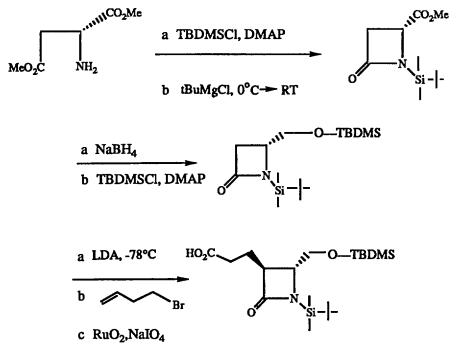

Me, methyl; TBDMSCl, *tert*-butyldimethylsilyl chloride; DMAP, dimethylaminopyridine; tBuMgCl, *tert*-butyl magnesium chloride; RT, room temperature; and LDA, lithium diisopropylamide.

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14. Supported by a grant from the National Eye Institute (to M.I.G.) and by an NSF grant (to M.K.). Additional support was from the Lucille P. Markey Charitable Trust (to M.I.G.) and the American Heart Association, Camille and Henry Dreyfus Foundation, and American Cyanamid (to M.K.). We thank W. V. Williams, G. N. Gaulton, and R. A. Chrusciel for helpful discussions and review of this manuscript; W. C. Still (Columbia University, New York) for a copy of Macromodel; and K. Koopferstock for assistance. Coordinates of the 87.1-mimetic structure will be submitted for deposit in the Brookhaven Protein Data Bank.

15 March 1991; accepted 4 June 1991

Photoperiodism and Effects of Indoleamines in a Unicellular Alga, *Gonyaulax polyedra*

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Mediation of photoperiodic effects by indoleamines, especially melatonin, is known in higher vertebrates. A similar mechanism may occur in a unicellular alga, the dinoflagellate *Gonyaulax polyedra*. This organism entered the dormant stage of a cyst upon short-day treatment at lowered temperatures. Interruption of darkness by 2 hours of light prevented cyst formation, even when the overall duration of light was the same as in cyst-inducing short days. When given in a noninducing photoperiod, melatonin and an analog, 5-methoxytryptamine, substances that had previously been shown to occur in *Gonyaulax*, provoked cyst formation. Methoxylated indoleamines may play a role as mediators of darkness in this unicellular, in a similar way as in vertebrates, suggesting a common biochemical basis of photoperiodism.

PHOTOPERIODIC TIME MEASUREMENT has evolved as a means for orientation within the year in organisms phylogenetically as distant as vertebrates, arthropods, and angiosperms. Whether or not a similar physiological basis for photoperiodic mechanisms exists, that can be attributed to the same evolutionary origin, is a matter of discussion.

In vertebrates, information about the relative lengths of photophase and scotophase is mediated by the indoleamine melatonin (*1–4*). However, the occurrence of melatonin is not restricted to these organisms. It exists also in insects, where this substance exhibits a circadian rhythm (*5–7*). In planarians, an effect of melatonin on reproduction has been described (*8*). In the dinoflagellate *Gonyaulax polyedra* we have recently detected two methoxylated indoleamines, melatonin and 5-methoxytryptamine, as well as enzymes involved in their formation, indoleamine *N*-acetyltransferase and hydroxyindole *O*-methyltransferase (*9, 10*). Moreover, melatonin exhibits a daily rhythm of high amplitude, with a nocturnal maximum as high as in vertebrate pineals (about 2.5 ng per milligram of protein) (*11*). We, therefore, tested this organism for a photoperiodic response and for its possible mediation by these indoleamines.

Gonyaulax polyedra was grown at 20°C in a light-dark cycle of 12:12 hours (LD 12:12) (*12*). Photoperiodic experiments were carried out at either 20° or 15°C, under various LD schedules. Light intensity was 400 lux. Cells were transferred to experimental conditions 3 hours after onset of light (circadian time 3 hours); this time point was chosen as the beginning of illumination in the new LD. Melatonin and 5-methoxytryptamine were dissolved directly before use in dimethyl sulfoxide (DMSO), at a concentration of 0.2 M, and then diluted in culture medium to give the desired molarity. DMSO in the molarities used was without effect. Five cultures were used for each data point, and cyst numbers were determined daily.

In the species investigated, photoperiodic responses were unknown. We detected that the cells can, in fact, react by entering a dormant stage. According to morphological criteria, the resting cells are of the type of so-called "thin-walled" or "temporary" cysts (*13*). The process of encystment involves cessation of movement, loss of flagella, retraction from the theca, and formation of a spherical cyst wall. The cyst is finally released from the theca. Cyst formation was quantitatively evaluated by calculating the ratio of cyst to total cell numbers. For definition of the stage of cyst, we used the most unambiguous criterion of a clearly visible cyst wall. Damaged cells were excluded from the evaluation.

