PART I

STUDIES ON THE ORGANIZATION OF THE EYE OF *APLYSIA CALIFORNICA*

PART II

STUDIES ON THE INTERRELATIONSHIP BETWEEN TWO NEURONAL CIRCADIAN OSCILLATORS IN *APLYSIA CALIFORNICA*

Thesis by

Gerald Joseph Audesirk

In Partial Fulfillment of the Requirements

for the Degree of

Doctor of Philosophy

California Institute of Technology

Pasadena, California

1974

(Submitted November 13, 1974)

To Terry

ACKNOWLEDGMENT

I would like to thank:

Dr. Felix Strumwasser, my advisor, for helpful advice, education, suggestions, and friendship during my stay at Caltech;

the other members of my committee, Drs. Wiersma, Lester, Russell, and Pettigrew, for encouragement and advice, especially in the writing of this thesis;

Barry Rothman, David Armstrong, and especially Duncan Stuart, for discussions, help, and good times;

Dennis and Karen Searcy, for introducing me to the Sierra;

Dave McConnell, for friendship and guitar playing with a newcomer;

and Terry, for everything.

ABSTRACT

PART I

The isolated eye of Aplysia californica produces a bursting pattern of spontaneous compound action potentials (CAPs) when recordings are made from the optic nerve in darkness. The CAP frequency varies with a circadian rhythm. The light response, also composed of CAPs, may be separated into an initial phasic response and a late tonic response similar in form to the dark discharge. Solutions containing La^{+++} or high Mg^{++} with low Ca^{++}, which are expected to block chemical synapses, stop the dark discharge and tonic light response but not the phasic light response. The suppression of dark discharge by high Mg^{++} with low Ca^{++} is usually temporary, lasting about 0.5 to 4 hours. Synchrony of the CAPs is not affected by either La^{+++} or high Mg^{++}, low Ca^{++}. These results indicate that the dark discharge is driven through chemical synapses, but the light response is not. Replacement of chloride in the bathing medium by propionate, which uncouples electrical junctions in the crayfish septate axon, abolishes all CAPs for varying periods of time, usually several hours. Propionate leaves the ERG intact and the optic nerve electrically excitable. A model for interneuronal connections in the Aplysia eye is constructed from these data. It is postulated that the light response is initiated in the photoreceptors, with the receptor depolarization passing through electrical synapses to higher order cells. Spikes are produced in these cells and pass down their axons in the optic nerve. Spontaneous dark activity

also represents spiking in these higher order cells, but is initiated through chemical synapses by pacemaker cell(s). Synchrony of the CAPs is facilitated by electrical synapses between higher order cells. In low Ca^{++} media, these higher order cells may become hyperexcitable to the point of autoactivity.

ABSTRACT

PART II

The circadian rhythm of spike output of the single neuron R15 in the isolated PVG of Aplysia californica can be entrained in vivo by light. The timing of the rhythm depends not only on the lighting schedule to which the animal was exposed prior to dissection, but also on the time of dissection relative to that light schedule. Entrainment of the rhythm by light proceeds very slowly, if at all, in Aplysia with their eyes removed. An indirect inhibitory neural pathway is shown to exist between the eyes and R15, but cutting nervous connections containing this and any other neural paths from the eyes to R15 does not prevent entrainment by light in a majority of animals. In vitro experiments show that the eyes can influence the activity of R15 even when the eyes and the PVG are not neurally connected. The eyes therefore must release a water soluble factor which can affect R15, either directly or through some other neurons in the PVG. If the eyes and PVGs from different animals are incubated together for several days and then separated, the subsequent spiking behavior of R15 is similar to that observed after in vivo entrainment to a light schedule equivalent in phase to the circadian rhythm of the eyes in vitro. It is a strong possibility that the factor released by the eyes can entrain the circadian rhythm of R15.

CONTENTS

General Introduction to Circadian Rhythms

Circadian rhythms are cyclically repeating biological events, for example locomotor activity, glandular secretions, and neuronal spike frequencies, which occur, in the absence of external time cues, with a period close to, but not exactly, 24 hours. This free running periodicity is, in nature, synchronized with the exact 24 hour period of the earth's rotation through reception of environmental time cues. The rhythms are reset daily so as to coincide with the 24 hour day. The most common cue used to entrain circadian rhythms is the alternation of night and day. An entrained rhythm usually maintains a characteristic phase relation to the light-dark cycle; i.e., some event of the rhythm, such as the onset of activity, occurs at a fixed time relative to dawn or dusk.

The study of circadian rhythms in animals has progressed rapidly since the demonstrations by von Frisch (1950) and Kramer (1950) that bees and birds, respectively, must possess an internal clock to aid in navigation. Several major areas of investigation have received particular attention: 1) the formal properties of circadian rhythms, presumably the same in most or all animals and plants; 2) the underlying molecular mechanisms; 3) the organs containing the endogenous oscillators which generate rhythmicity (especially under constant conditions); 4) the organs receiving environmental rhythmicity cues (usually light or temperature); and 5) the coupling between the receptor organs and the endogenous oscillator and this oscillator with other subordinate ones.

The study of the formal properties of rhythms has usually been undertaken in whole organism experiments, such as locomotor activity rhythms or insect eclosion rhythms. These investigations have yielded information about the timing of the rhythm by external cues (e.g., phase response curves; Pittendrigh, 1960, 1974); the endogenous, circadian nature of the rhythm in the absence of (detectable) external time cues; the stability of the rhythm in the face of changes in certain external conditions (especially temperature). Excellent general reviews of these and other properties of circadian rhythms have been written by Pittendrigh (1960 and 1974).

Far less is known about the molecular mechanisms underlying circadian rhythms. In several systems RNA and/or protein synthesis is implicated in the production of rhythmicity (Karakashian and Hastings, 1963; Strumwasser, 1965; Feldman, 1967; Cymborowski and Dutkowski, 1969, 1970; Rothman and Strumwasser, 1973). However, in Acetabularia a rhythm of photosynthetic capacity occurs even in the absence of the nucleus (vanden Driessche, 1971), although this rhythm apparently is normally controlled by a nuclear rhythm.

The search for the site of the driving oscillator in multicellular animals, presumably in the central nervous system, has resulted in more knowledge about where such oscillators are not than where they are. In mammals, recent results indicate the suprachiasmatic nucleus in the hypothalamus is the site of the major driving oscillator for several rhythms (Stephan and Zucker, 1972; Moore, 1974). Many subordinate circadian rhythmic centers are also known (Menaker, 1974). In birds,

a major role has been suggested for the pineal organ (Gaston and Menaker, 1968). Potential sites in other vertebrates have not been identified, for the most part. It is known that the pineal is not the driving site in lizards (Underwood, 1973). The data on invertebrates are even more scanty, despite a great many experiments, especially on insects. Reports have appeared claiming to demonstrate the master role of the subesophageal ganglion (Harker, 1956) and of the pars intercerebralis (Nishiitsutsuji-Uwo and Pittendrigh, 1968b) in cockroaches, with each report quite convincingly disposing of any role for the rest of the nervous system, including the part found vital in the other paper. (For a discussion of this controversy, see Harker, 1973.) Konopka (Ph.D. dissertation, California Institute of Technology, 1972), using mutant gynandromorph Drosophila, has apparently succeeded in localizing the eclosion and locomotor activity master clock(s) in the head of the fly.

The receptor for photoentrainment has also been difficult to locate in many cases. In mammals alone it is clearly known that the "obvious" route, the eyes, mediates entrainment of rhythms to light cycles (Browman, 1943; Halberg, Visscher, and Bittner, 1954). Entrainment is not accomplished through the form - movement visual pathways to the visual cortex, but rather through the accessory tract and a special retinohypothalamic route (Stephan and Zucker, 1972; Moore, 1974). In birds (Menaker, 1968), lizards (Underwood, 1973), frogs (Adler, 1971), salamanders (Adler, 1969), and fish (Reed, 1968; Erikson, 1972), the lateral eyes are not required for entrainment, although large

differences in the form of the rhythms measured are often seen after blinding. However, it is not known what the receptor organ is in any of these animals. In lizards, for example, Underwood (1973) removed lateral eyes, pineal organ, parietal eye, and painted the head black without abolishing entrainment to light in most cases.

The situation in insects has recently become fairly clear for those species that have been studied. As of a few years ago, the photoreceptor for entrainment of the locomotor rhythm in cockroaches was thought to be, depending upon the report read, 1) both the ocelli and the compound eyes (Cloudsley-Thompson, 1953); 2) the ocelli alone (Harker, 1956); or 3) the compound eyes alone (Nishiitsutsuji-Uwo and Pittendrigh, 1968a). Recently it has been agreed upon that the compound eyes are the receptor (Roberts, Skopik, and Driskill, 1971). Eclosion rhythms do not use the eyes (Truman and Riddiford, 1970); apparently the brain is directly light sensitive at normal environmental levels.

Finally, as might be expected, the nature of the coupling between receptor and master clock, and master with subordinate systems, remains quite obscure in most cases. See the introduction to Part II for a discussion of this point.

References

Adler, K. 1969. Extraoptic phase shifting of circadian locomotor rhythm in salamanders. Science 164: 1290-1292.

Adler, K. 1971. Pineal end organ: role in extraoptic entrainment of circadian locomotor rhythm in frogs. In: M. Menaker (ed.), Biochronometry. Washington, D.C.: National Academy of Sciences, pp. 342-350.

Browman, L.G. 1943. The effect of bilateral optic enucleation upon activity rhythms of the albino rat. J. Comp. Psychol. 36: 33-46.

Cloudsley-Thompson, J.L. 1953. Studies in diurnal rhythms. III. Photoperiodism in the cockroach Periplaneta americana (L.) Ann. Mag. Nat. Hist. 6: 705-712.

Cymborowski, B., and A. Dutkowski. 1969. Circadian changes in RNA synthesis in the neurosecretory cells of the brain and sub-esophageal ganglion of the house cricket. J. Insect Physiol. 15: 1187-1197.

Cymborowski, B., and A. Dutkowski. 1970. Circadian changes in protein synthesis in the neurosecretory cells of the central nervous system of Acheta domesticus. J. Insect Physiol. 16: 341-348.

Erikson, L.O. 1972. Tagesperiodik geblendeter Bachsaiblinge. Naturwissenschaften 59: 219-220.

Feldman, J.F. 1967. Lengthening the period of a biological clock in Euglena by cycloheximide, an inhibitor of protein synthesis. Proc. Nat. Acad. Sci. 57: 1080-1087.

Frisch, K. von. 1950. Die Sonne als Kompass im Leben der Bienen. Experientia 6: 210-221.

Gaston, S., and M. Menaker. 1968. Pineal function: the biological clock in the sparrow? Science 160: 1125-1127.

Halberg, F., M.B. Visscher, and J.J. Bittner. 1954. Relation of visual factors to eosinophil rhythm in mice. Am. J. Physiol. 179: 229-235.

Harker, J. 1956. Factors controlling the diurnal rhythm of activity in Periplaneta americana (L.). J. Exp. Biol. 33: 224-234.

Harker, J. 1973. Circadian rhythms in insects. In: J.N. Mills (ed.), Biological Aspects of Circadian Rhythms. New York: Plenum Press, pp. 189-233.

Karakashian, M.W., and J.W. Hastings. 1963. The effects of inhibitors of macromolecular biosynthesis upon the persistent rhythm of luminescence in Gonyaulax. J. Gen. Physiol. 47: 1-12.

Konopka, R. 1972. Circadian clock mutants of Drosophila melanogaster. Ph. D. dissertation, Calif. Inst. of Tech.

Kramer, G. 1950. Weitere Analyse der Faktoren, welche die Zugaktivität des gekäfigten Vogels orientieren. Naturwissenschaften 37: 377-378.

Menaker, M. 1968. Extraretinal light perception in the sparrow. I. Entrainment of the biological clock. Proc. Nat. Acad. Sci. 59: 414-421.

Menaker, M. 1974. Aspects of the physiology of circadian rhythmicity in the vertebrate central nervous system. In: F.O. Schmitt and F.G. Worden (eds.), The Neurosciences: Third Study Program. Cambridge: MIT Press, pp. 479-489.

Moore, R.Y. 1974. Visual pathways and the central neural control of diurnal rhythms. In: F.O. Schmitt and F.G. Worden (eds.), The Neurosciences: Third Study Program. Cambridge: MIT Press, pp. 537-542.

Nishiitsutsuji-Uwo, J., and C. Pittendrigh. 1968a. Central nervous system control of circadian rhythmicity in the cockroach. II. The pathway of light signals that entrain the rhythm. Z. Vergl. Physiol. 58: 1-13.

Nishiitsutsuji-Uwo, J., and C. Pittendrigh. 1968b. Central nervous system control of circadian rhythmicity in the cockroach. III. The optic lobes, locus of the driving oscillator? Z. Vergl. Physiol. 58: 14-46.

Pittendrigh, C. 1960. Circadian rhythms and the circadian organization of living systems. Cold Spring Harbor Symp. Quant. Biol. 25: 159-184.

Pittendrigh, C. 1974. Circadian oscillations in cells and the circadian organization of multicellular systems. In: F.O. Schmitt and F.G. Worden (eds.), The Neurosciences: Third Study Program. Cambridge: MIT Press, pp. 437-458.

Reed, B.L. 1968. The control of circadian pigment changes in the pencil fish: a proposed role for melatonin. Life Sci. 7(II): 961-973.

Roberts, S.K., S.D. Skopik, and R.J. Driskill. 1971. Circadian rhythms in cockroaches: does brain hormone mediate the locomotor cycle? In: M. Menaker (ed.), Biochronometry. Washington, D.C.: National Academy of Sciences, pp. 505-516.

Rothman, B.S., and F. Strumwasser. 1973. Aflatoxin B1 blocks the circadian rhythm in the isolated eye of Aplysia. Fed. Proc. 32: 365.

Stephan, F.K., and I. Zucker. 1972. Rat drinking rhythms: central visual pathways and endocrine factors mediating responsiveness to environmental illumination. Physiol. Behav. 8: 315-326.

Strumwasser, F. 1965. The demonstration and manipulation of a circadian rhythm in a single neuron. In: J. Aschoff (ed.), Circadian Clocks. Amsterdam: North-Holland Publ. Co., pp. 442-462.

Truman, J.W., and L.M. Riddiford. 1970. Neuroendocrine control of ecdysis in silkmoths. Science 167: 1624-1626.

Underwood, H. 1973. Retinal and extraretinal photoreceptors mediate entrainment of the circadian locomotor rhythm in lizards. J. Comp. Physiol. 83: 187-222.

Vanden Driessche, T. 1971. Structural and functional rhythms in the chloroplasts of Acetabularia: molecular aspects of the circadian system. In: M. Menaker (ed.), Biochronometry. Washington, D.C.: National Academy of Sciences, pp. 612-622.

Circadian Rhythms in *Aplysia*

It is widely assumed, and most data indicate, that the controlling circadian rhythms in animals are found in the nervous system. Thus the same properties of marine opisthobranchs that are advantageous for neurophysiological and neurochemical studies also make them useful subjects for investigations of circadian rhythms: very large, electrically excitable neuron somas; synapses sufficiently close to the cell body so that large postsynaptic events can be recorded there; distributed nervous systems; numbers of morphologically, physiologically, and biochemically identifiable cells; and relatively large body size.

Aplysia californica has so far been the only opisthobranch extensively used in circadian rhythm studies. Four rhythms have been described in *Aplysia*. Strumwasser (1963, 1965) discovered that the identifiable neuron R15 in the parietovisceral ganglion (PVG) possesses a circadian rhythm of spiking activity recorded in the isolated ganglion. Second, other, as yet unidentified, neurons in the PVG show circadian rhythmicity of spike activity for many days *in vitro* (Strumwasser, 1967). Strumwasser (1967) and Kupfermann (1968) independently showed that the locomotor activity of the intact animal occurs with a circadian rhythm. Fourth, Jacklet (1969b) found that the frequency of compound action potentials (CAPs) recorded from the optic nerve of the isolated eye in constant darkness also has circadian rhythmicity.

Strumwasser (1965) has shown that the circadian rhythm of R15 can

be entrained by the lighting regime to which the animal was subjected prior to dissection. He found that the peak activity of spiking in the cell, monitored continuously for 24 hours or more, usually occurred very near dawn (Fig. 1, from Strumwasser, 1965). Some cells showed more than one cycle of activity. A four hour advance in the lighting schedule of the animals' holding tank resulted, after a few days of entrainment, in a four hour advance in the timing of the cell's activity. Other experiments on this neuron have shown that the rhythm is affected by intracellular injection of the DNA-dependent RNA synthesis inhibitor actinomycin D and by the application of heat pulses to the whole PVG (Strumwasser, 1965); that the isolated soma is endogenously active (Alving, 1968); and that the rhythm persists for at least one cycle in the absence of spiking or synaptic input (Strumwasser, 1973). It has been reported that the timing of the peak activity of the R15 rhythm changes with time of year (Lickey, 1969), and that entrainment of the rhythm by light in vivo does not require the presence of the eyes (Lickey, Zack, and Birrell, 1971; Lickey and Zack, 1973). Little is known of the properties of the unidentified circadian oscillators in the PVG, except that, unlike R15, these cells can maintain rhythmicity for many days in vitro.

The locomotor activity of Aplysia also follows a circadian pattern. The animal, at least in the laboratory, is strongly diurnal. Nearly all of the locomotor activity occurs in the lighted portion of the light-dark cycle, and the animal is quiet, even appearing to "sleep" in preferred positions in the aquarium, in the dark

(Strumwasser, 1967, 1971). This is a true endogenous rhythm, as shown by its persistence under constant light (Strumwasser, 1971).

Since the animal has a distributed CNS, the dissection of the rhythm and its localization in restricted parts of the nervous system should be possible. So far, two main manipulations have been performed: removal of the eyes and removal of the PVG. Aplysia without their PVGs (which contain the known circadian oscillator R15 as well as others (Strumwasser, 1973, 1974)) show normal locomotor rhythms for months, once the trauma of the operation wears off (Strumwasser, Schlechte, and Bower, 1972). Thus the driving center for the locomotor rhythm must lie elsewhere, possibly in the circum-esophageal ganglia.

The situation with regard to the eyes is more complicated. Strumwasser, using a non-contact, optical tracking system which generates data which can be converted into an estimate of distance travelled per unit time, has found that most Aplysia, after blinding, are no longer capable of entraining their locomotor rhythms to an external LD cycle of 300 to 500 lux (Strumwasser, 1973, 1974). The data are analyzed for rhythmicity with periodograms and power spectra (see Strumwasser, Schlechte, and Streeter, 1967, for a description of these methods of analysis). A few blinded animals remain entrained, and most blinded Aplysia will entrain under high intensities of light (about 1800 lux; Strumwasser, 1973). Block and Lickey (1973), on the other hand, using a contact system connected to an event recorder, report that blind animals, although showing differences in their

locomotor patterns, still photoentrain to low intensities (170 lux). The analysis of these data is unfortunately only by visual inspection of the event recorder charts. Examples of the data collected by the two systems are shown in Figures 2 and 3. It seems likely to this writer that a statistical analysis of the data of Block and Lickey would reveal severely diminished rhythmicity in the activity of the blinded animals, and in some cases perhaps none at all or a free run (especially in the case of data such as that of Fig. 3ii-B). Also, the activity averages of Strumwasser (1973; Fig. 2) hint at the possibility of continued responsiveness of blinded animals to the onset of light, without entrainment of the entire activity cycle. It is possible that the analysis and interpretation of the data are more different in the two laboratories than the data themselves. Both groups do agree, at least, that blinding has large effects on the activity of the animals, even though there is no apparent effect on health.

The fourth rhythm described in Aplysia so far is that in the frequency of CAPs recorded from the optic nerve of the isolated eye in constant darkness. The eye of Aplysia is of the closed vesicle type, with the retina almost completely surrounding the lens (Jacklet, 1969a). The output of the nerve is in the form of compound action potentials, there is no apparent mechanism for focusing, and the receptors show morphological evidence for electrical coupling (Alvarez and Strumwasser, in preparation). Thus the eye is probably not used for form vision. This has not, however, been behaviorally tested. The eye responds nearly linearly to changes in light intensity

over about five log units (Waser, 1968; Jacklet, 1969a) in addition to the production of spontaneous impulses in darkness.

Many of the properties of circadian rhythms known from other animals are also known for Aplysia eyes. For example, the eyes can be phase shifted by light in vivo and in vitro (Eskin, 1971). Phase response curves for the free running rhythm in vitro have been obtained for pulses of high potassium (Eskin, 1972) and light (Jacklet, 1974). Phase shifts are also induced by pulses of the protein synthesis inhibitor puromycin (Rothman, Ph.D. dissertation, California Institute of Technology, 1974). Conflicting reports have appeared, ruling out (Jacklet, 1971) and confirming (Block, Hudson, and Lickey, 1974) extraoptic entrainment of the eye rhythm by light in vivo.

It is not known how the circadian rhythm of the eye is produced. Jacklet and Geronimo (1971) hypothesized that non-photoreceptor cells whose axons enter the optic nerve and which produce the CAP also are the origin of the rhythm. These cells were thought to be ultradian individually, but to produce a circadian rhythm through unspecified "interactions." However, the data upon which this hypothesis was based are not convincing and could not be reproduced (Sener, 1972; Strumwasser, 1973).

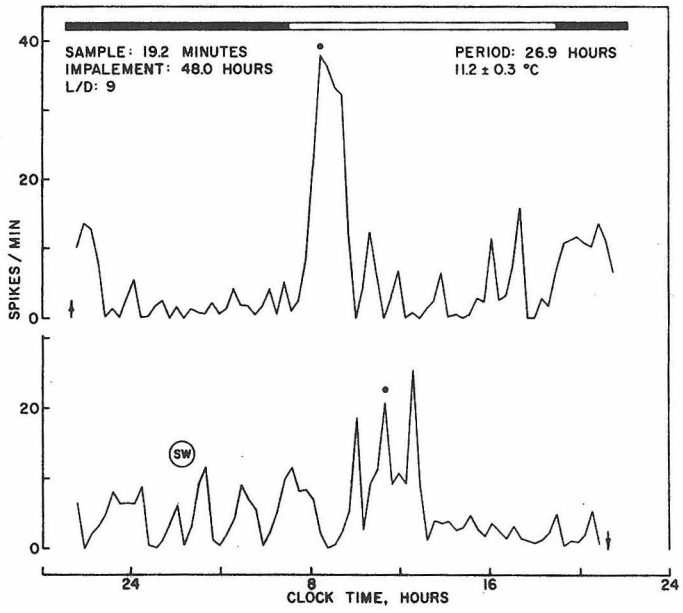
Aplysia rhythms thus relate to several of the topics mentioned in the general introduction. The formal properties of rhythms can be probed in the eye and locomotor rhythms, although the potential here is perhaps less than in some other animals, especially insects, due to the difficulties involved in obtaining, maintaining, and assaying large

numbers of Aplysia. The basic molecular mechanisms generating rhythms on a cellular level can be studied in the eye and in R15. The search for individual oscillators can be (and has been) narrowed to the single neuron level (R15). The eye is both a photoreceptor capable of entraining other rhythms, and also has one of its own, which, of course, it can also entrain, even in vitro. Finally, coupling between oscillators and between receptor and oscillator is perhaps easier to study in Aplysia than in most other animals.

This dissertation consists of two parts. The first is an attempt to learn something about neuronal interactions within the Aplysia eye. Until more is known about the morphological and physiological properties of intercellular connections within the eye, it will remain a black box not unlike the whole animal black boxes of many other circadian rhythm investigations. The second part is a study of the coupling of the eye and R15 rhythms. In particular, several questions have been asked, and, it is hoped, at least partially answered: Do the eyes entrain R15's rhythm? What connections exist between the eyes and R15 by which they might influence each other's activities? If the eyes entrain the R15 rhythm, what pathways might the entrainment take? Last, can an in vitro assay system for entrainment be constructed?

Figure 1. Upper: Spiking activity of neuron R15 recorded for 48 hours from the isolated PVG of Aplysia. The light schedule under which the animal was kept prior to dissection is given at the top of the graph. The open bar represents projected day. Note the strong activity peak near projected dawn, repeating on the second day. From Strumwasser, 1965.

Lower: Spiking activity of R15 in the isolated PVG. Open bar represents projected night. From Lickey, 1969.


SAMPLE: 19.2 MINUTES
IMPALEMENT: 48.0 HOURS
L/D: 9
PERIOD: 26.9 HOURS
11.2 ± 0.3 °C
SPIKES / MIN
40
20
0
20
0
SW
24
8
16
24
CLOCK TIME, HOURS

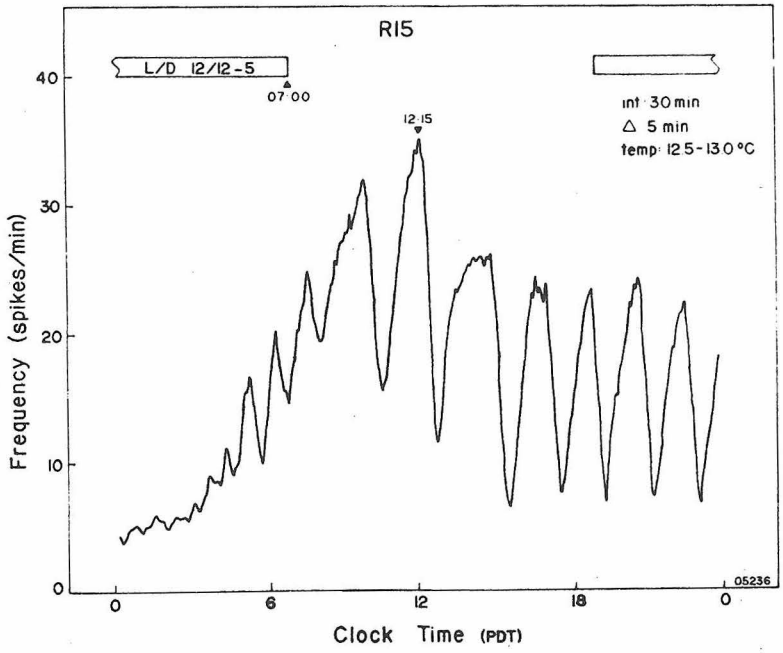

R15
L/D 12/12-5
07:00
12:15
int: 30 min
Δ 5 min
temp: 12.5-13.0 °C
Frequency (spikes/min)
40
30
20
10
0
0
6
12
18
0
05236
Clock Time (PDT)

Figure 2. Locomotor activity averages (left) and periodograms (right) from an Aplysia kept under a light-dark schedule of LD 12:12. The eyes were removed between the second and third frames. Note the strongly diurnal pattern of activity in the intact animal, with the periodogram showing a dominant 24 hour peak. After blinding, nocturnal activity becomes evident, and periodogram shows a gradual change from a free run (approximately a 27 hour period) to complete desynchronization and loss of rhythmicity. From Strumwasser, 1973.

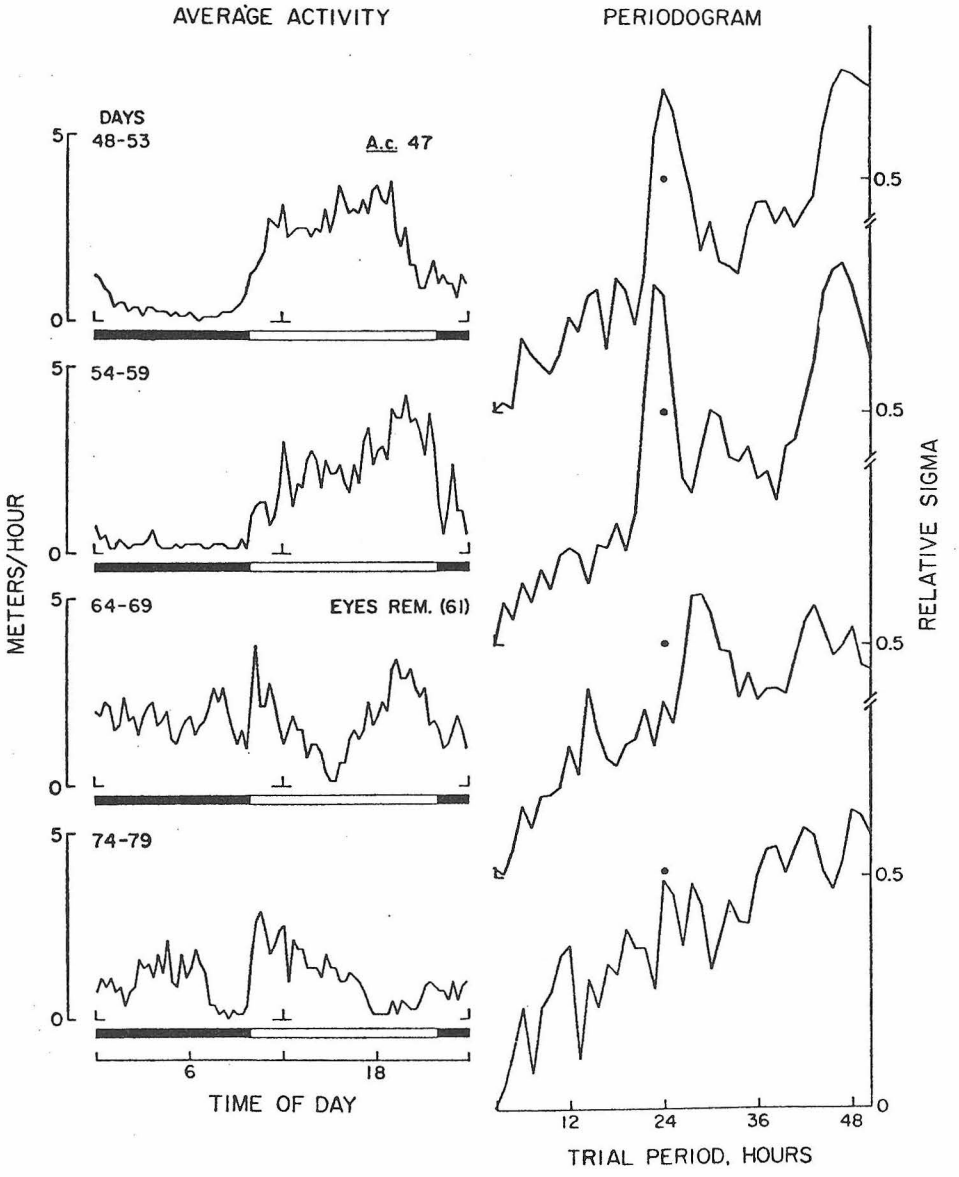
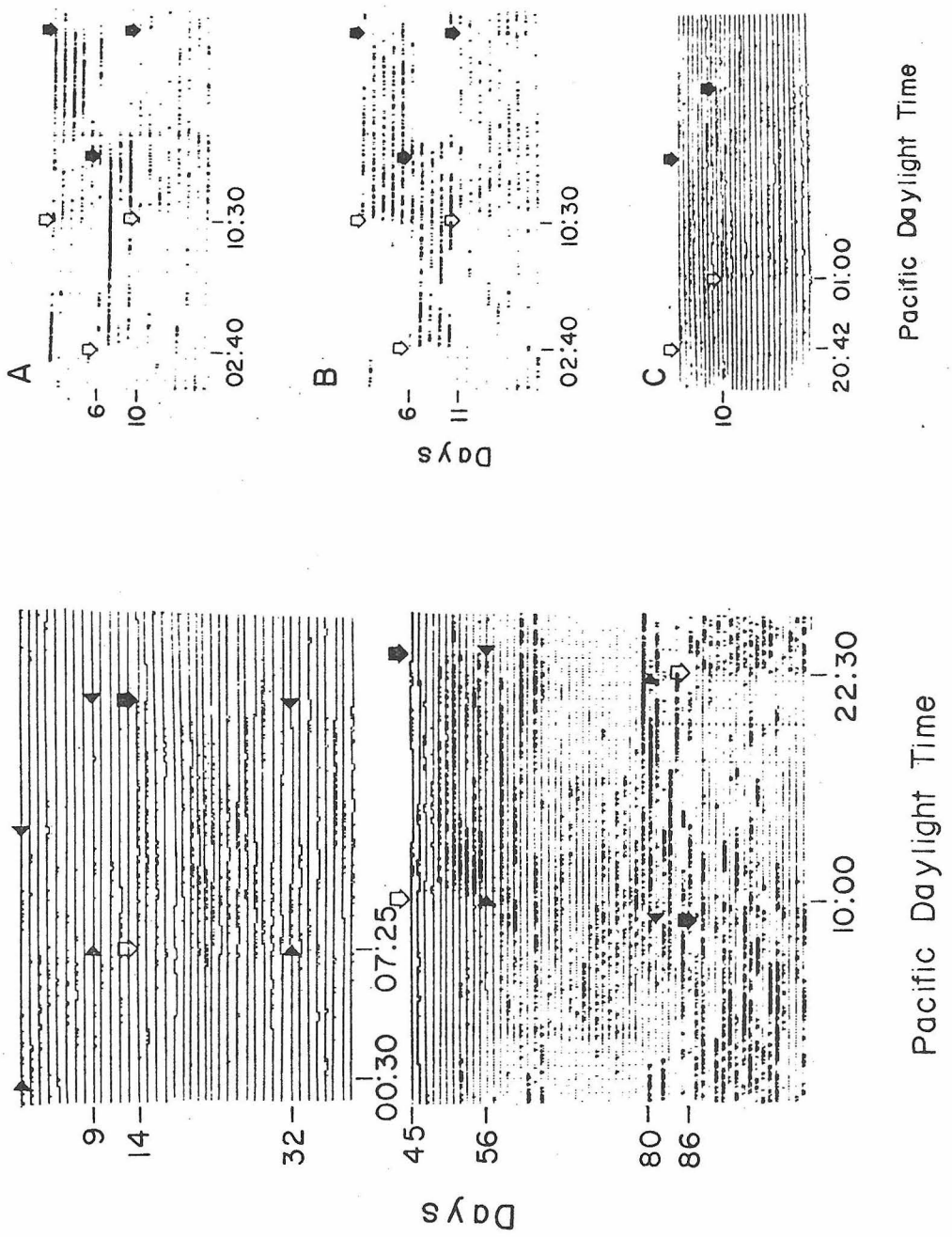

AVERAGE ACTIVITY
PERIODOGRAM
DAYS
48-53
A.c. 47
54-59
64-69
EYES REM. (61)
74-79
METERS/HOUR
5
0
6
18
TIME OF DAY
0.5
RELATIVE SIGMA
12
24
36
48
TRIAL PERIOD, HOURS

Figure 3. Event recorder charts of locomotor activity of Aplysia. Triangles represent "pseudo" LD cycle (the bulbs used to illuminate the aquarium were painted black, but still turned on with the schedule indicated by the triangles). Open and closed arrows represent true LD schedule, with lights on at the open arrow (LD 12:12, 170 lux: 0 lux).

Left: A. californica. Free run in pseudo LD, followed by imposition of true LD cycle. Animal blinded on day 23. Lower part of the chart is the same animal tracked with a different setup.

Right: Symbols as on left. Three different animals, two A. californica (A and B) and one A. vaccaria (C). Animals blinded on day 9 (A), day 10 (B), and day 9 (C). From Block and Lickey, 1973.


A
B
C
Days
6
10
9
11
10
02:40
10:30
02:40
10:30
20:42
01:00
Pacific Daylight Time
Days
9
14
32
45
56
80
86
00:30
07:25
10:00
22:30
Pacific Daylight Time

References

Alving, B.O. 1968 Spontaneous activity in isolated somata of Aplysia pacemaker neurons. J. Gen. Physiol. 51: 29-45.

Block, G.D., D.J. Hudson, and M.E. Lickey. 1974. Extraocular photoreceptors can entrain the circadian oscillator in the eye of Aplysia. J. Comp. Physiol., in press.

Block, G.D., and M.E. Lickey. 1973. Extraocular photoreceptors and oscillators can control the circadian rhythm of behavioral activity in Aplysia. J. Comp. Physiol. 84: 367-374.

Eskin, A. 1971. Properties of the Aplysia visual system: in vitro entrainment of the circadian rhythm and centrifugal regulation of the eye. Z. Vergl. Physiol. 74: 353-371.

Eskin, A. 1972. Phase shifting a circadian rhythm in the eye of Aplysia by high potassium pulses. J. Comp. Physiol. 80: 353-376.

Jacklet, J.W. 1969a. Electrophysiological organization of the eye of Aplysia. J. Gen. Physiol. 53: 21-42.

Jacklet, J.W. 1969b. A circadian rhythm of optic nerve impulses recorded in darkness from the isolated eye of Aplysia. Science 164: 562-564.

Jacklet, J.W. 1971. A circadian rhythm in optic nerve impulses from an isolated eye in darkness. In: M. Menaker (ed.), Biochronometry. Washington, D.C.: National Academy of Sciences, pp. 351-362.

Jacklet, J.W. 1974. The effects of constant light and light pulses on the circadian rhythm in the eye of Aplysia. J. Comp. Physiol. 90: 33-45.

Jacklet, J.W., and J. Geronimo. 1971. Circadian rhythm: population of interacting neurons. Science 174: 299-302.

Kupfermann, I. 1968. A circadian locomotor rhythm in Aplysia californica. Physiol. Behav. 3: 179-182.

Lickey, M.E. 1969. Seasonal modulation and non-24 hour entrainment of a circadian rhythm in a single neuron. J. Comp. Physiol. Psychol. 68: 9-17.

Lickey, M.E., and S. Zack. 1973. Extraocular photoreceptors can entrain the circadian rhythm in the abdominal ganglion of Aplysia. J. Comp. Physiol. 84: 361-366.

Lickey, M.E., S. Zack, and P. Birrell. 1971. Some factors governing entrainment of a circadian rhythm in a single neuron. In: M. Menaker (ed.), Biochronometry. Washington, D.C.: National Academy of Sciences, pp. 549-564.

Rothman, B.S. 1974. Biochemical studies of the effects of inhibitors of RNA and protein synthesis on the circadian rhythm of the eye of Aplysia californica. Ph. D. dissertation, Calif. Inst. of Tech.

Sener, R. 1972. Site of circadian rhythm production in Aplysia eye. Physiologist 15: 262.

Strumwasser, F. 1963. A circadian rhythm of activity and its endogenous origin in a neuron. Fed. Proc. 22: 220.

Strumwasser, F. 1965. The demonstration and manipulation of a circadian rhythm in a single neuron. In: J. Aschoff (ed.), Circadian Clocks. Amsterdam: North-Holland Publ. Co., pp. 442-462.

Strumwasser, F. 1967. Neurophysiological aspects of rhythms. In: G.C. Quarton, T. Melnechuk, and F.O. Schmitt (eds.), The Neurosciences: An Intensive Study Program. New York: Rockefeller Univ. Press, pp. 516-528.

Strumwasser, F. 1971. The cellular basis of behavior in Aplysia. J. Psychiat. Res. 8: 237-257.

Strumwasser, F. 1973. Neural and humoral factors in the temporal organization of behavior. Physiologist 16: 9-42.

Strumwasser, F. 1974. Neuronal principles organizing periodic behaviors. In: F.O. Schmitt and F.G. Worden (eds.), The Neurosciences: Third Study Program. Cambridge: MIT Press, pp. 459-478.

Strumwasser, F., F.R. Schlechte, and S. Bower. 1972. Distributed circadian oscillators in the nervous system of Aplysia. Fed. Proc. 31: 405.

Strumwasser, F., F.R. Schlechte, and J. Streeter. 1967. The internal rhythms of hibernators. In: K. Fisher (ed.), Proceedings of the Third International Symposium on Mammalian Hibernation. Edinburgh: Oliver and Boyd, pp. 110-139.

Waser, P.M. 1968. The spectral sensitivity of the eye of Aplysia californica. Comp. Biochem. Physiol. 27: 339-347.

PART I

STUDIES ON THE ORGANIZATION OF THE EYE OF *APLYSIA CALIFORNICA*

Brain Research, 59 (1973) 229–242

© Elsevier Scientific Publishing Company, Amsterdam – Printed in The Netherlands

SPONTANEOUS AND LIGHT-INDUCED COMPOUND ACTION POTENTIALS IN THE ISOLATED EYE OF *APLYSIA*: INITIATION AND SYNCHRONIZATION

GERALD AUDESIRK

Division of Biology, California Institute of Technology, Pasadena, Calif. 91109 (U.S.A.)

(Accepted February 13th, 1973)

SUMMARY

(1) The isolated eye of *Aplysia* shows a bursting pattern of spontaneous compound action potentials (CAPs) in the dark. The 'light response', also composed of CAPs, may be separated into a phasic initial response and a tonic late response similar in form to the dark discharges when the illumination is of low intensity.

(2) Blockage of chemical synapses with La^{3+} or hi Mg^{2+}–lo Ca^{2+} stops the dark discharge and tonic light response but not the phasic light response. Synchrony of the CAPs is not affected.

