J Comp Physiol A (1984) 154:583–589

Journal of Comparative Physiology A Sensory, Neural, and Behavioral Physiology

Induction of rapid testicular growth in Japanese quail by phasic electrical stimulation of the hypothalamic photosensitive area

Mitsuaki Ohta[1], Masaru Wada[2], and Kazutaka Homma[1]

[1] Department of Veterinary Physiology, Faculty of Agriculture, University of Tokyo, Yayoi 1-1-1, Bunkyo-ku, Tokyo 113, and
[2] Institute for Medical and Dental Engineering, Tokyo Medical and Dental University, 2-3-10, Kanda-Surugadai, Chiyoda-ku, Tokyo 101, Japan

Accepted January 4, 1984

Summary. Optic fibers were implanted stereotaxically into the brain of immature male Japanese quail reared under short-day photoperiod (lights on from 1000 to 1800 h), and photosensitive sites in the hypothalamus were examined using gonadal growth and associated hormonal changes as the indices.

In the subsequent experiments, bipolar (coaxial) electrodes were implanted chronically using predetermined coordinates for highly photosensitive sites. Henceforth the birds received brief electrical stimulation (square wave, 100 Hz, 100 μA, 2 min) once daily for 21 consecutive days. When the electrical stimulation was applied early in the dark period, marked gonadal growth was induced, but identical stimulation given in the light period resulted in no testicular growth. The response curve of testicular weight vs clock time of electrical stimulation has a prominent peak at 3 h after the onset of dark. Apparently, the neural complex in the photosensitive area of the quail hypothalamus responds to electrical stimulation as it does to light. We conclude that in photoperiodic birds the principal factor which determines the magnitude of gonadal responses is not the intensity of the stimulus but its timing (circadian phase).

Introduction

Sexual maturation in birds is markedly accelerated under long day photoperiods. This process involves two different types of photoreceptors, retinal and hypothalamic (Benoit 1974, 1975). Several recent studies in the chicken (Ohkawa 1970a, b), Japanese quail (Homma and Sakakibara 1971; Oishi and Lauber 1973), house sparrow (Menaker et al. 1970; Menaker 1971), and white-crowned sparrow (Yokoyama et al. 1978) all indicate that retinal photoreception is dispensable but encephalic photoreception is essential for the induction of gonadal growth by light. Our experiments with Japanese quail (Homma et al. 1979) revealed rhythmic changes in hypothalamic photosensitivity with a maximum between 10 and 12 h after the onset of the environmental light period. This time coincides with the photoinducible phase which had been detected by exposing the whole animal to light pulses at night (Follett and Sharp 1969; Follett et al. 1977; Wada 1981). In several mammalian species in which light applied directly to the brain is known to be completely ineffective, electrical lesioning and stimulation of the brain have been used routinely in studies of the central mechanisms regulating reproductive activity (Everett and Radford 1961; Terasawa and Sawyer 1969; Clemens et al. 1971; Kalra et al. 1971; Cramer and Barraclough 1971; Kalra and McCann 1973; Charlton et al. 1975; Fink and Aiyer 1974; Spies et al. 1977; Arendash and Gallo 1979). Similarly, in avian species electrical stimulation of discrete areas of the hypothalamus and preoptic area has been reported to induce gonadotropin release (Davies and Follett 1974, 1975). However the results of these acute experiments might involve the responses of photosensitive as well as photo-insensitive neurons, both of which may undergo rhythmic control, and it is difficult to draw conclusion about photoperiodic regulatory mechanisms from them.

The objective of the present study was to elucidate the rhythm of responsivity of the photosensitive neuronal complex in the quail hypothalamus by means of once daily application of direct electrical stimulation.