At a temperature of 15°C, encystment was strictly photoperiodically controlled (Fig. 1). At a subcritical day length of 11 hours, almost no cyst formation was observed. But a day length of 10 hours or less caused practically all cells to encyst within 4 days. Treatment with a single long dark period sufficed in many experiments to induce cyst formation in up to 20% of cells (Table 1).

A lowered temperature, as compared to normal growing conditions, appears to represent a prerequisite for enabling the cell to respond photoperiodically. At 20°C, cyst formation was negligible even at LD 6:18, whereas the same short day treatment at 15°C led to complete encystment. The inefficiency of short photoperiods at 20°C was supported by extended experiments lasting for several weeks (Table 1).

Photoperiodism cannot be demonstrated solely by comparing effects of long and short days, because the variation of day length is accompanied by changes in the amount of light. This is particularly important in photoautotrophic organisms such as *Gonyaulax*, in which light deficiency could become limiting for cell metabolism. Moreover, encystment has been attributed in dinoflagellates to unfavorable environmental conditions (*13*). Therefore, we carried out experiments in which we interrupted the dark period by

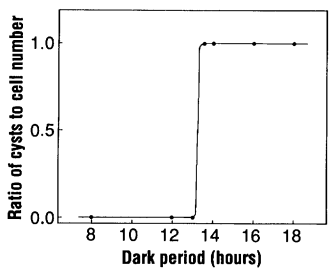

Fig. 1. Cyst induction by short days and decreased temperature. Cultures of mastigote cells were transferred from 20° to 15°C and from LD 12:12 to the experimental LD cycles; the relative number of cysts was determined 4 days after transfer. Experiments were carried out in five cultures with triplicate determinations, but statistical variation was negligible, because cells responded practically in an all-or-none fashion. Ordinate: ratio of cysts to total cell number; abscissa: length of dark period (hours) within an LD cycle of 24 hours.

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2 hours of light. The overall duration of light was the same as in a cyst-inducing short day. The comparison of LD 10:14 with these illumination patterns showed that light breaks largely inhibited cyst formation (Table 1). This was demonstrated for two different circadian phases of the light break (LDLD 8:2:2:12 and LDLD 2:2:8:12). Short day plus light break can, therefore, be interpreted by the cell as a subjective long day. Hence, the total amount of light is not decisive for induction, but rather the temporal distance between onset and end of light, a result demonstrating the involvement of a photoperiodic mechanism.

Since melatonin is known to mediate photoperiodic effects in vertebrates, and since we had found this substance to be present in *Gonyaulax*, we tested whether or not the indoleamine might be able to induce encystment. In the otherwise non-inductive conditions of LD 11:13, at 15°C (but not at 20°C), a single addition of 10^{-4} M melatonin given 1 hour prior to the onset of darkness caused all cells to encyst within 3 days (Table 1). Melatonin is, therefore, able to mimic effects of short day treatment, a remarkable parallel to what is known in mammals (*14, 15*). A rather high concentration of melatonin was required in our experiments to provoke cyst formation, a fact that may be due to the instability of this substance, especially when exposed to light. The indoleamine is destroyed by light-induced superoxide anions, a reaction that is largely catalyzed by complexed iron (*16*). This is of particular importance, because the culture medium contains iron-EDTA complexes. Additional destruction occurs within the cell under the influence of iron porphyrin compounds. When incubated with extracts from *Gonyaulax*, melatonin decays rapidly upon exposure to light (*17*). Thus, low concentrations of melatonin cannot be expected to be effective during photophase. Additions of melatonin earlier in the photophase would lead to losses of this substance at an unknown rate impeding the use of accurate concentrations.

Table 1. Effects of temperature (T), light breaks, and methoxylated indoleamines on cyst formation.