(3) Ca^{2+}-free solutions produce continuous rapid firing of CAPs, seldom coordinated into bursts.

(4) Replacement of chloride by propionate abolishes all CAPs for several hours, but leaves the ERG intact and the optic nerve electrically excitable.

(5) Low sodium levels (about 50% normal) suppress dark discharge and tonic light activity but allow a normal phasic light response.

(6) It is concluded that receptors transmit light-induced excitation to a higher order neuron population via electrical junctions, and that synchrony of the CAPs is also due to electrical junctions, interconnecting the higher order population. One or more pacemaker cells are postulated to drive the higher order neurons by chemical synapses, producing the dark discharge and the low intensity tonic light response. The pacemaker mechanism may be sodium dependent.

INTRODUCTION

The isolated eye of the sea hare *Aplysia californica* shows a circadian rhythm of spontaneous impulse activity in constant darkness[10]. This impulse activity consists of compound action potentials (CAPs) in the optic nerve, representing the synchroniza-

tion of the individual action potentials of presumably several hundred axons[11]. The eye is active during projected day (that part of the 24 h cycle during which the animal was under illumination) with a peak of CAP production near projected dawn. During projected night CAP production decreases and the eye becomes silent for a few hours before activity again increases near projected dawn[10]. In addition to the regular pattern of dark discharges, the eye also responds to illumination with a characteristic sequence of CAPs. Some of the properties of single cells in the eye are known from previous work[11], and substantial work has been done on the circadian properties of the eye[6,10,13].

In these experiments, an extracellular approach has been used in an attempt to infer the general organization of the main populations of neurons in the eye by the effect of various ionic treatments on the activity recorded from the optic nerve. In particular, the mode of synchronization of the compound action potentials and their initiation in light and dark were investigated with several synapse blocking agents.

A preliminary report of some of these experiments has been presented[3].

METHODS

Aplysia californica, obtained from Pacific Bio-Marine Supply Co., were maintained in the laboratory in sea water tanks at a constant temperature of 14 °C with a lighting regime of 12 h light and 12 h dark. Animals were dissected in a controlled temperature room kept at 14 °C. The operation, which required about 20 min to complete, consisted of removing the eye and the attached optic nerve from the animal. The optic nerve was cut away from its connection to the cerebral ganglion. Dissections were performed 1–3 h after the onset of light in the sea water tanks; all recordings were taken during the first day after dissection in the hours when the animals would have been in the light, at which time the spontaneous activity of the eye is at its maximum[10].

Recording was done from the optic nerve by means of a suction electrode, constructed from a needle and syringe. A piece of Intramedic PE 10 polyethylene tubing was placed over the needle tip, which contained a platinum wire protruding beyond the tip into the tubing. An input wire was soldered to the needle. Another piece of platinum wire, covered with PE tubing except at the end, served as the indifferent electrode. The signal was amplified by a Tektronix 122 preamplifier, and then monitored on a Tektronix 564B oscilloscope and/or recorded on a Grass Model 7 polygraph. During recording, the eye was kept in a light-tight box; illumination was with a 6 V microscope lamp. The bathing medium of the eye was at a constant 14 °C.

ERGs were recorded in the same manner as the optic nerve impulses. In this case the suction electrode was affixed to the corneal surface of the eye.

The bathing media were based on a modification of 'average' sea water[19]. The standard artificial sea water (ASW) was composed of: NaCl, 470 mM; KCl, 10 mM; $CaCl_2$, 10 mM; $MgCl_2$, 53.5 mM; with 10 mM Tris at pH 7.8–8.0. Other solutions used were:

High magnesium–low calcium sea water (hi Mg^{2+}–lo Ca^{2+}SW): NaCl, 375 mM; KCl, 10 mM; $CaCl_2$, 1 mM; $MgCl_2$, 125 mM; Tris, 10 mM, pH 7.8–8.0.

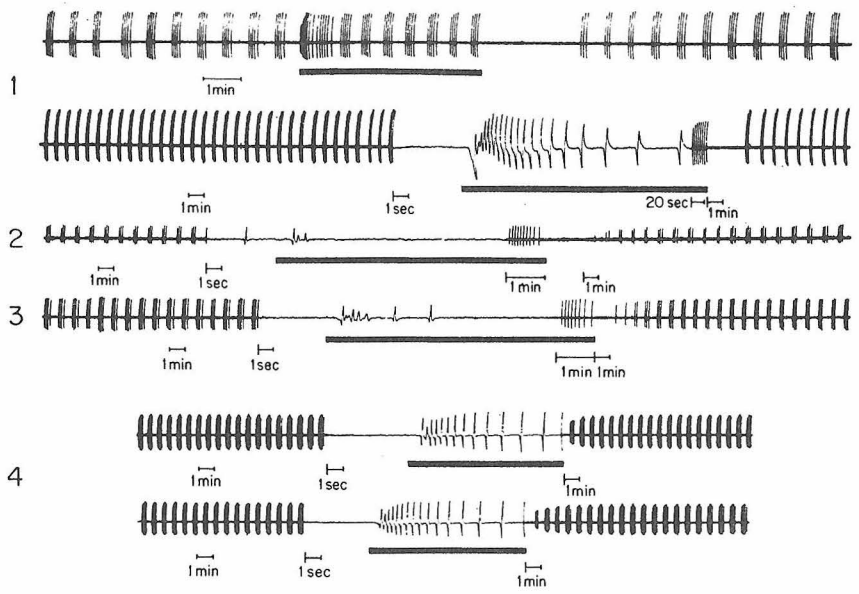


Fig. 1. Characteristic CAP patterns of the isolated eye of *Aplysia* in ASW. In all figures, dark bar represents illumination of the eye. The same illumination configuration was used in all recordings. Record speed is represented by time calibration beneath each line; speed changes are designated by larger (left) vertical line on calibration marker and speed remains constant until the next marker. Individual eyes are given numbers at left; thus the two initial lines are recordings from the same eye. Amplitudes of CAPs vary from about 30 μV for eye 2 to 90 μV for eye 1 (amplification factors are not the same from eye to eye). Half-amplitude bandpass filters are usually set at 1 (low) and 60 (high) Hz.

Lanthanum sea water ($LaCl_3$SW): standard ASW with 100 μM–4.5 mM $LaCl_3$ added, pH 7.8–8.0.

Propionate sea water (PrSW): sodium propionate, 470 mM; KCl, 10 mM; $CaCl_2$, 10 mM; $MgCl_2$, 53.5 mM; Tris, 10 mM, pH 7.8–8.0.

Calcium-free sea water (Ca^{2+}-free SW): NaCl, 470 mM; KCl, 10 mM; $MgCl_2$, 63.5 mM; Tris, 10 mM, pH 7.8–8.0.

Low sodium sea water (lo NaSW): KCl, 10 mM; $CaCl_2$, 10 mM; $MgCl_2$, 53.5 mM; NaCl + Tris, 470 mM (in the per cent Na^+ given), pH 7.8–8.0.

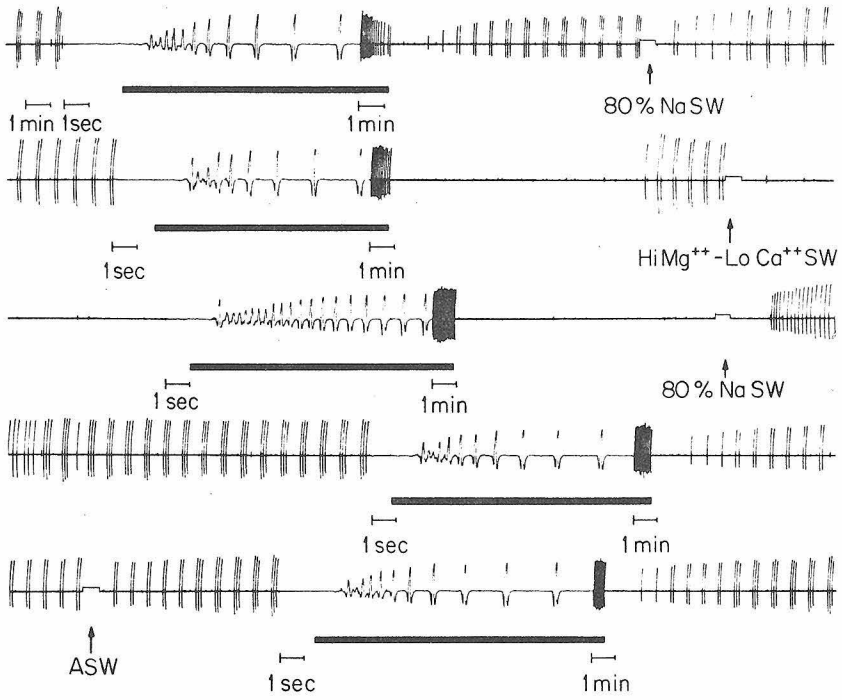
Solutions were changed by complete removal of the initial medium by suction, followed by injection of new medium. The eye remains held by the suction electrode during this process, and retains a thin film of fluid around it. The exchange takes 30 sec or less, and does not affect the output of the eye when both initial and final media are ASW, as judged by the patterns of impulses before and after the change (the eye cannot be recorded from during the exchange). Light conditions (darkness) are constant during the exchange.

RESULTS

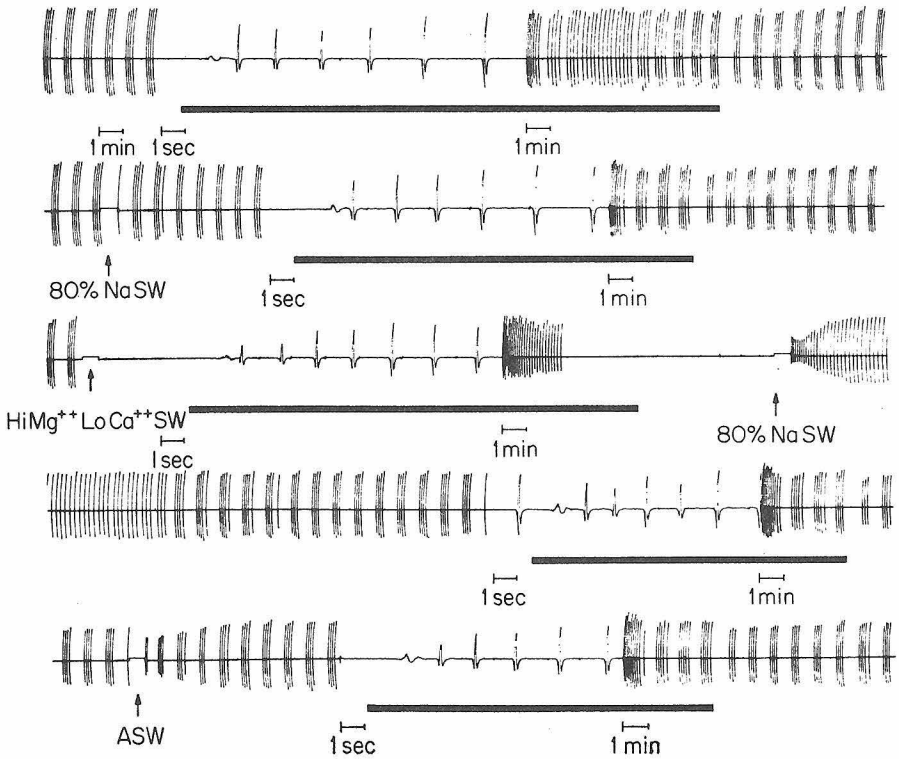
The typical output of normal eyes (Fig. 1) is composed of spontaneous impulses

Hi Mg^{++}-Lo Ca^{++} SW

150 LUX



10 LUX



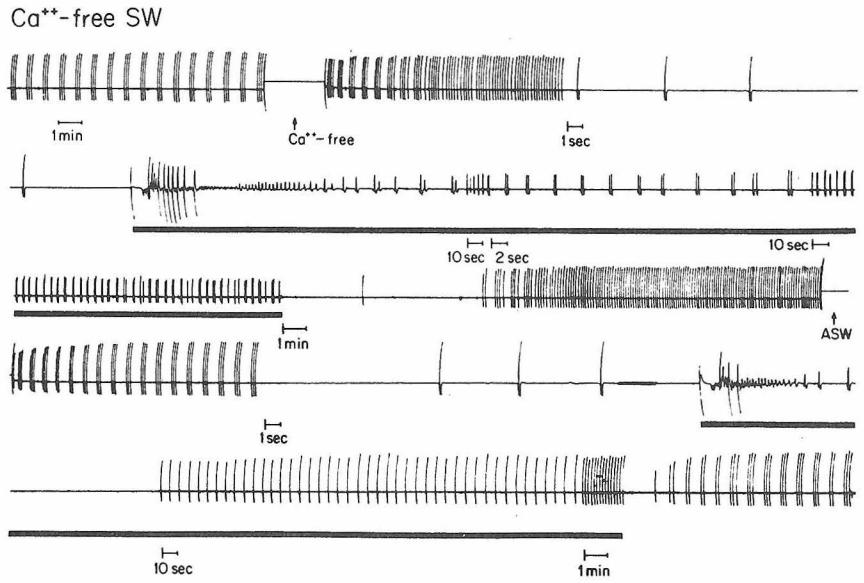


Fig. 3. CAP patterns in Ca^{2+}-free SW, compared to those in ASW. Lines are consecutive recordings. Eye initially is in ASW. Labeled vertical arrows beneath lines represent changes to the indicated solution.

in darkness, usually in bursts of 1–5 imp./burst, with a roughly constant interburst interval of 1–2 min. The long duration of the impulses (about 100–180 msec measured trough to trough of the triphasic spike at 14 °C), their variation in amplitude, and their disruption into numerous small units upon treatment with hypotonic sea water all indicate that they are compound action potentials[11]. Upon illumination, a rapid series of CAPs occurs. If the illumination is kept constant, this rapid initial firing (called here the 'phasic response') slows. Under low intensities of light, the impulses usually evolve into a bursting pattern (the 'tonic response') similar to that seen in the dark, but at a higher rate (line 1, Fig. 1). Higher intensities often produce evenly spaced impulses with no discernible grouping. After cessation of illumination, the eye is silent for a time, after which bursts reappear. Variation in the light response from eye to eye is considerable, but reproducibility for a given eye at a fixed light intensity is good.

Transmission at chemical synapses requires the presence of calcium in the bathing medium[7,15] and high concentrations of magnesium inhibit transmission[5], particularly in combination with reduced levels of calcium. 125 m*M* Mg^{2+} (about 2.5 × normal) with Ca^{2+} reduced to 1 m*M* (about 0.1 × normal) stops CAP production in the dark (Fig. 2). This effect persists overnight. With moderately high light inten-

Fig. 2. Hi Mg^{2+}–lo Ca^{2+}SW. Note different light intensities in top and bottom of figure, which are recordings from different eyes. Hi Mg^{2+}–lo Ca^{2+}SW contains 80% of the normal sodium concentration, and therefore the effect of 80% NaSW is also shown for each eye, as well as the activity pattern in ASW. The sequence of solutions is ASW → 80% NaSW → hi Mg^{2+}–lo Ca^{2+}SW → 80% NaSW → ASW.

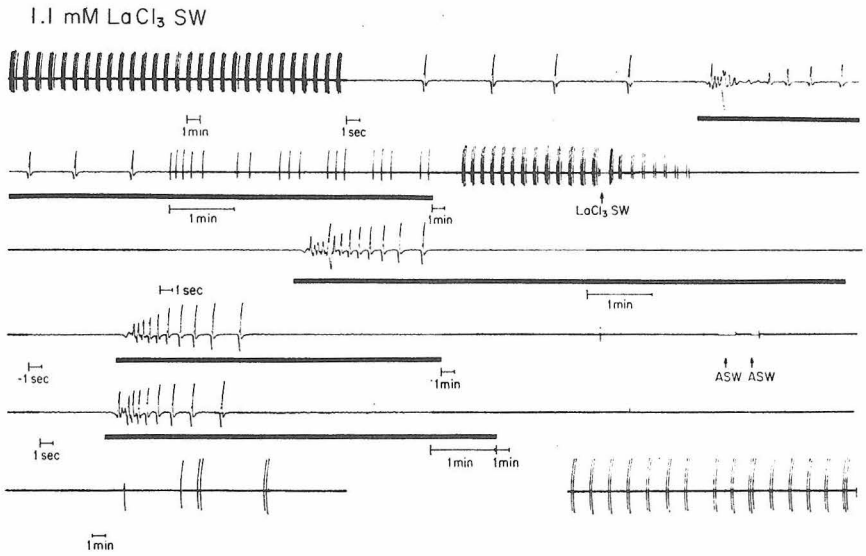


Fig. 4. CAP patterns in 1.1 m*M* $LaCl_3$SW. Eye initially is in ASW. First 4 lines are consecutive recordings; line 5 follows with a delay of approximately 1 h, line 6 follows line 5 with a delay of approximately 1.5 h, and the gap in line 6 represents approximately 1 h. The total time to recovery of normal bursting patterns in the dark is 3.5 h in ASW.

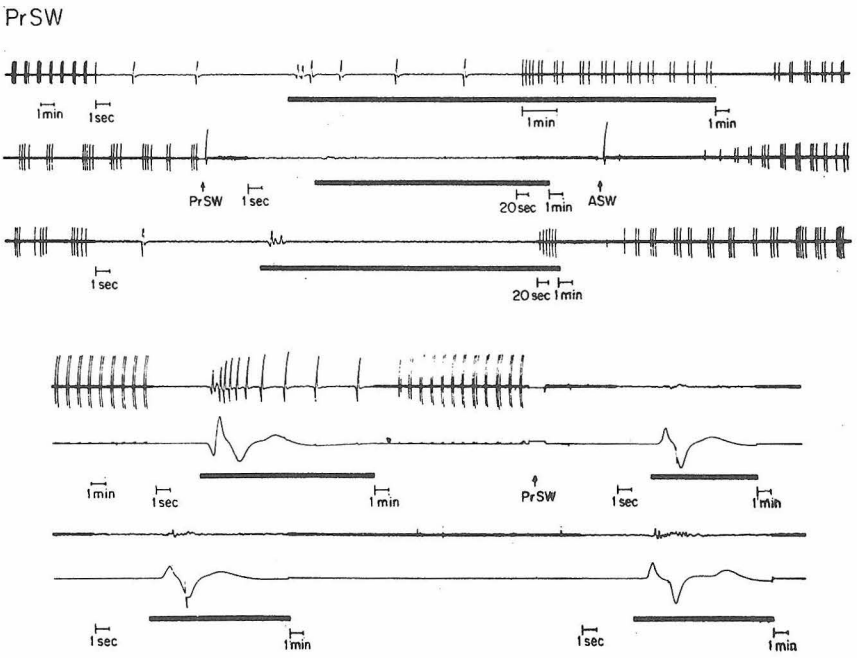


Fig. 5. Effect of replacing most of the chloride in ASW by propionate. First 3 lines are consecutive recordings from a single eye, beginning in ASW. The last 4 lines are in pairs, the upper line of each pair recording from the optic nerve, the lower line a simultaneous ERG (AC-coupled recording) from the corneal surface of the same eye (a different eye than in the first 3 lines of the figure). Bandpass is 0.2–60 Hz. Lower pair of recordings is consecutive with upper pair. Eye initially in ASW.

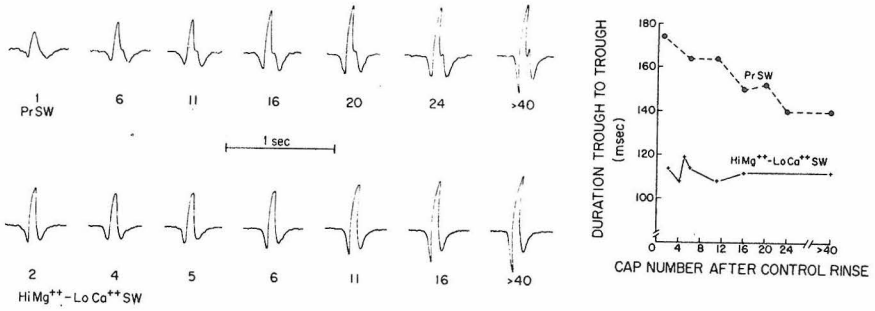


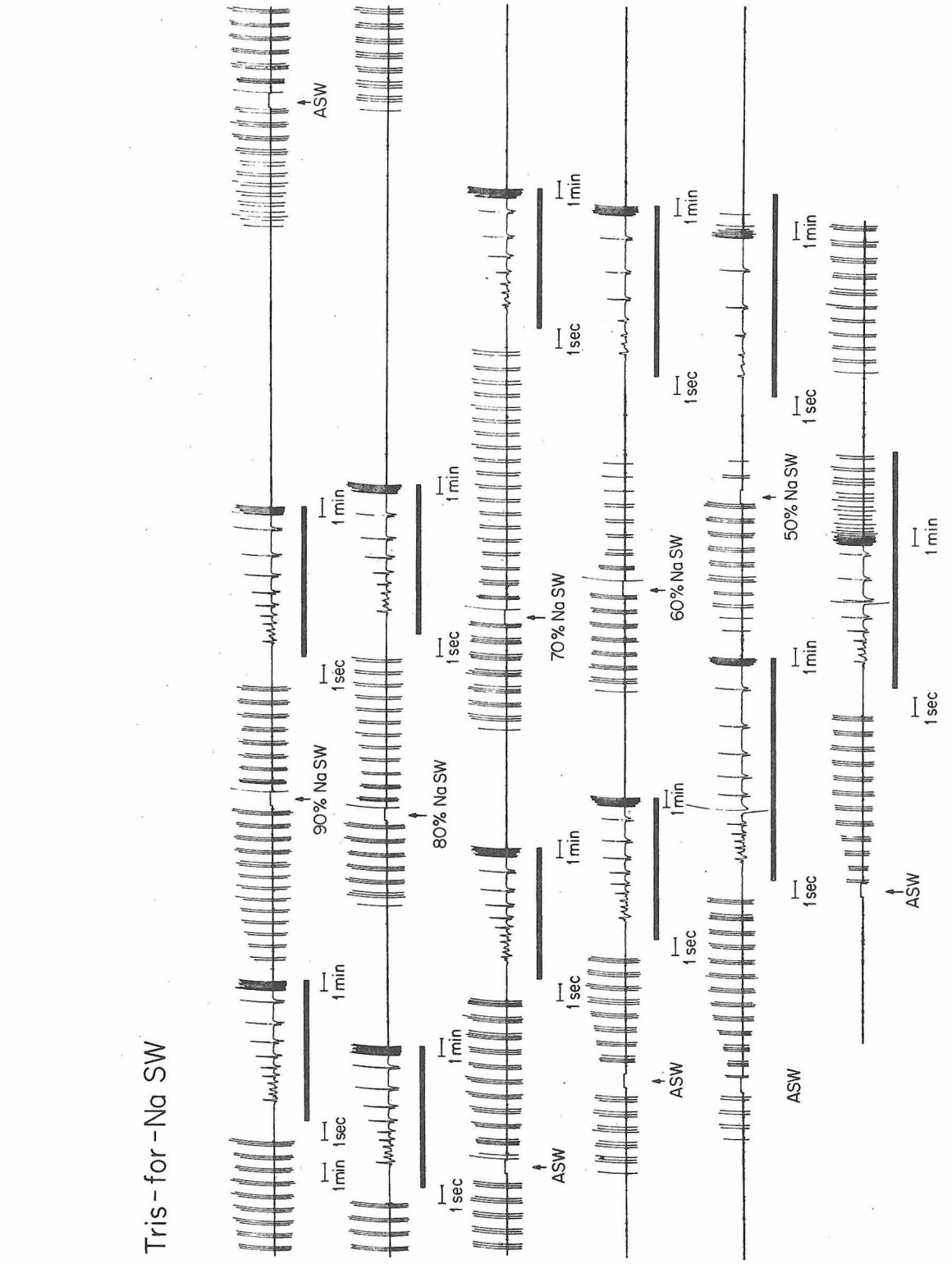
Fig. 6. Spontaneous CAPs recorded from eyes in darkness after a short period (8 min, PrSW; 7 min, hi Mg^{2+}–lo Ca^{2+}SW) in the indicated solutions. Recovery from PrSW is in ASW; recovery from hi Mg^{2+}–lo Ca^{2+}SW is in 80% NaSW. Graph is plotted from CAPs in left half of figure. Other experiments (3) give comparable results.

sities, the light response consists of a rapid train of impulses that persists as long as the light is left on. At low intensities the eye in ASW produces a bursting tonic light response similar to the normal dark discharge; at these intensities, hi Mg^{2+}–lo Ca^{2+}SW blocks the tonic discharge in light.

Ca^{2+}-free SW (Fig. 3) does not block the tonic discharge in light or in dark; on the contrary, continuous firing occurs, usually without any burst patterning. The light response is a high frequency series of impulses. Continuous activity persists in darkness as long as the eye lives. Further removal of calcium, as in Ca^{2+}-free SW with 2 m*M* EDTA added, causes initially continuous CAPs, followed by a rapid decrease in CAP amplitude, and then silence. In this condition the optic nerve itself is electrically inexcitable.

Low concentrations (about 1 m*M*) of lanthanum have been reported to block transmission rapidly in the frog neuromuscular junction[8,15] and to inhibit the inward calcium current in the presynaptic axon of squid giant synapse[16]. Prolonged treatment of the frog neuromuscular junction with $LaCl_3$ (in the absence of calcium) causes irreversible loss of synaptic vesicles[8]. In the *Aplysia* eye, $LaCl_3$ blocks CAP output in the dark and tonic activity in light for at least 3 h (Fig. 4). Recovery in ASW after long immersions in $LaCl_3$SW is only partial or may not occur at all.

Replacement of chloride in the bathing medium by propionate or acetate has been found by Pappas *et al.*[17] and Asada and Bennett[2] to uncouple electrical junctions in crayfish septate axon. This treatment, however, does not uncouple leech giant neurons[18]. When propionate is substituted for most of the chloride in ASW, all impulse activity in the optic nerve of the *Aplysia* eye ceases (Fig. 5), both in light and in darkness. However, as the lower traces of Fig. 5 show, a normal ERG still occurs in PrSW. In some eyes, spontaneous CAPs resume after several hours in PrSW (2 out of 5); the light response usually returns somewhat before the spontaneous dark discharge. Low amplitude impulses can be seen in the last light response of Fig. 5. In general, a series of light responses in PrSW show initially no CAP response in the optic nerve at all or a very small apparent increase in the background noise level. Later responses show low amplitude impulses which increase until near normal CAPs are seen. The


Tris-for-Na SW
ASW
90% Na SW
80% Na SW
70% Na SW
60% Na SW
50% Na SW
ASW
ASW
ASW
1 min
1 sec

optic nerve can be stimulated electrically with the eye still attached to the nerve in such a way that spontaneous activity from the eye can be recorded as well as the electrically evoked CAP. In PrSW, while the spontaneous activity of the eye and the light response are both absent, the optic nerve can still be stimulated to give a compound action potential identical to that which can be elicited in ASW. The threshold and amplitude of the elicited CAP are not changed by PrSW.

In recovery from suppression of impulse production by both hi Mg^{2+}–lo Ca^{2+}-SW (Fig. 2) and PrSW (Fig. 5), the amplitude of the CAP grades upward in most but not all experiments (6 of 7, hi Mg^{2+}–lo Ca^{2+}SW; 8 of 10, PrSW). High speed recording from eyes in recovery from short periods in each of these solutions (Fig. 6) shows that recovery from hi Mg^{2+}–lo Ca^{2+}SW involves no change in duration, while recovery from PrSW produces CAPs of duration at first considerably greater than normal, and approaching the normal value with increasing time in ASW.

During attempts to determine the levels of calcium and magnesium necessary to block output by the eye, experiments were run to control for the effects of the reduced sodium content necessitated by the raised magnesium levels. The dark discharges can be halted at least temporarily by reducing the concentration of sodium below normal (Fig. 7). Individual eyes vary in their sensitivity to reduced sodium, but usually become silent in 50% NaSW; the range is about 70–20% normal sodium. The output of the eye in low sodium resembles that in $LaCl_3$SW or hi Mg^{2+}–lo Ca^{2+}SW; that is, the phasic light response remains after the abolition of the dark discharges and the tonic light response.

DISCUSSION

Receptor–optic nerve relation

Early work by Jacklet[9] led him to hypothesize the structure of the eye as receptors synapsing on a population of secondary neurons in which spikes are produced, and whose axons comprise the optic nerve. Later, he found that intracellular recordings of receptors showed no spiking activity, while cells which did spike in synchrony with the optic nerve CAPs did not show any receptor-like generator potentials when the eye was illuminated[11]. He nevertheless concluded that the simplest hypothesis was that all recorded activity occurs in various parts of the receptors. More recently, citing these intracellular recordings, Jacklet has returned to his original position that secondary cells are responsible for the CAP in the optic nerve[13].

None of the chemical synapse blocking solutions, hi Mg^{2+}–lo Ca^{2+}SW (Fig. 2), Ca^{2+}-free SW (Fig. 3), or $LaCl_3$SW (Fig. 4), block the phasic light response. These data lend support for either continuity of receptor and optic nerve or for electrical junctions between receptors and higher order cells whose axons comprise the optic nerve.

Fig. 7. Low sodium effect on the eye. Varying concentrations of sodium (90–50%) in the medium, expressed as percentage of normal (470 m*M*), with ASW controls between each concentration. Tris is the replacement for the removed sodium.

Bathing media with most of the chloride replaced by propionate uncouple electrical junctions in crayfish septate axon[2,17] but not those between leech giant neurons[18]. If PrSW uncouples electrical junctions in *Aplysia* eyes, then it would be possible that the failure of the phasic response (Fig. 5) could be due to uncoupling of electrical junctions between receptors and higher order cells. No impulses at all, in light or in dark, are seen in PrSW, at least initially. In addition, this effect occurs in less than a minute, much faster than in the septate axon. Thus PrSW may cause simple loss of excitability, resulting in impulse failure in the *Aplysia* eye. However, during this failure of spontaneous or light-induced activity, electrically stimulated CAPs in the optic nerve appear identical in form, amplitude and threshold to those in ASW. The ERG also remains (Fig. 5).

Since neither the receptor membranes nor the electrically excitable axon membranes are significantly affected by PrSW, it must cause either an interruption of transmission between the two, or desynchronization of the CAP into units whose activity is too small to record. Even if eye CAPs are due to PrSW-sensitive coupling, the onset of light is simultaneous for all receptors, so at least one 'onset CAP' might be expected in PrSW which has eliminated this coupling. This does not occur. Therefore the data indicate that there are electrical junctions between receptors and higher order cells.

Pacemaker–optic nerve relation

The population of axons in the optic nerve is fairly homogeneous[12], and it is presumed that the same axons produce the CAPs seen in light and in dark. In dim light the tonic light response becomes virtually identical to the dark discharge (Fig. 1, line 1). Further, both $LaCl_3$SW and hi Mg^{2+}–lo Ca^{2+}SW block the dark discharge and tonic light response (although in hi Mg^{2+}–lo Ca^{2+}SW the tonic light discharge inhibition depends on light intensity). Therefore it is likely that the same mechanism drives the bursts in the higher order cells in both cases.

Both solutions with high magnesium levels[5] and those with low concentrations of lanthanum[8] block chemical synaptic transmission, apparently presynaptically, without blocking conduction of action potentials. This suggests that the tonic discharge in the *Aplysia* eye is mediated by chemical synapses, with a pacemaker(s) driving the higher order CAP-producing cells. The results with Ca^{2+}-free SW, of course, conflict with this interpretation, since with this solution continuous impulses occur. This conflict cannot be definitively resolved without intracellular recording from both the pacemaker and higher order cells. It is possible that Ca^{2+}-free SW does not remove all calcium from the synaptic cleft, and/or that it causes an increase in excitability, as in several other systems[1,4,14].

The impulses of the eye are produced in the absence of external stimuli, so an endogenously active neuron(s) must initiate the CAPs. The possibility thus arises that the lanthanum and high magnesium solutions could be blocking the pacemaker mechanism. Neither lanthanum nor high magnesium with low calcium (with TTX added to block spikes so as to improve the visibility of the membrane oscillations) block the sodium-dependent pacemaker oscillations of R15, an endogenously active

cell in the abdominal ganglion of *Aplysia* (Strumwasser, personal communication). Nevertheless, a calcium pacemaker could be inhibited by lanthanum or magnesium.

Synchronization of the CAP

The method of synchronizing the action potentials of the numerous small axons into a coherent CAP could be by chemical synapses, electrical synapses, or simultaneous input from receptors (in light) or pacemaker (dark). All of the chemical transmission blocking media still enable a series of fully synchronized CAPs to be produced in the light; in Ca^{2+}-free SW CAPs are also seen in the dark. Therefore it is unlikely that chemical synapses synchronize the CAP by interconnecting the higher order cells. The series of CAPs in a phasic light response, at a time of nearly constant depolarization of the receptors[11], argues against the need for simultaneous, temporally discrete inputs.

The remaining possibility is synchronization through electrical junctions interconnecting the higher order cells. If this is true, then the PrSW result of complete CAP suppression might be expected on the basis of the uncoupling of these junctions. It has been shown above that PrSW probably uncouples receptor–optic nerve transmission by uncoupling electrical junctions. In addition, PrSW blocks pacemaker oscillations in R15[20,21], and in a few experiments run with lower levels of propionate, the circadian rhythm of the eye seemed to be affected. Therefore, although the results with PrSW are consistent with the uncoupling of the higher order cells, the same results could be due to blockage at the receptor and pacemaker levels. However, the decreasing durations and increasing amplitudes observed in the CAPs of an eye recovering from a short immersion in PrSW and then returned to ASW (Fig. 6) are most easily explained as recoupling of the higher order cells. As individual action potentials are brought more nearly into synchrony, the duration of the CAP would be expected to decrease; the amplitude should increase as individual spikes are made to superimpose (temporally) more exactly.

Pacemaker

This interpretation of coupling of higher order cells by electrical junctions also has bearing on the problem of the location and type of the pacemaker. The higher order cells are not able to produce CAPs in the dark when chemical synapse activity is suppressed, so it is not likely that these cells act as their own pacemakers. Since the receptors show no tonic activity related to the CAP activity[11], they must not be the pacemakers either. It is most probable that a separate class of neuron(s) acts as pacemaker.

Preliminary experiments reported here (Fig. 7) provide some evidence as to the nature of the pacemaker mechanism. Reduction of the sodium concentration below about 50% (the range is about 20–70%) of the normal results in cessation of dark discharge and tonic light activity. The phasic light response remains. Either a requirement for sodium in the pacemaker (as in R15; see ref. 20) or in the production of the EPSP between the pacemaker and follower is indicated. An apparent circadian rhythm of sensitivity to sodium replacement (unpublished results) suggests the former.

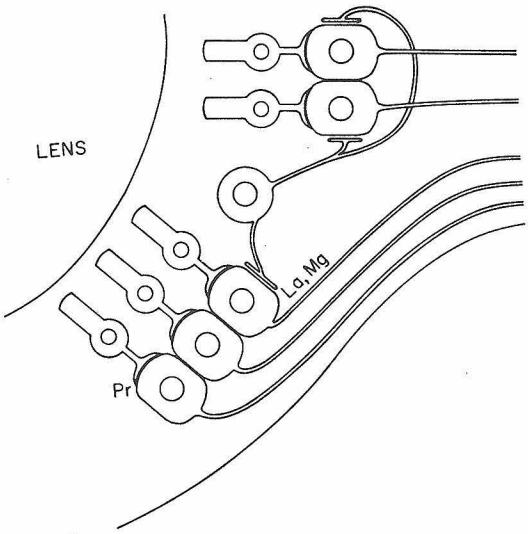


Fig. 8. A simplified model for the eye of *Aplysia*, showing interrelations among the major cell types. Receptors ring lens, and are electrically coupled (thickened lines) to secondary neurons, whose axons comprise the optic nerve. Round cell in the center represents a pacemaker cell, which chemically synapses on the secondaries (with gap). Secondaries are electrically coupled to one another. Pacemaker may provide input to every secondary (upper) or only to some (lower), with electrotonic coupling providing transmission of excitation to other secondaries. Electrical coupling probably interconnects all secondaries; separation into groups is only for convenience of illustration. Letters near synapses represent types of ions which likely interrupt transmission across them.

Model for the Aplysia eye

The neuronal relationships in the eye, as suggested by the data, can be represented by a model (Fig. 8). In the dark adapted eye, the pacemaker cell(s) excites the higher order neuron population through chemical synapses. Whether this excitation must be given directly to every neuron (upper group) or whether the electrical coupling is of low enough resistance that not all neurons need be innervated by the pacemaker (lower group) cannot be decided by the data. When the eye is illuminated, the receptors respond with a potential change which is transmitted to the higher order neurons by electrical junctions. Coordination of the individual spikes initiated by light or pacemaker action into the CAP occurs by means of electrical coupling between higher order neurons.

The circadian rhythm of the eye is expressed in changes in frequency of spontaneous dark discharges with time of day. These dark discharges, which occur in the higher order neurons, are initiated by the pacemaker cell(s). The two most probable mechanisms for the circadian rhythm, then, are a circadian fluctuation in the output of the pacemaker, or a change in the responsiveness of the higher order cell population to the pacemaker excitation.

Other investigations have found centrifugal regulation of the eye by the cerebral ganglion[6] and additional cell types in the eye that are not included in this model[11]. These indicate, not surprisingly, that the model presented here is oversimplified. A

definitive proof of the neuronal relationships hypothesized in this model must await the difficult task of simultaneous intracellular recordings from two higher order cells, from receptor and higher order cell, and from pacemaker and follower. However, experiments aimed at explaining the organization and circadian rhythm of the *Aplysia* eye must consider the general features of this model.

ACKNOWLEDGEMENTS

I would like to thank Drs. Felix Strumwasser and Arnold Eskin for their assistance and helpful suggestions in the course of these experiments, and for their critical reading of this manuscript. I would also like to thank Mr. James Gilliam for his technical assistance.

This work was supported by a predoctoral fellowship from NSF, by NIH Grant NS07071 and NASA Grant NGR 05-002-031 to Felix Strumwasser, and by the NIH predoctoral training grant of the California Institute of Technology.

REFERENCES

1 ADRIAN, E. D., AND GELFAN, S., Rhythmic activity in skeletal muscle fibres, *J. Physiol. (Lond.)*, 78 (1933) 271–287.
2 ASADA, Y., AND BENNETT, M. V. L., Experimental alteration of coupling resistance at an electrotonic synapse, *J. Cell Biol.*, 49 (1971) 159–172.
3 AUDESIRK, G., Neuronal interactions in optic nerve impulse production in *Aplysia*, *Physiologist*, 14 (1971) 105.
4 BRONK, D. W., LARRABEE, M. G., GAYLOR, J. B., AND BRINK, JR., F., The influence of altered chemical environment on the activity of ganglion cells, *Amer. J. Physiol.*, 123 (1938) 24–25.
5 DEL CASTILLO, J., AND ENGBAEK, L., The nature of the neuromuscular block produced by magnesium, *J. Physiol. (Lond.)*, 124 (1954) 370–384.
6 ESKIN, A., Properties of the *Aplysia* visual system: *in vitro* entrainment of the circadian rhythm and centrifugal regulation of the eye, *Z. vergl. Physiol.*, 74 (1971) 353–371.
7 HARVEY, A. M., AND MACINTOSH, F. C., Calcium and synaptic transmission in a sympathetic ganglion, *J. Physiol. (Lond.)*, 97 (1940) 408–416.
8 HEUSER, J., AND MILEDI, R., Effect of lanthanum ions on function and structure of frog neuromuscular junctions, *Proc. roy. Soc. B*, 179 (1971) 247–260.
9 JACKLET, J. W., Synchronized neuronal activity and neurosecretory function of the eye of *Aplysia*, *Proc. 24th int. Union Physiol.*, 7 (1968) 213.
10 JACKLET, J. W., A circadian rhythm of optic nerve impulses recorded in darkness from the isolated eye of *Aplysia*, *Science*, 164 (1969) 562–564.
11 JACKLET, J. W., Electrophysiological organization of the eye of *Aplysia*, *J. gen. Physiol.*, 53 (1969) 21–42.
12 JACKLET, J. W., ALVAREZ, R., AND BERNSTEIN, B., Ultrastructure of the eye of *Aplysia*, *J. Ultrastruct. Res.*, 38 (1972) 246–261.
13 JACKLET, J. W., AND GERONIMO, J., Circadian rhythm: population of interacting neurons, *Science*, 174 (1971) 299–302.
14 KISHIMOTO, U., Repetitive action potentials in *Nitella* internodes, *Plant Cell Physiol.*, 7 (1966) 547–558.
15 MILEDI, R., Strontium as a substitute for calcium in the process of transmitter release at the neuromuscular junction, *Nature (Lond.)*, 212 (1966) 1233–1234.
16 MILEDI, R., Lanthanum ions abolish the 'calcium response' of nerve terminals, *Nature (Lond.)*, 229 (1971) 410–411.
17 PAPPAS, G. D., ASADA, Y., AND BENNETT, M. V. L., Morphological correlates of increased coupling resistance at an electrotonic synapse, *J. Cell Biol.*, 49 (1971) 173–188.

