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PHASE SHIFTING THE CIRCADIAN RHYTHM FROM THE APLYSIA EYE: INVOLVEMENT OF 5-HT AND CYCLIC AMP

Rice University

Ph.D. 1980

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PHASE SHIFTING THE CIRCADIAN RHYTHM FROM THE
APLYSIA EYE: INVOLVEMENT OF 5-HT AND CYCLIC AMP

by

GEORGE CORRENT

A THESIS SUBMITTED
IN PARTIAL FULFILLMENT OF THE
REQUIREMENTS FOR THE DEGREE OF

DOCTOR OF PHILOSOPHY

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ABSTRACT

THE CIRCADIAN RHYTHM FROM THE APLYSIA EYE:
IN Volvement of 5-HT AND CYCLIC AMP IN PHASE SHIFTING

by George Corrent

The circadian rhythm (CR) of compound optic nerve potentials (CAPs) from the isolated eye of Aplysia californica can be phase shifted by 5-HT (serotonin). Data are presented in this thesis which suggest that 5-HT acts as a transmitter of temporal information to the circadian pacemaker (CP) in the Aplysia eye. Six h. 5-HT treatments produce both advance and delay phase shifts, are effective at low concentrations (10^{-7} M), and alter both the spontaneous activity of the eye and the response of the eye to light. In addition, evidence is presented that the eye contains 5-HT (50ng/mg protein), can synthesis 5-HT from tryptophan, and that the 5-HT stored in the eye can be depleted by Hi-K^+ (depolarizing) treatments. The effects of 5-HT on the eye also show a degree of stereospecificity since three structurally related indoleamines, LSD, Br-LSD, and bufotenine acted as agonists of 5-HT when added to the eye, while other amines (5-hydroxytryptophan, tryptamine, 7-methyltryptamine, and others) and two putative neurotransmitters (Dopamine, Acetylcholine) did not mimic the effects of 5-HT. These results suggest that 5-HT may act as a neurotransmitter in the Aplysia eye.

5-HT appears to be producing its effects on the CR by acting either directly on the CR cell(s), or on cells electrotonically coupled to the CR cell(s), since treatments which block transmitter release (HiMg^{2+}-LoCa^{2+}-EGTA) do not inhibit phase shifting by 5-HT.

The phase shifting effects of 5-HT may be mediated by changes in
cellular cAMP, since 8-benzylthio-cAMP (2 x 10^{-3} M), a cAMP analog, mimics 5-HT by producing both advance and delay phase shifts when given during the same phases as 5-HT. Papaverine (2 x 10^{-4} M), which is a phosphodiesterase inhibitor that has been shown to increase cAMP levels in Aplysia tissue, also mimicked the phase shifting effects of 5-HT. IBMX (5 x 10^{-4} M), another phosphodiesterase inhibitor, did not produce significant phase shifts when given alone, but did potentiate the phase shifting effects of subthreshold doses of 5-HT (10^{-8} M).

The results presented in this thesis are significant because 5-HT is the first endogenous substance shown to produce phase shifts in Aplysia and one of the few natural substances known to produce effects on the CR from any system. The data suggest that 5-HT may be part of a pathway which transmits entrainment information to the CP cell(s) in the eye, and that the effects of 5-HT may be mediated by changing levels of cAMP. 5-HT may provide a natural marker for investigating entrainment pathways and mechanisms in the Aplysia eye.
ACKNOWLEDGEMENTS

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INTRODUCTION

Circadian rhythms (CRs) are repetitive biochemical, physiological and behavioral cycles of about 24 h. duration. These rhythms, which appear to be ubiquitous among eucaryotic organisms, are driven by endogenous time keeping mechanisms variously called circadian oscillators (CO), circadian pacemakers, or circadian "clocks." Under constant conditions CRs are observed to "free-run" or oscillate with a period (free running period or FRP) which is characteristic of both the species and the individual and is usually slightly greater or less than 24 h. CRs can be entrained to external cycles which have periods which are not grossly different from the FRP. Although light-dark cycles appear to be the universal entraining agent, it has been shown that certain CRs can be entrained by temperature, food, pressure, or specific sounds (Bruce, 1960; Sweeny and Hastings, 1960; Menaker and Eskin, 1966; Hayden and Lindberg, 1969). The fact that the FRP is not identical to the period of natural external cycles, but is dependent on the genetic background is strong evidence for the existence of an endogenous time-keeping mechanism.

Another important characteristic of circadian systems is that while CRs can be entrained by temperature cycles, the FRP is relatively stable over a wide range of temperature and CRs are considered to be temperature compensated. The $Q_{10}$s of CRs over physiological temperature ranges are typically close to 1 and may even be less than 1. Although the degree of homeostatic control demonstrated by temperature compensation is essential for maintaining a reliable temporal organization in a variable environment, the homeostasis of circadian systems does not
appear to be a significant barrier to the biochemical manipulation of CRs, as was once feared. In the past ten to twelve years the use of drugs to alter the phase or period of CRs has become a common method for investigating circadian systems (for review see Pittendrigh, 1974; Menaker, et al., 1978). It is important at this point to distinguish between manipulation of the processes "driven" by the CO and changes in the timing of the CO itself. Since it is often possible to alter the output of the clock while having no effect on the clock mechanism itself, only changes in the FRP or the phase of the CR are accepted as evidence that the CO, by direct or indirect means, has been affected.

At the present time, the physical entity(s) that is the circadian "clock" has not been identified. A considerable amount is known about the properties of CRs though, and CRs are being investigated by researchers in a number of different disciplines. Genetic dissection of circadian systems appears to be a particularly promising approach, and mutants differing from wild type in FRP, phase angle, and possibly temperature compensation have been reported (Konopka and Benzer, 1971; Feldman and Waser, 1971; Bruce, 1972; Feldman and Atkinson, 1978). It may turn out, and much of the available data is consistent with this, that CRs are dependent upon and controlled by interactions among many systems within a cell. It is also quite possible that more than one system exists in nature for producing a CR.