Abbreviations: *GnRH* gonadotropin releasing hormone; *LD* light and dark; *LED* light emitting diode; *CRT* cathode ray tube; *LH* luteinizing hormone; *OD* outer diameter; *ID* inner diameter

Materials and methods

Animals and photoperiods. Male Japanese quail (*Coturnix coturnix japonica*) were purchased from a commercial source at the age of 3 weeks and reared in individual cages, 18 × 10 × 18 cm, under a non-stimulatory photoperiod of 8 h of light and 16 h of dark (LD 8:16, lights on from 1000 to 1800 h). White fluorescent lamps provided illumination of at least 300 lux at the floor of the cages. Birds used in experiments were 35–42 days of age (under short day quail are still sexually immature at this age; maturation occurs 5–6 weeks later). The short day photoperiod was maintained throughout the experiments.

Optic fibers and photic stimulation. Plastic optic fiber (Machida, Tokyo, OD 125 μm 9 mm long) was inserted into a fine stainless steel sheath (OD 0.4 mm, ID 0.2 mm, 8.5 mm long). The tip of the fiber protruded 0.3 mm beyond the lower end of the sheath. After urethane anesthesia (25–40 mg/kg B.W.) birds were held in a stereotaxic instrument modified for quail. A polished light-outlet of the optic fiber (emitting angle of light beam = 63°) was lowered down to the desired position in the hypothalamus referring to the coordinates in the brain atlas (Baylé et al. 1974) and the stainless sheath was cemented firmly to the skull with a glass-ionomer (a mixture of aluminoscilicate and polyacrylic acid, G-C Dental, Tokyo). A green light emitting diode (LED, International Rectifier, Kanagawa), peak at 560 nm, was employed as the light source and its lighting surface was glued to the upper end (light-inlet) of the implanted optic fiber with acrylresin dissolved in dichloroethane. With the wiring system we employed, birds were allowed to move freely in the cages and they seem to have no difficulty in feeding and drinking (Fig. 1).

In experiment 1, in order to locate the most photosensitive site, birds bearing optic fibers were subjected to 16 h of daily hypothalamic lighting starting at 1800 h for 21 consecutive days. Control birds received similar implants but were not illuminated with the fiber (sham operated, Table 1). Intact control birds (intact, Table 1) were kept in the cages beside the experimental birds. On-off time of the LEDs was regulated with a quartz clock, and the light intensity (290 μcd, at 2.0 V, 5 mA) with a solid state voltage stabilizer.

In experiment 2, a group of birds received hypothalamic illumination at site 9 in Fig. 2a. Blood samples for hormone assay were collected during and at the end of experiments.

Type of electrode and electrical stimulation. Wiring and implanting procedures used for photic stimulation were adapted for electrical stimulation with a slight modification. Bipolar (coaxial) electrodes consisting of epoxy coated stainless tube (OD 0.4 mm, ID 0.2 mm, 8.5 mm long) with a polyurethane coated platinum wire (OD 0.05 mm) as the core were used. Both anodal (platinum) and cathodal (stainless steel) ends of the electrode were bared of the coating for 0.2 mm at the tip and the anodal end was adjusted to protrude 0.3 mm beyond the cathodal end. The tip of the electrode was positioned, by a procedure similar to that used in the optic fiber implant, at site 9 in Fig. 2a which had best responded to the direct illumination with the optic fiber (coordinate X = 3.8, Y = −0.1 in brain atlas, Baylé et al. 1974). The stimulation parameters used in the electrical study were square waves of 100 Hz, 1.0 or 2.0 ms in pulse duration, 100 μA in peak to peak current. The current and waveforms were monitored by a set of microammeters and on a CRT screen. Two minutes of stimulation to each bird was given once daily. A different stimulation time was allotted to nine groups (A to I, Table 2). Two control groups were prepared (J and K in Table 2); one group of birds (group J) received stimulation at the time between 1800 and 2200 h to site 1 or more rostral area in Fig. 2a, and the other (group K) served as intact control. On day 5 and day 14, 1 ml blood samples were taken at 5 min before and 55 min after the stimulation in three groups (D, F and J).