L:D schedule	T (°C)	Treatment	Day after transfer	Cysts/total cells (±SEM)
LD 6:18	20		1	0.00 ± 0.00
LD 6:18	20		29	0.00 ± 0.00
LD 6:18	15		1	0.12 ± 0.00
LD 6:18	15		2	0.95 ± 0.02
LD 6:18	15		3	1.00 ± 0.00
LD 10:14	15		1	0.16 ± 0.03
LD 10:14	15		4	1.00 ± 0.00
LD 11:13	15		4	0.02 ± 0.01
LD 11:13	15		7	0.01 ± 0.00
LD 11:13	15	10^{-4} M melatonin	1	0.16 ± 0.09
LD 11:13	15	10^{-4} M melatonin	2	0.68 ± 0.17
LD 11:13	15	10^{-4} M melatonin	3	1.00 ± 0.00
LD 11:13	20	10^{-4} M melatonin	7	0.00 ± 0.00
LD 12:12	20		4	0.00 ± 0.00
LD 12:12	20		14	0.00 ± 0.00
LD 12:12	15		4	0.04 ± 0.00
LD 12:12	20	2×10^{-5} M 5-methoxytryptamine	1	1.00 ± 0.00
LD 12:12	20	10^{-5} M 5-methoxytryptamine	1	1.00 ± 0.00
LD 12:12	20	5×10^{-6} M 5-methoxytryptamine	1	1.00 ± 0.00
LD 12:12	20	2×10^{-6} M 5-methoxytryptamine	7	0.00 ± 0.00
LL	20	10^{-5} M 5-methoxytryptamine*	1	1.00 ± 0.00
LL	20	5×10^{-6} M 5-methoxytryptamine	1	1.00 ± 0.00
LD 11:13	15	10^{-4} M *N*-acetylserotonin	1	0.00 ± 0.00
LD 11:13	15	10^{-4} M *N*-acetylserotonin	2	0.01 ± n.d.
LD 12:12	20	10^{-4} M *N*-acetylserotonin	14	0.00 ± 0.00
LD 12:12	20	5×10^{-5} M *N,N*-dimethyl 5-methoxytryptamine	1	1.00 ± 0.00
LDLD 2:2:8:12	15		1	0.03 ± 0.01
LDLD 2:2:8:12	15		3	0.04 ± 0.02
LDLD 2:2:8:12	15		5	0.11 ± 0.02
LDLD 2:2:8:12	15		7	0.10 ± 0.00
LDLD 8:2:2:12	15		1	0.01 ± 0.01
LDLD 8:2:2:12	15		3	0.01 ± 0.01
LDLD 8:2:2:12	15		5	0.01 ± 0.00

*The same result was obtained at various circadian phases of addition: circadian time 0, 3, 12, 15, 18, and 21.

As compared to melatonin, its analog 5-methoxytryptamine proved to be even more potent. Its efficacy did not depend on a lowered temperature. At 20°C and in LD 12:12, 10^{-5} M 5-methoxytryptamine induced cyst formation within 6 hours in the entire cell population. In another experiment, 5×10^{-6} of this indoleamine led to complete encystment, even in LL (Table 1).

In these experiments we have shown both photoperiodism and short-day-like effects of methoxylated indoleamines in *Gonyaulax*. These findings may be relevant for our understanding of the temporal organization of dinoflagellates, especially with regard to the annual cycle. Unfavorable environmental conditions can be perceived and, perhaps, even anticipated by sensing a combination of low temperatures and short days. The amastigote cysts, once formed, will sink down to the sea bottom where they can be sampled in the sediments. In these depths, the dormant stages are effectively shielded from exposure to light; therefore, germination might be controlled by an endogenous circannual cycle, as in *Alexandrium tamarense* (*18*).

Our data suggest that methoxylated indoleamines may act as mediators of darkness in *Gonyaulax*. This constitutes a surprising similarity to the situation in mammals, birds, and reptiles (*1–4, 19*). The occurrence of circadian rhythms of melatonin in insects (*7*) and effects of this substance in platyhelminths (*8*) suggests similar roles in these groups of organisms. Extending this principle to unicellulars would imply a considerable broadening of a common biochemical basis of photoperiodic mechanisms. The findings that the unicellular *Gonyaulax polyedra* synthesizes melatonin, that this substance exhibits a daily periodicity (*11*) and can mimic a photoperiodic effect is of general relevance, especially in an organism belonging to the best studied objects of circadian clock mechanisms. The existence of same physiological mechanism does, however, not allow us to comment on a monophyletic origin. This would require a search for melatonin and 5-methoxytryptamine in many more taxa; such investigations would have to take account of the high instability of these substances and the need for preservative extraction procedures.

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12 February 1991; accepted 12 June 1991

Similar Neuronal Alterations Induced by Axonal Injury and Learning in *Aplysia*

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Learning in the marine mollusk *Aplysia* has been associated with enhanced sensory function, expressed in mechanosensory neurons as (i) decreases in action potential threshold, accommodation, and afterhyperpolarization, and (ii) increases in action potential duration, afterdischarge, and synaptic transmission. These alterations also occur, with a delay, after sensory axons are injured under conditions in which synaptic transmission is severely reduced. The latency and specificity of injury-induced alterations indicate that induction signals are generated at the site of injury and conveyed centrally by axonal transport. Similarities in neuronal modifications support the hypothesis that some memory mechanisms evolved from mechanisms of injury-induced sensory compensation and repair.

