mmol dm^{-3} solution of melatonin, formation of the head was retarded, and that regeneration of the tail was also inhibited in animals from which the tail was cut and kept in the same concentration of melatonin. These results seem to be consistent with the results in which melatonin inhibits gonad development and its functions in higher vertebrates. As mentioned above, endogenous melatonin has now been detected in two species of planarians, *Dugesia dorotocephala*[20] and *Dugesia japonica*[26], indicating the existence of a melatonin metabolic system in planarians. Although the actions of melatonin on the mechanism of fissioning (asexual reproduction) should be investigated comprehensively, we would postulate that melatonin may also be involved in the regulatory mechanisms of planarian sexual reproduction.

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