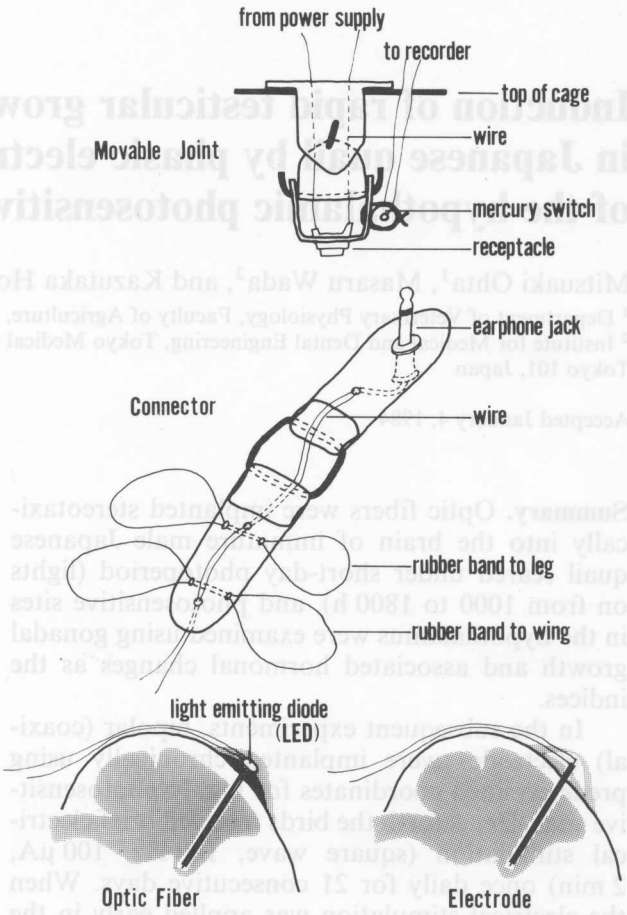

Fig. 1. The photic and electrical stimulation unit (see Materials and methods)

Confirmation of the stimulation sites. At the end of experiments, birds were killed and brain tissue was fixed in 10% formalin. The combined testes weights (fresh) were taken to the nearest mg. Fixed hypothalamic tissues were frozen, serially sectioned at 50 μm and stained with Nissl's reagent to locate the real site of stimulation.

Assay of hormones. Blood samples were collected by heart puncture. Heparinized samples were centrifuged with minimum delay and separated plasma specimens were stored at −20 °C until assay. Plasma testosterone was measured by the method of Coyotupa et al. (1972). Plasma luteinizing hormone (LH) was measured by post-precipitation double antibody radioimmunoassay. Anti-avian LH serum raised in rabbit, purified avian-LH for radioiodination (IEF-1 Gunma) and the reference standard (IRC-2 Gunma) were kindly donated by Drs. Hattori and Wakabayashi, Institute of Endocrinology, Gunma University.

Statistics. Statistical significance between the responses of different groups was calculated by multiple range test (Duncan 1955; Kramer 1956).