BEHAVIORAL SENSITIZATION AND classical conditioning in various species have been correlated with a persistent increase in the excitability of selected neurons, often expressed as a decrease in action potential threshold or an increase in firing rate during depolarizing test stimuli (*1, 2*). Increased excitability is also seen in diverse neurons after injury of their peripheral axons (*3*). These observations, and the presumed ability of hyperexcitability to compensate for loss of sensory function in an area of injury, led to the suggestion that some learning mechanisms evolved from long-lasting adaptive responses to cellular injury in primitive sensory neurons (*4*).

We have begun to test this hypothesis by comparing injury-induced and learning-induced plasticity in the same neurons: nociceptive mechanosensory neurons in the mollusk *Aplysia*. The most dramatic plasticity reported for these cells occurs when the animal receives noxious stimulation within the receptive field of the neuron during aversive training, and this plasticity is mediated in part by activity-dependent effects of neuromodulators released on or near the sensory neuron soma (*2, 5*). However, because noxious stimuli can damage peripheral axons, we wondered whether additional signals for long-term plasticity in these cells are generated at the sites of axonal injury.

Experiments were performed on sensory neurons that have their somata in the ventrocaudal (VC) clusters of the pleural ganglia (Fig. 1A). The axons of these neurons project exclusively through the adjacent pedal ganglion and ipsilateral pedal nerves to innervate the ipsilateral body surface (*6*). Mechanosensory neurons in *Aplysia* usually show marked action potential accommodation during prolonged depolarization (*7*); action potentials (spikes) can only be initiated at the beginning of a depolarizing pulse (Fig. 1B, control), even if large depolarizing currents are applied. To investigate the effects of axon injury on accommodation, we anesthetized each animal and, through a small incision, crushed all of the pedal nerves coming from one pedal ganglion (*8*). After 1 to 23 days the pedal-pleural ganglia were dissected, and sensory neurons in each VC cluster examined.

We tested action potential accommodation by injecting a 1-s depolarizing pulse into the soma (Fig. 1B) (*9*). Sensory clusters subjected to axon crush 4 days earlier responded with significantly more action potentials (exhibiting less accommodation and thus greater excitability) than contralateral control clusters with uncrushed axons by a paired comparison [$t(5) = 4.45$, $P = 0.0043$] (Fig. 1C). Sensory clusters also showed significantly less accommodation 3 days [$t(5) = 7.20$, $P = 0.0008$], 6 days [$t(4) = 6.45$, $P = 0.003$], 7 days [$t(3) = 6.36$, $P = 0.008$], and 10 days [$t(3) = 3.76$, $P = 0.035$] after crushing the ipsilateral pedal nerve than before (Fig. 1D). Our paired testing procedure (*10*) and the number of sensory neuron pairs (five to eight) examined within each animal also allowed comparisons of cell populations in individual animals. Sensory neurons with crushed axons displayed less action potential accommoda-

Table 1. Electrophysiological properties altered by axon crush. Data are expressed as mean ± SEM, except for the percentage of cells showing afterdischarge. Values of P were determined with two-tailed paired t tests (n.s., not significant), except for the incidence of afterdischarge (Fisher's exact test). The term AHP denotes afterhyperpolarization. The term n indicates pairs of sensory clusters (one per animal) except in the afterdischarge incidence, where it refers to the total number of sensory neurons in crushed axon and control clusters in the subset of animals ($n = 5$) that displayed afterdischarge during determination of spike threshold. Each EPSP data point was the average of the ten largest EPSPs recorded in the motor neuron from one sensory cluster.

Property	Control	Crushed axon	P	n
Accommodation (number of spikes)	5.9 ± 0.9	14.5 ± 1.0	0.001	26
Threshold (nA)	1.39 ± 0.05	1.30 ± 0.07	0.005	26
AHP (mV)	5.8 ± 0.2	4.4 ± 0.3	0.001	26
Resting potential (mV)	44.1 ± 0.5	43.9 ± 0.5	n.s.	26
Spike amplitude (mV)	78.6 ± 1.5	79.3 ± 1.5	n.s.	26
Spike duration (ms)	1.9 ± 0.2	2.4 ± 0.2	0.001	9
EPSP (mV)	4.5 ± 0.9	11.7 ± 2.3	0.01	8
Afterdischarge incidence	5%	33%	0.01	86

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