The study of CRs is important not only as a study of a particular kind of temporal organization, although that is an excellent reason in itself, but also because it has broad implications for other fields of
physiology. Many physiological systems oscillate within a narrow range of periods, both longer and shorter than 24 h., and elucidating how circadian systems work may provide clues to understanding other oscillatory systems. Circadian systems exert control and influence over a wide range of behavioral and physiological activities, and the study of circadian systems will thus contribute to the understanding of how diverse physiological systems are synchronized and controlled within individual organisms. Finally, information from several sensory systems can be recognized and transduced by circadian systems into meaningful temporal information. The study of how this process takes place in circadian systems may have general implications about how sensory information is integrated by other physiological systems.

The eye of the marine gastropod mollusk, Aplysia californica provides an excellent system for studying the reception of temporal information and the mechanisms involved in the translation of this information into changes in the timing (phase shifts) of the CR. The isolated eye produces a CR of compound optic nerve impulses (CAPs) and this rhythm can be recorded with little damping for over seven days (Jacklet, 1969; Eskin, 1971). The eye itself is comprised of about 5,000 cells, of 4-5 cell types, although the great majority of the cells appear to be receptor cells (Jacklet, et al., 1972). The eye has one neural pathway, the optic nerve, which connects the eye to the cerebral ganglion and contains both afferent and efferent neural pathways (Eskin, 1971).

Electrotonic synapses may be involved in the generation of the CR since treatments which are known to uncouple electrotonic junctions in other organisms produce phase shifts when given as pulses to the
isolated eye (Eskin and Corrent, 1977b; Eskin, 1977). Since treatments which uncouple electrotonic junctions are known to produce other cellular effects, phase shifting by these agents may have other plausible explanations. Chemical synapses do not appear to be involved in the production or expression of the CR since the rhythm is expressed in solutions which block chemical transmission and treatments which block chemical synapses do not produce phase shifts (Jacket, 1973c; Eskin, 1977).

The eye contains not only an endogenous CO, but also a complete entrainment pathway, since the CR from the eye can be entrained to light:dark (L:D) cycles in vitro (Eskin, 1971). Under certain conditions, eye CRs will re-entrain to shifted L:D cycles in one day in vivo, but require several days to entrain in vitro (Eskin, 1971). Red light, which has little effect on neurally isolated eyes, can entrain the CR of intact eyes in vivo (Block, et al., 1974). The eyes themselves may act to mutually entrain each other weakly in vivo, and there is evidence that eyes which have intact optic-nerve connections with the cerebral ganglion are phase-shifted differently than isolated eyes (Hudson and Lickey, 1977; Lickey and Pritchard, 1979). These results suggest that information from extra-ocular sensory systems and COs in Aplysia may be involved in the in vivo entrainment of the CR from the eye. Since evidence exists for both ocular and extra-ocular information pathways to the CO in the eye, and many of the effects of these inputs can be studied in vitro, the eye provides an excellent system for the study of the information pathways and mechanisms involved in the entrainment of CRs.
Significant work on the ocular entrainment pathway has already been accomplished. Application of 8 putative neurotransmitters to isolated eyes did not mimic the effects of light on the eye or produce phase shifts at the phase tested. However, one of the putative neurotransmitters, 5-HT (serotonin), did cause some changes in the waveform of the CR, although it produced no significant phase shifts when given during the phase of the rhythm at which light pulses produced large phase shifts (Eskin, 1977). By utilizing drugs and changes in the ion concentrations of the external media to block chemical synapses and regenerative potentials, it was determined that neither chemical transmission nor action potentials were required for transmission of light information to the CO in the eye (Eskin, 1977). From these and other investigations of the circadian system in the eye, models have been proposed for the location of the CO (Audersirk, 1973; Jacklet, 1973a; Eskin, 1977). The models which seem to be best supported by available evidence place the CO either in the receptor cells, or in secondary cells which receive light information from the receptor cells via electrotonic junctions (Fig. 1).

Although the ocular pathway has been investigated, less is known about the pathways by which extra-ocular temporal information reaches the CO of the eye. An intact optic nerve connection with the cerebral ganglion is necessary for phase shifting the eye CR by red light and by LL-DD transitions, under some conditions (Hudson and Lickey, 1977; Lickey and Pritchard, 1979). It is also possible that information could be transmitted to the eye CO via a humoral pathway, as several small blood vessels have been observed to terminate near the eye.
(Eskin, unpublished results). For the most part, however, the means by which temporal information from extra-ocular sources (or the contra-lateral eye) is transmitted to the CO in the eye is unknown.

This thesis reports the finding that 5-HT, a putative neurotransmitter in Aplysia, phase shifts the CR from the eye. This result may represent a key step in an entrainment pathway, and an important beginning in elucidating the physiological processes involved in phase shifting. 5-HT is also the first putative neurotransmitter (and represents one of the first unequivocal demonstrations of any natural messenger) to produce phase shifts in a CR. Since chemical synapses are unlikely to be involved in a strictly ocular entrainment pathway (although this has not been ruled out), it appears likely that 5-HT transmits temporal information from extraocular sources to the CO in the eye. Numerous potential sources for such extra-ocular information exist in Aplysia, and include the contra-lateral eye, extra-ocular COs in various ganglia and extra-ocular sensory organs (Fig. 1).

This thesis examined two hypotheses about the role of 5-HT in the Aplysia eye: First, 5-HT may act as a neurotransmitter in the Aplysia eye. Second, 5-HT may be involved in a pathway by which temporal information reaches the CO in the eye.

The first hypothesis represents a classical neurobiological study while the second hypothesis has important implications for neurophysiology in general, and entrainment of CRs in particular.
MATERIALS AND METHODS

I. Animals and Maintenance

*Aplysia californica* weighing from 100 to 300 g were obtained from Pacific Biomarine Laboratories Inc., Venice, California. The animals were kept in 100 gallon sea water tanks at 15°C, and were fed three times a week on fresh or dried algae (Pacific BioMarine).

Artificial sea water for the tanks and for control solutions was made from Instant Ocean and allowed to sit at least three days before use. The *Aplysia* were entrained to a 12:12 light:dark (L:D) cycle and were exposed to at least three complete cycles before being used in experiments. Circadian time 00 (CT00) corresponds to dawn, or lights on for the intact animals. For isolated eyes, circadian time corresponds to the projected portion of the L:D cycle to which the eyes would have been exposed if they had remained in the tanks.