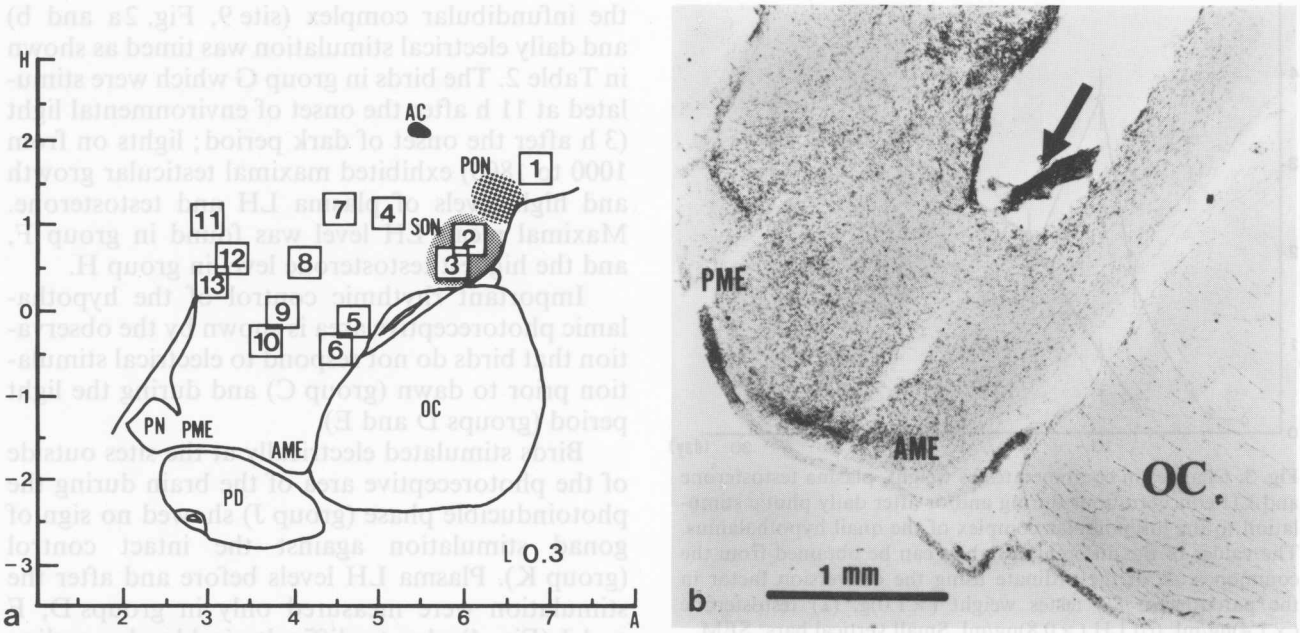

Fig. 2. a Diagrammatic representation of the positioning of the tip of the optic fiber in the quail hypothalamus: A square with number shows position of the tip. Anterior (*A*) and vertical (*H*) of stereotaxic coordinates at lateral (L = 0.3). *OC* optic chiasma; *PON* preoptic nucleus; *SON* supraoptic nucleus; *AME* anterior median eminence; *AC* anterior commissure; *PME* posterior median eminence; *PD* pars distalis and *PN* pars nervosa of the pituitary. **b** Sagittal section of hypothalamus of representative quail which showed a marked gonadal growth (×42). *Arrow*: implant tract of electrode

Results

Photic stimulation

Experiment 1. Optic fibers were implanted into various regions of the brain along the midsagittal plane and 21 days later the site of stimulation and the extent of gonadal growth were examined. Microscopically confirmed sites of local optic fiber illumination are illustrated schematically in Fig. 2a and the corresponding gonadal growth in Table 1. In this series of experiments illumination at site 9 resulted in maximal testicular growth, while illumination at sites 2 and 3 induced moderate growth, and at site 1 or other areas more rostral only slight testicular growth.

Experiment 2. Based on the results of experiment 1, an additional group of immature quail was prepared. They were implanted with optic fibers and subjected to local illumination as in the preceding experiment. The time course of testicular growth was determined by periodic autopsy, and that of plasma hormone levels, by frequent hormone assay (Fig. 3). Each point in Fig. 3 represents the mean of at least 3 birds in which the tips of the fiber were found on a line connecting sites 7 and 10 shown in Fig. 2a.

Plasma LH levels increased rapidly during the first week, peaked on day 10, then declined and stabilized after the third week. Negative feedback of the increased testosterone levels on LH may have been responsible for its decline after day 10. Testicular growth first became noticeable, concurrent with the sharp rise in plasma LH. Changes

Table 1. Combined testes weight, plasma testosterone and LH concentrations at day 21 of the daily photic stimulation to specified sites in and out of the quail hypothalamus

Site[a] number	Testes weight (mg)	Plasma testosterone (ng/ml)	Plasma LH (ng/ml)
1	224	<0.05[b]	0.37
2	939	0.58	2.61
3	1006	0.67	2.50
4	1735	1.50	2.36
5	1985	1.84	2.31
6	2224	1.83	2.29
7	2450	2.75	3.10
8	2357	1.85	2.34
9	3607	2.23	2.01
10	2387	2.01	1.98
11	1313	0.93	2.64
12	1950	1.90	2.45
13	2513	2.01	2.07
Controls			
Intact ($n=5$)	60.0±20.8[c]	<0.05	0.53±0.36
Sham ($n=8$)	33.0±10.2	<0.05	0.25±0.04