18 PAYTON, B. W., AND LOEWENSTEIN, W. R., Stability of electrical coupling in leech giant nerve cells: Divalent cations, propionate ions, tonicity, and pH, *Biochim. biophys. Acta (Amst.)*, 150 (1968) 156–158.
19 PROSSER, C. L., AND BROWN, F. A., *Comparative Animal Physiology*, Saunders, Philadelphia, 1965, p. 60.
20 STRUMWASSER, F., Membrane and intracellular mechanisms governing endogenous activity in neurons. In F. D. CARLSON (Ed.), *Physiological and Biochemical Aspects of Nervous Integration*, Prentice-Hall, Englewood Cliffs, N.J., 1968, pp. 329–341.
21 STRUMWASSER, F., AND KIM, M., Experimental studies of a neuron with an endogenous oscillator and a quantitative model of its mechanism, *Physiologist*, 12 (1969) 367.

Further Studies of the Effects of High Magnesium with Low Calcium on the *Aplysia* eye

Recently, Jacklet (1973a) has reported that high magnesium (with near normal calcium) decreases the amplitude of CAPs in the optic nerve, both light evoked and electrically stimulated. He also finds that with 100 mM Mg^{++} and 0.5 mM Ca^{++} the circadian rhythm of the eye is still expressed. He therefore concludes that the secondary cells are spontaneously active, and that they produce the circadian rhythm. He suggests that the blockage of spontaneous but not light evoked CAPs which I observed with 125 mM Mg^{++} and 1 mM Ca^{++} is probably due to a depressive effect of high Mg^{++} directly on spike generation, rather than to any synaptic effect. However, Jacklet's stimulation protocol only tested for effects on spike amplitude, not on excitability. Further, he stimulated in high Mg^{++} with 8 mM Ca^{++}, and the solution on which my conclusions were based had only 1 mM Ca^{++}, which might be expected to contribute to increased excitability. Jacklet also hypothesized that the similar effect of lanthanum on dark discharge is due to a non-synaptic suppression of spontaneous activity, but he offered no direct tests of this.

The further studies presented here find that the inhibition of dark activity by high Mg^{++} with low Ca^{++} is usually temporary (two to three hours). During this time, light-driven impulses are not suppressed; on the contrary, the impulse frequency of the light response is greatly enhanced. High Mg^{++} does not suppress spiking or spontaneous activity in several known pacemaker neurons in the PVG. The

overall effect of high Mg^{++} with low Ca^{++} is excitatory in these neurons. It is therefore likely that the secondary population in the Aplysia eye is not spontaneously active, but is driven by excitation through chemical synapses. High Mg^{++} with low Ca^{++} probably blocks transmission through these synapses located between a spontaneously active cell(s) and the otherwise silent secondary population.

Methods

Animal storage and optic nerve recordings were the same as has been previously described. Recordings from single neurons in the PVG were made intracellularly by conventional means, or extracellularly with 50 to 200 micron tip diameter fire-polished glass pipettes filled with artificial sea water and placed on the connective tissue sheath over the desired cell. Amplification for these extracellular electrodes was the same as for the suction electrodes used to record from the optic nerve.

The solutions used were:

Artificial sea water (ASW): NaCl, 470 mM; KCl, 10 mM; $CaCl_2$, 10 mM; $MgCl_2$, 53.5 mM; with 10 mM Tris at pH 7.8-8.0.

High magnesium sea water: NaCl, 375 mM; KCl, 10 mM; $CaCl_2$, 10 mM; $MgCl_2$, 125 mM; with 10 mM Tris at pH 7.8-8.0.

High magnesium, low calcium sea water: NaCl, 375 mM; KCl, 10 mM; $CaCl_2$, 0, 0.5, or 1 mM, as specified; $MgCl_2$, 125 mM; with 10 mM Tris at pH 7.8-8.0.

Lanthanum sea water ($LaCl_3SW$): ASW with 1 mM $LaCl_3$ added.

Low sodium sea water: NaCl, 375 mM; KCl, 10 mM; $CaCl_2$, 10 mM;

$MgCl_2$, 53.5 mM; choline chloride, 95 mM; with 10 mM Tris at pH 7.8-8.0.

Optic nerves were stimulated through a suction electrode attached to one end of the nerve. Another suction electrode at the other end recorded the evoked activity. Stimulation was by a calibrated Grass S4 stimulator. Voltage values are accurate to about 5%. Solutions were changed by completely removing one solution, replacing it with the next solution, and then repeating the process to ensure complete exchange.

Results

a) Stimulation of the optic nerve

In order to test the effect of various high Mg^{++} solutions on optic nerve excitability, three parameters were determined for a given optic nerve, in ASW and in the experimental solution: maximum CAP amplitude which could be elicited by electrical stimulation; minimum voltage required to elicit a CAP of this amplitude; and the threshold voltage. The values of these parameters should allow an estimate of effects on spike amplitude and excitability separately.

The results of these experiments are shown in Table I and Figure 1. In each experiment, the optic nerve was stimulated first in ASW, then in the experimental medium, and then again in ASW. Measurements were made 10 minutes after the solution exchanges, which is comparable to, but greater than, the time that the effects of these solutions on the spontaneous activity of Aplysia eyes is seen. The average ASW values for a given parameter before any treatment were extremely close to the average ASW values after the treatment. No difference exceeded 10%, and the average absolute value of the differences for all three

parameters taken over the five treatments was less than 4%.

When 125 mM Mg^{++} with 1 mM Ca^{++} (hi Mg^{++}-lo Ca^{++} of Audesirk, 1973) is used, there is clearly little effect (about 2% at most) on any of the parameters measured. If the Mg^{++} concentration is kept at 125 mM, and Ca^{++} is further reduced to 0 mM, some optic nerves show a slight decrease in the voltage needed to produce CAPs of all amplitudes. The maximum CAP amplitude is reduced by about 14%. High Mg^{++} with normal Ca^{++} (10 mM) produces reduced amplitudes (8% lower) and an increased threshold voltage, but the voltage required to elicit a CAP of maximum amplitude does not change. Reduction of Na^{+} in the same amount as in these high Mg^{++} solutions results in a similar effect on CAP amplitude (7% decrease). Lanthanum in 1 mM concentration added to ASW has the same effect as the high Mg^{++} or low Na^{+} solutions in decreasing the amplitude of the CAP.

b) Effects on Aplysia pacemaker cells

Intracellular recording from single neurons in the Aplysia eye is difficult, and penetrations usually hold only a few minutes (see Jacklet, 1969). Further, suspected pacemaker cells in the eye only constitute a fraction of the neurons present. Therefore the effects of the various high Mg^{++}, low Ca^{++} solutions on pacemaker activity was tested on known Aplysia pacemakers in the PVG.

None of the high Mg^{++} solutions used above interrupts bursting in R15 for even a short time, and activity continues for many hours (Figure 2). The same is true of neurons of the L2-4,6 and R3-13 clusters. The main effect of these solutions appears to be to stabilize

the burst pattern, producing nearly identical interburst intervals for hours at a time. High Mg^{++}, with any of the Ca^{++} levels used, reduces the burst frequency. Spike frequency increases with low Ca^{++} due to an increase in the burst size (Table II). This has been observed previously with normal Mg^{++} and zero Ca^{++} by Carpenter and Gunn (1970). The increased burst size can reach extremes of several hundred spikes per burst after an hour or more in high Mg^{++} with zero Ca^{++}. With normal Ca^{++} (10 mM), the burst size remains about the same as in ASW. Since the burst frequency declines, as in all of the high Mg^{++} solutions, the overall spike frequency declines also.

Lanthanum in 1 mM concentration has differing effects on different PVG neurons. R15 (Fig. 3) usually gives progressively longer bursts, often becoming continuously active, and then gradually stops firing. The spike frequency increases during the initial exposure to La^{+++} (an average of a 39% increase in the first half hour in 5 cells). Some other spontaneously active cells, such as those of the L2-4,6 cluster, are much less affected by La^{+++}, continuing to burst for hours after exposure (Fig. 3). The compound EPSP on R2 evoked by stimulation of the right pleurovisceral connective is usually blocked within 15 to 45 minutes. After an hour or more in 1 mM $LaCl_3$SW, a precipitate appears in the chamber.

c) Spontaneous eye activity in high Mg^{++}, low Ca^{++} solutions

Three solutions were used in this series of experiments. All had 125 mM Mg^{++}. The Ca^{++} concentrations were either 0, 0.5, or 1 mM. Table III shows the duration of suppression of spontaneous activity for

each of these solutions for several eyes. The blockage of dark activity by the various high Mg^{++}, low Ca^{++} solutions almost invariably occurs as a sudden failure, not preceded by any large decrease in CAP amplitude (Fig. 2 of Audesirk, 1973). During the period in which the eyes are silent in darkness, light can initiate impulse production at a higher frequency than in ASW. When CAPs return in any of these solutions, the amplitude is about the same as that before suppression occurred. Some eyes do not produce spontaneous impulses again (monitored over 40 hours) in the 1 mM Ca^{++} medium. When activity resumes, the rate of firing is greatly increased over the rate prior to immersion in the high Mg^{++}, low Ca^{++} solution. The spontaneous activity then proceeds in a circadian rhythm similar but not identical to that observed in a normal eye in ASW (Fig. 4 and Jacklet, 1973a).

When an eye is placed in 1 mM $LaCl_3$SW, it typically gives a few spontaneous CAPs and then ceases firing within a minute or two. Illumination, however, evokes a strong phasic light response (see Fig. 4 in Audesirk, 1973). None of seven eyes placed in this solution resumed spontaneous activity, although light responses could be elicited for at least several hours.

Discussion

In two different types of media presumed to block chemical synapses, the dark activity of Aplysia eyes is suppressed, while the phasic light response is not. In one of these, high Mg^{++} coupled with low Ca^{++}, spontaneous CAPs usually reappear, especially with Ca^{++} concentrations below 1 mM. When this occurs, the eye still shows a

circadian rhythm, albeit somewhat different in detail from that of a normal eye in ASW. This rhythmicity was observed by Jacklet (1973a), who, however, failed to note the period of silence following the change from ASW to these solutions. With 1 mM $LaCl_3$SW, spontaneous activity never returns. Eyes often fail to show normal activity when returned to ASW after an hour or more in this solution. These inhibitions are probably due to one of three causes: a blockade of chemical synapses as postulated by Audesirk (1973); an interference with pacemaker activity; or an interference with spike production itself by high Mg^{++} as proposed by Jacklet (1973a).

The last hypothesis seems unlikely, for the following reasons. First, the eyes give strong light responses in all of the solutions during inhibition of dark discharge (Figs. 2 and 4 in Audesirk, 1973). Second, electrical stimulation of the optic nerve does not require higher voltages to elicit CAPs in these solutions (Fig. 1 and Table I). Third, neither spontaneous nor induced spikes in identified single neurons in the Aplysia PVG are blocked by any of the high Mg^{++} solutions. If there is a depressive effect of high Mg^{++}, it must be slight. The main depressive effect observable on the eye in these experiments is a decrease in the maximum CAP size, and this seems to be accounted for by the reduced sodium in these media. In conjunction with low Ca^{++}, the overall effect is excitatory (Tables I and II, Fig. 2, and the light response of Fig. 2 in Audesirk, 1973).

As to the second possibility, high Mg^{++} with normal or reduced Ca^{++} levels has no suppressive effect on PVG pacemakers (Table II and

Fig. 2). Therefore it is unlikely that high Mg^{++} with or without low Ca^{++} suppresses pacemakers in the eye. In $LaCl_3$SW some pacemakers, such as R15, eventually become silent, often after passing through a phase of increased and continuous spiking lasting one to three hours. Although this increased spiking does not occur in the eye, the possibility is not excluded that La^{+++} interferes with eye pacemakers.

Therefore, the inhibition of dark activity, especially with high Mg^{++} with low Ca^{++}, is probably due to blocking chemical synaptic transmission, cutting off a spontaneously active cell(s) from its usually silent follower population. Unless further perturbations occur, the eye would be expected to remain silent indefinitely, if not stimulated by light. This is perhaps what occurs in 1 mM $LaCl_3$SW, and in some high Mg^{++}, 1 mM Ca^{++} experiments. There is a cell type which has been recorded by Jacklet (1973b) which seems to be a candidate for such a pacemaker, although it was not interpreted by him as such. This cell spikes in bursts, with each burst occurring synchronously with a CAP in the optic nerve.

Why does activity return in most high Mg^{++}, low Ca^{++} experiments? The answer cannot be given with certainty, but the return of firing and its high frequency may be caused by enhanced excitability in low Ca^{++} media. This is known to occur in several other systems (Adrian and Gelfan, 1933; Bronk, Larrabee, Gaylor, and Brink, 1938; Kishimoto, 1966), and a similar effect occurs with R15 and other pacemakers in Aplysia (Fig. 2 and Table II; Carpenter and Gunn, 1970). Perhaps a normally silent population of cells becomes active in the eye when Ca^{++}

levels are low enough. No assurances exist that the initial suppression of dark activity is not due to some presently unknown, nonsynaptic effect of high Mg^{++} and/or low Ca^{++}, from which the eye gradually recovers. However, there are two indications that the return of dark activity is related to reduced Ca^{++}. First, the suppression of activity sometimes lasts indefinitely in 1 mM Ca^{++}, but apparently never in the 0.5 or 0 mM Ca^{++} solutions. Second, the average duration of inhibition by the high Mg^{++}, low Ca^{++} media is least with 0 Ca^{++} and most with 1 mM Ca^{++}.

The continuation of the eye's circadian rhythm in these solutions would seem to indicate that a rhythmic ability resides in the follower population, one of the alternatives given by Audesirk (1973). There is a negative correlation between the previous activity level and the duration of inhibition by high Mg^{++} and low Ca^{++} (Table III; for 1 mM Ca^{++}, $n = 11$; Spearman rank correlation, $r = -0.67$, $p < 0.025$; for all solutions, $n = 23$; $r = -0.55$, $p < 0.01$). Thus a more active eye recovers more rapidly than a less active one. A system operating nearer to threshold initially, as manifested by a high activity level, might be expected to take less time for the excitability enhancement by low Ca^{++} to cause firing in the absence of synaptic transmission. If this is true for variations from eye to eye, perhaps it is also true for the variation in activity that constitutes the circadian rhythm of a given eye.

Perhaps, then, the follower population has a diurnal variation in threshold or membrane potential, which extracts a circadian rhythm out

of an arhythmic barrage of pacemaker EPSPs, or further refines a circadian rhythm from the pacemaker(s). This hypothesis would predict that the duration of inhibition by high Mg^{++}, low Ca^{++} would be negatively correlated with the activity level of a given eye at different times of day, as it is with the activity of different eyes at the same time of day. In practice, this would have to be tested with different eyes at the various time points, and correction made for differing activities between eyes.

It must be pointed out, however, that the circadian rhythm in high Mg^{++}, low Ca^{++} could also arise from a response of hyperexcitable follower cells to the residual EPSPs from the pacemakers. There are differences between the circadian rhythms of low Ca^{++} eyes and ASW eyes, which indicate that the method of rhythm production in the high Mg^{++}, low Ca^{++} solution is not entirely normal. For instance, there is a rhythm of bursts per unit time in normal eyes that the high Mg^{++}, low Ca^{++} eyes do not usually show. In fact, the eyes do not burst at all in high Mg^{++} with 0 Ca^{++}.

The eye of Aplysia is probably quite complicated, both in cell types and in interactions between cells. The model presented in the preceding chapter appears to be supported by the data presented here, but no simple model is likely to account for all of the observed phenomena. It is hoped, however, that this model can serve as a starting point for further research which will make additions and corrections to achieve increasingly accurate pictures of the neuronal interactions involved in rhythm production in the Aplysia eye.

Table I

Solution	n	Threshold Voltage (volts)	Maximum Amplitude (μ V)	Voltage for Max. Amp. (volts)	Stimulation Duration (msec.)
ASW	4	8.8	81	25.7	0.3
125 mM Mg^{++}, 1mM Ca^{++}		8.7	80	25.8	0.3
ASW	5	19.9	216	58.3	0.15
125 mM Mg^{++}, 0 mM Ca^{++}		19.3	189	51.0	0.15
ASW	4	22.9	687	38.1	0.15
125 mM Mg^{++}, 10 mM Ca^{++}		26.1	629	37.9	0.15
ASW	4	24.4	593	38.9	0.15
1 mM $LaCl_3$SW		25.0	555	38.2	0.15
ASW	4	22.1	569	41.5	0.15
80% NaSW		21.9	528	41.5	0.15

Each ASW measurement is the average of values before and after the experimental solution, with the exception of the $LaCl_3$SW eyes, in which the ASW value is only the average of the parameters measured before the change to $LaCl_3$SW.

Table II

125 mM Mg^{++}, low or normal Ca^{++}, on R15

Solution	n	Spikes	Bursts	Spikes/burst
ASW		156	52	3.0
0 mM Ca^{++}	2	600	38	15.8
ASW		404	33	12.2
ASW		302	42	7.2
1 mM Ca^{++}	3	401	30	13.4
ASW		283	35	8.1
ASW		380	48	7.9
10 mM Ca^{++}	4	271	33	8.2
ASW		379	44	8.6

Each number is an average of counts for one half hour: before, during a half hour immersion in the experimental solution, and immediately after.

Table III

125 mM Mg^{++}, low Ca^{++} on isolated Aplysia eye

Ca^{++} Level	Duration of Inhibition (hr)[a]	CAP Number 1200-1230 hr[b]
	0.3	235
	0.4	169
	1.1	200
	1.3	121
	1.4	148
1 mM	3.2	137
	3.4	102
	4.0	92
	4.5	124
	9.0	151
	9.0[c]	91
	0.1	148
	0.9	223
0.5 mM	0.9	166
	1.5	190
	3.2	52
	7.5	155
	0.1	176
	0.3	167
0 mM	0.5	135
	0.6	150
	0.8	147
	2.1	162

a) Duration of suppression of spontaneous dark activity following immersion in the high Mg^{++}, low Ca^{++} solution. All changes to high Mg^{++}, low Ca^{++} occurred between 1330 and 1630 hr on the day of dissection. Normal eyes become silent on the day of dissection at about 2300 hr, so the times are measured until recovery occurs or until 2300 hr, whichever is least.

b) Number of spontaneous impulses in darkness at a fixed time of day,

Table III (con't)

before immersion in high Mg^{++}, low Ca^{++}.

c) These two eyes never gave spontaneous dark impulses again in 125 mM Mg^{++}, 1 mM Ca^{++}, although a light response was evident 40 hours later.

Figure 1. Electrical stimulation of Aplysia optic nerve. In all examples, the left member of the pair is recorded in ASW, the right member in the experimental solution. A) 125 mM Mg^{++}, 1 mM Ca^{++} (hi Mg^{++}-lo Ca^{++} of Audesirk, 1973). B) 125 mM Mg^{++}, 10 mM Ca^{++}. C) 1 mM $LaCl_3$SW. D) 80% NaSW (choline substitution). Vertical calibration: 20 μV for A, 200 μV for B, C, and D. Horizontal calibration: 20 msec. for all.

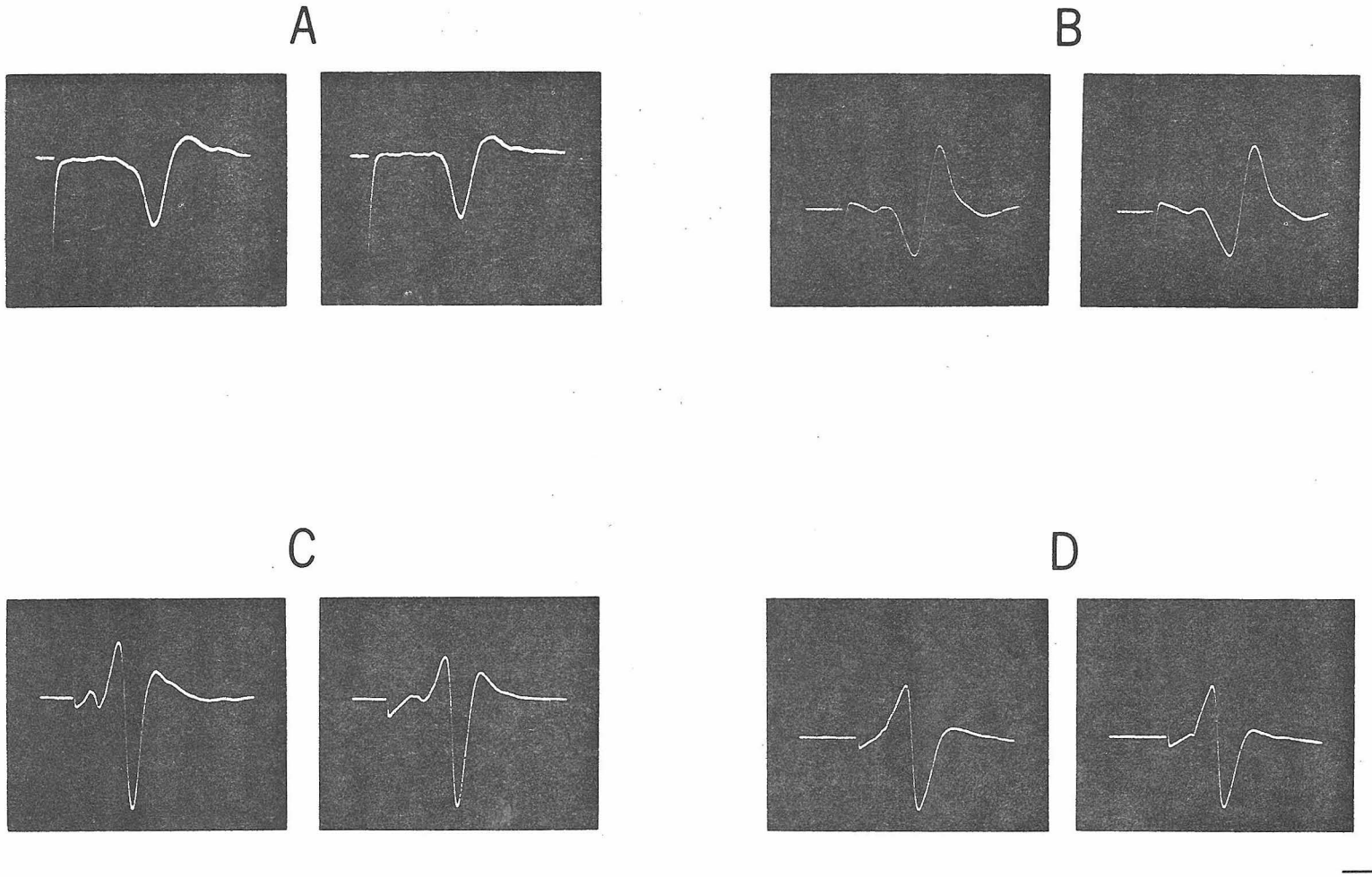

A
B
C
D

Figure 2. Spontaneous firing of R15, a bursting neuron in the Aplysia PVG. Experimental solutions have 125 mM Mg^{++} with the indicated Ca^{++} concentrations, in mM. All recordings in the experimental solutions begin 10 minutes after the change to the indicated solution, with the exception of the last line in 10 mM Ca^{++}. This line of recording begins about 11 hours after the change. Vertical calibration: 50 μV; horizontal calibration: 30 sec.

125 mM Mg^{++}SW on R15

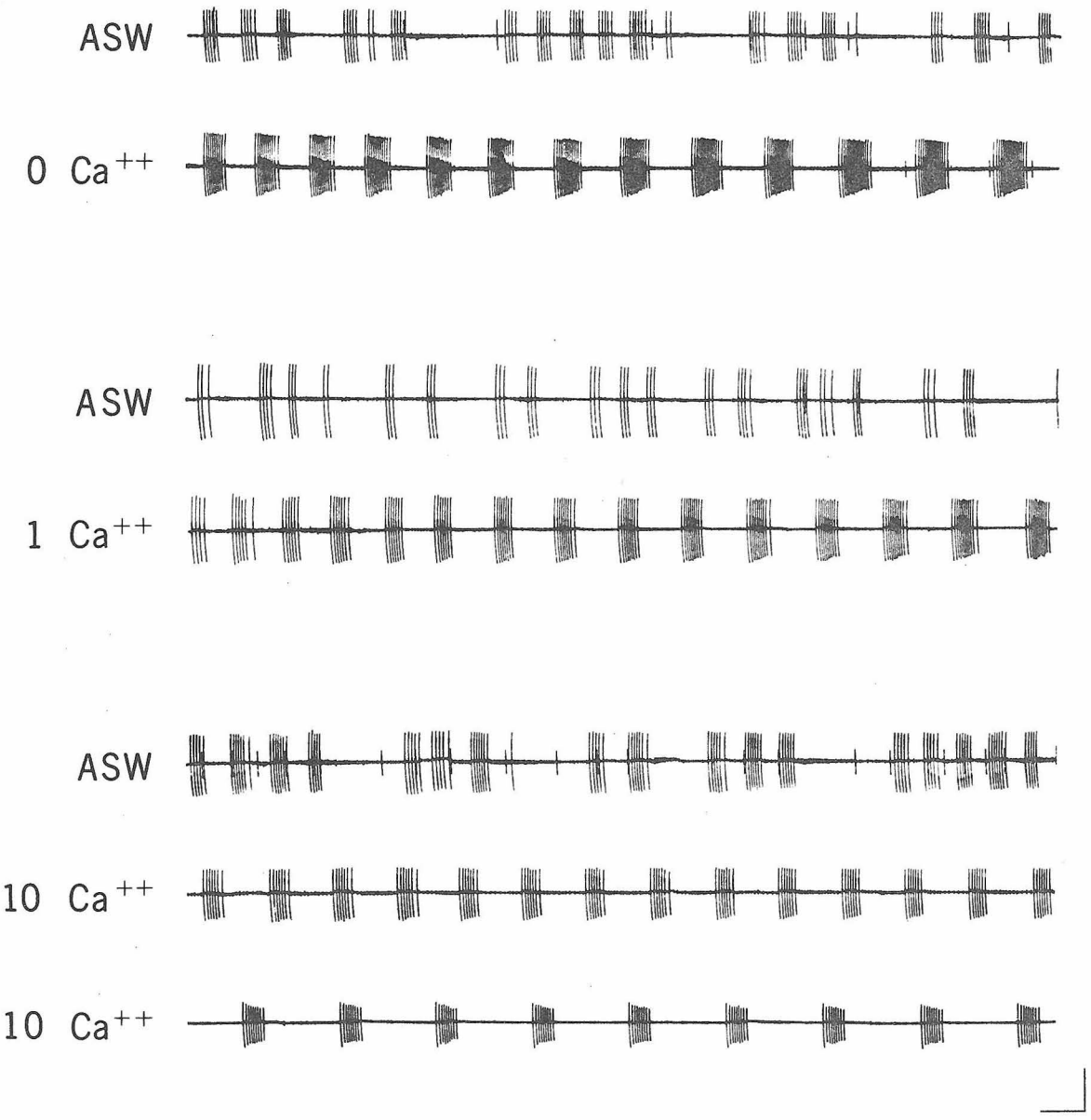


Figure 3. Spontaneous firing of R15 and a member of the L2-4,6 cluster of cells in the PVG in 1 mM $LaCl_3$SW. R15 recordings are continuous in $LaCl_3$SW; one minute occurs between the ASW and $LaCl_3$SW recordings. L2-4,6 recording in $LaCl_3$SW begins nine hours after the ASW recording. R15 calibrations: vertical, 100 μV; horizontal, 20 sec. L2-4,6 calibrations: vertical, 50 μV; horizontal, 30 sec.

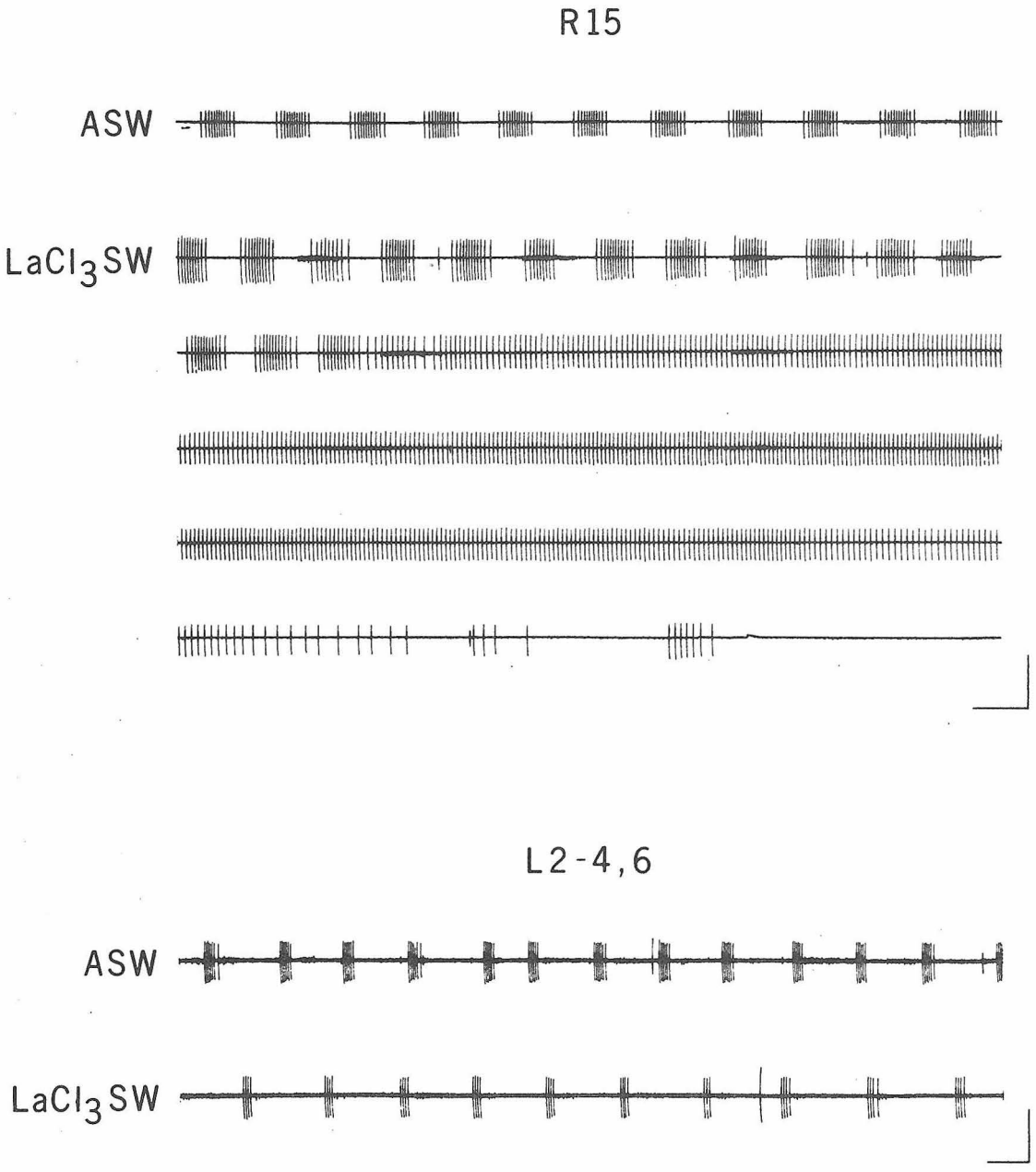
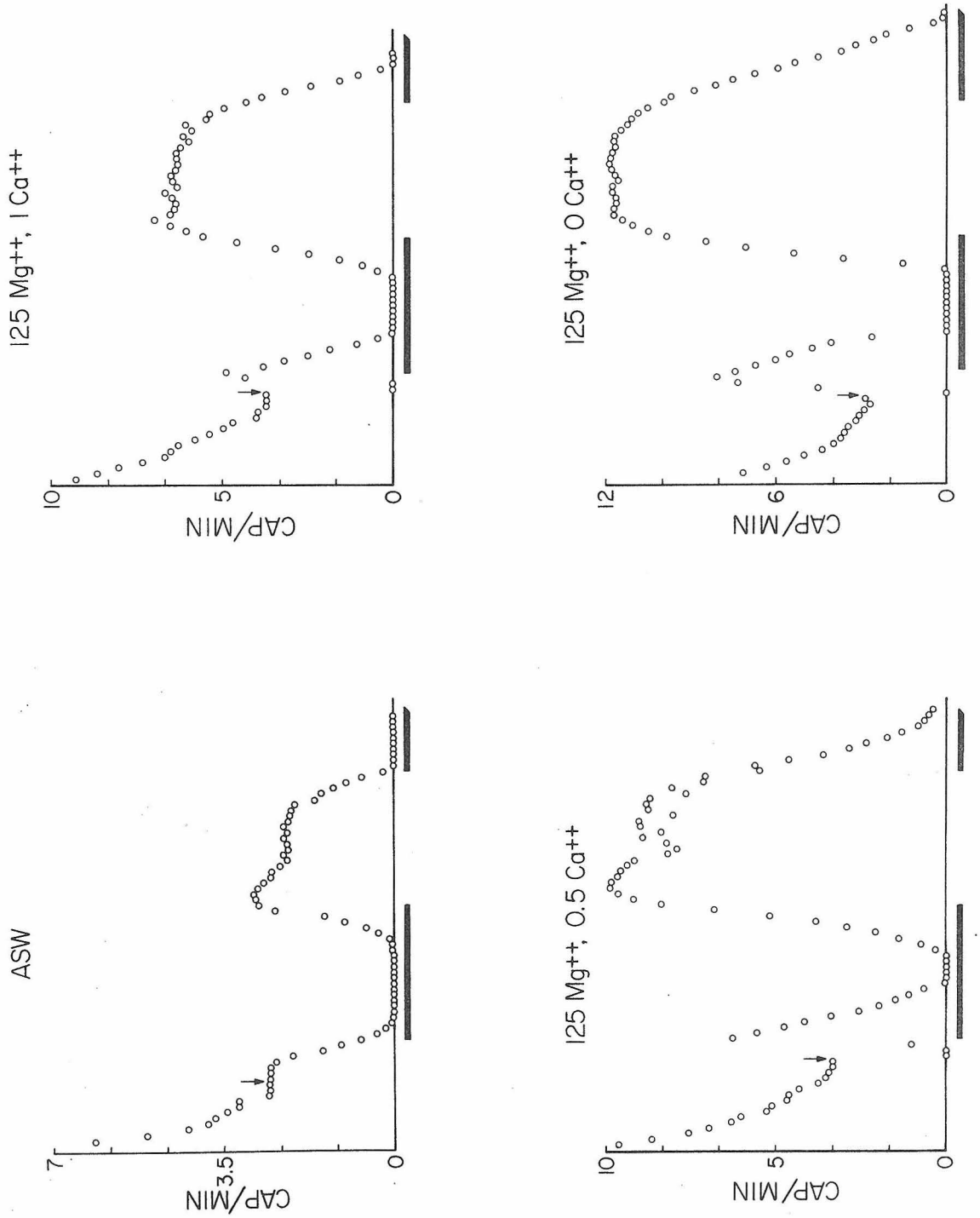

R15
ASW
LaCl3SW
L2-4,6
ASW
LaCl3SW

Figure 4. Spontaneous activity of Aplysia eyes in high Mg^{++}, low Ca^{++} solutions. All start in ASW, and change to the indicated solution at the arrow. Note the suppression of activity in all of the high Mg^{++}, low Ca^{++} solutions immediately after the arrow (zero frequencies), and the subsequent recovery to greater than normal activity. Some eyes in 125 mM Mg^{++} with 1 mM Ca^{++} do not recover spontaneous activity again (see Table III). All recordings are in constant darkness. Dark bars represent projected night.


ASW
125 Mg++, 1 Ca++
125 Mg++, 0.5 Ca++
125 Mg++, 0 Ca++
CAP/MIN
7
3.5
0
10
5
12
9

References

Adrian, E.D., and S. Gelfan. 1933. Rhythmic activity in skeletal muscle fibres. J. Physiol. 78: 271-287.

Audesirk, G. 1973. Spontaneous and light-induced compound action potentials in the isolated eye of Aplysia: initiation and synchronization. Brain Res. 59: 229-242.

Bronk, D.W., M.G. Larrabee, J.B. Gaylor, and F. Brink, Jr. 1938. The influence of altered chemical environment on the activity of ganglion cells. Am. J. Physiol. 123: 24-25.

Carpenter, D., and R. Gunn. 1970. The dependence of pacemaker discharge of Aplysia neurons upon Na^+ and Ca^{++}. J. Cell Physiol. 75: 121-128.

Jacklet, J.W. 1969. Electrophysiological organization of the eye of Aplysia. J. Gen. Physiol. 53: 21-42.

Jacklet, J.W. 1973a. The circadian rhythm in the eye of Aplysia. Effects of low calcium and high magnesium. J. Comp. Physiol. 87: 329-338.

Jacklet, J.W. 1973b. Neuronal population interactions in a circadian rhythm in Aplysia. In: J. Salanki (ed.), Neurobiology of Invertebrates, Tihany 1971. Budapest: Hungarian Academy of Sciences, pp. 363-380.

Kishimoto, U. 1966. Repetitive action potentials in Nitella internodes. Plant Cell Physiol. 7: 547-558.

PART II

STUDIES ON THE INTERRELATIONSHIP BETWEEN TWO NEURONAL CIRCADIAN OSCILLATORS IN *APLYSIA CALIFORNICA*

Introduction

a) Photoentrainment of circadian rhythms

The circadian rhythms of most organisms are entrained by some aspect of their environment; that is, some regularly occurring feature of the environment is used as a timer to "set" the biological clock. Entraining agents can vary widely, from social stimuli (Halberg, Visscher, and Bittner, 1954; Gwinner, 1966; Pöppel, 1968; Reinberg, 1971) to the internal rhythms of humans for some parasites such as the Microfilariae (Conroy and Mills, 1970; Pittendrigh, 1974). The most common and most powerful agent, however, is light (Pittendrigh, 1974). In temperate and tropic regions, the difference in light intensity between night and day is an unmistakeable cue that occurs precisely on a 24 hour schedule, far more reliably than other cues, such as temperature or humidity differences.

Much attention has been given to the problem of the entrainment of circadian rhythms by light. The questions asked are usually two: what structure receives the photic stimulation and passes it on to rhythmic centers in the body; and how does the transmission from receptor to oscillator occur. Underlying these questions is a more fundamental problem, for which these are the necessary preliminaries: how does a circadian rhythm become timed, i.e., how can the clock be set?

The preliminary questions are usually approached in a straightforward way. The suspected receptor is ablated, and a manifestation of rhythmicity (usually locomotor activity in a light-dark cycle) is assayed for entrainment by light. As simple as this procedure appears,

the results and their interpretation are often confusing. There are several reasons for this confusion. First, it is not always as easy to blind an animal (usually the first experiment) as it may seem (Nishiisutsuji-Uwo and Pittendrigh, 1968a). Second, there may be additional effects of blinding. For example, in some animals blinding may cause scar tissue to form over the site of the eye, which may be less transparent for underlying nervous structures than the eye was. In others, a window may be opened to the brain by removal of pigmented tissue along with the eye. Either of these effects may be important if direct light sensitivity of the brain is suspected. Third, the assay for rhythmicity is many times done by visual examination of event marker recordings, and experimenters may differ in their interpretation of the same records. Fourth, the type of activity monitor (perches, running wheels, balanced cages) can affect the expression of rhythmicity (Harker, 1973). Last, the fact that an animal without eyes still shows rhythmicity does not prove that the eyes are not used for entrainment in the intact animal, especially if the rhythm is weaker in the blinded animal.

These difficulties notwithstanding, it has been reported that the eyes are not required as photoreceptors for entrainment of circadian rhythms (locomotor rhythms unless otherwise specified) in: sparrows (Menaker, 1968); salamanders (Adler, 1969); frogs (Adler, 1971); lizards (Underwood, 1973); chromatophore color change in pencil fish (Reed, 1968); various insects, such as cockroaches (Harker, 1956) and Drosophila (Zimmerman and Ives, 1971); and Aplysia (locomotor rhythm:

Block and Lickey, 1973; spike rhythm in the neuron R15: Lickey, Zack, and Birrell, 1971, and Lickey and Zack, 1973). The authors of many of these reports also find that the rhythms are weaker, or changed in timing or form, or have a higher threshold intensity of light required for entrainment. Animals which have been reported to require their eyes for entrainment include: rat (drinking rhythm: Browman, 1943); mice (eosinophil level in blood: Halberg, Visscher, and Bittner, 1954); fiddler crabs (chromatophore color: Stephens, Halberg, and Stephens, 1964); cockroaches (locomotor activity: Nishiitsutsuji-Uwo and Pittendrigh, 1968a; Roberts, 1965; Roberts, Skopik, and Driskill, 1971); and Aplysia (locomotor activity: Strumwasser, 1973, 1974). Note that some organisms appear on both lists.