Eyes with attached optic nerves (O.N.) were dissected from *Aplysia* after making a ventral incision and pinning the animal out flat in a wax dish filled with seawater (15°C). All dissections were performed between CT 08-12. Isolated eyes were rinsed two times in Instant Ocean which had been filtered through a .22u millipore filter (FSW). The cut end of the O.N. was then sucked into a "J" shaped piece of polyethylene (PE) tubing (PE 20, Clay-Adams) set in a layer of Sylgard (Dow Corning) approximately one cm deep, in the bottom of a no. 7 PE stopper (Mallinkrodt). This eye cup was filled with FSW to which had been added 30 mM Hepes buffer (Sigma) and 100 units/ml penicillin-streptomycin mixture (Microbiological Associates), and buffered to pH 7.8 at 15°C (BFSW). The cups were then placed in a temperature
controlled water bath (15° ± .2°C), in a light-tight box. The temperature of the BFSW in the cups was monitored with a vinyl-polymer covered temperature probe (Yellow Springs Instruments). Compound optic nerve potentials (CAPs) were recorded from the end of the O.N. by placing a platinum wire coated with platinum black into the open end of the J-tube. To record differentially, a second electrode, identical to the recording electrode, was placed in the BFSW in the eye cup. A platinum wire connected to ground was also placed in each cup. A Grass Polygraph recorder was used to record electrical activity from the eye. Solutions were changed through PE 40 tubing which was set into the Sylgard in the cups and was connected to syringes outside the box. The bathing solution was first removed, and the sea water solution with the dissolved drug was immediately added. Drug effects were terminated with five exchanges of FSW and two exchanges of BFSW over a one hour period. Experimental solutions and rinses were kept cold (9-13°C) until added in order to minimize temperature variations. All wires and tubes which passed into the box from outside entered the box through light-tight fittings.

Phase shifts of eyes were calculated by taking the difference between the time of peak CAP activity of the experimental eye and the time of peak CAP activity of a control eye from the same animal. The CRs of two eyes from the same animal recorded under identical conditions have been observed to differ by only 2 ± 50 minutes (S.D., N=6) over four cycles (Rothman and Strumwasser, 1976). Phase shifts of 1 h. or less were generally not considered to be significant. Some treatments consistently produced small advance phase shifts of 0.5 to 1.5 h., and
these are noted in the text of the Results section. Except where otherwise noted, phase shift measurements were taken during the second full cycle after treatment to allow for complete expression of the effects of treatments on the CR. CAPs were counted and recorded for each hour.

II. The drugs utilized in the experiments of this thesis are listed below, and are preceded by the company from which they were purchased.

Sigma Chemical: 5-HT creatinine sulfate; Tryptamine HCl; L-Tryptophan; 5-hydroxy-L-tryptophan; dopamine (3-Hydroxytyramine HCl); d-Tubocurarine-chloride (dTC); adenosine 3':5' - cyclic monophosphoric acid (cAMP); papaverine HCl; 3-Isobutyl-1-methylxanthine (IBMX); 5-Hydroxyindole 3-acetic acid (5HIAA); Nicotine-sulfate; Atropine-sulfate; Ethyleneglycol-bis-(β-aminoethyl ether) N, N'-tetraacetic acid (EGTA); Tris (hydroxy-methyl) aminomethane (TRIS); N^6, O^2'-dibutyryl adenosine 3':5' - cyclic monophosphoric acid (db-cAMP); L-Ascorbic acid-sodium salt; Creatinine sulfate; 7-methyl tryptamine.

ICN Biochemicals: 8-(benzylthio) Adenosine 3'-5' cyclic monophosphoric acid (8-bt-cAMP).

Aldrich: 5-methoxygramine.

We wish to thank the following companies for the gifts of experimental drugs: Sandoz, Inc.: Methysergide maleate (UML)

E.R. Squibb and Sons: Cinanserin-HCl

Eli Lilly Company: Fluoxetine-HCl

Bufotenine, lysergic acid diethylamide tartrate, and bromo-lysergic acid diethylamide were obtained through the National Institute on Drug Abuse; Alcohol, Drug Abuse, and Mental Health Administration, Rockville,
Maryland.

III. Artificial sea water solutions were made up according to the following table.

<table>
<thead>
<tr>
<th></th>
<th>NaCl</th>
<th>KCl</th>
<th>CaCl₂</th>
<th>MgCl₂</th>
<th>Na₂SO₄</th>
<th>Hepes</th>
<th>Other</th>
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</thead>
<tbody>
<tr>
<td>High K⁺–Low Na⁺</td>
<td>7</td>
<td>150</td>
<td>10</td>
<td>50</td>
<td>0</td>
<td>0</td>
<td>Tris 320</td>
</tr>
<tr>
<td>Lok⁺</td>
<td>427</td>
<td>1</td>
<td>10</td>
<td>50</td>
<td>28</td>
<td>30</td>
<td>–</td>
</tr>
<tr>
<td>Low Na⁺–Low Cl⁻</td>
<td>7</td>
<td>10</td>
<td>10</td>
<td>125</td>
<td>0</td>
<td>30</td>
<td>Mannitol 527mm</td>
</tr>
<tr>
<td>High Mg²⁺–Low Ca²⁺</td>
<td>320</td>
<td>10</td>
<td>0.1</td>
<td>125</td>
<td>28</td>
<td>30</td>
<td>–</td>
</tr>
<tr>
<td>High Mg²⁺–Low Ca²⁺</td>
<td>210</td>
<td>10</td>
<td>0.1</td>
<td>200</td>
<td>28</td>
<td>30</td>
<td>–</td>
</tr>
<tr>
<td>Low Ca²⁺–EGTA</td>
<td>412</td>
<td>10</td>
<td>2.0</td>
<td>50</td>
<td>28</td>
<td>30</td>
<td>EGTA 10mm</td>
</tr>
</tbody>
</table>