[a] See Fig. 2a (one bird per site)
[b] Lower limit of assay
[c] Mean±SEM; n = number of birds

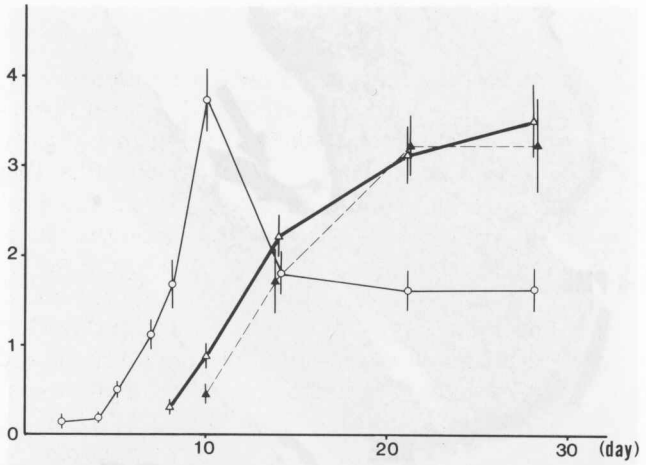

Fig. 3. Changes in combined testes weight, plasma testosterone and LH concentrations during and/or after daily photic stimulation to the infundibular complex of the quail hypothalamus. The values of the different variables can be obtained from the common scale in the ordinate using the conversion factor in the parenthesis; (△) testes weight (×1.0)g, (▲) testosterone (×1.4)ng/ml, (○) LH (×0.8)ng/ml. Small vertical bars: SEM

in plasma testosterone paralleled changes in testicular weight.

Electrical stimulation

A new group of immature birds were prepared for electrical stimulation as described in the Methods section. The tips of electrodes were positioned in the infundibular complex (site 9, Fig. 2a and b) and daily electrical stimulation was timed as shown in Table 2. The birds in group G which were stimulated at 11 h after the onset of environmental light (3 h after the onset of dark period; lights on from 1000 to 1800) exhibited maximal testicular growth and high levels of plasma LH and testosterone. Maximal mean LH level was found in group F, and the highest testosterone level in group H.

Important rhythmic control of the hypothalamic photoreceptive area is shown by the observation that birds do not respond to electrical stimulation prior to dawn (group C) and during the light period (groups D and E).

Birds stimulated electrically at the sites outside of the photoreceptive area of the brain during the photoinducible phase (group J) showed no sign of gonad stimulation against the intact control (group K). Plasma LH levels before and after the stimulation were measured only in groups D, F and J (Fig. 4), due to difficulty in blood sampling in complete darkness within a limited time. On day 5, mean plasma LH in group F before the stimulation was still comparable to that of groups J and D, and its increase after stimulation was not significant compared to either group. On day 14 the basal LH level of group F was significantly elevated over that of groups D and J ($P<0.001$) but temporal increase in response to stimulation was not obvious. Perhaps a longer delay in blood

Table 2. Combined testes weight, plasma testosterone and LH concentrations after daily electrical stimulation (2 min) to the infundibular complex of the Japanese quail for 21 days. Lights were on from 1000 to 1800 h