The next step, that of determining the oscillators into which the photic information is transmitted, is more difficult. Attempts to find at least some apparent sites for neuronal oscillators have met with best success in birds, mammals, and insects. In birds, the pineal organ seems to be required for free-running rhythmicity in daily locomotor activity in constant darkness (Gaston and Menaker, 1968). In rodents, both the pineal (Klein, 1974; Moore, 1974) and the hypothalamus - pituitary - adrenal cortex complex (Menaker, 1974) have been found to be rhythmic in content and secretion of various products and to receive entrainment information from the eyes. The integrity of the suprachiasmatic nucleus in the hypothalamus appears to be critical for various rhythms in rats (Stephan and Zucker, 1972; Moore, 1974). Neurosecretory cells in the brain of insects have been implicated in

activity and eclosion rhythms (Nishiitsutsuji-Uwo and Pittendrigh, 1968b; Rensing, 1971; Truman, 1974) although some of this evidence has been disputed (Brady, 1969; Roberts, et al., 1971).

The most interesting question, that of the mechanism of entrainment of the oscillators, is furthest from an answer. Only in the rodents are the receptors, the site of at least some central oscillators, and a complete pathway between them known with certainty (Stephan and Zucker, 1972; Klein, 1974; Moore, 1974).

b) Photoentrainment in Aplysia

Aplysia californica would appear to be well suited for the investigation of some of these matters. It has a single neuron known to be a circadian oscillator (Strumwasser, 1965), easily accessible eyes, which also possess a circadian rhythm (Jacklet, 1969b), a strong locomotor rhythm (Strumwasser, 1967; Kupfermann, 1968), and a distributed central nervous system which makes discrete lesions relatively simple.

The search for the receptor involved in the photoentrainment of the circadian locomotor rhythm of this animal has unfortunately been plagued by controversy (Strumwasser, 1973, 1974; Block and Lickey, 1973; see the chapter on circadian rhythms in Aplysia in Part I above). Only two points will be made here regarding this controversy. First, the reports of extraoptic entrainment show that the locomotor rhythm is much weaker in blinded animals compared to normal ones (Block and Lickey, 1973, Figs. 1 and 2), but the emphasis has been placed on the ability of extraoptic receptors to entrain the rhythm. Second,

Strumwasser's data (1973, 1974) show bursts of activity in blinded animals near the dark-light transition, but statistical analysis reveals little or no rhythmicity over the whole daily cycle. It might be suspected that these bursts at light onset represent much of the "entrainment" seen by Block and Lickey in their visual analysis of event recorder charts. A blinded Aplysia might still respond to a light onset without entraining its endogenous circadian oscillators.

The neuron R15 shows a circadian rhythm of spike activity when recorded in the isolated PVG (Strumwasser, 1965). The timing of the peak of activity is related to the timing of the lighting schedule of the animal prior to dissection. It has been reported that the eyes are not necessary for photoentrainment of the R15 rhythm in vivo (Lickey, Zack, and Birrell, 1971; Lickey and Zack, 1973).

In both sets of experiments from the Lickey laboratories, Aplysia were left intact, blinded, or sham blinded, and placed in aquaria. The light schedule in the aquaria was LD 12:12, but the time of lights-on varied from tank to tank. Blinded animals were not separated from sighted ones. Dissection times were not reported and presumably were not controlled. In the latter paper (Lickey and Zack, 1973), the intensity of the light during entrainment was given as 170 lux. This series of experiments also included a group of blinded Aplysia exposed to an entraining LD cycle of only 20 lux. These animals were isolated from sighted animals, although apparently not by special design. The conclusions from both papers were that blinded Aplysia can still have their R15 rhythms entrained by light cycles, unless the light is very

dim (20 lux). Normal animals were not tested at the 20 lux intensity to see if their R15s entrained.

There are several flaws in the designs of these blinding experiments. The potentially greatest one is the housing of sighted and blinded animals in the same aquaria. Social entrainment of blind mice in the presence of sighted mice has been reported (Halberg, Visscher, and Bittner, 1954). Social interactions can entrain rhythms in men kept in constant conditions (Pöppel, 1968; Reinberg, 1971), and song can entrain bird activity rhythms (Gwinner, 1966). Similar phenomena could also occur in Aplysia. Second, dissection times were not controlled. Dissection time relative to light onset turns out to be an important parameter in the timing of the R15 rhythm in the isolated PVG (see Appendix A). Third, the phase of the entraining cycle was not, or at least was not reported to be, controlled or related pre- and postoperatively. Fourth, the dim light experiments tested two variables at once: isolation from sighted animals (which is dismissed by the authors as highly unlikely to be significant) and intensity of light. Fifth, normal animals were run only in the 170 lux tanks, and not at 20 lux, so it is not known whether they would have entrained at the lower intensity. (Aplysia eyes can respond to light at least as dim as 10 lux; see Audesirk, 1973, Fig. 2.)

The reports themselves contain no numerical listing of the data (such as peak times) and almost no statistical analysis. Both reports present their data graphically (Fig. 1, from Lickey, Zack, and Birrell, 1971, and Lickey and Zack, 1973), and conclude that the activity peaks

of R15s from blinded Aplysia do not differ from those of sighted animals. Lickey, et al. (1971) base this conclusion on the occurrence of most peaks during the projected night, both in blinded and in sighted cases. Lickey and Zack (1973) state that "peaks were most likely to occur shortly following the middle of the projected day or the middle of the projected night" for R15s from both sighted and blinded animals. Inspection of the data of both experiments reveals, first, a large spread in peak times of R15s, regardless of the source, and, second, a greater scatter of peaks in the eyeless cases.

The large spread in peak times is significant in view of data to be presented here (Part II and Appendix A). As stated, the time of dissection was not controlled in the Lickey experiments, and (using peak times approximated from their graphs to within 0.5 hours) the standard deviations were very large (for the data of Lickey, et al., 1971: normal Aplysia, $s = 5.4$ hours; blinded, $s = 6.1$ hours; for the data of Lickey and Zack, 1973: normal, $s = 5.2$ hours, blinded, $s = 4.9$ hours). These large standard deviations make most statistical tests of differences between blinded and sighted animals doomed to failure. Even the peak average of R15s from blinded Aplysia exposed to the 20 lux light cycles, which Lickey and Zack consider to be unentrained, cannot be statistically distinguished from the controls used in that report ($t = 0.49$; $p > 0.5$). The greater variability of the blinded R15 peaks comes closer to being, but is still not, statistically significant ($F = 2.25$; $0.1 < p < 0.2$). (When dissection time in relation to light onset is kept constant, the standard deviations in

peak times are half the values obtained in these reports; see Part II and Appendix A.)

Since, as Lickey and Zack (1973) point out, most peaks of R15s from sighted animals occur near but normally after noon or midnight, perhaps a better way of measuring time is in 12 hour blocks, relative to noon or midnight (hereafter called "midperiod"). Then, for example, noon = midnight = 0 hr; or two hours after noon = two hours after midnight = +2 hr. In this fashion, the average normal R15 peak time for both sets of the Lickey experiments taken together is +1.2 hr, s = 2.2 hr; for blinded animals, average peak is +0.2 hr, s = 2.8 hr. Using this method of determining peak time, the difference in average peak time between the 20 lux blinded animals and the controls (Lickey and Zack, 1973) is almost significant ($t = 2.0$; $0.05 < p < 0.1$) and the variability difference is highly significant ($F = 5.0$; $p < 0.005$). Despite the authors' interpretation, using midperiod times shows that the peaks of blinded animals exposed to 170 lux (the same as the controls) are significantly more variable than the control peaks ($F = 2.7$; $p < 0.05$).

The midperiod timing of the R15 peaks of blinded animals raises an interesting point. If a series of events are randomly distributed in a period of time, then the average time of an event would be expected to occur at the middle of the time period. Both normal and blinded Aplysia (both Lickey reports) had peak averages near midperiod, but the normal time differs from midperiod ($t = 2.7$; $p < 0.025$), while the blinded time does not ($t = 0.5$; $p > 0.5$). Another consequence of a

random distribution of peaks in time would be equal numbers of peaks before and after the midperiod. The normal animals showed a strongly skewed distribution (20 after midperiod, 4 before), while the blinded ones do not (18 after, 15 before). These distributions are significantly different (chi square = 4.0; $p < 0.05$).

In summary, the data of Lickey, et al. (1971) and Lickey and Zack (1973) are open to conflicting interpretation. Two indications of entrainment of blinded animals' R15s are the nocturnality of the peaks (occurrence during the projected night) and the clustering of the peaks near midnight. However, the blinded animals' R15s have somewhat different peak times, greater variability, and a nearly randomly distributed pattern of peaks relative to midperiod.

What can be concluded from this confusion of facts and interpretation? It is clear that, first, the experiments should be better designed and controlled. Animals should be isolated from one another during entrainment, and dissection time should be controlled or at least taken into consideration. Second, nocturnality is not a sufficient measure of entrainment. There could be any number of reasons for nocturnality, not the least of them the fact that the cell will be deteriorating from the moment of dissection and might be expected to have a preferred peak time <u>after dissection</u> even if it had no rhythm at all. Dissections with appropriate phase relative to the entraining light cycle could give the appearance of rhythmicity to an endogenously arhythmic cell. Third, a free run of the blinded animals may occur, and if the correct phase relation holds between the

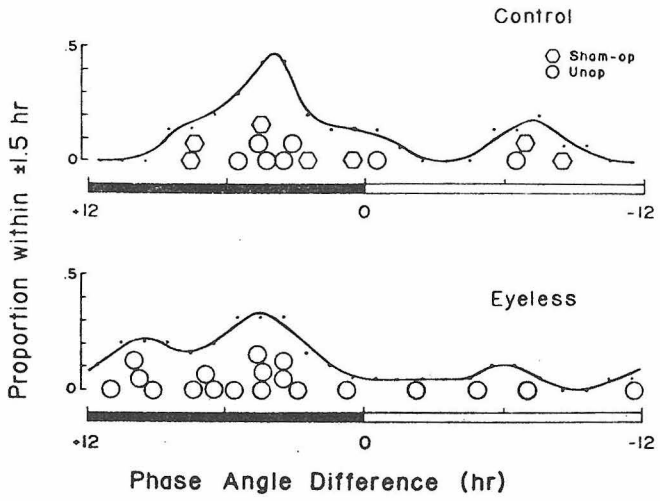
pre- and postoperative light schedules, this free run may give the appearance of entrainment. Finally, a visual inspection of the data, especially in the 1971 paper, shows that the same results could be obtained if a fraction, say one fourth to one third, of the blinded animals entrained, while the rest did not. This would account for both the clustering of peaks near the control cluster time (the entrained fraction) and the variable and nearly random distribution of the peaks (due to the unentrained majority).

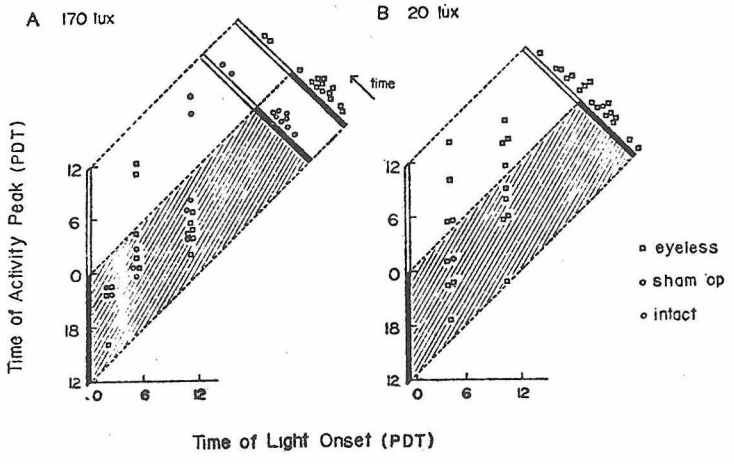
To summarize photic entrainment in Aplysia, the eyes seem vital to the entrainment of the locomotor rhythm. Also, from the reports discussed above, it would appear that, contrary to the authors' conclusions, the eyes are probably important for the photoentrainment of the R15 rhythm. Experiments were therefore designed to: 1) definitively determine the role of the eyes in R15 entrainment in vivo; 2) determine what routes for the transmission of photic information exist between the eyes and R15 in vivo and in vitro; 3) identify which of these routes mediates entrainment or at least rule out some pathways; and 4) develop, if possible, an in vitro system for the entrainment of R15.

Figure 1. Peak times of spike activity in R15s recorded in the isolated PVG. Projected light schedule represented as black (night) and open (day) bars.

Upper: From Lickey, Zack, and Birrell, 1971.

Lower: From Lickey and Zack, 1973. Actual peak times relative to projected night and day are given by the symbols above the diagonal bars.


Control
Sham-op
Unop
.5
0
+12
0
-12
Eyeless
Proportion within ±1.5 hr
Phase Angle Difference (hr)


A 170 lux
B 20 lux
time
12
6
0
18
12
eyeless
sham op
intact
Time of Activity Peak (PDT)
Time of Light Onset (PDT)

References

Adler, K. 1969. Extraoptic phase shifting of circadian locomotor rhythm in salamanders. Science 164: 1290-1292.

Adler, K. 1971. Pineal end organ: role in extraoptic entrainment of circadian locomotor rhythm in frogs. In: M. Menaker (ed.), Biochronometry. Washington, D.C.: National Academy of Sciences, pp. 342-350.

Audesirk, G. 1973. Spontaneous and light-induced compound action potentials in the isolated eye of Aplysia: initiation and synchronization. Brain Res. 59: 229-242.

Block, G.D., and M.E. Lickey. 1973. Extraocular photoreceptors and oscillators can control the circadian rhythm of behavioral activity in Aplysia. J. Comp. Physiol. 84: 367-374.

Brady, J. 1969. How are insect rhythms controlled? Nature 223: 781-784.

Browman, L.G. 1943. The effect of bilateral optic enucleation upon activity rhythms of the albino rat. J. Comp. Psychol. 36: 33-46.

Cloudsley-Thompson, J.L. 1953. Studies in diurnal rhythms. III. Photoperiodism in the cockroach Periplaneta americana (L.). Ann. Mag. Nat. Hist. 6: 705-712.

Conroy, R.T.W.L., and J.N. Mills. 1970. Human Circadian Rhythms. London: J. & A. Churchill.

Gaston, S., and M. Menaker. 1968. Pineal function: the biological clock in the sparrow? Science 160: 1125-1127.

Gwinner, E. 1966. Periodicity of a circadian rhythm in birds by species-specific song cycles (Aves, Fringellidae: Carduelis spinus, Serinus serinus). Experientia 22: 765-766.

Halberg, F., M.B. Visscher, and J.J. Bittner. 1954. Relation of visual factors to eosinophil rhythm in mice. Am. J. Physiol. 179: 229-235.

Harker, J. 1956. Factors controlling the diurnal rhythm of activity in Periplaneta americana L. J. Exp. Biol. 33: 224-234.

Harker, J. 1973. Circadian rhythms in insects. In: J.N. Mills (ed.), Biological Aspects of Circadian Rhythms. New York: Plenum Press, pp. 189-233.

Jacklet, J.W. 1969. A circadian rhythm of optic nerve impulses recorded in darkness from the isolated eye of Aplysia. Science 164: 562-564.

Klein, D.C. 1974. Circadian rhythms in indole metabolism in the rat pineal gland. In: F.O. Schmitt and F.G. Worden (eds.), The Neurosciences: Third Study Program. Cambridge: MIT Press, pp. 509-515.

Kupfermann, I. 1968. A circadian locomotor rhythm in Aplysia californica. Physiol. Behav. 3: 179-182.

Lickey, M.E. 1969. Seasonal modulation and non-24 hour entrainment of a circadian rhythm in a single neuron. J. Comp. Physiol. Psychol. 68: 9-17.

Lickey, M.E., S. Zack, and P. Birrell. 1971. Some factors governing entrainment of a circadian rhythm in a single neuron. In: M. Menaker (ed.), Biochronometry. Washington, D.C.: National Academy of Sciences, pp. 549-564.

Lickey, M.E., and S. Zack. 1973. Extraocular photoreceptors can entrain the circadian rhythm in the abdominal ganglion of Aplysia. J. Comp. Physiol. 84: 361-366.

Menaker, M. 1968. Extraretinal light perception in the sparrow. I. Entrainment of the biological clock. Proc. Nat. Acad. Sci. 59: 414-421.

Menaker, M. 1974. Aspects of the physiology of circadian rhythmicity in the vertebrate central nervous system. In: F.O. Schmitt and F.G. Worden (eds.), The Neurosciences: Third Study Program. Cambridge: MIT Press, pp. 479-489.

Menaker, M., and A. Eskin. 1966. Entrainment of circadian rhythms by sound in Passer domesticus. Science: 154: 1579-1581.

Moore, R. 1974. Visual pathways and the central neural control of diurnal rhythms. In: F.O. Schmitt and F.G. Worden (eds.), The Neurosciences: Third Study Program. Cambridge: MIT Press, pp. 537-542.

Nishiitsutsuji-Uwo, J., and C. Pittendrigh. 1968a. Central nervous system control of circadian rhythmicity in the cockroach. II. The pathway of light signals that entrain the rhythm. Z. Vergl. Physiol. 58: 1-13.

Nishiitsutsuji-Uwo, J., and C. Pittendrigh. 1968b. Central nervous system control of circadian rhythmicity in the cockroach. III. The optic lobes, locus of the driving oscillation? Z. Vergl. Physiol. 58: 14-46.

Pittendrigh, C. 1974. Circadian oscillations in cells and the circadian organization of multicellular systems. In: F.O. Schmitt and F.G. Worden (eds.), The Neurosciences: Third Study Program. Cambridge: MIT Press, pp. 437-458.

Pöppel, E. 1968. Desynchronizationen circadianer Rhythmen innerhalb einer isolierten Gruppe. Pflüg. Archiv. Ges. Physiol. 299: 364-370.

Reed, B.L. 1968. The control of circadian pigment changes in the pencil fish: a proposed role for melatonin. Life Sci. 7 (II): 961-973.

Reinberg, A. 1971. Methodologic considerations for human chronobiology. J. Interdiscipl. Cycle Res. 2: 1-15.

Rensing, L. 1971. Hormonal control of circadian rhythms in Drosophila. In: M. Menaker (ed.), Biochronometry. Washington, D.C.: National Academy of Sciences, pp. 527-540.

Roberts, S.K. 1966. Circadian activity rhythms in cockroaches. III. The role of endocrine and neural factors. J. Cell Physiol. 67: 473-486.

Roberts, S.K., S.D. Skopik, and R.J. Driskill. 1971. Circadian rhythms in cockroaches: does brain hormone mediate the locomotor cycle? In: M. Menaker (ed.), Biochronometry. Washington, D.C.: National Academy of Sciences, pp. 505-516.

Stephan, F.K., and I. Zucker. 1972. Rat drinking rhythms: central visual pathways and endocrine factors mediating responsiveness to environmental illumination. Physiol. Behav. 8: 315-326.

Stephens, G.W., F. Halberg, and G.C. Stephens. 1964. The blinded fiddler crab: an invertebrate model of circadian desynchronization. Ann. N.Y. Acad. Sci. 117: 386-406.

Strumwasser, F. 1965. The demonstration and manipulation of a circadian rhythm in a single neuron. In: J. Aschoff (ed.), Circadian Clocks. Amsterdam: North-Holland Publ. Co., pp. 442-462.

Strumwasser, F. 1967. Neurophysiological aspects of rhythms. In: G.C. Quarton, T. Melnechuk, and F.O. Schmitt (eds.), The Neurosciences: An Intensive Study Program. New York: Rockefeller Univ. Press, pp. 516-528.

Strumwasser, F. 1973. Neural and humoral factors in the temporal organization of behavior. Physiologist 16: 9-42.

Strumwasser, F. 1974. Neuronal principles organizing periodic behaviors. In: F.O. Schmitt and F.G. Worden (eds.), The Neurosciences: Third Study Program. Cambridge: MIT Press, pp. 459-478.

Truman, J.W. 1974. Circadian release of a prepatterned neural program in silkmoths. In: F.O. Schmitt and F.G. Worden (eds.), The Neurosciences: Third Study Program. Cambridge: MIT Press, pp. 525-529.

Underwood, H. 1973. Retinal and extraretinal photoreceptors mediate entrainment of the circadian locomotor rhythm in lizards. J. Comp. Physiol. 83: 187-222.

Zimmerman, W.F., and D. Ives. 1971. Some photophysiological aspects of circadian rhythmicity in Drosophila. In: M. Menaker (ed.), Biochronometry. Washington, D.C.: National Academy of Sciences, pp. 381-391.

Photoentrainment of R15 *In Vivo*

Methods

Aplysia californica were kept in a community tank in a closed recirculating sea water system. The temperature was maintained at 14 ± 0.5°C. The light schedule in this system was LD 12:12, with lights-on at 0800 hr PDT ("dawn") and lights-off at 2000 hr PDT ("dusk"). Both control and experimental animals spent at least one week in these tanks before use.

Experimental animals were taken from the community tanks, and one of three procedures was carried out: blinding, sham-blinding, or no operation. Blinding was accomplished by seizing the skin of the animal near the eye with rat-tooth forceps, lifting up slightly, and cutting off the eye along with a small patch of skin. The underlying muscular body wall was left intact. Microscopic examination of the removed piece and post-sacrifice autopsy were used to verify removal of the entire eye. The sham blinding operation consisted of removal of a similar sized piece of skin just anterior to the eye.

After the appropriate operation, the experimental animals were housed in individual 57 liter aquaria, isolated from each other and from the rest of the sea water system. The temperature was less precisely controlled than in the main system. During the lighted portion of the light-dark cycle, the temperature in these aquaria rose to between 15 and 15.5°C.; the night temperature fell to 13.5 to 14°C. The lights used were 15 watt fluorescent bulbs placed about 15 cm above the water surface. Light intensity in these aquaria varied

from about 250 to 2000 lux, depending on position and orientation in the tank. The lighting schedule was again LD 12:12, but six hours advanced relative to the main system, from which the control animals were taken. Lights-on occurred at 0200 hr PDT, and lights-off at 1400 hr PDT. Experimental animals were sacrificed after one to two weeks in these aquaria.

All final dissections, both for control animals from the main system (dawn = 0800 hr) and the individual experimental aquaria (dawn = 0200 hr), were performed at 0900 $\pm$ 0100 hr (usually within 15 minutes of 0900). The PVG was isolated from the rest of the nervous system, and pinned out on a silastic stage in a 100 ml chamber filled with Millipore filtered (0.22 microns) sea water. The branchial, genital, pericardial, and siphon nerves, and the pleurovisceral connectives were cut one to two cm from their junction with the PVG and used to stretch out and hold down the PVG to the stage. The temperature in this chamber at the level of the ganglion was kept at 15 $\pm$ 1°C with a Haake/ Brinkmann KT-62 cooler. The spike activity of R15 was recorded for the following 24 hours or more by conventional intracellular techniques using glass micropipettes filled with 0.6 M K_2SO_4.

Spikes were counted either by hand from polygraph recordings, or with a Sodeco counter driven by a standardized pulse triggered by the spikes. The spikes were counted in 10 minute bins, and these were averaged over 30 minutes. Peak times are therefore reported to the nearest half hour.

The experiments were performed June through August, 1973.

Results

Four groups of five animals each were used. One group (controls) was taken after at least one week in the community tank, with lights-on at 0800 hr PDT. The average peak time for the five R15s was -2300 ± 0233 hr (Table I). (The minus sign indicates a peak occurring on the day of dissection; plus signs are used for peaks occurring on the day after dissection.) Examples of the rhythms are given in Figure 1. If one week on the six hour phase advanced light cycle was sufficient to completely entrain the R15 rhythm, then the average peak time for the experimental animals' R15s should be six hours earlier than the control average, i.e., -1700 hr. The R15s of both the intact and sham-operated animals showed complete entrainment. The intact animal peak average was -1648 ± 0136 hr, and the sham operated average was -1624 ± 0309 hr. The R15s of the blinded animals, on the other hand, had not appreciably shifted from the timing of the controls. This may be significant, since these animals were entrained to the control light schedule for at least one week prior to blinding. Examples of the experimental rhythms are shown in Figure 2.

Discussion

There are two immediate conclusions to be drawn from these data. The first conclusion in that R15s truly show activity after dissection that is related to the lighting schedule under which the animal was kept. The second is that this relation is destroyed if the animal is blinded prior to exposure to the new entraining light cycles.

The first point is readily seen by an examination of the R15 peaks

of the control and intact or sham operated Aplysia (Table I). The light schedules were six hours different in phase between control and experimental conditions, and the R15 peaks preserved this difference. Dissections, however, were performed at the same absolute time of day. Control animals, therefore, were dissected about one hour after their dawn, and the experimentals about seven hours after their dawn. The average peaks occurred about 14 hours after dissection for the controls, but only 8 hours after dissection for the experimentals. This shows that the timing of the R15 rhythm is related to the lighting schedule to which the animal was exposed, and not to the time of dissection. (Dissection time does, however, play a major part in the timing of R15 peaks at other times of day; see Appendix A.)

Blind animals do not appear to be able to photoentrain their R15 rhythms. The average peak activity of the R15s of blinded Aplysia is significantly different from that of both the intact (difference = 0506 hr; $t = 4.8$; $p < 0.01$) and the sham operated phase shifted animals (difference = 0530 hr; $t = 3.4$; $p < 0.01$). The average spike frequencies for all of the phase shifted animals are very similar (normal animals, 19.2 spikes/minute; sham operated, 22.9; blinded, 20.0), and somewhat less than the average frequency of the controls taken from the main community tank (average, 28.0 spikes/minute). The difference between the isolated animal spike frequency and the community animal frequency is not statistically significant ($t = 1.45$; $p > 0.2$).

Interestingly, the timing of the R15 peaks of the blinded animals was not very different from the controls, although the peaks of the

blinded animals are shifted in the same direction as the light cycle phase shift. There are two implications of this. First, either the blinded animals' R15s free run very near to 24 hours, or the rhythm can pick up non-photic cues that enable the cell to maintain about a 24 hour period without altering phase, or another receptor system can receive the photic information and transmit it to R15. If this last alternative is the case, then the phase induced in the R15 by this photic information is different from that induced by the eyes, and the similarity of the timing of the R15 peaks of the blinded animals and unshifted controls is merely due to coincidence. The first two alternatives would suggest that if animals are blinded and then returned to the same lighting schedule, they might appear to entrain their R15 rhythms. The second implication of the timing of the blinded animals' R15 peaks is that blind Aplysia may in fact by able to entrain their R15 rhythms to the same phase as in intact animals. This is suggested by the fact that the timing is shifted in the correct (advanced) direction, although not by very much. If such entrainment can occur, these data indicate that it must be much slower than photo-entrainment mediated by the eyes.

A major difference in protocol between the experiments reported here and those of Lickey, et al. (1971) and Lickey and Zack (1973) is that my animals were isolated during entrainment and the Lickey animals were not. This suggests that social stimuli from normal animals may be able to entrain the R15 rhythms of blinded Aplysia. Social stimuli can entrain rhythms in some other animals and in man (see introduction to

Part II). Perhaps the locomotor rhythms of the normal Aplysia in the Lickey experiments, causing the blinded animals to be bumped into and crawled upon with a circadian rhythm, can entrain the R15s of the blinded animals. Another possibility is pheromone or metabolite release by the normal animals. In addition, as has been shown in the introduction, there are major differences in distribution and variability of peak timing between their control and blinded animals of which they apparently were not aware.

The reanalysis of the data of Lickey, et al. (1971) and Lickey and Zack (1973), and the data presented here both indicate that the eyes are important in the photoentrainment of the circadian rhythm of R15 in vivo.

Table I

R15: Isolated PVG

	Control[b]	Normal Shift[c]	Blind Shift[c]	Sham Shift[c]
Time of Peak[a] (PDT)	+0030	-1530	-2030	-1430
	+0230	-1800	-2130	-1530
	-2230	-1530	-2400	-1800
	-2130	-1600	-2000	-2100
	-2000	-1900	-2330	-1300
Mean ± S.D.	-2300 ±0233	-1648 ±0136	-2154 ±0147	-1624 ±0309
Predicted Peak[d]	--	-1700	-1700	-1700

a) Positive times are those peaks which occurred on the day after dissection; negative times are those peaks which occurred on the day of dissection.

b) Control light schedule: LD 12:12; lights-on at 0800 hr; off at 2000 hr (PDT).

c) Shifted light schedule: LD 12:12; lights-on at 0200 hr; off at 1400 hr (PDT).

d) Predicted peak time based on a six hour advance over the control peak average.

Figure 1. Spike activities of R15s recorded in the isolated PVG. PVGs are taken from intact Aplysia kept under the indicated LD schedule for at least one week in community tanks. Dark bars in this and the following spike activity graphs represent projected night.

R15: ISOLATED PVG

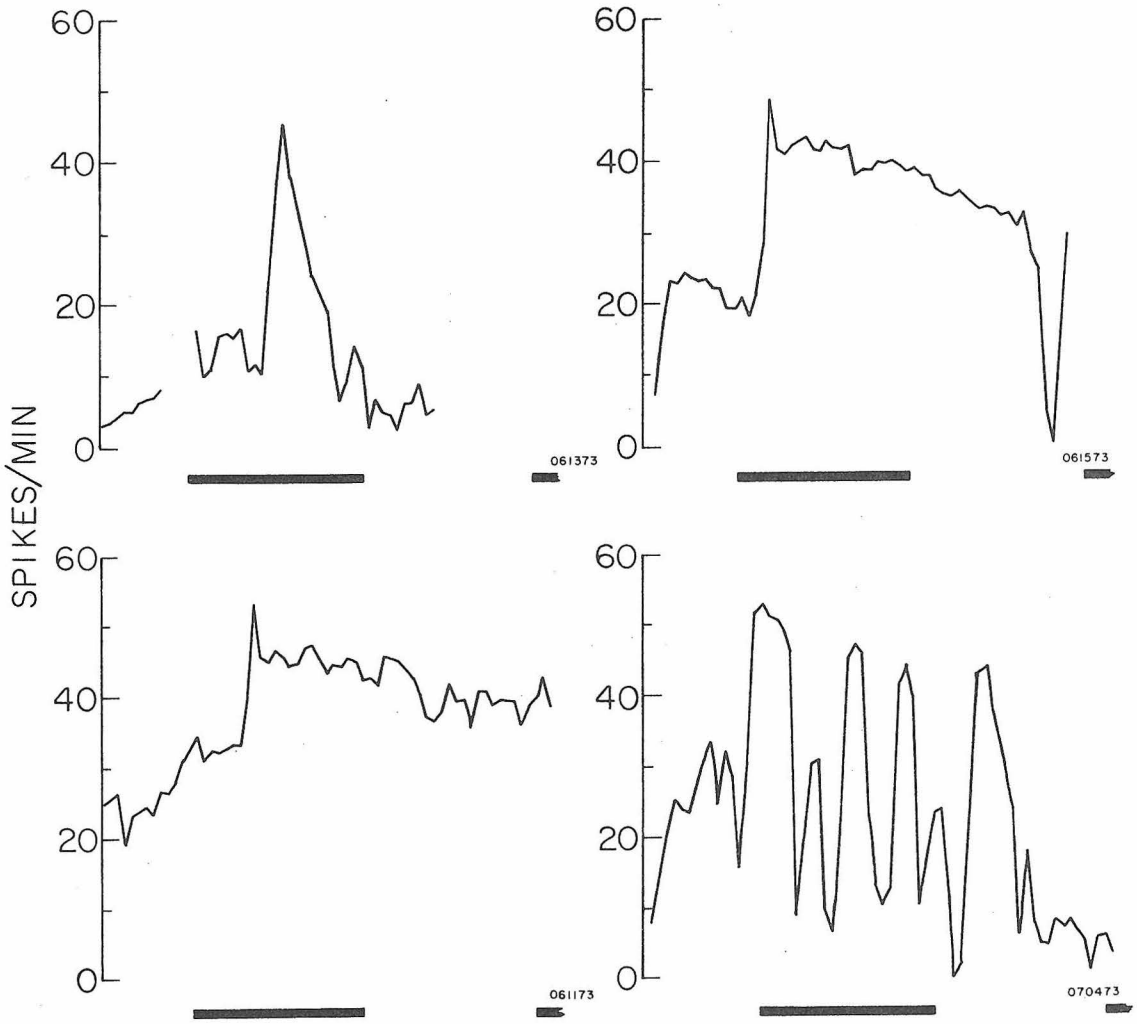
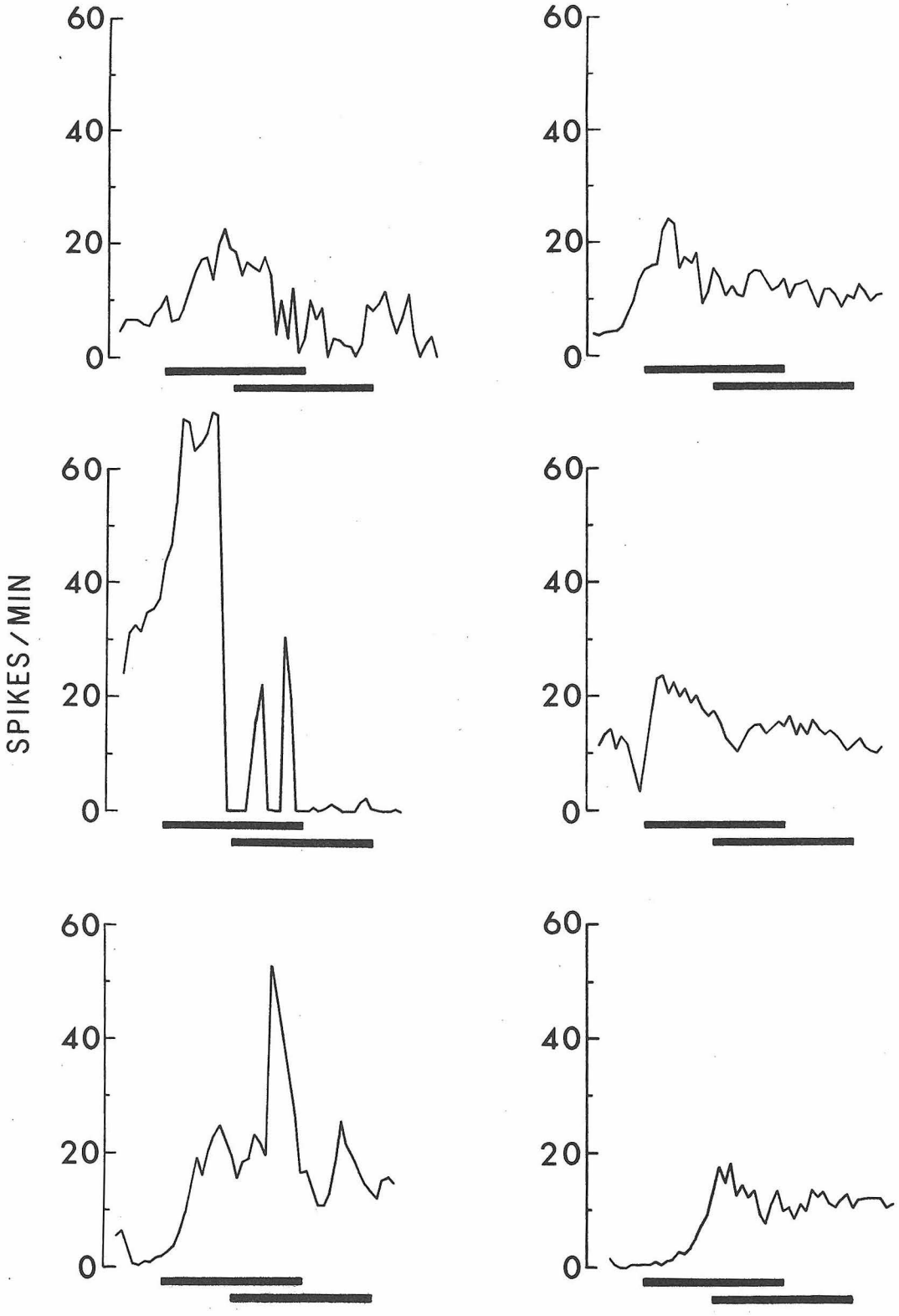


Figure 2. Spike activities of R15s in isolated PVGs taken from intact (top), sham blinded (center), and blinded (bottom) Aplysia. Bars represent two lighting regimes: upper bar on each graph denotes projected night of the postoperative schedule; lower bar denotes projected night of the preoperative schedule. Animals were kept under the postoperative LD cycle for at least one week prior to the final dissection.

R15: ISOLATED PVG



References

Lickey, M.E., and S. Zack. 1973. Extraocular photoreceptors can entrain the circadian rhythm in the abdominal ganglion of Aplysia. J. Comp. Physiol. 84: 361-366.

Lickey, M.E., S. Zack, and P. Birrell. 1971. Some factors governing entrainment of a circadian rhythm in a single neuron. In: M. Menaker (ed.), Biochronometry. Washington, D.C.: National Academy of Sciences, pp. 549-564.

Pathway for Photoentrainment of R15 *In Vivo*

The nervous system of *Aplysia* is a distributed one. Figure 1 shows the arrangement of the ganglia of the central nervous system of the animal. The eyes send their optic nerves into the cerebral ganglion, one of the circumesophageal ring. The PVG, which contains R15, has nervous connections to the circumesophageal ganglia only through the pleurovisceral connectives. Thus severing those connectives should interrupt any neural paths that may exist between the eyes and R15. Accordingly, experiments were designed to test whether such neural connections are necessary for the photoentrainment of R15, or whether a hormonal route between the eyes and R15 might be sufficient.

Methods

The experimental protocol was essentially identical with the previous study. New control animals were taken from the community tanks to correct for possible seasonal effects on the time of peak activity of the R15 rhythm (Lickey, 1969).

Experimental animals were removed from the community tank, an operation was performed to cut the pleurovisceral connectives, and then the animals were placed in individual aquaria as before. The lighting schedule in these aquaria was again six hours advanced relative to the main sea water system.

In the initial operations, mortality was high, apparently due to loss of blood and infection. In later operations, using the protocol described below, blood loss was kept to a minimum by using small

incisions, and infection was controlled with antibiotics. The final method of operating, which resulted in only one fatality in the eight operations in which it was used, was as follows.

An Aplysia was prepared for the operation by setting it on a cork board, foot down, and allowing it to relax. Then a sterilized dissecting needle was put through the tail of the animal into the board. Hooks were placed through the anterior parts of the parapodia and pulled to the side to keep the dissection area open. The animal, apparently reacting to the needle through its tail, invariably stretched out full length, even during the operation, precluding any need for pinning the head.

A small incision was made between the anterior attachment points of the parapodia, just to the left of the midline. The PVG and pleurovisceral connectives usually were readily visible. If not, they were easily located by gentle probing with a blunt instrument, care being taken not to cut the delicate wall of the digestive tract. The connectives were then cut, and the incision sutured closed with three or four stitches of surgical thread. The animal was injected with approximately 20,000 units each of penicillin and streptomycin (Microbiological Associates) in 10 ml of filtered sea water. Properly done, the operation could be performed by one person in 5 to 10 minutes, and the animal lost little blood.

The operated animals were kept on the phase advanced light schedule for one to two weeks. At the final dissection (always performed, as before, at 0900 $\pm$ 0100 hr PDT), the PVG was removed and

the successful cutting of the connectives confirmed. The PVG was then transferred to a 10 ml chamber filled with Millipore filtered (0.22 microns) sea water, kept at 15 ± 1°C.

The spike activity of R15 was recorded extracellularly for both controls and experimentals in these experiments. A 50 to 150 micron tip diameter fire polished glass pipette, filled with sea water, was placed on the connective tissue capsule of the PVG over the R15 soma. Amplification of the spikes was by Textronix 122 preamplifiers, and the resulting signals were recorded on a Grass Model 7 polygraph. Spikes usually ranged from 30 μV to 100 μV in amplitude. Activity was counted by hand from the polygraph record or with a Sodeco counter.

These experiments were run January through April, 1974.

Results

The five control R15s again showed peak activity in the first half of the projected dark, and the average peak time was close to that obtained the previous August (-2342 ± 0255 hr). As previously, a complete phase shift would result in a peak six hours earlier than the control average, or -1742 hr. (Perhaps a slightly later average might be expected, since one out of the five control R15s had a much earlier peak time than the other four (Table I); the peak average for these four was +0100 ± 0024 hr.)

The R15s of the operated animals (8) fell into three groups (Table I, Figure 2). The majority of the rhythms (5) showed clear evidence of entrainment to the postoperative light cycle. Their average peak time was -1748 ± 0234 hr, very close to the predicted time

of about -1800 hr. One of these five had a very early peak, as had happened with the controls.