Solutions were made to approximate, within ± 40 milliosmoles, the osmolarity of a standard FSW solution. The osmolarity of the solutions was measured on a model 31 freezing point depression osmometer (Advanced Instruments Inc.). One hundred units/ml of penicillin-streptomycin (Microbiological Associates) was added to all solutions. Ascorbic acid (1mM) was added to dopamine solutions and occasionally to 5-HT solutions to reduce spontaneous oxidation. Drugs, except as noted below, were dissolved directly in BFSW. Bufotenine (free base) was first dissolved in a small volume of dimethyl-sulfoxide (DMSO) prior to adding to BFSW. Final concentration of DMSO was .01% for these solutions. DMSO concentrations as high as .1% had no effect on the eye when applied at CT 05-11. Previous control experiments with DMSO at other phases also showed a lack of effect in concentrations as high as .2% (Eskin and Corrent, 1977a).
IV. ERGs. Electroretinograms (ERGs) were recorded using platinum wire microelectrodes with tip resistances from 200-400 kΩ (measured DC). The electrodes were coated with either glass or with Epoxy-lite. Glass covered electrodes were made by sealing thin walled glass capillary tubing (Drummond Scientific Company) to the platinum wire under low heat. This tubing was chosen because it had a coefficient of expansion similar to that of the platinum wire. The exposed end of the electrode wire was then etched to the desired taper and tip diameter by running 0.1-0.4 amps (AC) through the electrode while dipping it in 8M NaCN/1.5M NaOH solution. Epoxy-lite covered electrodes were etched first and then covered with epoxy-lite and baked for approximately 30 minutes in an oven in gradually increasing temperatures from 70-130°. Electrode tips of both types of electrodes were coated with platinum black (5% platinum chloride solution, H.H. Thomas Co.) prior to use. For recording from isolated eyes, the electrodes were held in Brinkman micro-manipulators and the tips were inserted into eyes tightly held in the previously described J-tubes. A microelectrode with a similar tip resistance was used as an indifferent electrode for differential recording. ERGs were recorded through the normal AC preamplifier inputs of a Grass recorder. Recordings were frequency filtered above 35 hz and below 0.3 hz. The ERG was measured as the height of the first wave following the onset of the light pulse. Light pulses were 6.5 s. in length and were repeated every 8 minutes. The light source was a 6.5 volt DC microscope lamp (Bausch-Lomb) suspended 50 cm above the eyes. Eyes were dark adapted for 1 to 2 h. before light pulses were initiated. No
changes in solution temperature were recorded during the periods of
the light pulses. This is particularly important since temperature
has been shown to affect ERG amplitude (Jacklet, 1969b). After 5-HT
treatment, the response to light was considered to be increased if the
amplitude of the first wave of the ERG was greater than pre-treatment
levels for the three light pulses (24 min.) after treatment. Changes
in ERG amplitude due to control solution exchanges never lasted for
more than one light pulse (8 min.), so changes lasting 24 min. or more
after a treatment were considered to be due to the effects of the
treatment. 5-HT solutions were applied only after a stable ERG
amplitude (±5%) was obtained for at least three consecutive responses,
since in control eyes this indicated that the ERG response to light
had reached a stable plateau. Changes in ERG amplitude of less than
6% were not considered to be significant, since spontaneous changes
less than or equal to 5% of the total ERG amplitude were commonly
measured.

Tonic light activity was recorded from the O.N.s of isolated eyes
with platinum wire electrodes in the same way that spontaneous CAP
activity was recorded. In experiments in which the tonic response
of the eye to light was recorded from the O.N., 5-HT was added to the
eyes 10-15 min. before the light was turned on.

V. 5-HT release. Dissected eyes with attached O.N.s were placed in
(15°C) eye dishes in a light tight box. After a 1 h. period to allow
the spontaneous eye activity to stabilize, eyes were treated with 2
changes of HiK⁺-LoNa⁺ solution during a 50 min. period. About 15-20
min. prior to treating the eyes with HiK⁺-LoNa⁺ solutions, a low Na⁺
solution was substituted for BFSW to insure that equilibration to LoNa\(^+\) had occurred throughout the tissue. When solutions which contained low Ca\(^{2+}\) were used, a low Ca\(^{2+}\) solution was always added 15-20 minutes prior to exchange with the experimental solution. After treatment, the eyes were rinsed for 1 h. with 4 changes of BFSW. The above 1 h. and 50 min. procedure was repeated 3 times for all solutions. After the third treatment, the eyes were rinsed and then removed from the light-tight box in dim light. The eyes and O.N.s were then severed and blotted dry, and placed in 1 ml capacity conical vials (Pierce) on dry ice. The vials were then placed in a -76\(^\circ\)C freezer until the 5-HT content of the eyes was assayed by gas chromatography-mass spectrometry (Corrent, et al., 1978).

VI. 5-HT measurements. Eyes were dissected from groups of 3-4 Aplysia, rinsed 2 times in FSW and severed from their O.N.s. The eyes were then blotted on paper towels to remove excess salt water and divided into groups, alternating right and left eyes from each animal, and placed in conical vials on dry ice. The eyes were then stored at -76\(^\circ\)C until assayed.

The 5-HT content of eyes was analysed by gas chromatography-mass spectrometry selected ion monitoring performed by Dr. D.J. McAdoo, Marine Biomedical Inst., Galveston, Tx. (see McAdoo and Coggeshall, 1976 and Corrent, et al., 1978 for details).