Groups	Number of birds	Body weight (g)		Testes weight (mg)	Plasma testosterone (ng/ml)	Plasma LH (ng/ml)
		Initial	Final			
A(0000 h)[1]	5	109.1±3.2[2]	104.6±3.5	395.4±82.0^{g}	0.26±0.11^{h}	1.21±0.54^{i}
B(0200 h)	5	109.2±3.5	113.8±4.0	376.0±110.2^{g}	0.26±0.15^{h}	1.19±0.63^{i}
C(0800 h)	6	110.0±2.8	120.6±3.8	94.7±35.5^{g}	<0.05[3]	0.69±0.13^{i}
D(1400 h)	5	103.5±1.2	110.9±4.3	28.4±17.2^{g}	<0.05	0.17±0.06^{i}
E(1800 h)	5	113.9±3.1	108.9±9.9	70.0±35.6^{g}	<0.05	0.66±0.07^{i}
F(2000 h)	5	91.3±4.5	105.3±3.7	1323.0±122.5^{b}	0.18±0.07^{h}	3.78±0.03^{f}
G(2100 h)	5	102.8±2.5	107.3±2.1	3075.6±241.7^{a}	0.35±0.12^{h}	3.18±0.15^{e}
H(2200 h)	5	108.9±4.0	119.6±3.9	1628.4±290.2^{b}	1.36±0.22^{d}	2.80±0.10^{e}
I(2300 h)	5	109.2±7.4	108.1±8.9	799.0±194.0^{c}	0.45±0.38^{h}	1.99±0.80^{i}
J(2000±2 h)[4]	9	99.6±3.4	100.9±1.7	46.7±10.3^{g}	<0.05	0.54±0.23^{i}
K(Control)	5	95.6±2.0	101.9±3.7	51.8±10.2^{g}	<0.05	0.45±0.10^{i}

[1] Time of stimulation
[2] Mean±SEM
[3] Lower limit of assay
[4] Birds stimulated outside of the photosensitive area (see text)
[a] Significantly greater than all other groups ($P<0.01$)
[b] Significantly greater than other groups except G ($P<0.01$)
[c,e] Significantly greater than any group of A–E, J and K ($P<0.05$)
[d,f] Significantly greater than other groups except I ($P<0.01$)
[b,c,g–i] Identical superscripts indicate no significant difference ($P>0.05$)

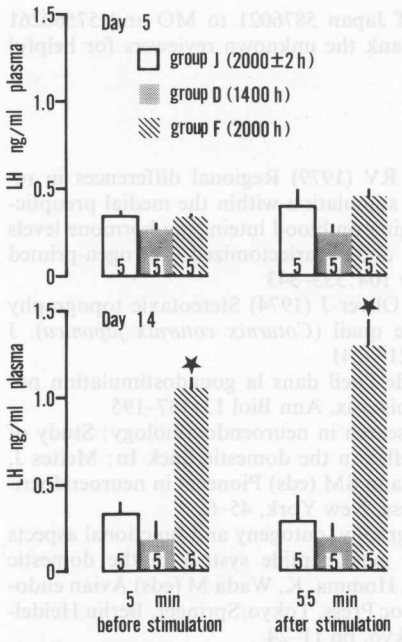

Fig. 4. Changes in plasma LH concentration after day 5 and day 14 of the daily electrical stimulation. Blood samples of each group were taken 5 min before and 55 min after stimulation. Values are mean±SEM; number of birds in each group at the base of bars. ★ Significantly greater than groups D and J ($P<0.001$)

sampling would have yielded a significant post stimulation increase (cf. Davies and Follett 1975).

Discussion

Photic experiments

The first part of the present study with optic fibers was intended to locate stereotaxically the light sensitive site in the quail brain. Green light was used because of its poor penetrance into living tissues (van Tienhoven and Plank 1973; Glass and Lauber 1981). The photoperiod delivered to the brain by the fiber was set at 16 h based on our previous observation that when the brain is illuminated from the surface, lighting for 16 h is even more stimulatory to the gonads than continuous lighting (Homma et al. 1979). Possible use of high intensity red LED (peak emission 700 nm, 360 μcd) and thick optic fiber (800 μm) was tested to shorten the illumination time, but this was found unsuitable for detecting the sites of photoreception owing to fanning out of illuminated area (cf. Yokoyama et al. 1978). The profiles of plasma hormone levels and testicular growth, induced by fiber illumination (Fig. 3) closely resembled those reported in quail exposed to LD 16:8 photoperiod (Wada 1981).