The R15s of two of the operated animals did not seem to have moved the phase of their rhythms from the old preoperative setting. The remaining R15 peaked in the projected morning, unlike any of the other control or experimental R15s. This peak occurred approximately 14 hours later than the average time of the entrained class. It has been previously found that some R15s can show a peak about 180° out of phase relative to the usual peak (see Fig. 1 in Lickey, 1969). These, however, are rare, and this R15 must remain unclassified.

As with the blinding study, the aquarium isolated animals R15s (operated) showed a lower average spike frequency (11.5 spikes/minute) than the community tank controls (17.6), but again the difference was not statistically significant.

Discussion

In five out of eight Aplysia with both pleurovisceral connectives cut in vivo, the circadian rhythm of R15 could still be entrained by light. Previously it has been shown that, under identical entrainment conditions, the eyes are required for entrainment to occur. Since the pleurovisceral connectives are the only neural path between the circumesophageal ganglia and the PVG, it appears that neuronal connections between the eyes and R15 are not necessary for the entrainment of the cell. A hormonal mechanism must be sufficient.

It should not be concluded too quickly from these experiments that neural inputs to R15 have no role in entrainment, or no effect on the

R15 rhythm. In the first place, the R15s of two (or three) of the eight animals with both connectives cut did not entrain to the new light cycle. While one of these animals had been poorly sutured and had lost most of its blood by the day of final dissection, the other appeared to be in perfect health. In addition, a neural route does exist for information, especially about dawn and dusk, to pass from the eyes to R15 (see the following chapter). This route and/or others as yet undiscovered may also be able to entrain the rhythm of R15.

It must also be pointed out that this experiment says little about the details of the pathway involved in the entrainment by light. While the eyes may release a hormone which directly affects R15, it is also possible that neurons in the circumesophageal ring receive the eye information neurally, and that they in turn release the entraining hormone. These experiments also cannot rule out that entrainment of R15 might come via other cells in the PVG, and that the hormonal link is between the eyes and/or circumesophageal ganglia and these neurons.

Table I

	Control		Operated[b]	
Time of Peak[a] (PDT)	+0130	-1700	-2400[c]	+0800
	+0100	-1830	+0030	
	-1830	-2100		
	+0100	-1400		
	+0030	-1830		
Mean ± S.D.	-2342 ±0255	-1748 ±0234		
Predicted Peak[d]	--	-1742		

a) Times and light schedules as given in Table I of the previous chapter.

b) Operated Aplysia had both pleurovisceral connectives cut on the day that they were placed in the phase shifted light cycle.

c) This animal had been sutured poorly, and had lost most of its blood by the day of sacrifice.

d) Predicted peak based on a six hour advance over the control peak average.

Figure 1. Diagrammatic representation of the central nervous system of Aplysia californica. Anterior is to the top of the page.

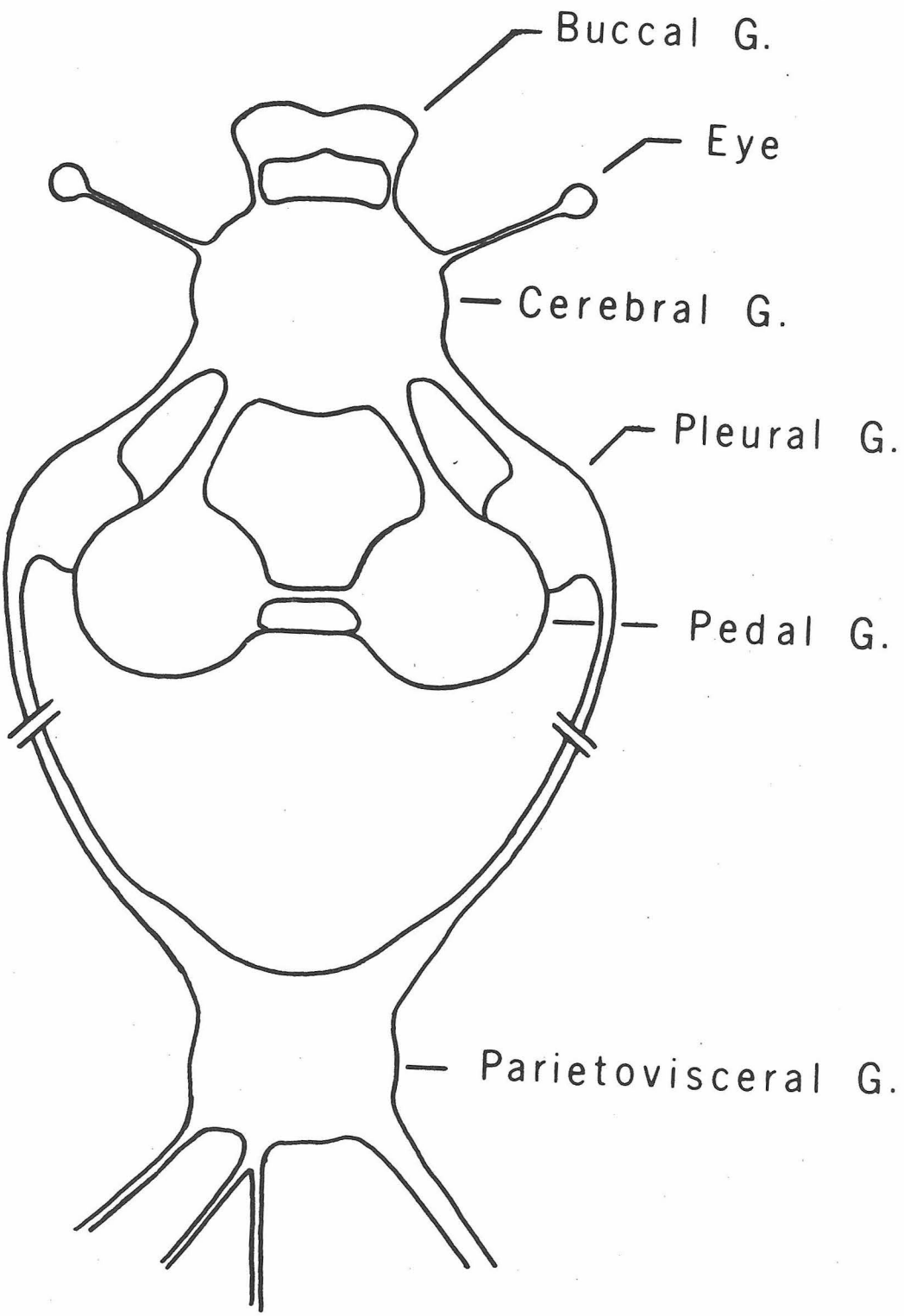
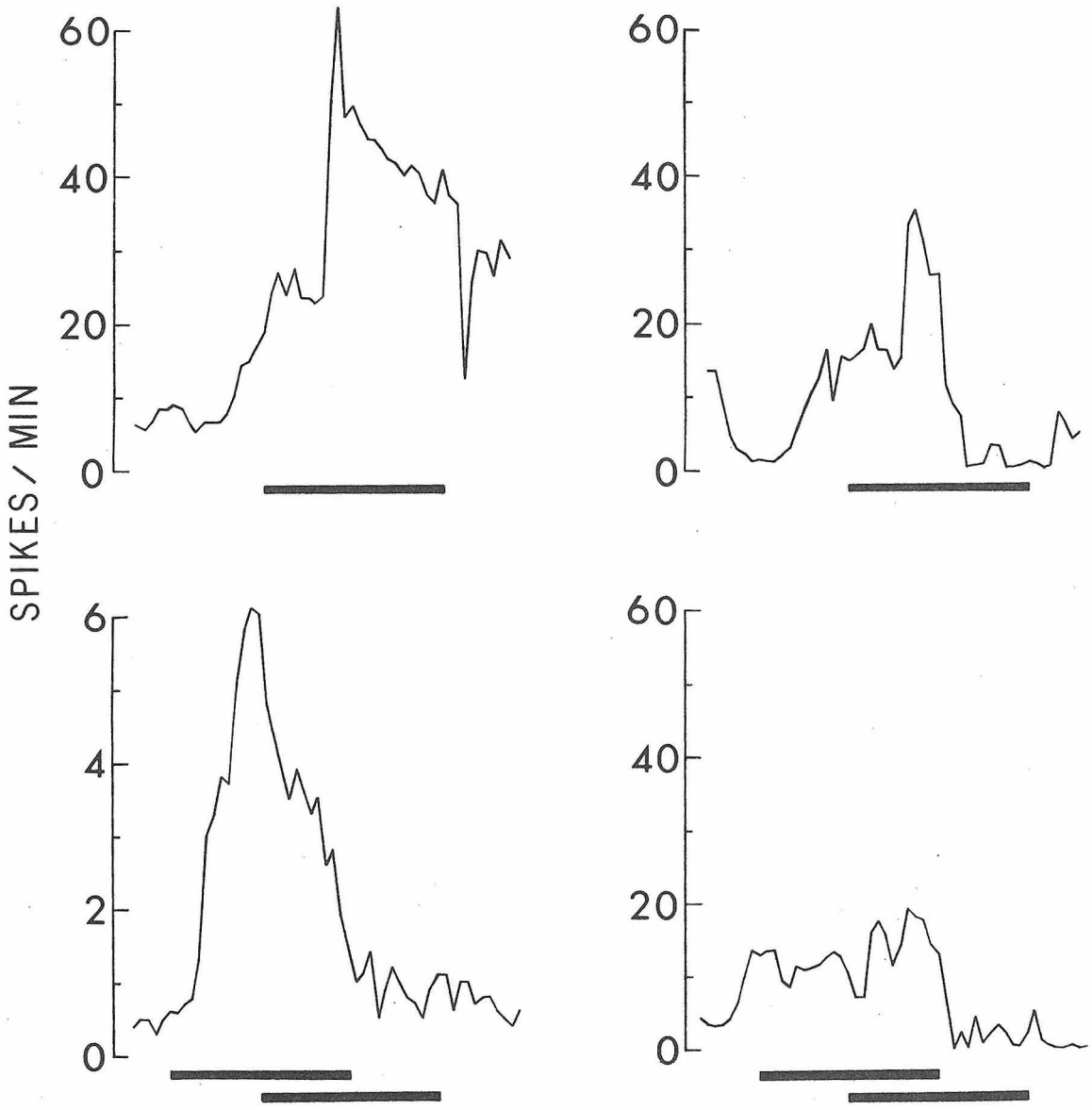

Buccal G.
Eye
Cerebral G.
Pleural G.
Pedal G.
Parietovisceral G.

Figure 2. Spike activities of R15s in isolated PVGs from (top) control and (bottom) operated (both pleurovisceral connectives cut *in vivo*) *Aplysia*. Dark bars represent projected night. In the lower half of the figure, the lower bar represents the preoperative projected night, and the upper bar the postoperative projected night. Two examples of operated animals' rhythms are shown. On the left the R15 showed peak activity at a time appropriate to the postoperative light schedule. On the right the R15 activity was broadly distributed and peaked at a time characteristic of the preoperative schedule.

R15: ISOLATED PVG



References

Lickey, M.E. 1969. Seasonal modulation and non-24 hour entrainment of a circadian rhythm in a single neuron. J. Comp. Physiol. Psychol. 68: 9-17.

Neural Interactions between the Eyes and R15

The eyes are required for the entrainment of R15 by light. It appears that a hormonal method of entrainment is sufficient, as shown by the entrainment of a majority of R15s in animals with both pleuro-visceral connectives cut. However, neural entrainment may also be possible. The sufficiency of this latter method of entrainment would be much more difficult to demonstrate in vivo because of the problems involved in trying to humorally isolate the PVG from the rest of the central nervous system. In addition, the actual spiking rate of the cell in vivo could easily be determined more by the synaptic stimulation impinging upon the cell than by endogenous factors.

Therefore neural pathways from the eyes to R15 may be important both in entrainment in intact Aplysia and in the instantaneous spiking rate of the neuron. Three types of experiments were designed to investigate neural influences of the eyes on R15: 1) long term recordings were made from the eyes and R15 in intact isolated central nervous system preparations; 2) electrical stimulation of optic nerves, light stimulation of the eyes, and spontaneous discharge of the eyes in intact central nervous systems were monitored for effects on R15; and 3) electrical stimulation of the inputs from the eyes to R15 discovered in (1) and (2) was performed to try to entrain R15 in vitro.

Methods

a) Long term recording from intact isolated central nervous systems

The major ganglia, with the eyes attached, were dissected from

Aplysia. The preparation consisted of the buccal, cerebral, pedal, pleural, and parietovisceral ganglia together with the eyes. All nerves interconnecting the ganglia were left intact, as were the optic nerves joining the eyes to the cerebral ganglion. The central nervous system was mounted on a silastic stage in a 100 ml chamber, care being taken that none of the interganglionic connectives were damaged. The chamber was filled with Millipore filtered (0.22 microns) sea water to which the following had been added: 2 gm/liter glucose; 3 mM $CaCl_2$ (to bring the Ca^{++} concentration in sea water (as listed in Prosser and Brown, p. 60) up to the approximate level in Aplysia blood (13.3 mM; Prosser and Brown, p.60)); and 150 units each per ml penicillin and streptomycin (Microbiological Associates). The temperature was controlled at $15 \pm 1^{\circ}C$.

Intracellular recordings were made from R15 by conventional means, and the resulting signals recorded on a Grass Model 7 polygraph, both DC and high gain AC, and monitored on a Textronix 5103 oscilloscope. Spike frequencies were counted by hand or with a Sodeco counter. Extracellular recordings were often made from one optic nerve via suction electrode en passant on the nerve. The signals from the optic nerve were amplified by Textronix 122 preamplifiers and recorded on the polygraph and oscilloscope. The entire preparation was kept in darkness by enclosure in a black box except for the times necessary to re-impale R15 if the electrode slipped out of the cell. Recordings were usually made for two to four days continuously following dissection.

b) Correlation of eye and R15 activities

In addition to observation of simultaneous activities of the eyes and R15 in the long term *in vitro* experiments described above, two other protocols were used. Both additional experiments also utilized intact isolated central nervous system preparations.

In one series of experiments, the optic nerve and/or other nerves of the cerebral ganglion were stimulated electrically with a Grass Model S4 stimulator. In some of these experiments, one or both eyes were first cut off their optic nerves, and the nerve stimulated via suction electrode on the stump. In other trials, the nerve was stimulated by en passant suction electrode with the eye left attached.

The effect of illumination of the eyes was also tested. A fiber optic light guide was used to deliver low intensity illumination to one eye; otherwise the preparation was kept in darkness. The effectiveness of the restriction of the light to the eye in question was tested by examination of the impulse pattern of the other eye, and by cutting off the eye and illuminating the resulting optic nerve stump. In general, both optic nerves were recorded en passant by suction electrode, and the activity of R15 was recorded intracellularly.

c) Neural entrainment *in vitro*

Isolated PVGs were maintained in 10 ml organ culture chambers for up to 13 days, at 15 $\pm$ 1°C. Two hundred fifty ml of organ culture medium (modified from Strumwasser and Bahr, 1966) consisted of:

5 ml Eagle's minimum essential amino acids (50x)
2.5 ml non-essential amino acids (100x)
2.5 ml vitamins (100x)

0.5 ml glutamine
150 units each per ml penicillin and streptomycin
(all of the above supplied by Microbiological Associates)
2.5 gm glucose
50 ml Aplysia blood
1 mM Tris, pH 7.8
brought up to 250 ml with sea water, pH adjusted to 7.6 to 7.8 at 15°C., and filtered (0.22 microns Millipore) in a sterile flask.

The right pleurovisceral connective was drawn through a tunnel containing two platinum cuff electrodes, for stimulation by Grass Model S4 stimulator. The rest of the PVG was pinned to a silastic platform in the chamber. The chamber was covered with a thin plexiglas plate to prevent evaporation. The plate had a small hole drilled through it for the recording electrode. Spiking activity of R15 was recorded extracellularly as described previously.

Results

a) Long term activity of the eyes and R15 of Aplysia recorded in vitro from the isolated intact central nervous system

Intracellular recordings of spikes and synaptic potentials from R15 in the intact isolated CNS were made for two to four days. The preparations were maintained in darkness, in order that the endogenous circadian rhythm of compound action potentials from the eyes could be expressed. In many experiments, recordings were also made from the optic nerve of one of the eyes. The expectation was that the rhythmicity of the eyes, which persists for many days in vitro (Jacklet, 1969; Eskin, 1971), might serve to enhance or maintain rhythmicity in R15.

Three synaptic events can usually be seen in R15 in an intact CNS

preparation. One is a small EPSP from L10 (another identifiable neuron in the PVG; Kandel, et al., 1967). The second is a slow, long-lasting IPSP, presumed to be from interneuron II, an unidentified neuron or group of neurons, also in the PVG (Frazier, et al., 1967). Both of these events are also seen in R15 in an isolated PVG preparation. The third event, and the most obvious one, is a large (up to 25-30 mV) EPSP which is not seen in isolated PVG recordings. This EPSP arises from an unidentified neuron in the right pleural ganglion, and the axon of this cell enters the PVG through the right pleurovisceral connective (Strumwasser, personal communication). In the terminology of Parnas, Armstrong, and Strumwasser (1974), this EPSP will be referred to as input I.

Figure 1 shows a graph of spiking activity of R15 in an intact CNS experiment run for over three days. Several features should be noted. First, both morning and evening peaks appear; this is especially prominent on the first and second days. This is usually not the case in recordings from R15 in isolated PVGs (see the chapter on photoentrainment of R15 <u>in vivo</u>, Fig. 1; also Strumwasser, 1965). Second, the peaks appear to be double, and to become more distinctly so as the run progresses. Again, this is not usually the case in isolated PVG recordings.

On this run the microelectrode remained in R15 for the entire three days. In most experiments, the electrode had to be positioned back into the cell several times during the run (see, for example, the gaps in Fig. 2). Further, most R15s do not show as clean a rhythm

as this one does. However, rhythms are observable for two or more days in about half of the experiments. There are usually peaks near projected dawn and in the first half of the projected night, although occasionally one will be missing or minor peaks will be added (Fig. 2). These spiking peaks invariably coincide with very high frequencies of input I in R15 (see Fig. 5).

Figure 2 also demonstrates one of the striking features of the intact CNS preparations. When the eye activity is recorded as well as the R15 spiking, it becomes apparent that strong peaks in R15 spiking (and often even minor ones) coincide with troughs in eye CAP frequency. The dotted lines in Fig. 2 illustrate a few such cases.

In a few (4) experiments, intact CNS preparations were run without the eyes. None of the R15 recordings showed any circadian rhythmicity in these experiments (Fig. 3). Usually the spike frequency cycled with a variable four to eight hour period. However, several of the intact CNS preparations with eyes also failed to show R15 rhythmicity, although none had the short period characteristic of the eyeless preparations. It cannot be completely ruled out that some rhythmic R15s would have been observed in a sufficiently large number of experiments with eyeless preparations.

b) Correlation of optic nerve and R15 activity

The activity of an isolated eye does not, in general, show irregularities (see Jacklet, 1969, and the second half of Part I, Fig. 4). The isolated eye rhythm is a smooth curve, monotonically increasing from about three or four hours before projected dawn until

projected dawn, then flattening off for a few hours, and finally monotonically decreasing until impulses cease near projected dusk. However, Eskin (1971) found that irregularities in the eye rhythm did occur if the eyes were left attached to the cerebral ganglion. He found that small single unit activity from the cerebral ganglion directed centrifugally to the eye occurred in these preparations. In general, when the eye was more active (spontaneously or due to light stimulation), these efferent units were relatively silent. When the efferents (which he called centrifugal fibers) were very active, the eye was less so. If the efferents were silenced temporarily by cooling the ganglion but not the eye, the pattern of eye CAPs became essentially identical to that of an isolated eye (see Figs. 4a and 4b, from Eskin, 1971).

It was strongly suspected, then, that the troughs in eye activity in the intact CNS preparation coinciding with peaks in R15 activity would be related to the frequency changes in efferent activity out to the eye. This indeed proved to be the case. Figure 5 shows both optic nerve and R15 recordings during spontaneous periods of low optic nerve efferent activity (top) and high efferent activity (bottom), both in darkness. Clearly, high frequencies of efferent spiking in the optic nerve coincide with high frequencies of input I EPSPs and spikes in R15, and low numbers of CAPs from the eye. When the efferent activity is less, eye CAPs are more frequent and R15 EPSPs and spikes are fewer.

Of course, it cannot be determined from these experiments whether input I cell firing causes increased centrifugal activity, or vice

versa, or whether a third neuron influences both of these. However, electrical stimulation of the optic nerve (Fig. 6) elicits input I in R15. Since spontaneous CAPs do not do so (see Fig. 5), it would appear that the effective stimulation is of axons of the efferent neurons. The input I cell, then, receives inputs, perhaps through interneurons, from some or all of the centrifugal cells.

Eskin (1971) found that illumination of the eyes inhibits the activity of the centrifugal cells, in general (see Fig. 4b). To test whether the eyes inhibit the particular set of centrifugal cells which project to the input I cell, the eyes were illuminated in the intact CNS preparation. A fiber optic light guide was placed so that it would illuminate, as far as possible, only one eye. Activity in both optic nerves and in R15 was recorded simultaneously. Except for the periods of light guide illumination, the preparation was kept in darkness. It had already been found, in a similar experiment with only the eyes and cerebral ganglion, that illumination of one eye would inhibit most efferent cerebral ganglion activity in both optic nerves (Eskin, 1971, and Eskin and Audesirk, unpublished results). Removal of the illuminated eye would abolish the inhibition by light, ruling out any direct effect of the light on the cerebral ganglion cells themselves (which are light sensitive, but less so than the eyes).

When only one eye of an intact CNS preparation is illuminated, efferent cerebral ganglion activity is inhibited (Fig. 7). Simultaneously, there is a decrease in the frequency of input I EPSPs on R15. When the light is turned off, the eye CAPs are temporarily

suppressed (this also happens in isolated eyes; see Fig. 1 in Audesirk, 1973). At the same time, somewhat increased activity of the optic nerve efferents also occurs, and a higher frequency of EPSPs and spikes in R15. If the illuminated eye is removed, and the light guide left to illuminate the optic nerve stump, there is no effect on efferent cerebral ganglion activity or on the frequency of input I in R15.

It must be pointed out that the eyes and cerebral ganglion units are not the only influences on input I frequency in R15. On the contrary, spontaneous efferent activity in some other cerebral ganglion nerves also influences the frequency of firing of the input I cell (Fig. 8). In addition, electrical stimulation of many cerebral ganglion or pedal ganglion nerves result in EPSPs in R15 (Fig. 9). It is not known whether the effective stimulation is of efferent or afferent fibers or both.

c) Attempts to entrain R15 <u>in vitro</u> by stimulation of input I

Since the eyes are needed for the photoentrainment of R15, and since the large EPSP of input I is the final element of at least one pathway by which the eyes can influence R15, an attempt was made to entrain the rhythm of R15 <u>in vitro</u> by stimulation of input I. These experiments were carried out with David Armstrong.

Isolated PVGs were maintained in organ culture for several days. The right pleurovisceral connective, which contains the axon of the input I cell, was placed through a tunnel containing stimulation electrodes. It had been previously shown by Parnas, Armstrong, and

Strumwasser (1974) that the axon of the input I cell invariably has the lowest stimulation threshold of any unit in the connective that produces a visible postsynaptic event in R15.

Several different stimulation protocols were attempted, but all were basically as follows. The R15 was allowed to fire spontaneously for one to several days. Then input I was evoked at frequencies of one to four per second, continuously or intermittently for one or two hours. Stimulation was performed for one to three successive days at the same time of day. The relatively long-lasting enhancement of the R15 spike frequency after stimulation previously described by Parnas, et al. (1974) was observed (Fig. 10). However, in no case was a spontaneously occurring peak produced in R15 on the day following stimulation which could be considered to be a circadian aftereffect of stimulation.

Discussion

The spike frequency of R15 can be observed to show circadian rhythmicity both in isolated PVG and intact CNS with eyes preparations. In the latter case, a large EPSP (input I) occurs spontaneously in R15, and the frequencies of spikes and EPSPs closely parallel each other (Fig. 5). In order for the spike frequency to have circadian rhythmicity in the intact CNS case, it would appear to be necessary for there to be a circadain rhythm in the frequency of spikes in the input I neuron. This neuron has never been identified, and little is known of its properties. It is located in the right pleural ganglion. If the other ganglia are systematically cut off, leaving only the PVG and

the right pleural ganglion connected to each other, and input I is monitored in R15, the frequency of EPSPs falls from several per second to near zero (Audesirk, unpublished observations). This seems to indicate that the activity of this cell is predominantly determined by inputs it receives from other neurons.

One group of neurons which influences the activity of the input I neuron (perhaps through interneurons) is now known. These are cells, probably located in the cerebral ganglion, which send axons out the optic nerve to the eye and which Eskin (1971) called centrifugal cells (Figs. 4 and 5). These cells, or at least some of them, are excitatory to the input I cell. In turn, they receive predominantly inhibitory input from the eyes. These cells are particularly well suited for the transmission of lights-on and lights-off information to R15. They are inhibited by illumination of the eyes (Fig. 7) and at higher threshold by direct illumination (Eskin, 1971). Of course, in vivo these cells are covered by the darkly pigmented epithelium of the animal, and receive far less light than do the eyes. Lights-off to the eyes produces a rebound of firing in these cells. It is not known if these neurons receive information through any other sensory modalities, but electrical stimulation of several of the other nerves entering the cerebral ganglion suppresses firing of the centrifugal cells (Eskin, 1971).

In the isolated CNS preparations without eyes (Fig. 3), the spike frequency of R15 did not occur in any recognizable circadian rhythm. Although this is only negative evidence, it does suggest that the eyes

are important in maintaining or enforcing rhythmicity in the input I cell. The information at hand indicates that the eyes do so through the cerebral ganglion centrifugal neurons. If this is so, then the centrifugal fibers might also be expected to show some circadian rhythmicity. It is not easy to recognize any rhythm in the overall efferent activity in the optic nerve, and single units have not been distinguished and followed throughout an entire day. It must be remembered that the indirect data at hand do not reveal the number of centrifugal units that project to the input I cell; it may be only one. This would make it very difficult to rule out a circadian input from such a cell to the input I neuron.

Although the eyes are needed for the photoentrainment of R15 *in vivo*, the integrity of neural connections from the eyes to the PVG is not. Further, attempts to entrain the circadian rhythm of R15 *in vitro* by stimulation of the input I axon failed (Fig. 10). However, neither of these experiments proves that the input I path has nothing to do with entrainment of R15 in the intact animal. The neural route may not be necessary, but it, like the humoral route, may be sufficient. The failure of the *in vitro* entrainment experiments appears to argue against this, but it is impossible to be sure that a different protocol would not have succeeded.

The activity of R15 *in vivo* is determined by the interaction of many factors. The list of known inputs to R15 activity is fairly long: endogenous rhythm; EPSP and IPSPs through the right pleuro-visceral connective (inputs I, II, and III of Parnas, Armstrong, and

Strumwasser, 1974); IPSP from interneuron II (Frazier, et al., 1967), probably identical with input III of Parnas, et al. (1974); EPSP from L10 (Kandel, et al., 1967); osphradium (Stinnakre and Tauc, 1969). In vivo, then, the instantaneous spike frequency of the cell may depend considerably on non-endogenous factors. Even if the circadian rhythm of R15 cannot be entrained by the eye-input I neural route, the daily functioning of the cell may nevertheless be strongly influenced by this input channel.

Figure 1. Long term recording from R15 in an intact CNS preparation with the eyes, kept in constant darkness.

Upper: Spike activity of R15. Note peaks occurring in early projected night and near projected dawn, free running with a period less than 24 hours. Dark bars represent projected night.

Lower: Periodogram of the above data. Note strong peaks at 11 and 22 hours. Black dot represents trial period of 24 hours and relative sigma of 0.5.

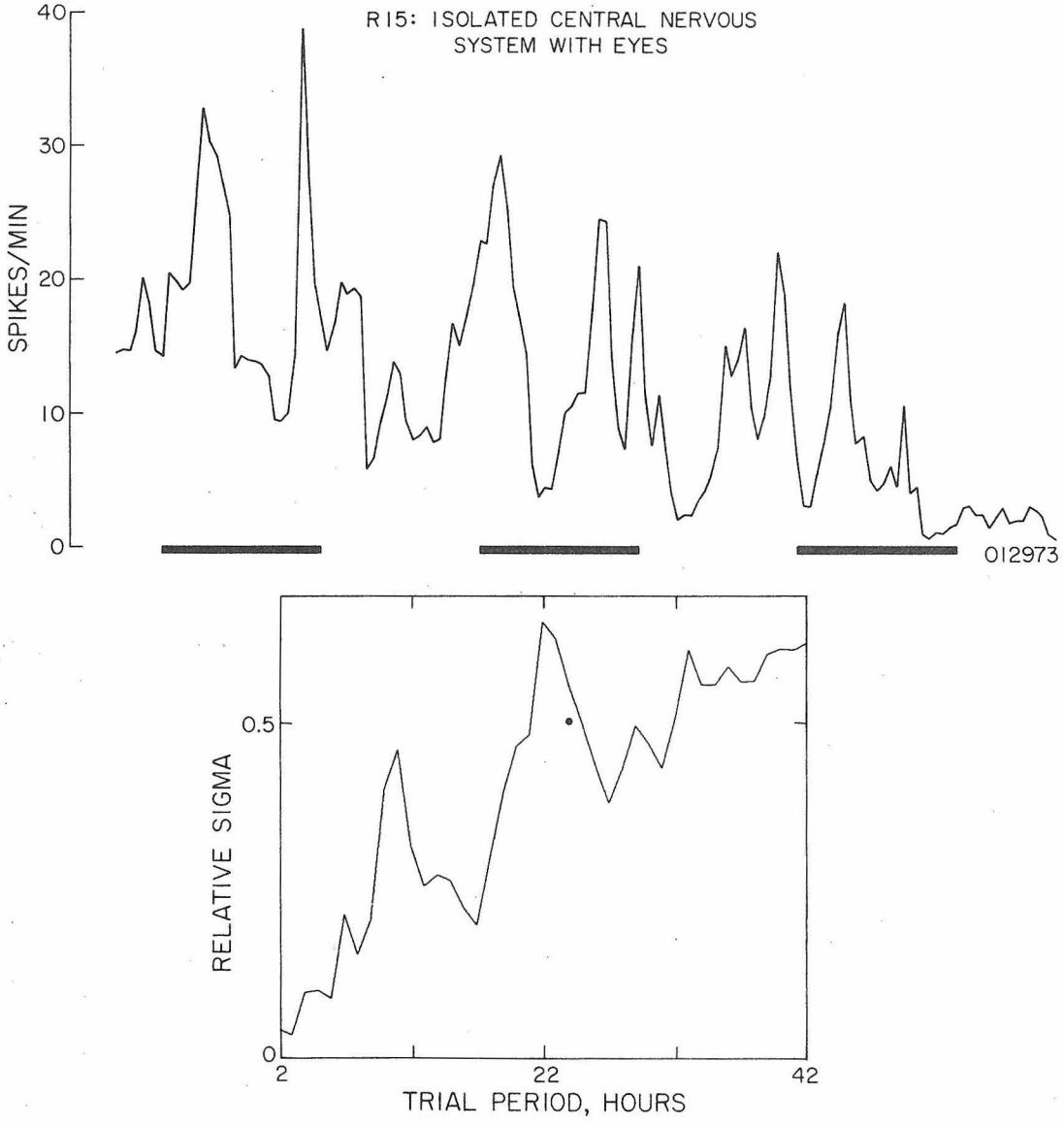

R15: ISOLATED CENTRAL NERVOUS SYSTEM WITH EYES
SPIKES/MIN
40
30
20
10
0
012973
RELATIVE SIGMA
0.5
0
2
22
42
TRIAL PERIOD, HOURS

Figure 2. Long term recording from R15 and eyes in an intact CNS preparation with the eyes attached, in darkness. Animals from which the nervous systems were taken were kept in LD 12:12 (left) and LD 16:8 (right) for at least one week prior to dissection. Gaps represent periods during which the microelectrode had slipped out of the cell. The dotted lines connect some cases of peaks in R15 spiking coinciding with relative troughs in eye activity.

Left: R15 has multiple peaks per day, one near projected dawn and one in the early projected night, as in Fig. 1, but also peaks in the middle of the day and night.

Right: R15 has two major peaks per day, as in Fig. 1. Note that the eye activity is extended to cover the longer projected day period.

ISOLATED CENTRAL NERVOUS SYSTEM WITH EYES

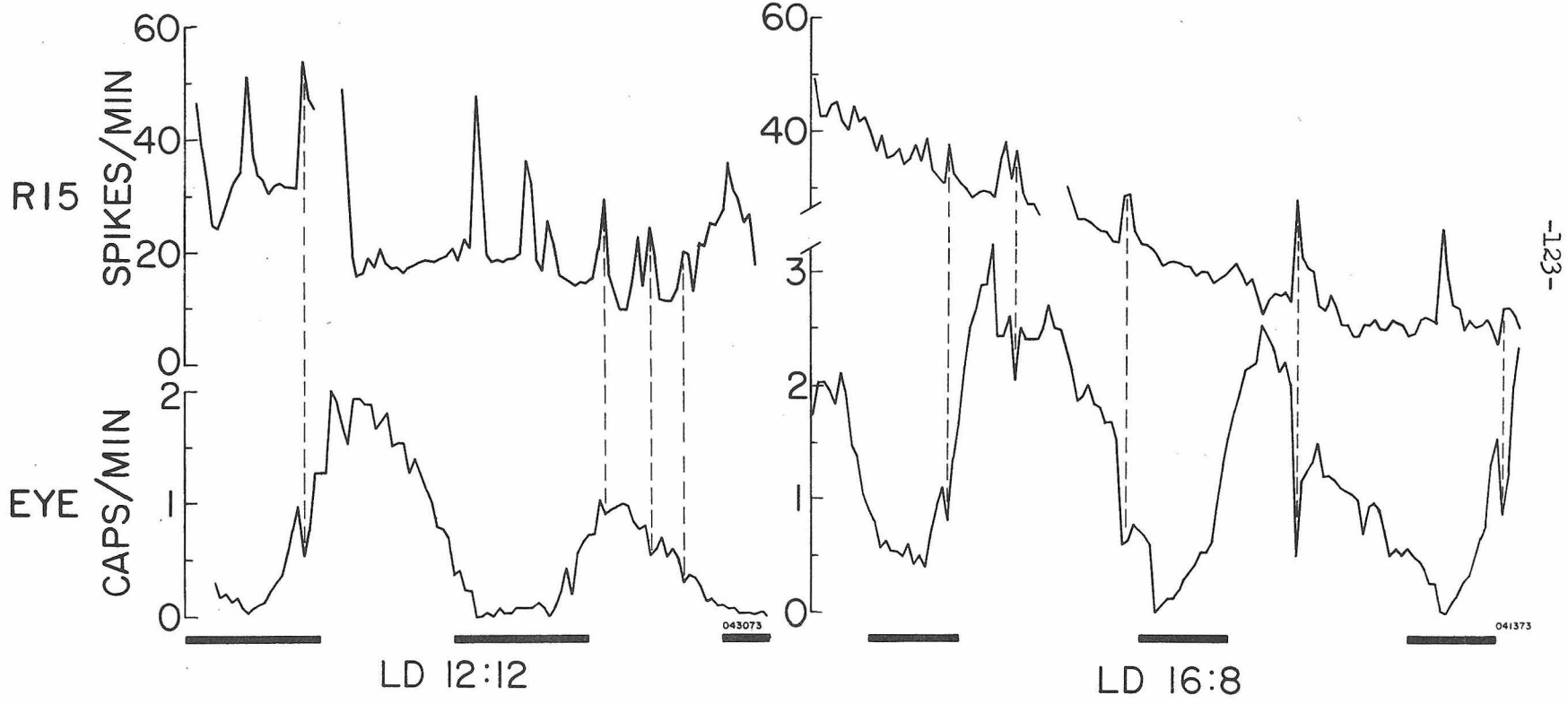


Figure 3. Long term recording from R15 in an intact CNS preparation without the eyes, in darkness. Conventions are the same as in Fig. 1. Note the lack of circadian rhythmicity and the appearance of four to eight hour periodicities.

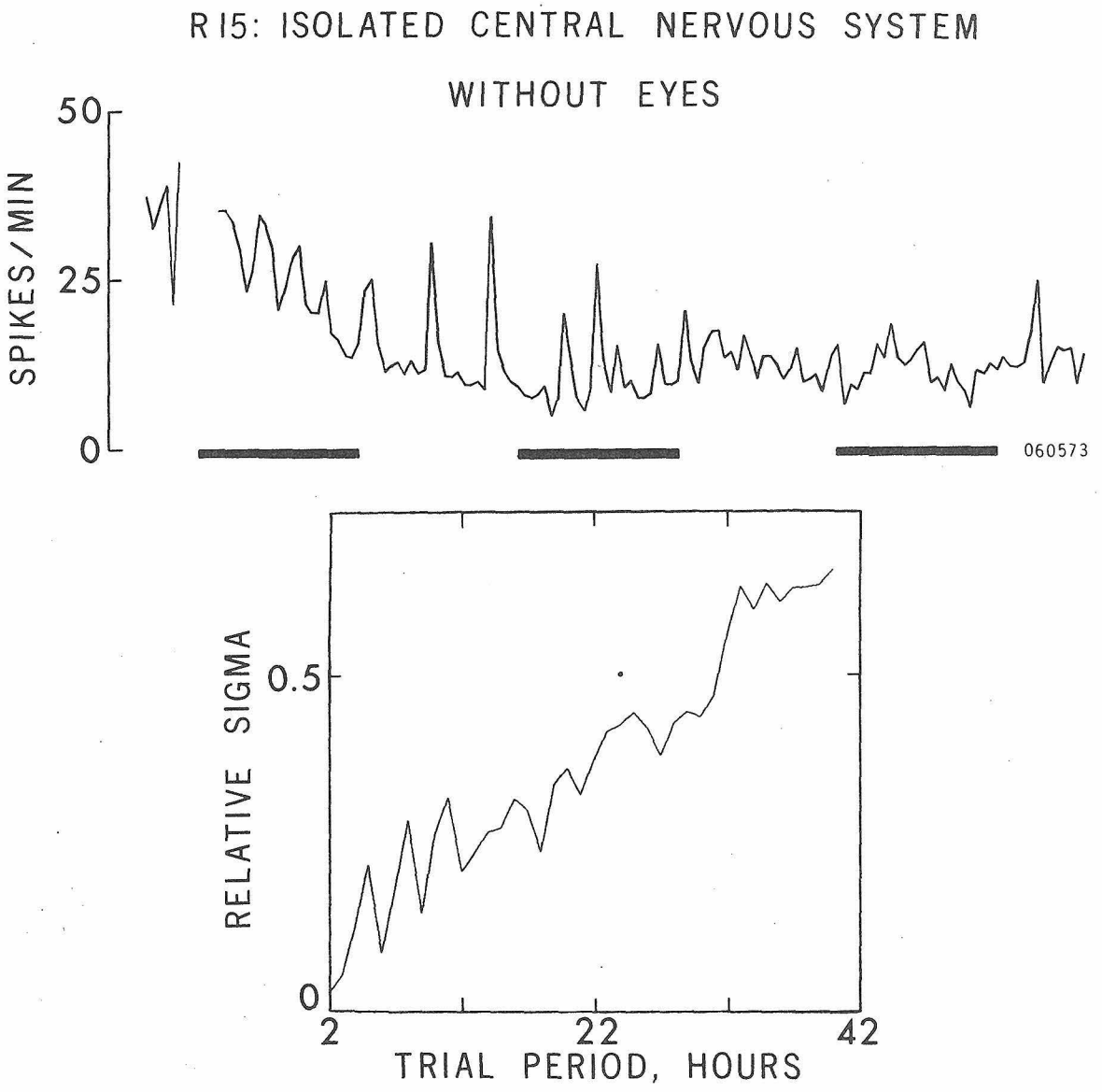
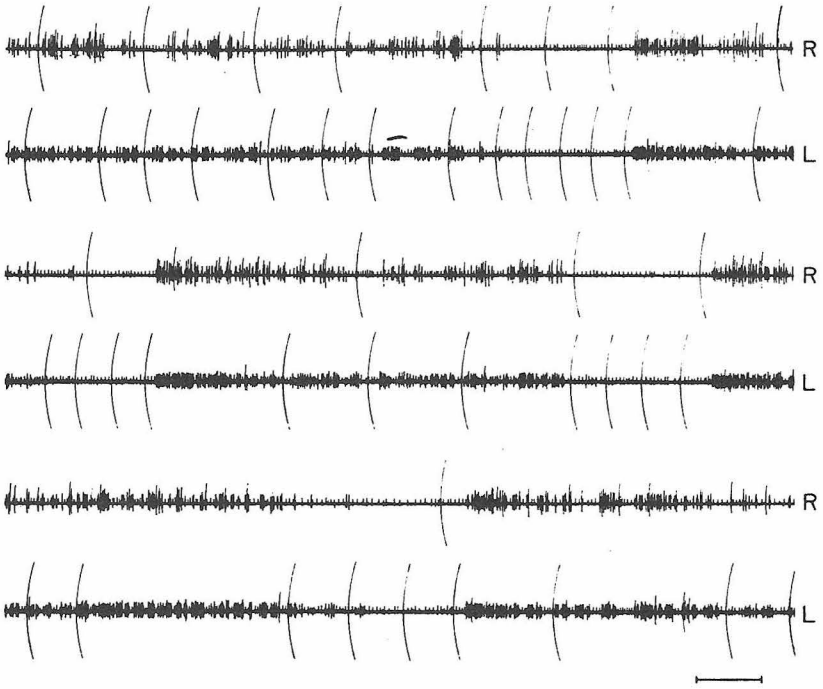

R15: ISOLATED CENTRAL NERVOUS SYSTEM
WITHOUT EYES
50
25
0
SPIKES/MIN
060573
RELATIVE SIGMA
0.5
0
2
22
42
TRIAL PERIOD, HOURS

Figure 4a. Upper: Recordings from right and left optic nerves of eyes attached to the cerebral ganglion. The CAPs (afferent from the eye to the ganglion) are the large impulses; the small impulses are single unit spikes from the efferent cerebral ganglion centrifugal fibers. The efferent activity is generally correlated in the two nerves, but apparently not one-to-one.