The 5-HT measurement for each group of three or four eyes was used as an independent result in calculating the between group variance. This number was then used to calculate the 95% confidence interval for the mean 5-HT measurement.
VII. **Synthesis.** Eyes were dissected from *Aplysia californica* during CT 08-12, placed in light-tight boxes, and treated with HIK⁺-LoNa⁺, as described in section V. These eyes were then removed in dim light and transferred to a size 0 PE stopper containing .4 ml of BFSW with .1% glucose and (10⁻⁵ M)³H labeled Tryptophan (18 Ci/mM, New England Nuclear). After incubating the eyes for varying amounts of time, eyes were removed, severed from their O.N.s and placed in 1 ml conical PE centrifuge tubes in .1 ml of 1 M formic acid in 50% EtOH and frozen at -26°C overnight. Before assay, the eyes were ground in a ground glass microhomogenizer over ice for 3-5 minutes, returned to the centrifuge tubes, and spun at 8,000xg for 1 minute. The supernatant was removed, .2 ml of fresh 1 N formic acid was added, and the eyes were shaken on a Vortex Genie Mixer for 1 minute and re-centrifuged. This process was repeated 3 times. The supernatant was dried down on a vacuum evaporator at 0-5°C and then resuspended in 1 ml (.2mM) formic acid with \(^{14}C\)-5-HT (10⁻⁸ Ci) and 2 ug of 5-HT (creatinine sulfate) as carrier. This was added dropwise onto a 6 x .4 cm column of Sephadex G-10 (Sigma) suspended in .5M formic acid. The column was formed by adding 1 g of Sephadex G-10 to 5 ml of .5 M formic acid (Boireau, et al., 1976). The Sephadex was allowed to swell for 15-30 minutes in the formic acid before pouring it into a pasteur pipette plugged with glass wool. The plugged pipette was pre-cleaned with 3 rinses of .5 M formic acid. After the sample was added, the column was rinsed with 9 x 1 ml of deionized-distilled H₂O. The 5-HT was then eluted with 4 x 1 ml of (5M) formic acid. The eluant was dried down and resuspended in .1 ml of 1M formic acid in 50%
ethanol and spotted on 3 x 57 cm strips of Whatmann 3 MM paper. The spots were dried under a stream of cool air, and 5 ul of a 5% Methyl Green solution was spotted as a marker. The strips were then placed in a Savant Hi-voltage electrophoresis tank, (Savant Instruments) and run in either pH 1.9 buffer (.47M formic acid: 1.5M glacial acetic acid) or pH 4.7 buffer (25 ml glacial acetic acid: 25ml pyridine:950ml H₂O). Sample strips run in pH 1.9 buffer were run at 2.5 Kv for 80-100 minutes. Samples run at pH 4.7 were run at 1.2 Kv for 180-200 minutes. Strips were then dried with cool air and examined under long and short wave U.V. light. Control strips, on which pure 5-HT and other amines had been spotted, were frequently sprayed with .1% Ninhydrin in acetone with (1mM) MnCl₂ and developed at 110° for 15 min. before being viewed under U.V. light (Singh, et al., 1978). In some experiments, 5-HT peaks from pH 1.9 electropherograms were cut out, eluted in 1M formic acid and 50% ethanol and respotted for a second run at pH 4.7. The peak of tritiated amine which ran exactly with authentic ¹⁴C-5HT (R.P.I.) through the Sephadex column and high voltage electrophoresis at 2 different pH's, was considered to be newly synthesized 5-HT (Fig. 6). In analyses run without ¹⁴C-5-HT, newly synthesized 5-HT was identified by U.V. flourescence and comparing the rf values to simultaneously run authentic 5-HT controls.

All strips, after examination under U.V. light, were counted on a Packard (model 7201) Radiochromatogram scanner (Packard Instruments). Selected strips from representative experiments were cut into 1 cm sections which were then burned in a Packard model 306 sample oxidizer and counted on a Packard Prias Liquid scintillation counter (courtesy
of Packard Instruments, Downers Grove, Illinois). Using this method, recovery of radioactivity spotted on electrophoresis strips was greater than 98%.

VIII. Statistics. The phase shifts produced by experimental solutions which were tested three times or less are expressed as the mean followed by the range of obtained results. Experiments run four or more times are expressed as the mean ± 95% confidence interval. Differences between the mean results of different treatments were compared using Student's "t" two-tailed distribution. For t values greater than the 95% confidence interval (p > .95) the hypothesis that no difference existed between the means (null hypothesis) was rejected. For p < .9 differences were not considered to be significant.

Depression of spontaneous CAP activity was calculated by dividing the number of CAPs during the hour following treatment by the CAPs/h. prior to treatment, and expressed as a percentage. A change in CAP frequency was considered to be significant only if the difference between the change in the treated eye was 20 percent greater than the change observed in the matched control eye during the same period, since such changes were observed to occur spontaneously in less than 5% of paired eyes during the time prior to treatment.

Correlations were assessed by calculating the Pearson product moment correlation coefficient (r).
RESULTS

I. 5-HT effects on the circadian rhythm (CR) of the Aplysia eye.

1) Phase Shifts

Treatment of isolated Aplysia eyes with the putative neurotransmitter, 5-HT, produced significant phase shifts in the CRs from these eyes. Six hour pulses of 5-HT (10^{-5} M) produced phase advances (+3.0 ± 0.4 h., N=14) in the CR of CAPs from the eyes, when administered during a late projected day phase (CT 05-11) (Fig. 2). 5-HT treatment also produced a rapid and marked depression in the CAP activity from the eye, with the CAP frequency exhibiting some recovery toward the end of a 6 h. pulse. These results suggested that 1) 5-HT is acting as a neurotransmitter in the Aplysia eye, and 2) 5-HT is involved in a pathway by which temporal information reaches the CO cells in the eyes.

A dose response curve was generated for 5-HT at CT 05-11 to find the most effective concentration of 5-HT for phase shifting, and to compare the range of concentrations of 5-HT which produced phase shifts in the Aplysia eye with concentrations of 5-HT effective in other "physiological" systems (Fig. 3). 5-HT produced 4 h. phase advances at concentrations as low as 10^{-7} M (Fig. 4). This compares favorably to concentrations of 5-HT which produced effects on other molluscan systems (Hidaka, et al., 1967; Trembley, et al., 1976; Brunelli, et al., 1976; Weiss, et al., 1978; Hill and Yantorno, 1979; Levitan and Drummond, 1979). The lower threshold for the response was between 10^{-7} and 10^{-8} M, since no phase shifts significantly different from controls were observed at 10^{-8} M (N=6). The abrupt increase (occurring over about
1 order of magnitude) from small effects to nearly maximum effects of 5-HT is characteristic of some cyclic nucleotide mediated systems (Berridge and Patel, 1968; Weiss, et al., 1979). At intermediate concentrations of 5-HT (10^{-3} to 10^{-6} M), the phase shifting response appeared to saturate, because there were no significant differences among the average phase shifts produced by any concentration of 5-HT within this range (Fig. 3). High concentrations of 5-HT (2 \times 10^{-3} M) did not produce phase advances of the CR (-0.9 \pm 1.4 h., N=4). It is possible that the reduced effects of high concentrations of 5-HT are due to desensitization of 5-HT receptors. Desensitization to high concentrations of 5-HT has been shown to occur in *Aplysia* and other invertebrate nerve tissue (Gershenfeld and Stephani, 1966; Tremblay, et al., 1976; Florey and Rathmayer, 1978).