Electrical experiments

Prior to conducting the main experiments with electrical stimulation, the effects of several different kinds of electrical pulses, varying in intensity and duration, were compared. We were thus able to maximize the stimulation parameters to be used routinely for the detection of possible phasic changes in hypothalamic sensitivity. In the course of these test trials, it was found that application of long and intense stimuli which have been reported to be effective for induction of ovulation in the rat and monkey (30 to 60 min in total duration, 100 to 400 μA, Terasawa and Sawyer 1969; Fink and Aiyer 1974; Spies et al. 1977) are not gonadostimulatory at all in immature quail. Furthermore neither reversal of the polarity of the electrode (outer sheath as anode, cf. Terasawa and Sawyer 1969), nor the use of unipolar electrode produced any significant gonadal stimulation in spite of repeated trials using various stimulation parameters. Our failure to obtain a response using the above stimulation protocol does not seem to be due to tissue damage, since on histological examination tissues around the electrode tips were seemingly normal even after long stimulation with 100 μA.

In contrast, stimulation of the hypothalamic area by bipolar electrodes under conditions similar to those described in the methods section, unfailingly stimulated gonadal activity provided that the time of application was within the photoinducible phase and that the duration of the daily stimulus was not more than 10 min. Square waves of 20 to 50 Hz have often been used for hypothalamic stimulation (Douglas 1974). Because we did not test the effects of altering the frequency, our choice of 100 Hz may not be optimal. Application of electrical pulses, which are higher than the optimal frequency, to the hypothalamic nuclei have been found to diminish the degree of response without affecting its basic pattern (Douglas 1974). It therefore seems likely that our conclusions, obtained by using 100 Hz, would be confirmed by experiments in which lower frequences were used.

The physiological meaning of the experimental factors that determine the success or failure of electrical stimulation given to the hypothalamus is still largely unknown. Spreading of excitation from the primary site of stimulation to inhibitory neurons located in the surround (cf. Oliver et al. 1978), might explain ineffectiveness of prolonged or unipolar stimulation to the photosensitive area.

Gonadal recrudescence and photorefractoriness are the chain of events induced by long day; both are primarily conditions of the hypothalamus

(Farner et al. 1983) and are rhythm dependent (Turek 1972). In this context future assessment of the threshold value, at which electrical stimulation turns into gonadoinhibitory would be helpful in the elucidation of the mechanism of photorefractoriness.

Davies and Follett (1975) have documented transient LH release following electrical stimulation of the hypothalamus of immature quail in acute experiments. Their results might indicate the location where GnRH neurones reside (Blähser 1983) but would not predict the rapid testicular growth and significant elevation of basal hormone levels found in the present study. We would emphasize that for weak, brief stimuli to induce conspicuous LH release in young quail, in harmony with the external coincidence model (Pittendrigh and Minis 1964) proper timing of stimulation is essential. This does not necessarily mean that the releasing effect is immediate or that the GnRH neurons are directly stimulated; rather such a weak stimulus might function as the signal to trigger a regulatory process or localized oscillator.

In spite of the paramount importance of the phase of stimulation, little attention has been paid to this aspect of electrical stimulation in acute experiments in immature quail (Davies and Follett 1975). The importance of the timing of electrical stimulation in photoperiodic studies has also been suggested recently in the hamster (Earnest and Turek 1983).

In our study, electrical stimulation of the non-photoreceptive area of the quail brain did not produce conspicuous testicular growth. Furthermore the phase of photo- and electro-inducibility at the photoreceptive area were in good chronological agreement (Homma et al. 1979). Taken together, these results indicate that the same neural complex may be responsible for induction of gonadal growth in response to either photic or electrical stimulation. Thus results obtained by one of these kinds of stimulation may increase our understanding of the effects of the other. The suspected insensitivity of the quail hypothalamus to direct photic stimulation during the environmental light period in short-days is matched by our finding of concurrent refractoriness of this area to electrical stimulation. The method of phasic electrical stimulation, which has enabled us to describe the temporal sensitivity of the hypothalamus explicitly, might also be used as a powerful tool for future studies of more complicated systems such as the entraining mechanisms of the circadian clock.

Acknowledgements. This research was supported in part by grants for Scientific Research from the Ministry of Education, Science and Culture of Japan 5876021 to MO and 57560261 to KH. We wish to thank the unknown reviewers for helpful comments.

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