Lower: Recording from the optic nerve of an eye attached to the cerebral ganglion. At the arrow in line A, the cerebral ganglion is cooled but the eye is not. Centrifugal activity drops out and the eye CAPs become regular, as in an isolated eye. At the arrow in line B, the cerebral ganglion is rewarmed.


R
L
R
L
R
L

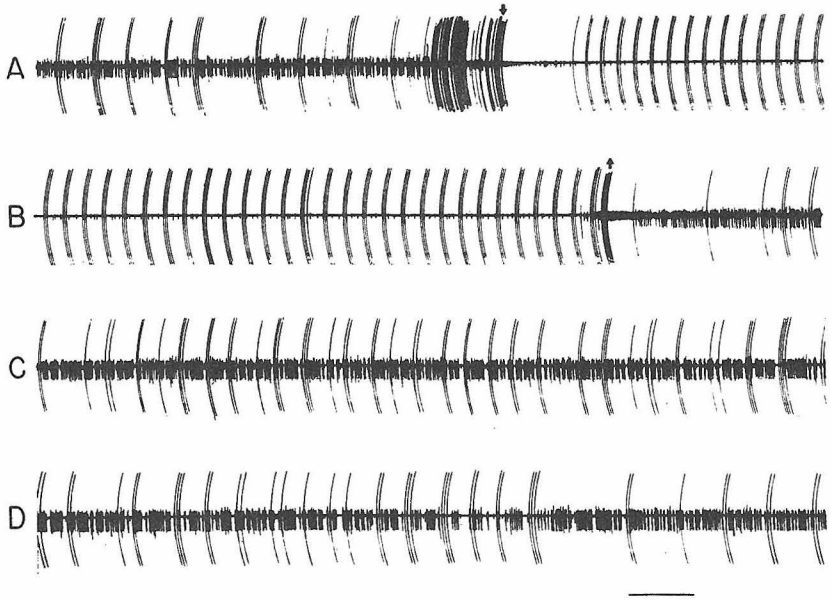
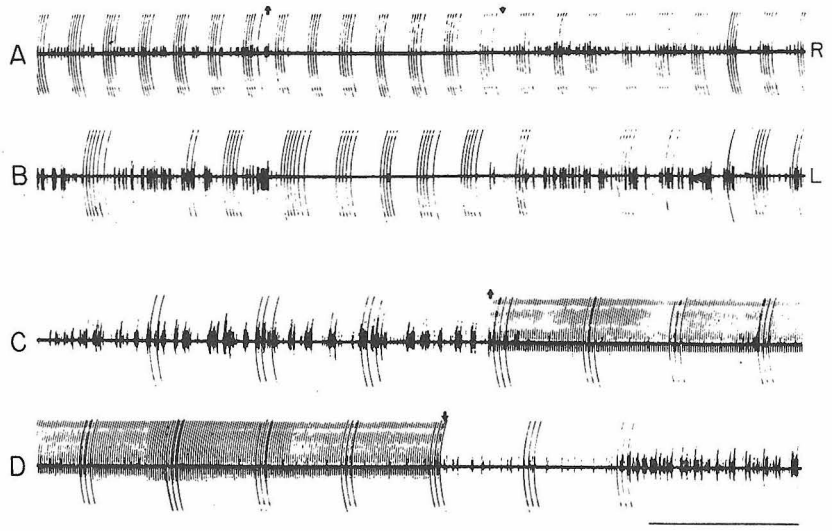

A
B
C
D

Figure 4b. Upper: A and B: Recordings from the optic nerves of one preparation, with the eyes attached to the cerebral ganglion. One eye (A) is illuminated by fiber optic light guide for the period between the arrows. Note suppression of cerebral ganglion efferent activity in both nerves. C and D: Continuous recordings from one optic nerve. Between the arrows the other optic nerve is electrically stimulated at 0.5/sec. Again the efferent activity is suppressed. Lower: Each pair of lines is a concurrent recording from both optic nerves attached to the cerebral ganglion. Other cerebral ganglion nerves are stimulated between the arrows. Nerves stimulated are: A) right rhinophore; B) left rhinophore; C) left rhinophore with the nerve crushed between the stimulation electrode and the cerebral ganglion; D) right tentacle nerve.

Figures 4a and 4b from Eskin, 1971.


A
R
B
L
C
D

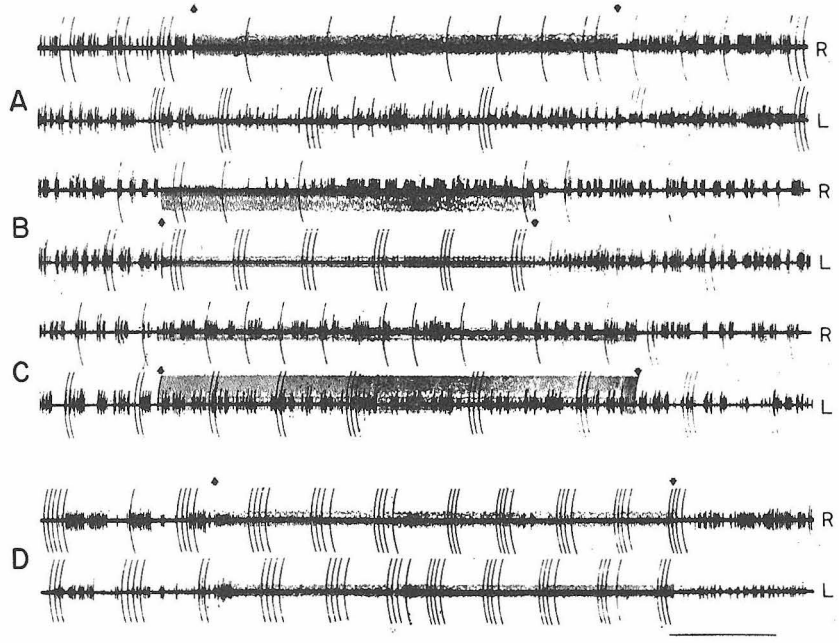

R
A
L
R
B
L
R
C
L
R
D
L

Figure 5. Simultaneous recordings from R15 and an optic nerve of an intact CNS preparation with the eyes, in darkness. The top line is a high gain AC recording and the second line a DC recording from R15. The third line is from the optic nerve. The EPSP visible in the R15 recording is input I. In the top half of the figure, the efferent activity in the optic nerve (small impulses) is low; input I EPSPs in R15 number approximately 520, and spikes in R15 are 295. The lower half shows a period of high efferent activity in the optic nerve; there are about 910 EPSPs in R15, and 422 spikes. The numbers of eye CAPs in the two cases are 24 and 12, respectively.

ISOLATED CENTRAL NERVOUS SYSTEM WITH EYES

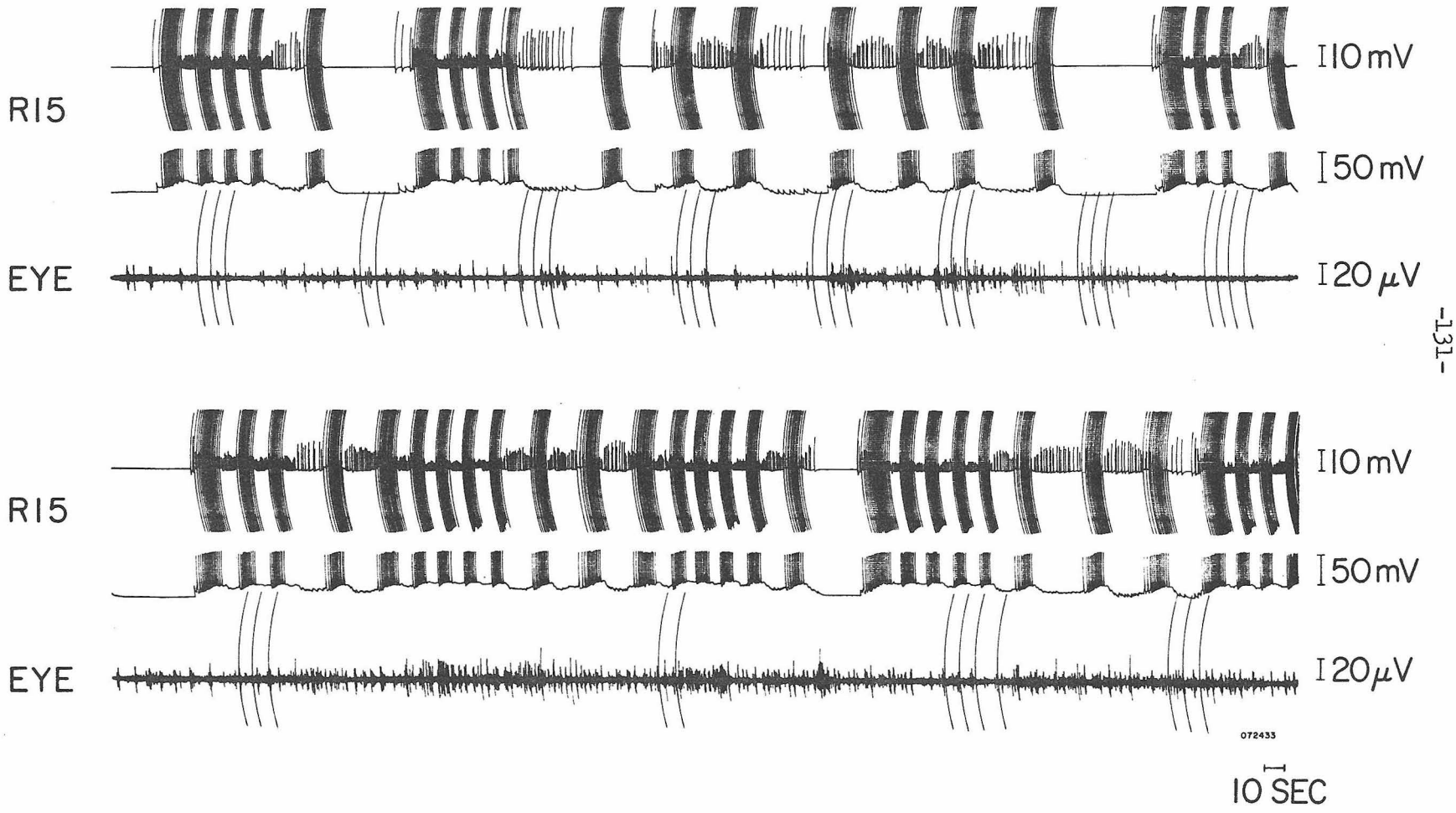


Figure 6. Stimulation of the left optic nerve at the arrows while recording from R15. The top line is DC, the lower line AC, intracellular recording from R15.

R15: ISOLATED CENTRAL NERVOUS SYSTEM WITH EYES
OPTIC NERVE STIMULATION

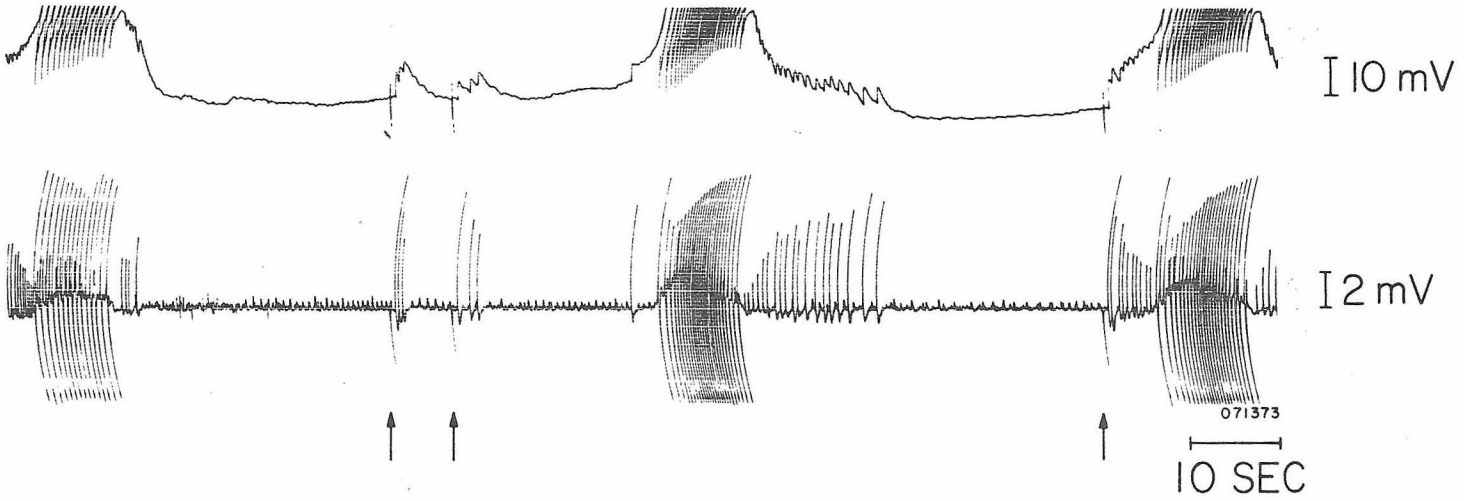


Figure 7. Simultaneous recordings from R15 and the right optic nerve in an intact CNS preparation with eyes. The CNS is kept in darkness except for periods of illumination of the right eye with very dim light via a fiber optic light guide, represented by the dark bars. The top two lines in each half of the figure are intracellular recordings from R15; the third line is from the optic nerve.

	Upper Half		Lower Half	
Light Status	EPSPs	Spikes	EPSPs	Spikes
Off before	256	55	183	49
On	168	17	144	28
Off after	234	59	205	80

Vertical calibration: R15 DC: 100 mV; R15 AC: 50 mV; eye: 50 μV.

Horizontal calibration: 30 sec.

R15: ISOLATED CENTRAL NERVOUS SYSTEM WITH EYES

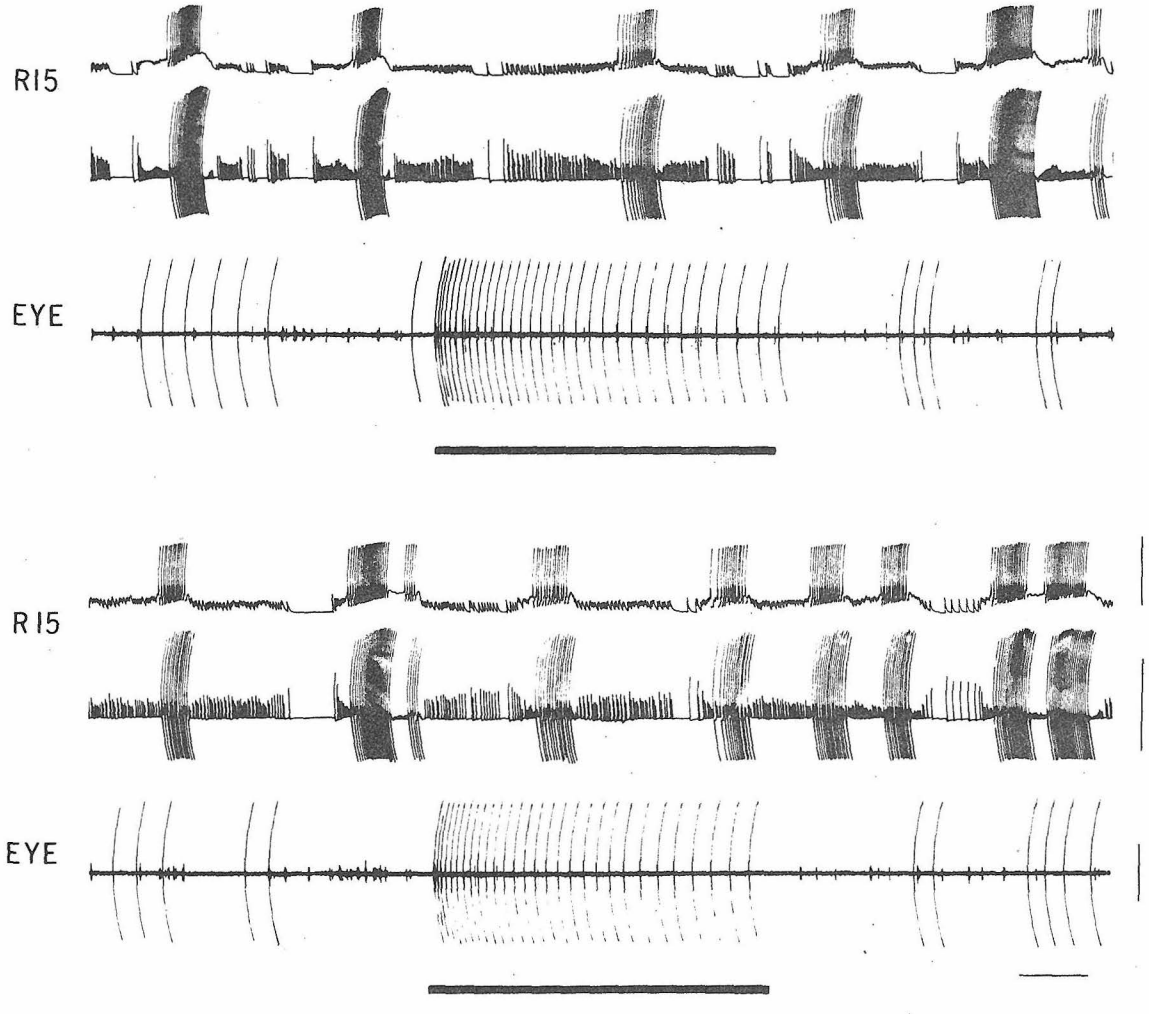
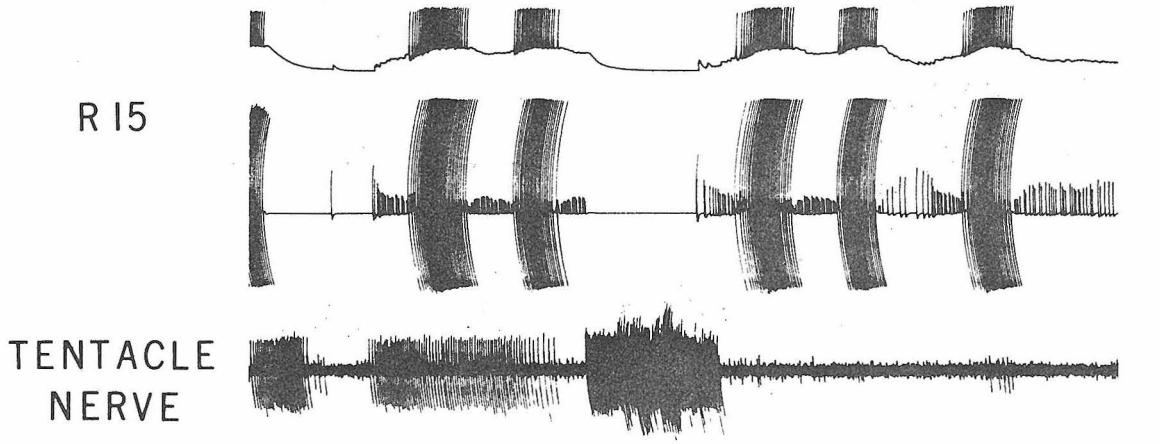


Figure 8. Simultaneous recordings of spontaneous activity of R15 (top two traces of each half of figure) and the right tentacle nerve (bottom trace in each half) in an intact CNS preparation. Note the abrupt cessation of input I EPSPs in R15 during high frequency bursts of activity in the tentacle nerve. Vertical calibration: R15 DC: 100 mV; R15 AC: 25 mV; tentacle nerve 25 μV. Horizontal calibration: 30 sec.

R15: ISOLATED

CENTRAL NERVOUS SYSTEM



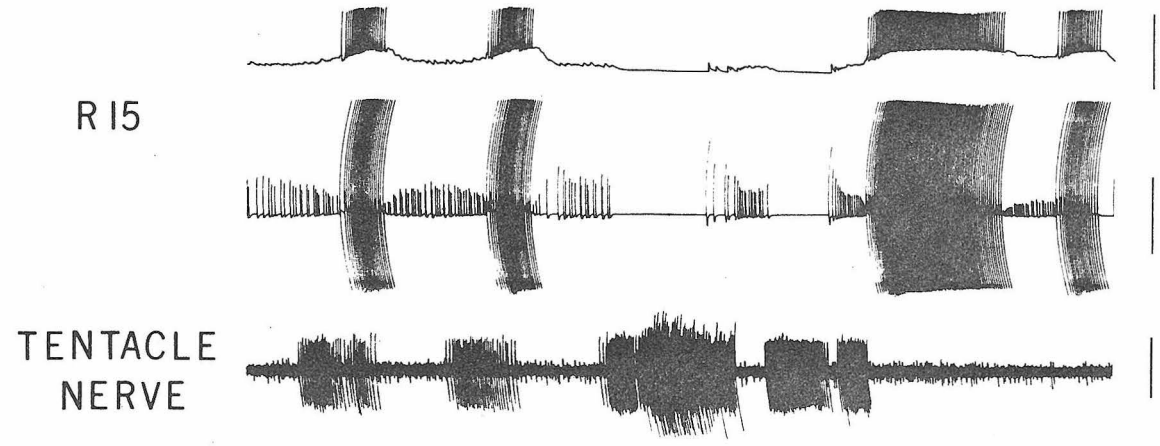


Figure 9. Stimulation of cerebral ganglion nerves (at arrows) of an intact CNS preparation while recording from R15. A) Right tentacle nerve. B) Left tentacle nerve. C) Right rhinophore nerve. Vertical calibration: R15 DC: 25 mV; R15 AC: 5 mV. Horizontal calibration: 10 sec.

R15: ISOLATED CENTRAL NERVOUS SYSTEM

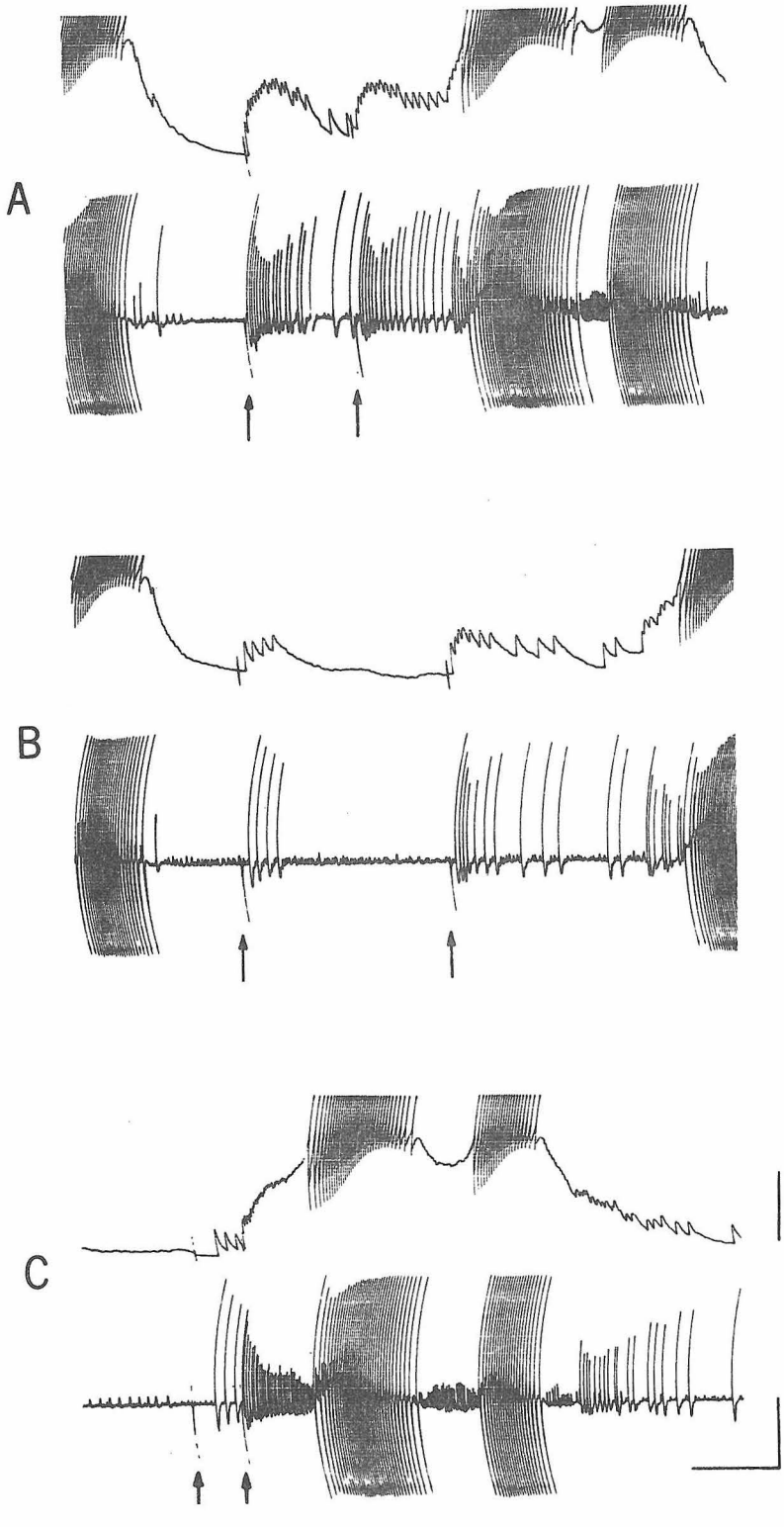
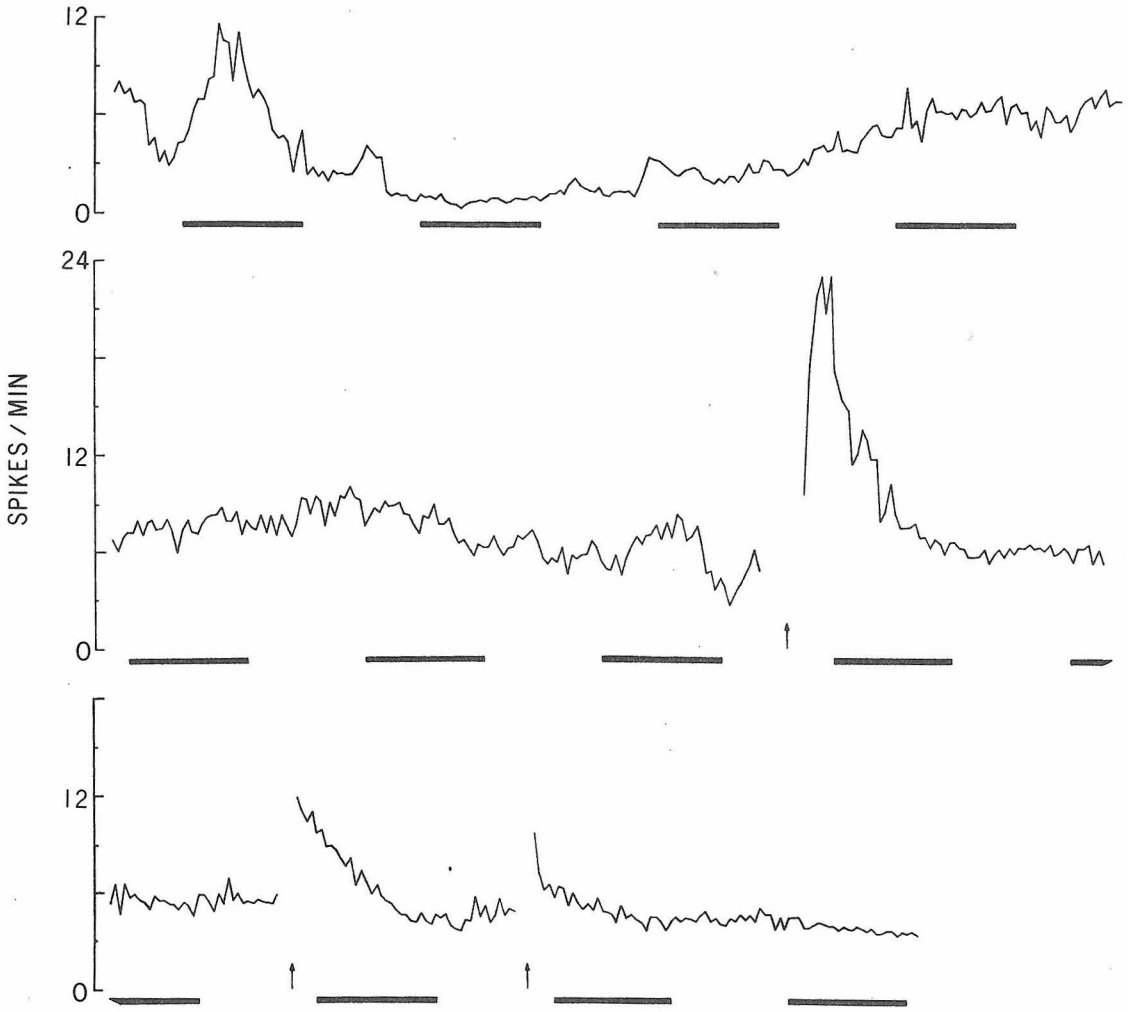


Figure 10. Long term recording of R15 spiking activity in the isolated PVG. Input I EPSP was evoked in R15 by stimulation of the right pleurovisceral connective at 1/sec. during the gaps marked with arrows. Dark bars represent projected night.

R15: ISOLATED PVG



References

Audesirk, G. 1973. Spontaneous and light-induced compound action potentials in the isolated eye of Aplysia: initiation and synchronization. Brain Res. 59: 229-242.

Eskin, A. 1971. Properties of the Aplysia visual system: in vitro entrainment of the circadian rhythm and centrifugal regulation of the eye. Z. Vergl. Physiol. 74: 353-371.

Frazier, W.T., E.R. Kandel, I. Kupfermann, R. Waziri, and R.E. Coggeshall. 1967. Morphological and functional properties of identified cells in the abdominal ganglion of Aplysia californica. J. Neurophysiol. 30: 1288-1351.

Jacklet, J.W. 1969. A circadian rhythm of optic nerve impulses recorded in darkness from the isolated eye of Aplysia. Science 164: 562-564.

Kandel, E.R., W.T. Frazier, R. Waziri, and R.E. Coggeshall. 1967. Direct and common connections among identified neurons in Aplysia. J. Neurophysiol. 30: 1352-1376.

Parnas, I., D. Armstrong, and F. Strumwasser. 1974. Prolonged excitatory and inhibitory synaptic modulation of a bursting pacemaker neuron. J. Neurophysiol. 37: 594-608.

Prosser, C.L., and F.A. Brown, Jr. 1961. Comparative Animal Physiology. Philadelphia: W.B. Saunders, p. 60.

Stinnakre, J., and L. Tauc. 1969. Central neuronal response to the activation of osmoreceptors in the osphradium of Aplysia. J. Exp. Biol. 51: 347-361.

Strumwasser, F. 1965. The demonstration and manipulation of a circadian rhythm in a single neuron. In: J. Aschoff (ed.), Circadian Clocks. Amsterdam: North-Holland Publ. Co., pp. 442-462.

Strumwasser, F., and R. Bahr. 1966. Prolonged in vitro culture and autoradiographic studies of neurons in Aplysia. Fed. Proc. 25: 512.

Humoral Effects of the Eyes on R15 *In Vitro*

It has been shown above that the circadian rhythm of R15 can be entrained *in vivo* even when the neural paths between the eyes and PVG have been severed. Therefore a humoral method of entrainment must be sufficient. The data do not define the stage at which the humoral intervention occurs. However, the eyes contain cells that have inclusions morphologically similar to neurosecretory granules (Jacklet, 1968). It is thus a reasonable assumption that the eyes release a humoral factor into the blood of the animal. This released substance may be able to entrain the circadian rhythm of R15. To test this hypothesis, isolated eyes and PVGs, from the same or different animals, were incubated together. If the eye is neurosecretory, and if R15 can be entrained by the product released by the eye, then it might be possible to observe an *in vitro* effect of the presence of the eyes on the activity of R15 even though the two are not neurally connected.

Methods

Long term recordings from the separated PVGs and eyes were done as described previously for PVGs in the nerve stimulation entrainment attempts (see the Methods of the last chapter). The spike activity of R15 was monitored extracellularly, and the CAPs in the optic nerve of one of the eyes were recorded via suction electrode. The preparations were kept in darkness except when repositioning of the R15 electrode was necessary. The eyes were approximately one to two cm from the PVG

in the incubation chamber, which had 6 ml of organ culture medium in it.

In one group of experiments, the eyes and PVG were removed from the same animal. These animals were taken from the community sea water tanks, and were dissected, as before, at 0900 ± 0100 hr, that is, one hour after lights-on. In a second group of experiments, eyes and PVGs were taken from different animals. In these experiments, the eye donors were entrained to a lighting schedule which was six hours advanced relative to the schedule of entrainment of the PVG donors. Dissections were performed on hour after lights-on for the PVG donors. In some of these experiments, eyes from two or more animals were used simultaneously.

A last group of experiments utilized the same protocol as the second group, with a few changes. First, after two days of incubation of the eyes and PVG together in vitro (the eyes, but not always the R15s, of these preparations were recorded for these two days), the eyes were removed and the PVG left in culture for one additional day. In some otherwise similar experiments, the PVG was removed to a new container of filtered sea water and recorded there for the extra day. Second, the timing of the eye and PVG donor animals' light schedules was varied. Third, dissections were made more random throughout the day. In these runs, the activity of the R15s was recorded for the 24 hours after eye removal, to see if the presence of the eyes in vitro could be shown to have any persistent effect on the neuron.

Results

a) Eyes and PVG from the same donor animal

When the isolated eyes and PVG from the same animal were incubated together, the first day R15 peak occurred very near projected dusk (Table I, Fig. 1). Except for the presence of the eyes in the medium, the protocol of these experiments was identical to that used to record the rhythm of R15 in isolated PVGs. Such recordings, at the same time of year (January to April, 1974), revealed R15 peak times considerably later when the eyes were not present (Table I). The average phase advance of the R15s with eyes was 0342 hr (Student's t test, $t = 3.0$; $p < 0.025$). When the eyes were kept in the medium with the PVG, the first day R15 peak was usually very sharp (Fig. 1), and in one instance a strong dusk peak even occurred on the third day in vitro.

This was the first experiment indicating that a factor was released spontaneously by the eyes, and that it affected the activity of R15. This eye factor appeared to induce a peak in R15 activity which was different in timing than the peak that the R15 would have produced if the eyes were not present. The exact timing of this peak could be a result of the interaction of the free running rhythm of R15 and the timing and amount of release of the humoral factor by the eyes. However, the simplest interpretation on which to base further experiments was that the peak was timed by the eye factor alone.

b) Eyes and PVG from different donor animals

If this hypothesis were true, a consequence would be that if eyes

and PVGs entrained to different light schedules were incubated together, the R15 peak should occur at a time determined by the eye, not the PVG, schedule. To test this, eyes and PVGs were taken from different animals, entrained to light schedules differing in phase by six hours (eye donor schedule advanced). The expected relationship between the eyes and R15, based on the previous experiments, would yield R15 peaks near the end of the eye activity cycle, regardless of where this is placed with respect to the R15 donor entrainment schedule.

Many, but not all, experimental runs agree quite well with this predicted outcome (Figs. 2 and 3). In the best cases, the second and third days of the experiment show strong R15 peaks near the end of the eye activity cycle. The spread of timing and amplitudes of these R15 peaks, not unexpectedly, is greater than in the case of eyes and PVG from the same animal.

Control R15s in isolated PVGs tend to show peaks (on the first day after dissection) around four hours after projected dusk (Table 1). Since the eyes were entrained to a six hour advanced light schedule, the end of the eye activity cycle (usually near projected dusk) should be about six hours earlier than projected dusk of the R15 schedule. This is, then, about ten hours earlier than the predicted R15 isolated PVG peaks. The actual R15 peaks (Figs. 2 and 3) observed during the latter half of the eye cycle are more than ten hours earlier than would be predicted under the assumption of a free run of R15's own rhythm with the same period as the eye. Further, the oscillator of

R15 is so heavily damped that the cell is seldom observed to have a properly timed peak for more than one or occasionally two days *in vitro*. The presence of a sizeable R15 peak on the second or third day *in vitro* is somewhat unusual for an isolated PVG recording. The presence and prominence of the R15 peaks appear to be related to the level of activity of the eyes.

The eye factor may influence the ongoing R15 spike rate while present and yet have no role in the entrainment of the R15 rhythm. However, a substance active in entrainment might be expected to show an aftereffect following removal. Therefore, in a series of otherwise similar experiments, the eyes were removed after two days *in vitro*, and the R15 recording maintained for another day. In most of these experiments, eyes were used from more than one animal.

These last experiments could be considered to have the form of a mimic "dissection." In standard isolated PVG recordings from R15, the animal is sacrificed, the PVG removed, and the spiking activity of R15 recorded for the next 24 hours or more. An eye hormone entrainment hypothesis would consider the main features of that dissection to be removal of the R15 from the influence of the eye factor, followed by recording for the next day. This is exactly what was done in the *in vitro* humoral entrainment attempts. Therefore, if the eye factor *in vitro* perfectly entrained R15, the neuron would be expected to show a peak time which is the same as that of an R15 freshly taken from an animal whose entraining light schedule was timed the same as the rhythm of the eyes *in vitro*.

It has been found that the timing of the peak of R15 activity in an isolated PVG freshly dissected from an Aplysia depends both on the previous lighting schedule under which the animal was kept and on the time of dissection relative to that lighting schedule (see Appendix A for a full discussion of this point). The timing of the R15 spiking peak can then be expressed in terms of the time of day of dissection (Figs. 1 and 4 of Appendix A). Figures 4 and 5 show two runs in which the eyes were removed on the fourth day of the experiment. As a further test, a few experiments were run in which the PVG was removed to a separate chamber filled with filtered sea water for the recording on the extra day. Figure 6 shows the extra day of recording in two such experiments, with freshly dissected R15 runs for comparison. The peak time - dissection time graphs of Appendix A are reproduced in Figures 7 and 8 with the times of R15 peaks after eye removal in these and other experiments added. The absolute number of hours between eye removal in vitro and the R15 peaks, and the circadian times of the eye activity cycles at which eye removal occurred are given in Table II. Although both the circadian time of eye removal and the length of time until R15 peak activity varied substantially, the time relationship between these two factors is very similar to that obtained in the fresh dissection control experiments. The R15 peaks in the in vitro entrainment experiments, however, are often not as prominent as the peaks in the fresh dissection experiments (see Fig. 5).

Discussion

Aplysia eyes have been suspected for some time to be neuro--secretory. Cells in the eye contain inclusions which have been considered to be neurosecretory granules on morphologic grounds (Jacklet, 1968). Little work has been done, however, to investigate this possibility further. Arch and Eskin (1971) have done the only experiments which support a secretory role for the eye. They found that they could induce the release of tritium in a TCA-precipitable form by high potassium treatment of an eye previously labelled with tritiated leucine. This work is currently being extended (Stuart and Audesirk, experiments in progress).

The results of the present experiments provide the first evidence of a spontaneously released substance from Aplysia eyes, and also give evidence of possible functions for that substance. First, the ongoing rate of spiking in R15 is changed when eyes are present in the same medium as the PVG. The peaks of spike frequency are shifted, in the presence of the eyes, to times other than those at which they would have occurred in the absence of the eyes. Since the eyes were not neurally connected to the PVG in these experiments, they must release a substance capable of influencing the activity of at least one neuron in the PVG. This neuron may be R15, or it may be another cell(s) in the PVG which then affects R15.