2) **Controls**

Since 5-HT was used in the form of a creatinine sulfate complex, and often dissolved in solutions with ascorbic acid (1 mM) to reduce spontaneous oxidation of 5-HT, these two drugs were tested for their effects on *Aplysia* eyes. Neither (10^{-3} M) ascorbic acid (+0.2 h., range 0 to 0.5 h., N=3), nor 10^{-5} M creatinine sulfate (-0.1 \pm 0.6 h., N=4) had any effects on the CR from the eye when given during the phase (CT 05-11) at which 5-HT produced its largest phase shifts. Ascorbic acid did produce a slight increase in the spontaneous CAP frequency when added, while creatinine sulfate had no effects on CAP activity.

3) **Phase Response Curve (PRC)**

5-HT treatments (10^{-5} M) were given at different phases of the CR
to produce a phase response curve (PRC) (Fig. 5). The information from this curve aids both in finding the interval during which 5-HT produces a maximum effect on the CR and in comparing the effects of 5-HT with other treatments which produce phase shifts. Comparison of PRCs can be used to relate the effects of 5-HT on the CR to other treatments which produce similar PRCs. If, for example, 5-HT was being released on the CO by impulses in the efferent fibers of the optic nerve, then it might be expected that the PRC generated by 5-HT should be similar in form to a PRC generated by stimulation of efferent optic nerve fibers by the proper input. In general 6 h. 5-HT pulses produced advances when given during the projected day and delayed the rhythm when treatments were started during the projected night. The PRC generated by 5-HT treatments is shifted in relation to phase, but is similar in magnitude and shape to the PRC generated by 1 h. and 6 h. light pulses (Jacklet, 1974; Eskin, unpublished results). This suggests that 5-HT, like light pulses, is capable of being an entraining stimulus on the CO in the eye.

4) **The Duration of the 5-HT Pulse**

Eyes were treated with 5-HT pulses both longer and shorter than 6 h. to acquire information about the length of treatment required for maximum response. All pulses were administered during the late projected day. A preliminary 5-HT (10^{-5}M) treatment of 2 h. (CT 07-09) had no effect on the CR (0 h., N=1). 5-HT (10^{-5}M) treatments of 3.5 h. (CT 0500-0830) produced no significant phase shifts (-0.75 ± 0.25 h., N=4) and pulses (10^{-4} to 10^{-7}M) longer than 6 h. (8, 9, and 12 h. pulses started at CT 04 to 05) all produced phase shifts not significantly
different from the advances caused by 6 h. pulses (range, + 2.0 to + 3.0 h., N=6). These results suggest that there is a critical length of time, greater than 3.5 h., during which 5-HT must be applied to the eye to produce phase advances. However, results from preliminary experiments using "skeleton" 5-HT pulses, (2 pulses, 1.5 h. in length, separated by a three hour rinse period, CT 05-0630 and 0930-11) have indicated that absolute duration may not be as important as the phase of onset and offset of the 5-HT pulse. The CR of one of two eyes treated with skeleton 5-HT (5 x 10^{-7} M) pulses was advanced by 1.5 h. and 3.0 h., 2 and 3 cycles after the 5-HT pulses respectively. The second eye was not significantly phase shifted (+ 0.5 h.). The possibility that skeleton 5-HT pulses may phase shift is interesting because in all circadian systems investigated so far, the rhythm can be entrained by skeleton light pulses (Pittendrigh and Minis, 1964; Minis, 1965). Thus, the above data indicate that 5-HT shares many of the phase shifting properties of light, such as advance-delay PRCs and phase shifting by skeleton pulses.

II. **Neurotransmitter-like Aspects of 5-HT**

1) **5-HT storage, release and synthesis by the eye.**

The effects of 5-HT on the spontaneous activity of the eye and the CR suggested that this compound might perform a neurotransmitter or neurosecretory function in the eye. Since a prerequisite for demonstrating a transmitter role for 5-HT is to show that it is contained in the eye, isolated eyes and optic nerves were assayed for 5-HT content using gas chromatography-mass spectrometry (GC-MS) in collaboration with Dr. D. McAdoo (Corrent, et al., 1978).
Groups of 3 or 4 eyes or optic nerves were measured as a single sample (N = # samples). Eyes averaged $0.26 \pm 0.12$ ng 5-HT/eye (52 eyes, N=14) while optic nerves contained $0.23 \pm 0.09$ ng 5-HT/nerve (32 nerves, N=8). Since an *Aplysia* eye contains about 5 ug retinal protein, and optic nerves about 14 ug protein, the concentration of 5-HT in the eyes is about $52 \pm 0.08$ng/mg protein, and in the nerves, about $16.4 \pm 0.06$ ng/mg protein. This may be compared to the 58 ng 5-HT/mg protein which was reported for the *Aplysia* cerebral ganglion, a ganglion which contains two giant serotonin cells as well as other smaller serotonergic cells (McCaman, et al., 1973). Histological and biochemical evidence has been reported for the presence of dopamine in the eye, so isolated eyes were also assayed for dopamine (Luborsky-Moore and Jacklet, 1976b; Loh and Jacklet, 1977). Eyes contained $4.0 \pm 2.0$ ng dopamine/mg protein (30 eyes, N=8), while $1.8 \pm 0.8$ ng/mg protein (32 nerves, N=8) of dopamine was measured in optic nerves. Octopamine, another putative neurotransmitter found in *Aplysia*, was also investigated. Eyes contained $1.0 \pm 0.6$ ng/mg protein (14 eyes, N=4) octopamine.