The net effect of the eye factor on the ongoing spike rate of R15 appears to be inhibitory. Aqueous extracts of homogenized Aplysia eyes exert a suppressive effect on the spike rate of R15 (Audesirk,

unpublished results). The peaks of R15 spiking near the end of the eye activity cycle may represent unusually long lasting rebounds from inhibition.

In addition to its effect on the instantaneous spike rate of R15, the eye factor also appears to play a role in the entrainment of the circadian rhythm of the neuron. The timing of the peak spiking frequency of R15 after removal of the eyes *in vitro* appears to be predictable in terms of the previous rhythmic output of the eyes and the time of eye removal (Figs. 7 and 8, and Table II). The predicted interval between eye removal and R15 peak is correlated with the actual interval (Pearson product moment, $r = 0.73$; $p < 0.025$). Further, the average absolute value of the differences between the peaks predicted and those actually obtained in the *in vitro* experiments, 2.5 hours, is only slightly greater than the average absolute value of the differences of the fresh dissection data used to generate the prediction, 1.9 hours.

Before concluding too strongly that the timing of the R15 peak is only determined by the timing of the release of the eye factor, however, other methods of predicting the peak time must be examined as well. Other factors which might be able to produce the appearance of entrainment include the free running rhythm of R15, uninfluenced by the eyes, and the amount of eye factor release.

The peaks could be timed by the free running rhythm originally entrained *in vivo* by the light-dark cycle. If we assume that the R15 free running rhythm has the same period as that of the eyes in the

same medium, then the expected and observed peak times are those given in Table III. (Using other periods for the R15 rhythm, such as 24 to 27 hours, results in similar or larger differences between the expected and observed peaks, ranging from 5.1 to 7.0 hours.) The magnitudes of the differences between the predicted and observed peak times under the free running R15 rhythm hypothesis (average, 5.1 hr) are much greater than the differences under the eye factor entrainment hypothesis (average, 2.5 hr; paired t test, $t = 2.3$; $p < 0.025$).

The amount of eye factor released could also affect R15 peak times. It was hypothesized above that the factor was inhibitory. If so, then the interval between eye removal and R15 peak activity might be related to the amount of eye hormone released before the eyes were taken out of the chamber. Since the amount of hormone cannot be directly assayed at the present time, indirect methods must be used to approximate the amounts released in the various experiments (Table IV). Both the number of eyes in the incubation medium with the PVG (correction made for incubation volume; see Table IV) and the product of the number of eyes and the peak activity of the recorded eye on the last day <u>in vitro</u> before eye removal are positively correlated with the interval between removal and R15 peak (Pearson product moment; for number of eyes, $r = 0.63$; $p \cong 0.05$; for product, $r = 0.45$; $0.05 < p < 0.1$). Entrainment by pulses of light in many organisms results in greater shifts in the phase of the rhythm for long light pulses than for short ones; see Fig. 14 in Pittendrigh (1960). A greater amount of eye hormone might be expected to act similarly to

long light pulses.

In conclusion, then, it appears that the Aplysia eye releases a substance which can affect the ongoing and longer term activity of R15. In vitro experiments are capable of essentially duplicating the peak time - dissection time relation which holds with in vivo entrained R15s. This eye factor is a strong candidate for the agent mediating the photoentrainment of R15 in vivo.

Table I

	Control	Eyes + PVG
Time of Peak	+0130	-2030
	+0100	-2000
	-1830	-1830
	+0100	-2100
	+0030	
Mean ± S.D.	-2342	-2000
	±0255	±0105

Table II

Date	Remove Eyes[a]	Predicted Peak[b]	Actual Peak[c]	Predict - Actual (hr)
5/20	CT 0000	CT 1248	CT 0730	5.3
5/24	CT 1800	CT 0248	CT 0100	1.8
6/18	CT 2230	CT 0906	CT 0900	0.1
7/14	CT 0100	CT 1521	CT 1830	-3.1
7/22	CT 0300	CT 1506	CT 1600	-0.9
8/13	CT 0030d	CT 1406	CT 0930	4.6
8/18[1]	CT 0830d	CT 1636	CT 1530	1.1
8/18[2]	CT 0830	CT 1636	CT 1200	4.6
9/03[1]	CT 0000d	CT 1248	CT 1000	2.8
9/03[2]	CT 2330d	CT 1136	CT 1030	1.1

a) The time of eye removal is determined by the following procedure. First, the midpoint of the silent period of the eye between the second and third days in vitro is assigned the value of CT 1800 (middle of the circadian night). Then the free running period of the eye is calculated as the number of hours between the midpoint of the silent period between days two and three and between days one and two. Then the circadian time of eye removal is found by: CT_{ER} = (Hr after CT 1800) - 6 - (Free running period - 24).

b) Predicted R15 peak time is determined by linear interpolation between the averages of the R15 times of recordings from isolated PVG controls of Appendix A.

c) Actual R15 peak times are found by adding the number of hours between eye removal and R15 peak to the circadian time of eye removal.

d) In these experiments, the PVG was removed to a separate chamber of filtered sea water for the extra day of recording. In all others, the eyes were removed and the R15 activity on the extra day recorded in the same chamber and medium in which the joint incubation was performed.

Table III

Date	Dissection Time	$FR_{,eye}$ (hr)	Predicted Peak[a]	Actual Peak	Predict - Actual (hr)
5/20	CT 0100	24	CT 1524	CT 1330	1.9
5/24	CT 0100	27	CT 0024	CT 1000	-9.6
6/18	CT 0100	23.5	CT 1354	CT 1430	-0.6
7/14	CT 0100	24	CT 1524	CT 0030	-9.1
7/22	CT 0100	25	CT 1824	CT 0100	-6.6
$8/13_1$	CT 1100	26	CT 0112	CT 0930	-8.3
$8/18^1$	CT 0200	26.5	CT 2112	CT 0330	-6.3
$8/18^2$	CT 0800	26	CT 2054	CT 0200	-5.1
$9/03^1$	CT 1200	26.5	CT 0154	CT 2330	2.4
$9/03^2$	CT 1200	26.5	CT 0154	CT 0030	1.4

a) Predicted R15 peak determined by linear interpolation between the averages of R15 peak times from isolated PVG controls of Appendix A, corrected for the period of the free running rhythm, which is assumed to be the same as that of the eye recorded in the same chamber.

Table IV

Date	Number of Eyes	Peak CAP Frequency[a]	Product[b]	Interval[c]
5/20	4	150	600	7.5
5/24	4	48	192	7.0
6/18	4	112	448	10.5
7/14	8	141	1128	17.5
7/22	6	64	384	13.0
8/13	4d	20	160	9.0
8/18[1]	2d	20	80	7.0
8/18[2]	4	56	224	3.5
9/03[1]	2d	102	408	10.0
9/03[2]	2d	126	504	11.0

a) Peak CAP frequency of the recorded eye on the day before eye removal.

b) Product = Peak CAP frequency x Number of eyes (corrected for volume)

c) Interval = Number of hours between eye removal and R15 peak

d) Volume one half of other experiments (3 ml)

Figure 1. Recordings from one eye (of two) and R15 in the neurally isolated PVG from the same animal, incubated together in the same chamber in darkness. Note the strong R15 activity peaks near the end of the eye cycle on days one and three. In this and subsequent figures, the dark bars represent projected night.

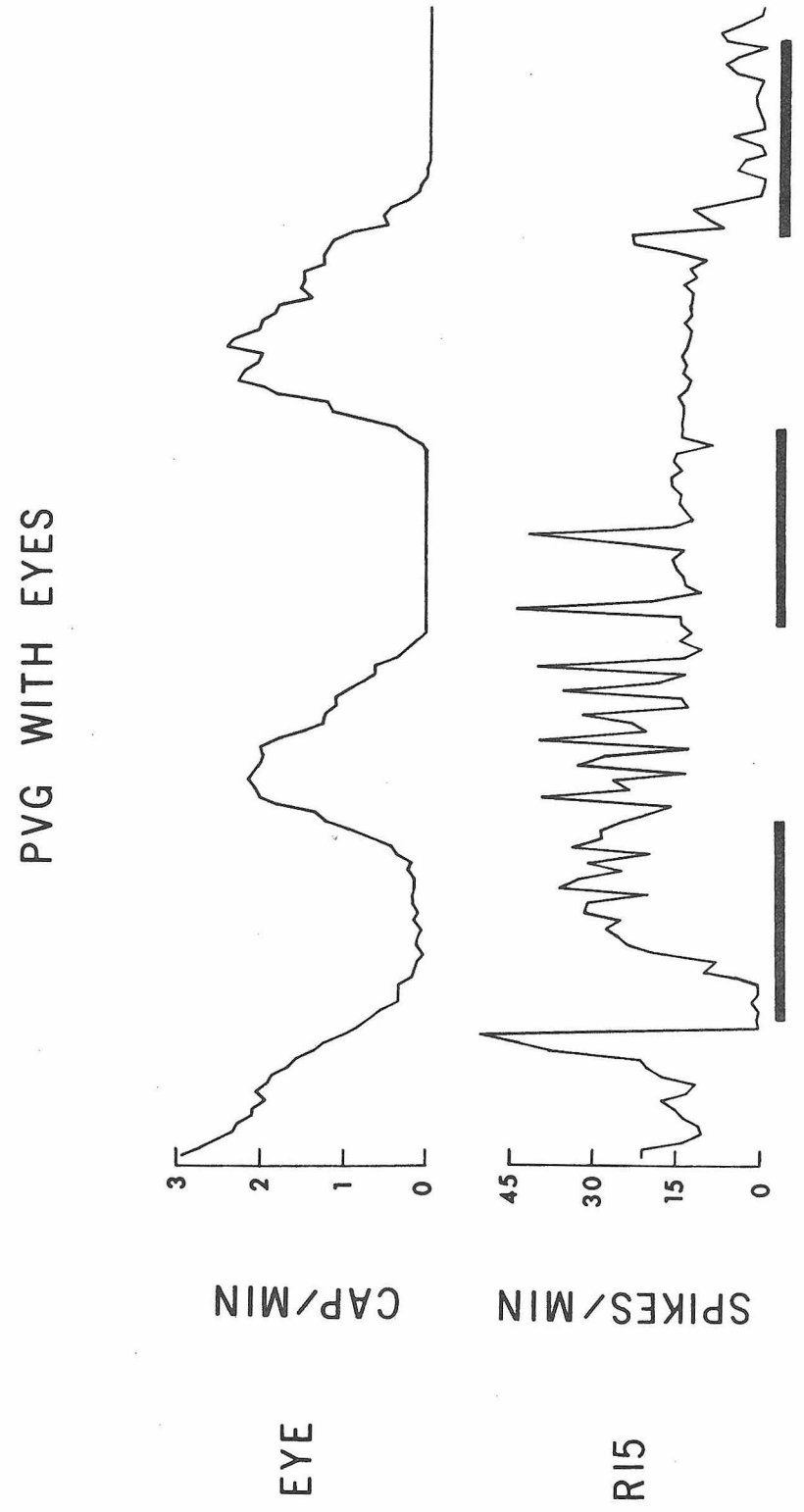

PVG WITH EYES
EYE
CAP/MIN
3
2
1
0
R15
SPIKES/MIN
45
30
15
0

Figure 2. Recordings from one eye (of two) and R15 in the isolated PVG of different animals, incubated together in darkness. The LD schedule of entrainment of the eye donor animal was six hours advanced relative to the PVG donor schedule.

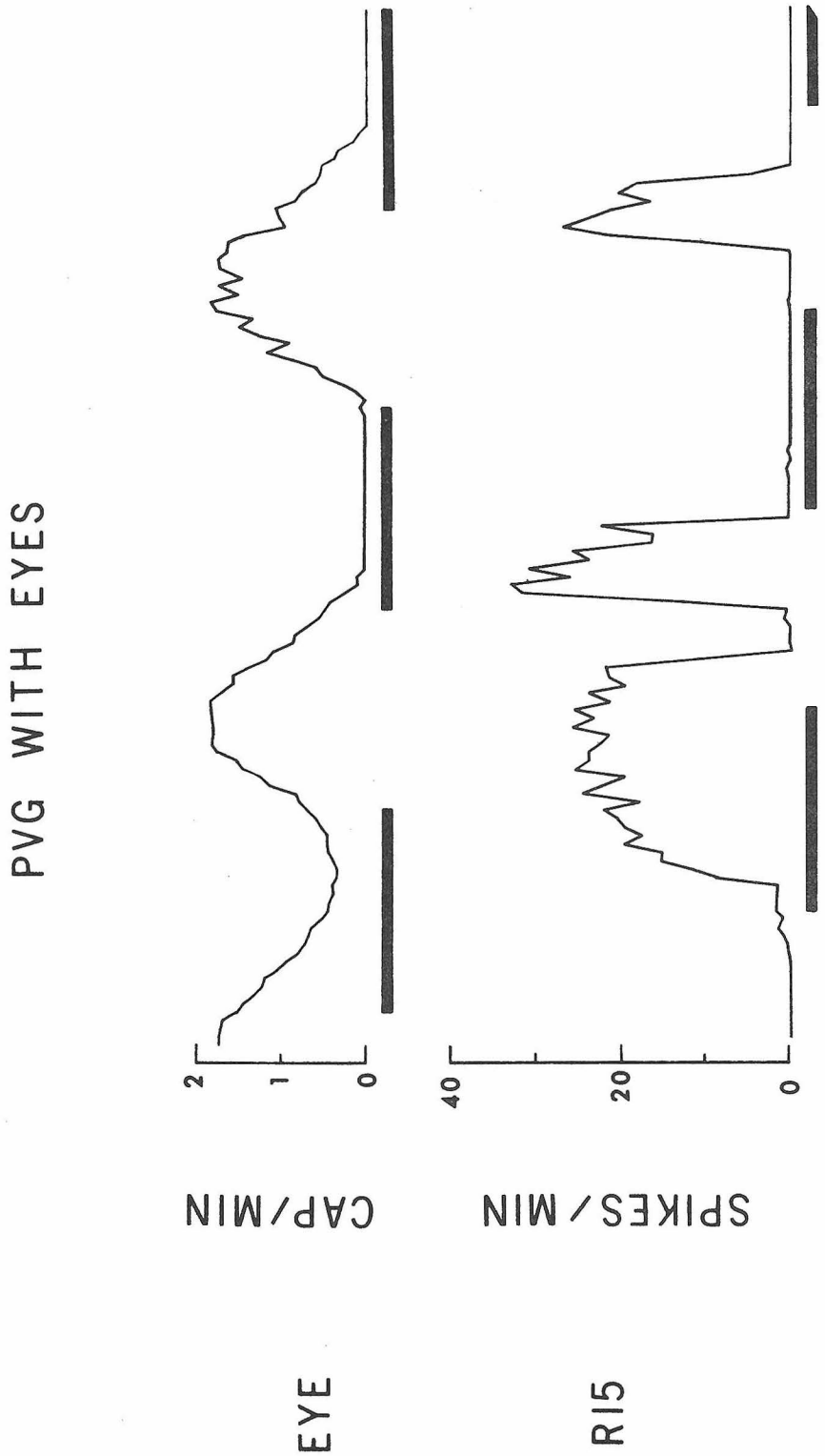

PVG WITH EYES
EYE
CAP/MIN
2
1
0
R15
SPIKES/MIN
40
20
0

Figure 3. Recordings from one eye (of two) and R15 in the isolated PVG of different animals, incubated together in darkness. The LD schedule of entrainment of the eye donor animal was six hours advanced relative to the PVG donor schedule.

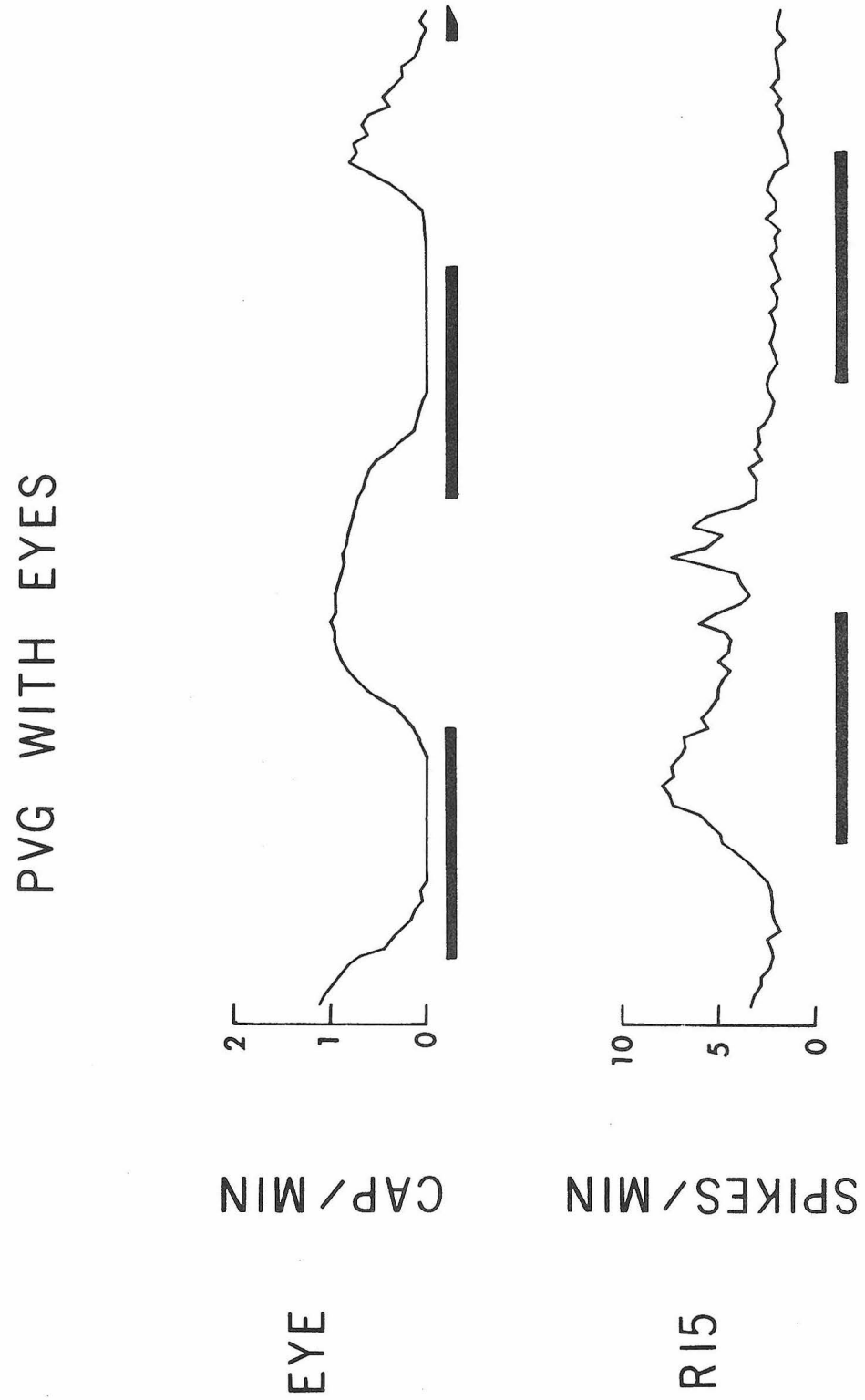

PVG WITH EYES
EYE
CAP/MIN
2
1
0
R15
SPIKES/MIN
10
5
0

Figure 4. Recordings from one eye (of four) and R15 in the isolated PVG from different animals, incubated together in darkness. At the end of the eye graph, the eyes were removed and the R15 activity recorded for another day. R15 peak activity after eye removal occurred at 7.5 hours after eye removal.

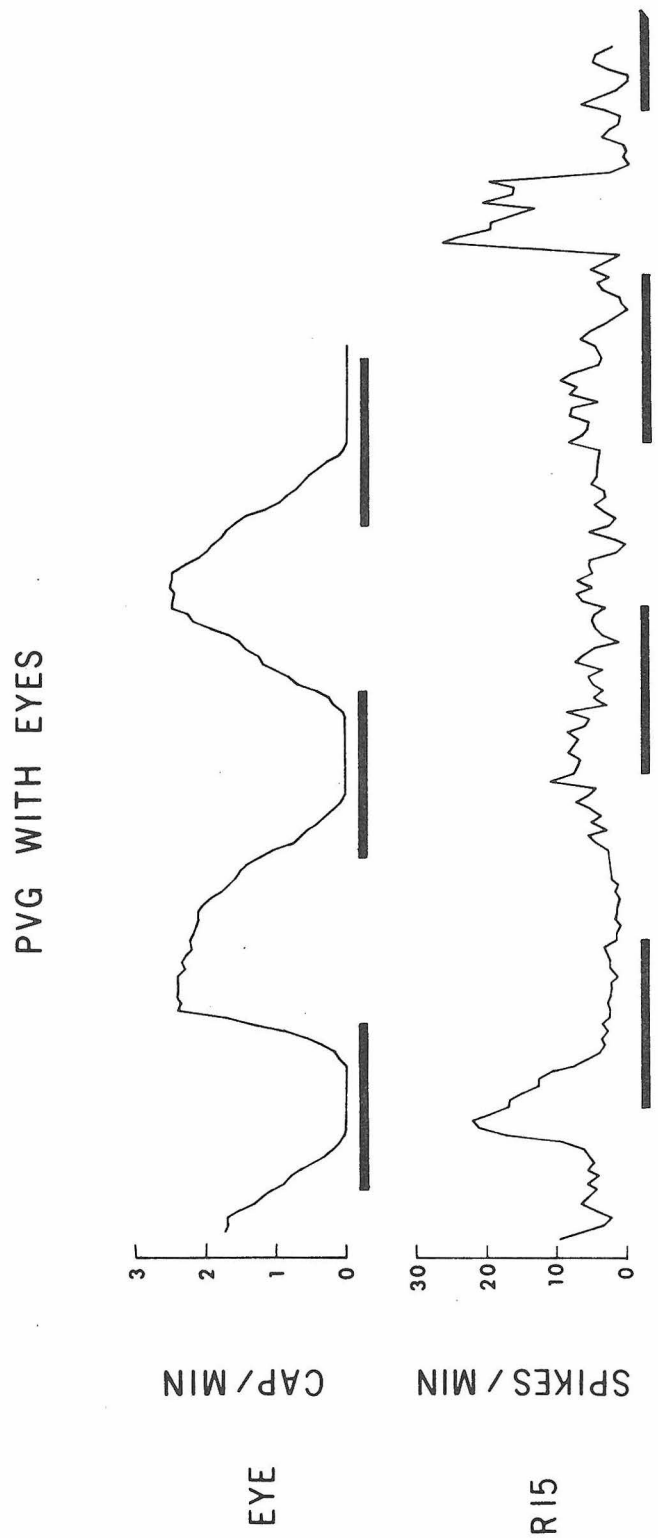

PVG WITH EYES
EYE
CAP/MIN
3
2
1
0
R15
SPIKES/MIN
30
20
10
0

Figure 5. Recordings from one eye (of four) and R15 in the isolated PVG from different animals, incubated together in darkness. At the end of the eye graph, the eyes were removed and the R15 activity recorded for another day. R15 peak activity after eye removal occurred at 10.5 hours after eye removal.

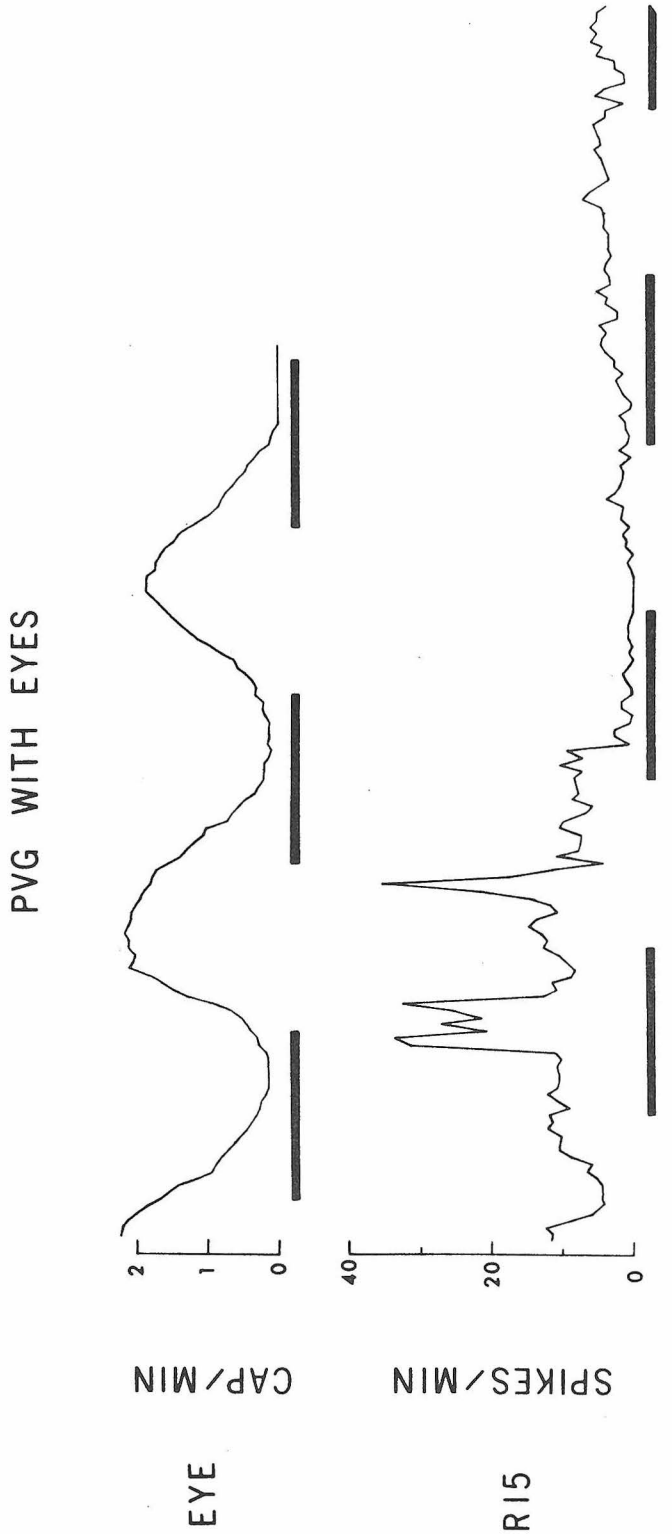

PVG WITH EYES
EYE
CAP/MIN
2
1
0
R15
SPIKES/MIN
40
20
0

Figure 6. Recordings from R15s in isolated PVGs.

Top: R15s from PVGs removed from three days of incubation with eyes in vitro. Left: PVG removed at CT 0000 of eye free running rhythm (9/03/74[1]). Right: PVG removed at CT 0830 of eye rhythm (8/18/74[1]).

Bottom: R15s of PVGs freshly dissected at times closely equivalent to PVG removal times above. Left: Dissection at CT 0100. Right: Dissection at CT 0700.

The spiking activity graphs are aligned vertically to facilitate comparison between R15 activities in the PVG removal and fresh dissection cases. Note that the PVG removal peaks are earlier than the corresponding fresh dissection peaks, as is the general case (see Table II).

OC = organ culture PVG removed from incubation with eyes

FC = freshly dissected PVG

RI5: ISOLATED PVG

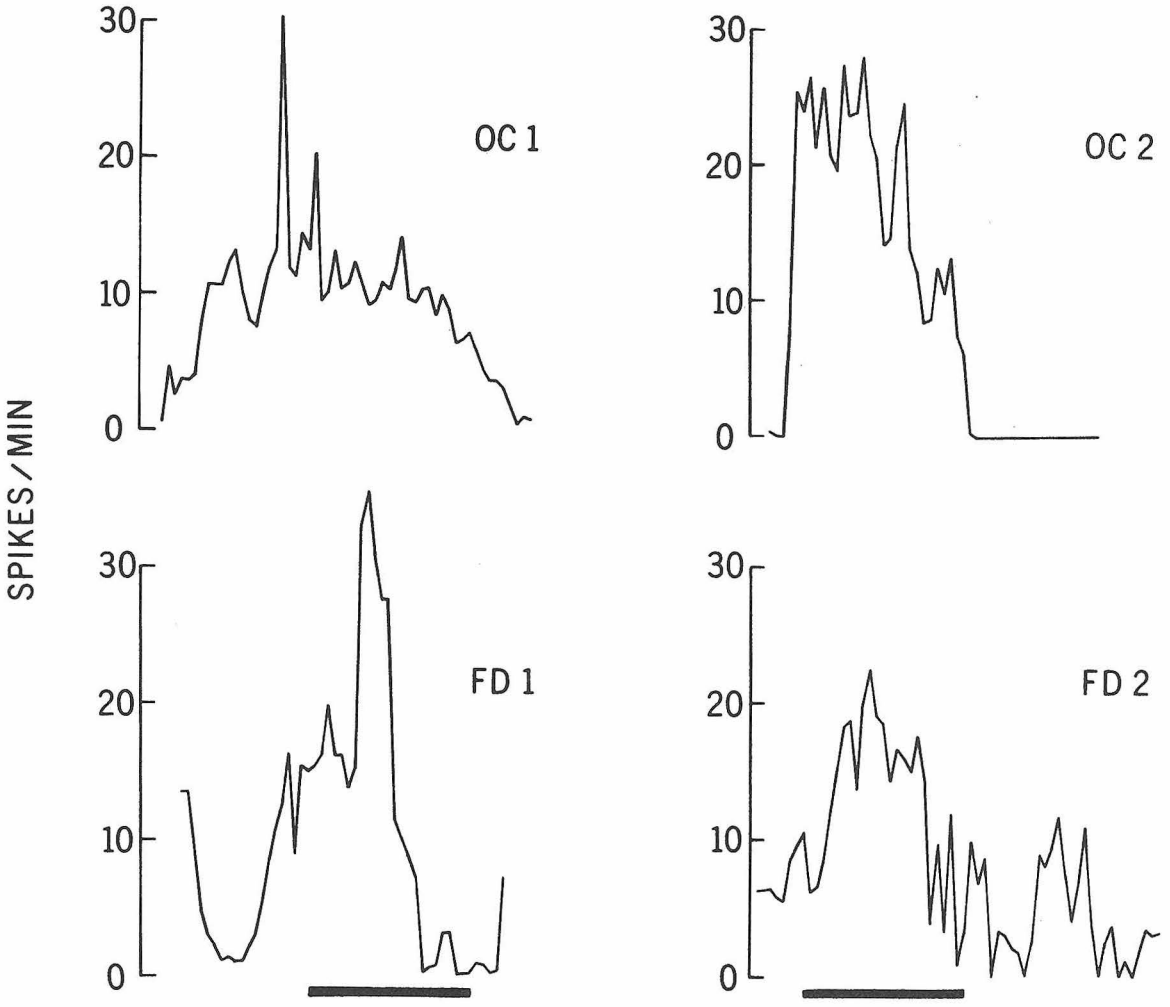


Figure 7. Reproduction of Fig. 1 of Appendix A, showing time of peak activity of R15 in the isolated PVG as a function of time of dissection (triangles). The squares represent peak timing of R15s after eye removal from in vitro incubations of eyes and PVGs together. Eye removal is taken as the "dissection time" and is assigned as equivalent to a point in the LD cycle of the graph based on the previous day's eye activity cycle. The midpoint of the silent period of the eye cycle is taken as the middle of the free running subjective night, and thus is set equal to CT 1800, six hours before dawn. Since the R15 rhythm is being tested for entrainment by the eyes, the period of the entraining cycle is taken as the free running period of the eyes. Therefore an eye removal occurring 24 hours after the midpoint of the silent period of an eye running at a 27 hour period, for example, would not be assigned the time of CT 1800 but rather CT 1500. See Table II for the times and formula for computing the time of eye removal.

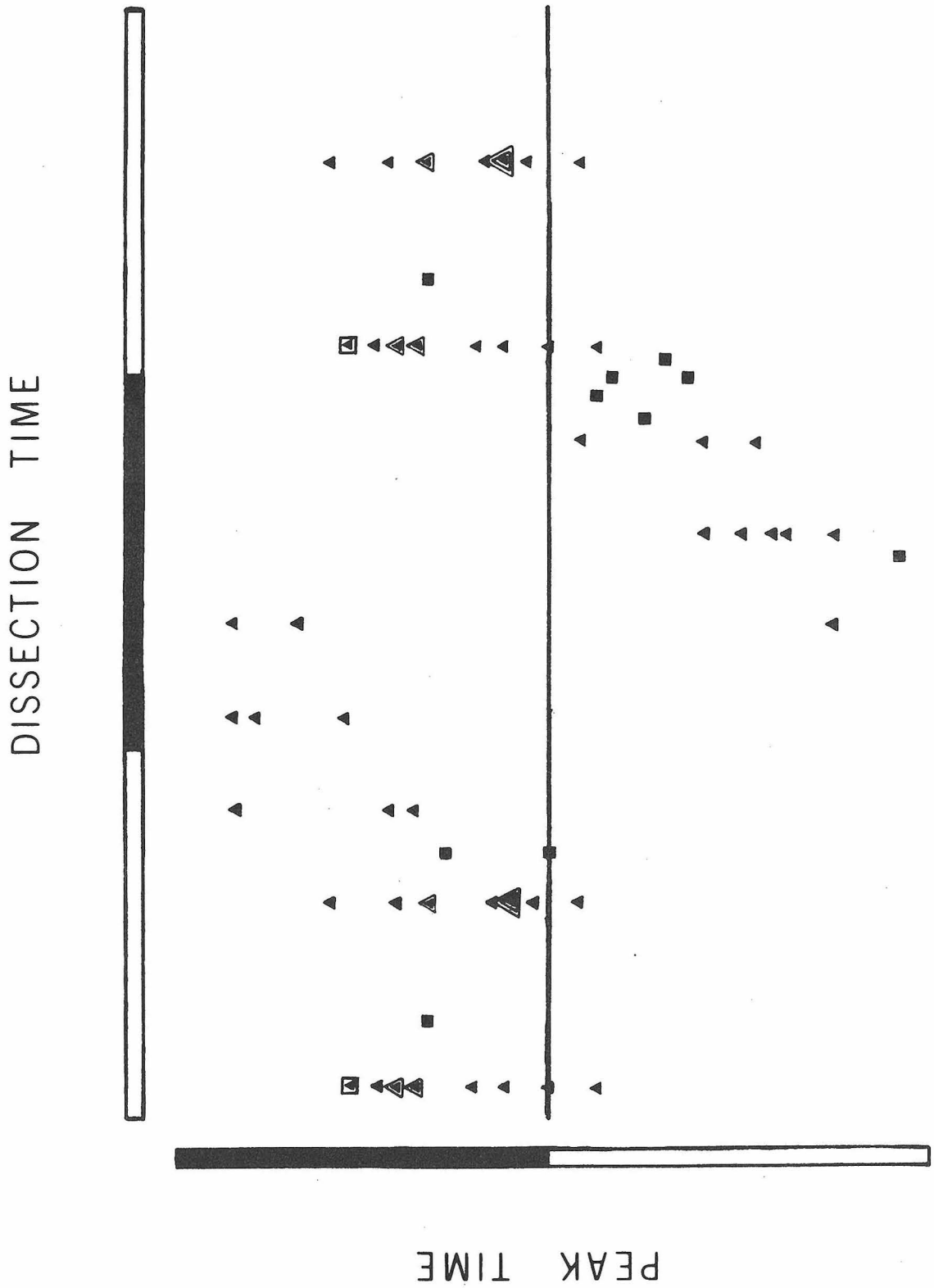
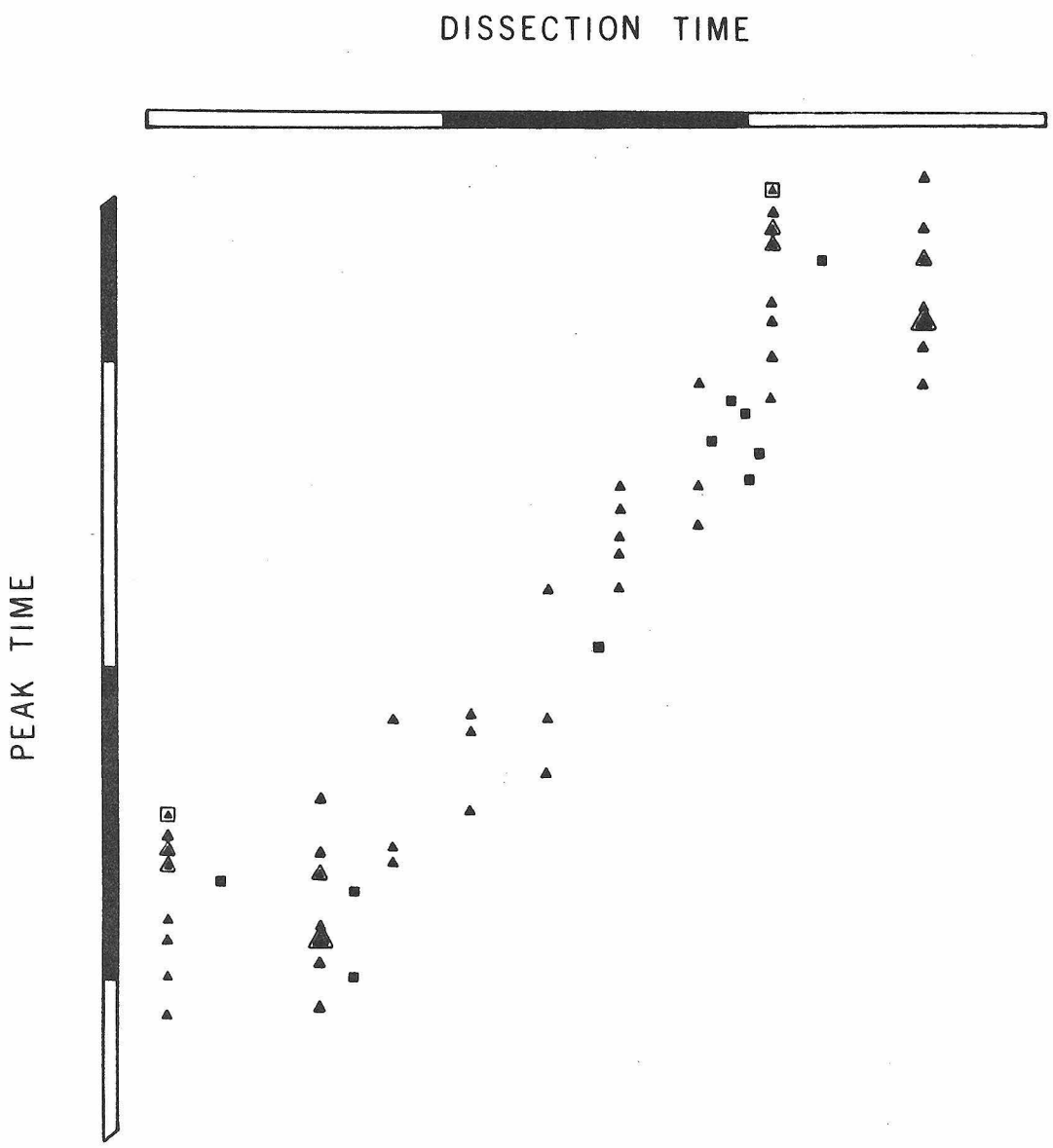

DISSECTION TIME
PEAK TIME

Figure 8. Reproduction of Fig. 4 of Appendix A with the in vitro entrainment points added, as in Fig. 7.


DISSECTION TIME
PEAK TIME

References

Arch, S., and A. Eskin. 1971. Neurosecretory activity in the eye of Aplysia californica. Biol. Ann. Rep., Calif. Inst. of Tech., p. 109.

Jacklet, J.W. 1968. Synchronized neuronal activity and neurosecretory function of the eye of Aplysia. Proc. Int. Union. Physiol. Sci. 7: 213.

Pittendrigh, C.S. 1960. Circadian rhythms and the circadian organization of living systems. Cold Spring Harbor Symp. Quant. Biol. 25: 159-184.

General Discussion

In several organisms, there have been indications of a hormonal link between suspected master circadian oscillators and subordinate systems. In sparrows, for example, severing the pineal stalk, which contains the only known neural output path from the pineal, the presumed master oscillator in birds, does not change the circadian locomotor rhythm (Menaker, 1974). The eclosion of adults in a population of Pernyi moths occurs with a circadian rhythm, which is abolished by removal of the brain. The rhythm is re-established by implantation of brains in the abdomens of the pupae (Truman, 1972, 1974). Both of these experiments suggest hormonal control of the systems actually causing the observed circadian behaviors. The location of the master and subordinate systems is not known on the cellular level in either of these organisms.

This dissertation has provided evidence of both hormonal and neuronal links between a master and a subordinate circadian oscillator in Aplysia: the eyes and R15, respectively. The diagram of Figure 1 presents the main data in pictorial form.