A characteristic of neurotransmitters is that they are released when the terminals in which they are contained are depolarized or invaded by action potentials in the presence of external Ca$^{2+}$. The 5-HT in the eyes appeared to be present in a releasable form. In a preliminary experiment, eyes treated with raised K$^+$ solutions (10 x normal) with normal Na$^+$ concentration, had 20% less 5-HT than control eyes. Depolarizing the eye with raised K$^+$ (10 to 20 x normal) and lowering Na$^+$ to block transmitter re-uptake, depleted from 30 to 49%
of 5-HT from eyes, relative to matched controls (N=3). Treating eyes with fluoxetine (10^{-4} M), a blocker of 5-HT uptake in mammals, depleted 5-HT in two of three trials (range +15 to -42%). These results suggest that depolarizing stimuli may cause release of 5-HT from cells in the eye, although alternative possibilities have not been ruled out. More experiments must be performed to determine if the differences in the amounts of depletion produced by these solutions are significant, and to test if the release of 5-HT is Ca^{2+} dependent, since external Ca^{2+} is required for depolarization induced release of neurotransmitters or neurohormones.

Another criteria for confirming that a substance is a neurotransmitter is to show that the cells which release the neurotransmitter also synthesize it. Experiments were run to test if eye tissue could synthesize 5-HT from its precursor amino acid, tryptophan. Ten eyes were depleted of 5-HT with H\text{I}K^{+}-LoNa^{+}, and then incubated in 5 \times 10^{-5} M \text{^{3}H-tryptophan for 24 h. These eyes showed significant synthesis of} \text{^{3}H-5-HT (Fig. 6). An average of 50 pg} \text{^{3}H-5-HT/eye, or about 20% of the mean 5-HT content of Aplysia eyes, was measured in eyes incubated in} \text{^{3}H-tryptophan. In an earlier experiment, groups of 4 eyes were incubated in a lower concentration (10^{-5} M) tryptophan solution for 1, 4, or 22 h. The 22 h. eyes synthesized 22 pg} \text{^{3}H-5-HT/eye, or about 8.5% of their total 5-HT content. The eyes incubated for 4 h. still converted a measurable (9.0 pg} \text{^{3}H-5-HT/eye) quantity of} \text{^{3}H-tryptophan to} \text{^{3}H-5-HT, but less than the 22 h. eyes. Eyes incubated for one hour did not synthesize sufficient 5-HT to be detected (<2 pg/eye).}

In all higher organisms, 5-HT appears to be synthesized from
tryptophan by a specific enzymatic pathway involving two enzymes, tryptophan-hydroxylase and an aromatic amino acid decarboxylase. If 5-HT in *Aplysia* eyes is actively synthesized from $^3$H-tryptophan by this pathway, then inhibiting either of these enzymes should reduce the amount of newly synthesized 5-HT. Parachlorophenylalanine (p-CPA) has been shown to block 5-HT synthesis in gastropod mollusks as well as in a number of mammalian tissues (Osborne and Neuhoff, 1974; Sanders-Bush and Massari, 1977). Addition of $10^{-3}$ M p-CPA to the incubation media (22h.) blocked the synthesis of 5-HT to below measurable levels (< 2 pg 5-HT/eye). P-CPA also decreased the total amount of radio-activity recovered from the eyes, suggesting that it may also have interfered with $^3$H-tryptophan uptake in addition to blocking synthesis of 5-HT. The amounts of radioactive label recovered from p-CPA treated eyes ranged from 75 to 50% less than the $^3$H recovered from simultaneously run groups of eyes incubated in $^3$H-tryptophan without p-CPA (N=3).

About 7-9% of the radiolabeled material from "long" incubation eyes (22-24 h.), which eluted from the column with 5-HT, ran with authentic 5-HT during high voltage electrophoresis. This material was considered to be newly synthesized 5-HT. Several other radioactive peaks different from the tryptophan peak were observed on the electropherograms. Although these radioactive peaks were quite consistent from experiment to experiment, no attempt was made to identify these compounds.

2) **Effects on CAPs**

The physiological effects of 5-HT on the spontaneous and circadian
activity of the eye, and the synthesis, storage, and depolarization induced release of 5-HT by the eye, are all data consistent with the possibility of a transmitter role of 5-HT in the eye. If a classical membrane-bound receptor mediates the effects of 5-HT on the CR, the same receptor type might mediate the other 5-HT effects on the eye, such as depression of CAP activity, changes in the response of the eye to light, and changes in the CAP peaks of the CR. An attempt was made to correlate the concentrations and durations of 5-HT treatments which produce phase shifts with the conditions required to produce other 5-HT mediated effects. A positive correlation between the conditions required to produce two different effects would suggest that a common element was involved. Such a correlation could provide a starting point for investigating the cell types and cellular mechanisms involved in phase shifting by 5-HT.

5-HT treatments have previously been reported to increase the peak frequencies of CAPs during the CR for two or three circadian cycles after termination of 5-HT treatment (Eskin, 1977). This effect of 5-HT was also observed regularly (50-60% frequency) during the course of these experiments, but only after 5-HT treatments of $10^{-5}$M or greater. 5-HT concentrations below $10^{-5}$M elicited increased peak CAP frequencies in less than 27% of the treated eyes. Although the 5-HT treated eyes which exhibited amplified CAP peaks also frequently exhibited large phase shifts, there is only a slight positive correlation ($r = .28$) between increases in peak frequency and the magnitude of phase shifts produced by $10^{-5}$M 5-HT. Thus, increases in the amplitude of the CAP rhythm can account for only 8% of the size of the phase shifts. Even
this small correlation does not hold true for 5-HT treatments with concentrations less than $10^{-5}$ M. Increases in CAP peak frequency were occasionally noted after (5 x $10^{-7}$ M) 5-HT treatments (Fig. 7), but the phase shifts of these eyes were not significantly larger than the shifts of eyes which did not show this phenomenon ($p < .9$), and there was no significant correlation between the increases in peak CAP frequency and the size of the phase advance ($r = + .1$). At higher concentrations of 5-HT (2 x $10^{-3}$ M), three of four treated eyes showed 30-70% increases in peak CAP frequency, although none were significantly phase shifted.