Although not much is known about the details of the wiring diagram or rhythm production in the eyes, information of a general nature about various properties of the eyes has been presented here, in Part I. It is not known what, if any, relation the neurosecretory cells in the eye bear to the cell types deduced from the experiments of Part I. It would be of particular interest to know if the higher order cells which produce the CAP in the optic nerve are identical with, or include, the

neurosecretory cells.

There are two methods of information transfer from the eyes to R15 that are now known. The first is neuronal, via the centrifugal cells of the cerebral ganglion. These eventually connect with the cell which produces the large EPSP of input I in R15. It is not known what role this input plays in the normal functioning of R15 in vivo, but, as pointed out previously, this pathway should be strongly influenced by large changes in light intensity, as at dawn or dusk. The other connection to R15 is via a neurosecretory product released by the eyes. This neurohormone appears to be able to entrain the circadian rhythm of R15. Entrainment may occur either by the hormone acting directly on R15, or indirectly via actions on other cells in the PVG. Data have not yet been obtained to allow a distinction between these two possibilities.

The eye is a difficult, but not impossible, preparation for intracellular work. R15 is not only easy to record from with intracellular electrodes for long periods of time, but is also large enough for biochemical work to be done on the individual neuron (Wilson, 1971). Thus the unique possibility now presents itself to study entrainment of and hormonal action on the single neuron. Questions open to investigation include: the nature and manner of release of the neurohormone; the cell on which the hormone acts (R15 and/or other neurons); the cellular site of action (e.g., membrane or nucleus); the neuronal properties altered by the hormone. The answers to these questions may yield valuable insights into the generation and entrainment of metazoan

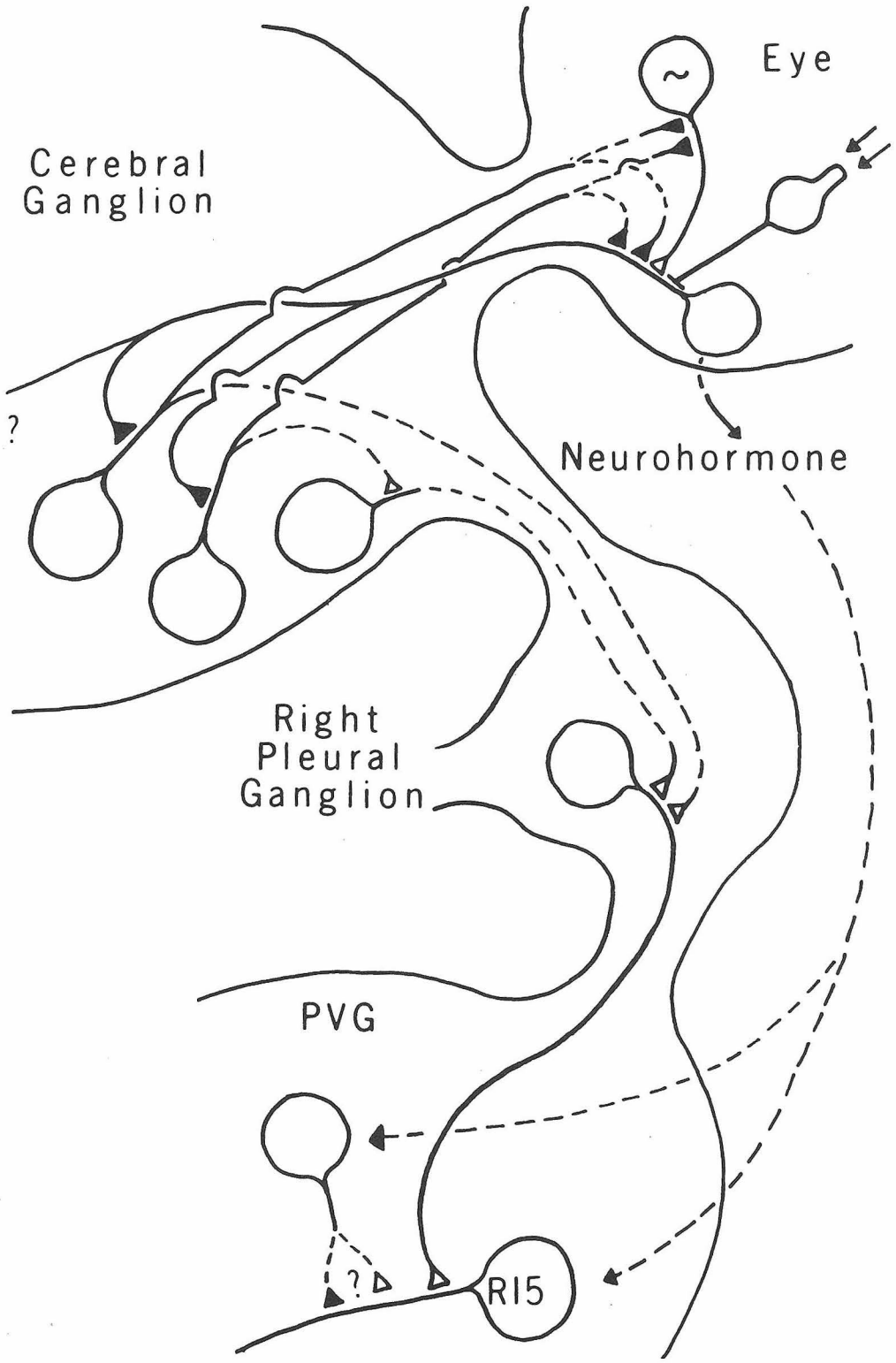
rhythms in general at the cellular level.

Figure 1. Diagram of interneuronal connections within the eye and between the eye and R15. In the eye, light (arrows) excites the photoreceptors, which communicate with higher order cells via electrical synapses (straight line synapse). Spikes are produced in these higher order cells and travel down their axons in the optic nerve to the cerebral ganglion. In darkness, spikes are initiated in these neurons via EPSPs from pacemaker cell(s) (open triangle: excitatory chemical synapse). The higher order cells may be somewhat autoactive, at least to the extent of responding differently to the pacemaker excitation at different times of day.

There are cells in the cerebral ganglion which receive inhibitory input from the eyes (closed triangle: inhibitory chemical synapse) and which in turn inhibit output from the eyes. This inhibition of spontaneous eye activity would seem to be most efficient if done at the pacemaker level, but may be done at the follower level (dotted lines: uncertain or alternate connections). Some of these cerebral ganglion cells also send excitation, perhaps through interneurons, to a cell in the right pleural ganglion. This cell in turn makes an excitatory synaptic connection onto R15 (input I).

Some cells in the eye release a neurohormone which can influence the activity and circadian rhythm of R15. The cells in the eye releasing the hormone are not known, but are possibly a subset of the follower cells. The hormone may act directly on R15, or it may act on other neurons in the PVG which act on R15. The net effect of the hormone is probably inhibitory, but this is not certain. It is

possible that the ongoing effect and the entrainment effect are due to different hormones or to different modes or sites of action of the same hormone.


Eye
Cerebral Ganglion
?
Neurohormone
Right Pleural Ganglion
PVG
?
R15

References

Menaker, M. 1974. Aspects of the physiology of circadain rhythmicity in the vertebrate central nervous system. In: F.O. Schmitt and F.G. Worden (eds.), The Neurosciences: Third Study Program. Cambridge: MIT Press, pp. 479-489.

Truman, J.W. 1972. Circadian rhythms and physiology with special reference to neuroendocrine processes in insects. In: Circadian Rhythmicity. Wageningen, Netherlands: Center for Agricultural Publishing and Documentation, pp. 111-136.

Truman, J.W. 1974. Circadian release of a prepatterned neural program in silkmoths. In: F.O. Schmitt and F.G. Worden (eds.), The Neurosciences: Third Study Program. Cambridge: MIT Press, pp. 525-529.

Wilson, D.L. 1971. Molecular weight distribution of proteins synthesized in single, identified neurons of Aplysia. J. Gen. Physiol. 57: 26-40.

Appendix A

The Timing of the Circadian Rhythm of R15

Previous reports concerning the circadian rhythm of R15 have contained a curious inconsistency. The timing of the rhythm has been different in nearly every paper describing the phenomenon. The rhythm is measured by the time at which the maximum spiking activity of the cell occurs, which has been reported to be predictable from knowledge of the light-dark schedule under which the animal was kept prior to dissection. The original report (Strumwasser, 1965) described the neuron's spiking activity as usually peaking very near projected dawn. Most of these experiments were performed in the spring of the year. Some cells were later found to peak at projected dusk (Strumwasser, 1967). Lickey (1969) reported that the neuron's peak activity could occur at dawn or dusk, but also near midday or midnight, depending on the season of the year at which the experiment was performed. Dawn/dusk peaks occurred during the winter and spring, and midday/midnight peaks occurred during the summer and fall. There was no obvious preference for dawn as opposed to dusk (six to five) but there were more midday peaks (eight) than midnight peaks (four).

Later experiments by Lickey (Lickey, Zack, and Birrell, 1971; Lickey and Zack, 1973), performed during the midday/midnight season, found, as expected, very few dawn/dusk peaks. However, unlike the 1969 data, most of these peaks now fell near midnight, with only a few at midday. Even more strangely, whereas the 1969 data show eight peaks

before midperiod (midday or midnight), three after, and one exactly at midnight, the 1971 data have nine peaks after midperiod and two before (all of these peaks were within three hours of midperiod; otherwise they would be closer to, and considered as, endperiod peaks in this analysis) and the 1973 data show seven or eight after and only one before. These distributions of 1969 on the one hand and 1971 and 1973 on the other are, of course, considerably different (Fisher's exact probability, $p < 0.01$ for before vs. after midperiod; $p < 0.05$ for midday vs. midnight).

The net outcome of these experiments, if all are taken at face value, is that R15 is a cell that peaks at dawn or dusk, or midday or midnight, depending on the season of the year. Sometimes both peaks of a pair may be present in the same run (see Lickey, 1969, Fig. 1). In addition, depending on the year (?), most midperiod peaks occur after or before the midperiod, and at midday or midnight. The conclusions which could be drawn from this welter of data are varied, and none very satisfactory. For example, if peaks occur both at dawn or dusk, or midday or midnight, or both members of a pair can be present, then perhaps the rhythm is not circadian but hemicircadian. Again, perhaps there is a rhythm related to the light-dark entrainment schedule of the animal, but the exact relation depends also on more subtle factors differing from laboratory to laboratory. Or perhaps there is no rhythm at all, but each investigator has introduced his own individual, unnoticed artifacts which gave the appearance of rhythmicity.

The real reason for this variation in peak timing, however, may lie in uncontrolled variables not taken into account by the investigators. One such variable, which goes virtually unmentioned in all of these reports, is the time of dissection relative to the light-dark schedule. Experiments which control for the effects of dissection time, reported below, have led to a surprising result: the phase of the R15 spiking rhythm is set by dissection. Moreover, the kinetics of the response of the rhythm to dissection time are strikingly similar to the kinetics of phase shifting of the circadian rhythms of virtually all organisms by light pulses.

Methods

The dissection and recording techniques were as previously described for normal animals (Part II). The Aplysia were entrained either in the community tanks or in individual aquaria for at least one week prior to dissection. Points from dissection times of CT 0100 and CT 0700 were derived from the control and sham blinded animals of Part II. Except for those of the blinding experiments, all recordings were extracellular. Recordings were done at various times of the year, from June 1973 to August 1974 (see Table I).

---

*CT = circadian time. Dawn = CT 0000, dusk = CT 1200 for an LD 12:12 light cycle, regardless of the position of these events in relation to the solar day.

Results

Dissection times and dates, and subsequent R15 spiking peaks, are given in Table I. These peaks were used to generate graphs of peak time vs. dissection time (Fig. 1), and interval between dissection and peak vs. dissection time (Fig. 2). For comparison purposes, graphs similar to the plot of Fig. 1, for steady state phase shifts produced by short light pulses applied to free running animals of various species, are shown in Fig. 3 (from Pittendrigh, 1965).

Several features of the R15 peak times and graphs should be noted. First, although CT 0100 dissections were performed during the summer of 1973 and the winter/spring of 1974, no seasonal change in the timing of the peaks was observed (summer average, CT 1500 $\pm$ 0233 hr; winter average, CT 1542 $\pm$ 0255 hr). This is contrary to the findings of Lickey (1969) that summer animals yield midperiod peaks (middle of the projected night or day; i.e., near CT 1800 or CT 0600) while winter ones yield endperiod peaks (near CT 0000 or CT 1200).

Second, the time of peak spiking activity is strongly dependent upon dissection time, with, in my experiments at least, all peaks occurring 4 to 18 hours after dissection. With this large number of experiments, the omission of any peaks in 10 hours of the day strictly by chance is vanishingly small ($p \cong 10^{-14}$).

Third, the timing of the peaks is nevertheless predictable on the basis of the predissection light schedule, when dissection time is also taken into account. This is most easily seen with dissections

performed at CT 0100 and CT 0700. In these two cases, the average R15 peak times were CT 1521 and CT 1436, respectively. The peaks averaged 15.35 hours after CT 0100 dissections, but only 7.6 hours after CT 0700 dissections. The peak times based on the predissection light schedule were nearly the same (difference = 0.75 hours; $t = 0.7$; $p > 0.4$), but the intervals between dissection and peak were widely different (difference = 7.75 hours; $t = 6.9$; $p \ll 0.001$). However, at a different time of dissection, CT 1900, for example, both the time of R15 peak and the interval between dissection and peak are significantly different from those of CT 0100 dissections (time of peak: difference = 10.25 hours; $t = 8.0$; $p \ll 0.001$; interval: difference = 5.25 hours; $t = 4.1$; $p < 0.001$). Thus the peak spiking time can be adequately predicted only by taking both dissection time and lighting schedule into account.

It should be pointed out here that, since more than half of the data was taken using extracellular recording, artifacts due to impalement damage cannot explain the results obtained. Nor is the trend of later peak times following later dissections (after CT 1000; see Fig. 4) merely a trivial consequence of peaks occurring a constant time after dissection. The slope of the regression fit to the points between dissections at CT 1300 and CT 2200 is not one; rather, it is about 1.4. There is, then, a greater delay in peak time than there is in dissection time in this region of the graph. The slopes at other parts of the graph, of course, are generally much less than one, due to the flat region generated by dissections at CT 0100 to CT 0700.

Discussion

a) Interpretation of past data

Since the past reports make little mention of the time of dissection, it is impossible to tell with certainty whether some or most of the R15 rhythms reported would agree with the dissection time - peak time curve shown in Figs. 1 and 4. However, the graphs of spiking activity included in some of the previous papers do show the entraining LD schedule and the time of impalement of R15 with the intracellular microelectrode. If we assume that the experiments were performed without interruption, then the impalement of the cell should have followed dissection rather quickly. Granted this assumption, we can then examine some of the past experiments in the light of the present data.

Strumwasser (1965) shows activity graphs of three R15s from animals previously exposed to 9, 3, and 2 days of entrainment, respectively (see Fig. 1 of the introduction to circadian rhythms in Aplysia in Part I of this dissertation for one of these graphs). In all of these experiments, impalement occurred between about CT 1330 and CT 1700, that is, 1.5 to 5 hours after dusk. Allowing one to two hours between dissection and impalement, the dissections would have taken place CT 1200 to CT 1600. Fig. 1 or 4 would predict R15 peaks at about CT 1800 to CT 0200. The actual peaks in these graphs (Strumwasser, 1965) occurred approximately at CT 0130, CT 2330, and CT 0000. These are entirely in the last half of, but within the range of, the prediction.

Turning to Lickey (1969), we again find three R15 activity graphs for LD 12:12 entrained Aplysia (one of these graphs is also reproduced in Fig. 1 of the introduction to circadian rhythms in Aplysia). Impalements ranged from CT 1300 to CT 1700, implying dissections from about CT 1100 to CT 1600. Figure 1 predictions would call for peaks between about CT 1700 and CT 0200. Actual peaks were CT 0300, CT 2200, and CT 0500. These peaks are slightly later than the prediction. Strumwasser ran his experiments at about 11.5°C., and Lickey at 12 to 13°C., as compared to the 15°C. for the experiments of Fig. 1. A slightly longer period of the rhythm at the colder temperatures could easily account for the later peaks.

It was mentioned that no seasonal effect on the timing of the peak of R15 activity was observed in the present experiments, at least with CT 0100 dissections. In fact, the difference in peak times (42 minute average) was in the opposite direction to that found by Lickey (1969). In all of the Lickey experiments, several differently phased LD 12:12 cycles were used, so that animals taken for different experimental runs were exposed to different times of light onset relative to the solar day. It is possible that both the seasonal effect and the variation in peak timing seen in those experiments were due to an interaction of the timing of the lighting schedules and the experimenters' own work schedules.

It is of interest to note that the three published spiking records of R15s taken from Aplysia kept in constant light prior to dissection do not fit into the dissection time - peak time curve. The peaks

occurred about 21 hours (Lickey, 1969) and no more than two to three hours (Strumwasser, 1965, Figs. 5 and 6) after dissection, with the latter two having the appearance of the dissection having occurred during an ongoing peak which continued after impalement. No peaks in the present experiments (Fig. 2) occurred either this long after or this soon after dissection. This indicates that the dissection time - peak time relation depends on the presence of the LD cycle, and that it is not the dissection per se, but the time of dissection within the LD cycle, that produces the relation.

b) Relation between peak time and dissection time

The circadian rhythms of almost every organism free running in constant darkness can be phase shifted by a short (minutes to a few hours) pulse of light. The amount and direction of the phase shift is dependent upon the phase of the free running rhythm at which the light pulse is applied. The resulting curve of phase shift vs. phase pulsed invariably assumes roughly the same shape for all organisms. Only small changes in phase result from light pulses applied during subjective day. The largest changes are seen for pulses given during the subjective night, with delays (activity peak or onset occurs later) induced early in the subjective night, and advances induced late in the subjective night. A changeover from delays to advances occurs somewhere near the middle of the subjective night (Fig. 3). For a complete discussion of phase response curves, see the reviews by Pittendrigh (1965, 1974).

A comparison of the graphs of Figs. 1 and 3 shows that the timing

of the rhythm of R15 can be interpreted in an exactly analogous way. The time of dissection acts as a resetting event for the rhythm of the cell. If the dissections occur during the day, little change in the timing of the subsequent activity peaks results from a change in the time of dissection (CT 0100 to CT 0700). However, dissections late in the day and into the night result in peaks which are later (delayed) than those following day dissections. For dissections shortly after the middle of the night and continuing to dawn, the peaks are earlier than those of day dissected R15s. In Fig. 1, R15 activity peaks are grouped in 12 hour blocks before and after projected dawn. In a heavily damped oscillator such as R15 (at least in vitro), properly timed peaks seldom occur after more than one day in vitro, and, if present, are of very low amplitude by the second day. This makes it essentially impossible to distinguish between delays early in the night and advances later in the night on the one hand, and continued delays throughout the night until the rhythm catches up with the day dissection peak time on the "next" day. Fig. 4 more clearly illustrates this point.

Nevertheless, the resemblance of the R15 dissection time - peak time curve to the phase response curves of other rhythms is striking, and requires some attempt at explanation. It has previously been shown that the rhythm of R15 is entrained by light-dark cycles via the eyes, and that the eyes release a substance which affects the activity of the neuron. Several other inputs to the cell exist as well, which have not as yet been related to its rhythmicity. If the rhythm of the

cell is entrained by the eye factor, then the phase of the R15 rhythm would depend on the time and perhaps amount of factor release. In this interpretation, at any given dissection time, the phase at which the R15 rhythm will be found will be determined by the point which has been reached in the eye release cycle and in the reaction of the neuron to the factor. Other inputs may play a role in phase setting in the intact animal, but the phase response curve can be essentially duplicated in vitro with only the eyes and PVG present. Therefore it would appear that the interaction of substances released by the eye, the endogenous rhythm, and inputs to R15 from within the PVG are sufficient for phase setting.

There are several practical consequences of this dissection time - peak time relation. Any attempt to test the effects of various experimental manipulations, such as blinding, on the entrainment of the R15 rhythm also tests for the effects of dissection time. The experiments on the photoentrainment of the R15 rhythm in vivo reported in this dissertation were fortuitously performed in the flat region of the curve. At any other time, in the absence of detailed knowledge of the curve, constant solar dissection time superimposed upon varying phase of light schedule would have rendered the results uninterpretable. Uncontrolled dissection time would, of course, lead to a wide scatter of peaks.

Isolating the factors occurring during a dissection which reset the phase of R15 would appear to be almost a hopeless task. If the eye factor can indeed entrain and phase shift the R15 rhythm, and if

it can be isolated and purified, however, many in vitro experiments would become possible which might be able to clarify this matter. For example, the range of possiblities can be narrowed if it can be determined whether the hormone acts directly on R15, that is, can both the short term and phase setting effects of the factor be exerted in a medium which synaptically isolates R15? If this is true, then a detailed analysis of the events behind the phase response curve presented here can be undertaken. If successful, this analysis may also give insights into the mechanisms underlying the phase response curves of other circadian rhythms.

Table I

Date	Dissection Time	Peak Time
06/11/73	0100	-1630
06/13/73	"	-1830
06/15/73	"	-1430
06/26/73	"	-1330
07/04/73	"	-1200
01/17/74	"	-1700
01/29/74	"	-1730
02/11/74	"	-1030
02/14/74	"	-1700
02/16/74	"	-1630
06/24/73	0700	-1330
06/28/73	"	-1600
06/30/73	"	-1330
07/15/73	"	-1400
07/17/73	"	-1700
07/29/73	"	-1230
08/02/73	"	-1330
08/07/73	"	-1600
08/08/73	"	-1900
08/09/73	"	-1100
07/19/74	1000	-1700
07/20/74	"	-1630
08/13/74	"	-2200
07/12/74	1300	-2200
07/13/74	"	-1830
07/15/74	"	-2130
07/25/74	1600	-2200
08/17/74	"	+0300
08/18/74	"	-2000
06/01/74	1900	+0600
06/02/74	"	+0500
06/19/74	"	+0430
06/20/74	"	+0300
06/21/74	"	+0700
07/17/74	2200	+1100
07/23/74	"	+0700
08/15/74	"	+0530

Figure 1. Peak spiking activity of R15 in the isolated PVG as a function of the time of dissection within the light-dark cycle. The dark bars represent the dark portion of the LD cycle (projected dark for the peak time axis). Multiple triangles indicate several R15s peaking at the same time. Note that the early day dissection points are repeated to emphasize the near zero slope during this time.

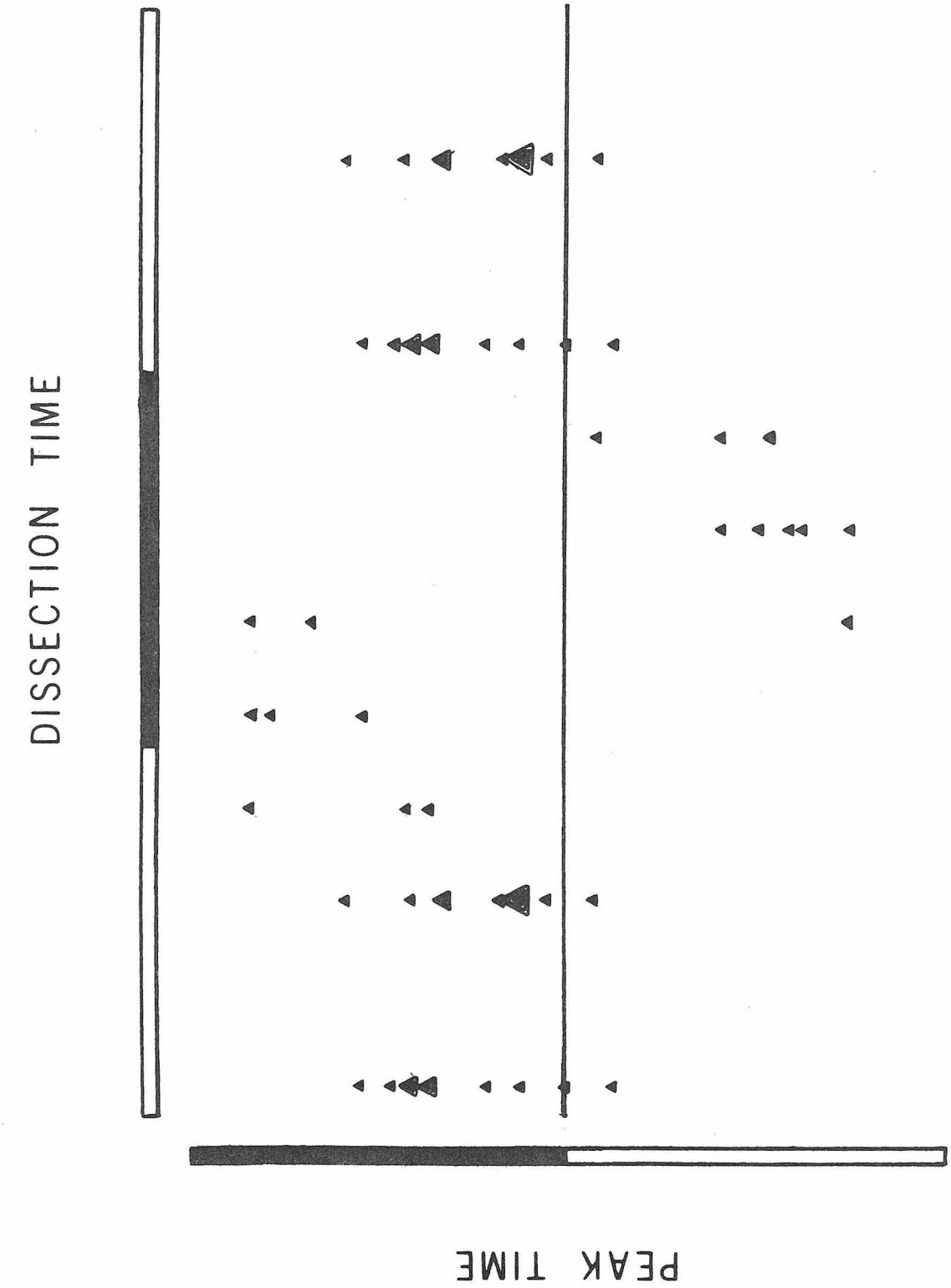

DISSECTION TIME
PEAK TIME

Figure 2. Interval between dissection and time of peak spiking activity of R15 in the isolated PVG as a function of the time of dissection. Conventions as in Fig. 1.

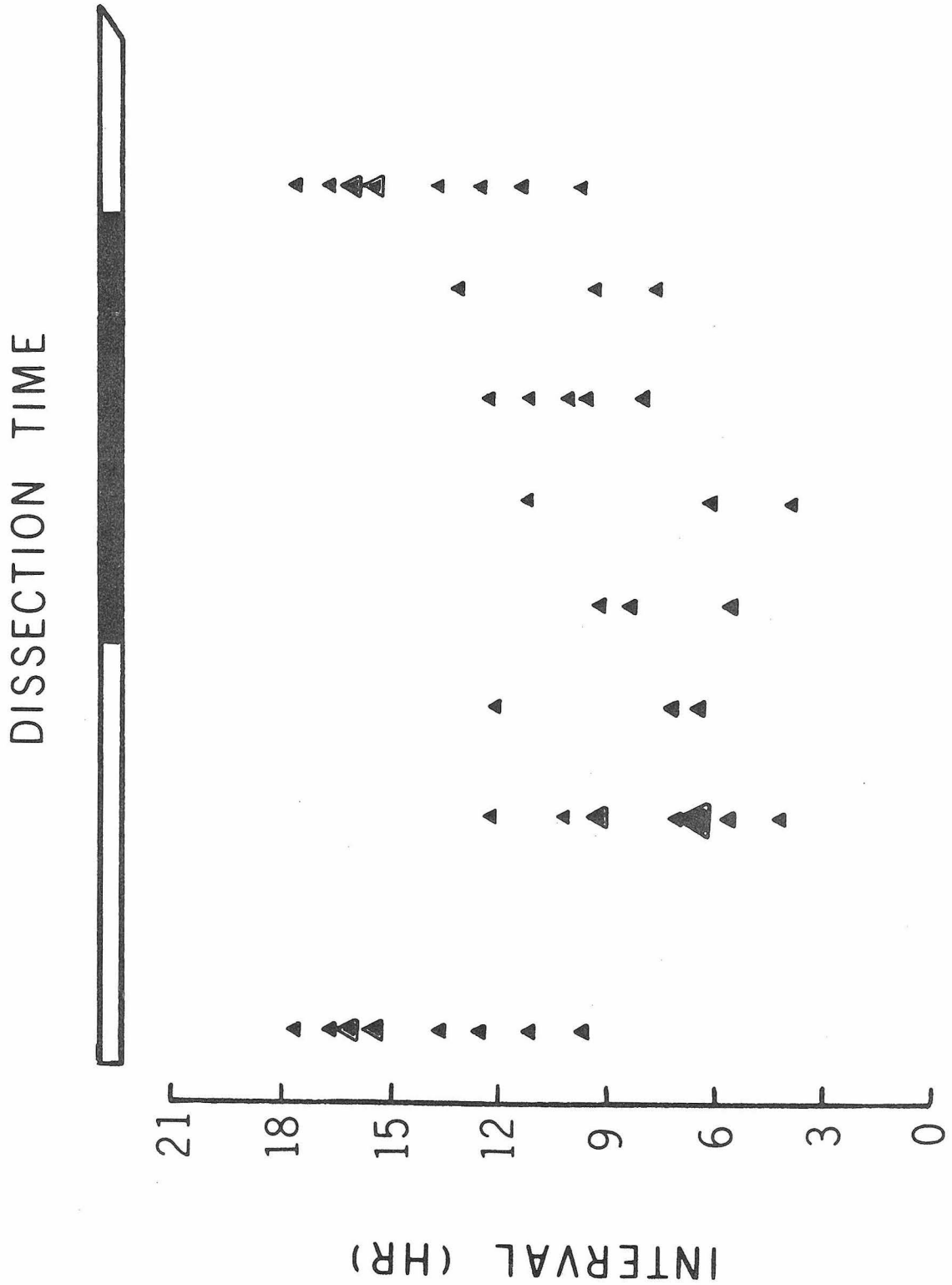

DISSECTION TIME
21
18
15
12
9
6
3
0
INTERVAL (HR)

Figure 3. Phase response curves for circadian rhythms of several organisms. The animals are kept in constant conditions and in darkness, and short (minutes to a few hours) pulses of light are presented at different times in the free running activity cycle. Direction and magnitude of the resulting steady state phase shift is a function of the phase pulsed. From Pittendrigh, 1965; compiled by him from data from the authors shown on the graphs.

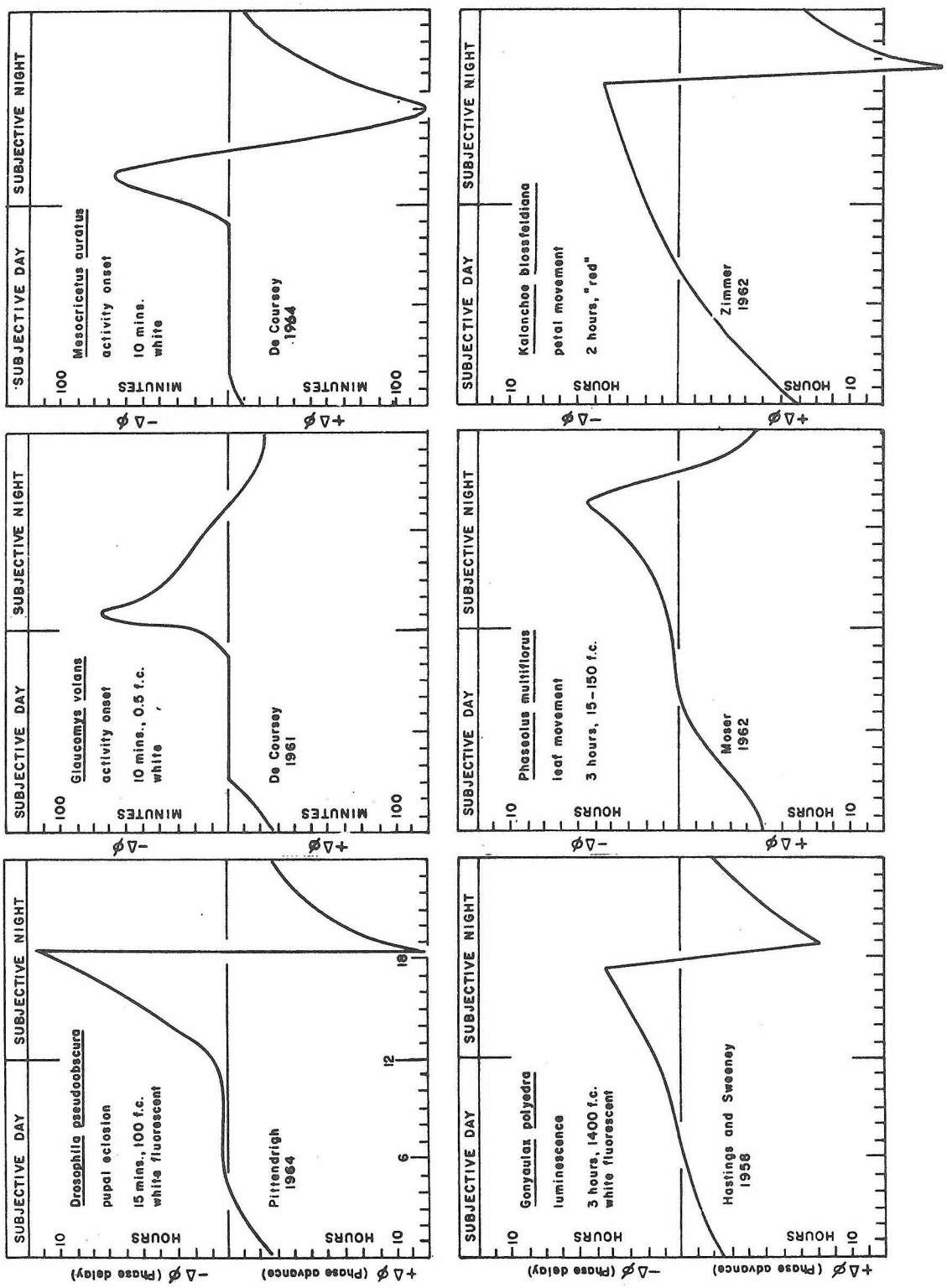
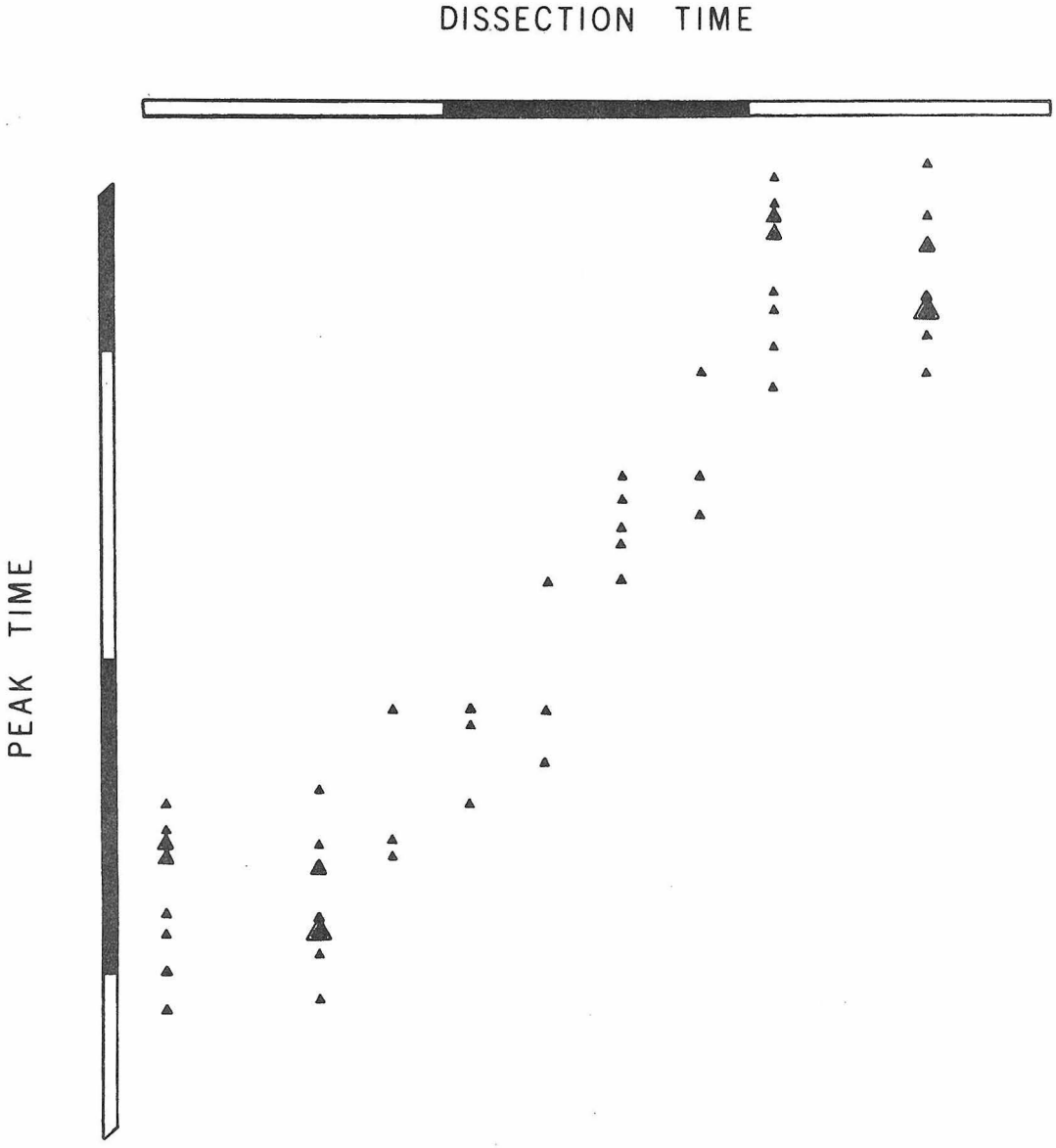

SUBJECTIVE DAY
SUBJECTIVE NIGHT
Drosophila pseudoobscura
pupal eclosion
15 mins., 100 f.c.
white fluorescent
Pittendrigh
1964
Glaucomys volans
activity onset
10 mins., 0.5 f.c.
white
De Coursey
1961
Mesocricetus auratus
activity onset
10 mins.
white
De Coursey
1964
Phaseolus multiflorus
leaf movement
3 hours, 15-150 f.c.
Moser
1962
Kalanchoe blossfeldiana
petal movement
2 hours, "red"
Zimmer
1962
Gonyaulax polyedra
luminescence
3 hours, 1400 f.c.
white fluorescent
Hastings and Sweeney
1958
HOURS
MINUTES
-Δφ (Phase delay)
+Δφ (Phase advance)

Figure 4. Peak spiking activity of R15 in the isolated PVG as a function of the time of dissection. Instead of restricting the ordinate to one 24 hour cycle starting at dawn, the ordinate has been extended to show the "constant delay" interpretation which is an alternative to the "delay and advance" curve of Fig. 1. Again, note that the early day dissection points are repeated.


DISSECTION TIME
PEAK TIME

References

Lickey, M.E. 1969. Seasonal modulation and non-24 hour entrainment of a circadian rhythm in a single neuron. J. Comp. Physiol. Psychol. 68: 9-17.

Lickey, M.E., and S. Zack. 1973. Extraocular photoreceptors can entrain the circadian rhythm in the abdominal ganglion of Aplysia. J. Comp. Physiol. 84: 361-366.

Lickey, M.E., S. Zack, and P. Birrell. 1971. Some factors governing entrainment of a circadian rhythm in a single neuron. In: M. Menaker (ed.), Biochronometry. Washington, D.C.: National Academy of Sciences, pp. 549-564.

Pittendrigh, C. 1965. On the mechanism of the entrainment of a circadian rhythm by light cycles. In: J. Aschoff (ed.), Circadian Clocks. Amsterdam: North-Holland Publ. Co., pp. 277-297.

Pittendrigh, C. 1974. Circadian oscillations in cells and the circadian organization of multicellular systems. In: F.O. Schmitt and F.G. Worden (eds.), The Neurosciences: Third Study Program. Cambridge: MIT Press, pp. 437-458.

Strumwasser, F. 1965. The demonstration and manipulation of a circadian rhythm in a single neuron. In: J. Aschoff (ed.), Circadian Clocks. Amsterdam: North-Holland Publ. Co., pp. 442-462.

Strumwasser, F. 1967. Neurophysiological aspects of rhythms. In: G.C. Quarton, T. Melnechuk, and F.O. Schmitt (eds.), The Neurosciences: An Intensive Study Program. New York: Rockefeller Univ. Press, pp. 516-528.