Other indoles which phase shifted, bufotenine ($10^{-3}$ M) and to a lesser extent LSD ($10^{-4}$ M), also caused increases in the amplitude of the CAP rhythm after their removal (Fig. 8). However, in addition, these drugs produced phase shifts in eyes which did not exhibit amplified CAP peaks. Significant phase shifts have also been recorded from 5-HT treated eyes which expressed peak CAP frequencies which were decreased relative to controls. These observations indicate that increased peak CAP frequencies are not a direct cause of phase shifting the CR or vice versa.

In addition to its effects on CAP peak frequencies, 5-HT also depressed spontaneous CAP activity during treatment periods. The threshold for this effect was between $10^{-8}$ and $10^{-7}$ M, similar to the concentration threshold for phase shifting. Significant depression of CAP generation was observed in several seconds to 1 minute after treatment with high concentrations of 5-HT ($> 10^{-6}$ M). During treatment with low concentrations of 5-HT ($10^{-7}$ M) depression occurred more slowly,
and was obvious only 10 to 30 min. after the start of treatment.

Although the observation that 5-HT depresses CAP activity seems to disagree with previously published reports that 5-HT excites the eye (Luborsky-Moore and Jacklet, 1976a), depression of spontaneous CAP activity has been observed in this laboratory after all 5-HT treatments (\(> 10^{-8} \text{M}\)) on eyes in darkness (DD). This depressant effect of 5-HT is mimicked by bufotenine (\(10^{-3}\text{M}\)) and 8bt-cAMP (2 x \(10^{-3}\text{M}\)), and more weakly by tryptamine (5 x \(10^{-4}\text{M}\)), LSD (\(10^{-4}\text{M}\)) and papaverine (2 x \(10^{-4}\text{M}\)).

The magnitude of depression of CAP activity caused by these drugs is not correlated \((r = + .1)\) with phase shift magnitude. Nor do treatments (LoCa\(^2+\) - EGTA; IBMX) which increase CAP frequency, when applied during 5-HT treatments, block 5-HT induced phase shifts. Thus, the depression of CAPs is not required for phase shifting. The above observations indicate that while the receptors which mediate the phase shifting effects of 5-HT may be similar or even identical to the receptors which mediate the effects of 5-HT on CAPs, the pathways involved in the production of these effects are probably not related. The study of the CAP effects of 5-HT and its agonists would thus seem unlikely to yield any pertinent information about the mechanisms by which 5-HT produces phase shifts.

3) **Properties of 5-HT receptors in the eye**

The literature on the properties of cellular receptors which mediate 5-HT effects in mollusks, and specifically in *Aplysia*, is quite extensive. A large number of drugs have been found to be specific antagonists of 5-HT in certain systems but not in others (see Gerschenfeld, 1973; Ascher and Kehoe, 1975, for reviews). Six
drugs which have been reported to block 5-HT induced responses in Aplysia tissue were tested for their potency in inhibiting phase shifts produced by 5-HT (Table 1). Tryptamine, d-tubocurarine (d-TC), cinanserin, methysergide (UML), lysergic acid diethylamide (LSD) and bufotenine were all tested on isolated eyes both alone and in conjunction with 5-HT. All blocker treatments were applied during the late projected day phase (CT 05-11) at which 5-HT caused the largest advances. Tryptamine (5 x 10^{-5} to 5 x 10^{-4}M) depressed spontaneous CAP activity, although the effects were weaker than the depression caused by 5-HT (tryptamine, 44% depression, N=8; 5-HT, 5 x 10^{-5} to 10^{-4}M, 89% depression, N=10). Raising the concentration of tryptamine did not appear to enhance the depression of CAPs. Tryptamine (5 x 10^{-5} - 10^{-4}M) also consistently produced small (+ 0.75 ± .1 h., N=8) advances. This effect did not appear to be increased at higher concentrations. When applied together with 5-HT, tryptamine did not block 5-HT induced phase shifts, even at tryptamine concentrations 3 magnitudes greater than the 5-HT concentrations used. Eyes treated simultaneously with either tryptamine (2 x 10^{-4}M) plus 5 x 10^{-7}M 5-HT (+ 1.8 ± .4 h., N=6), or 5-HT (5 x 10^{-7}M) alone (+ 2.0 ± .3 h., N=6), showed equal phase advances (p < .8). Concentrations of tryptamine as high as 10^{-3} M did not inhibit 5-HT induced phase shifts.

D-Tubocurarine (d-TC), in addition to its well known blocking effect on acetylcholine receptors, has been reported to block the effects of 5-HT on Aplysia cells (Gerschenfeld and Paupardin-Tritsch, 1974). Alone, d-TC (10^{-3} - 10^{-4}M) had no effect on spontaneous CAPs or on the CR (N = 3, range + 0.5 to - 0.5h.). The phase shifts of eyes (N=5) treated with various concentrations (10^{-3} - 10^{-4}M) of d-TC before
and during 5-HT treatments ($10^{-5}$ & $5 \times 10^{-7}$M) were occasionally larger (+3.0 ± 1.6 h., N=5), but did not significantly differ ($p < .08$) from phase shifts produced in eyes treated simultaneously with 5-HT alone ($10^{-5}$M, +4.5 h., N=1, and $5 \times 10^{-7}$M, +2.25 h., range 2.0 - 2.5 h., N=2).

Methysergide-bimaleate (UML) has been shown to be an effective antagonist of 5-HT both in certain tissues in *Aplysia* as well as in a variety of other invertebrate and vertebrate systems. In *Aplysia* abdominal ganglia, UML blocks increases in cAMP concentration and increases in protein phosphorylation produced by 5-HT treatments (Levitan, et al., 1974). When applied to the isolated *Aplysia* eye, UML ($10^{-4}$M, N=2) weakly inhibited CAP frequency during the treatment period (-25 to -40%). Higher concentrations of UML ($10^{-3}$M, N=5) with or without 5-HT, severely decreased the amplitude of the CR both during and after the treatment period and caused irregularities in the CR waveform during subsequent cycles. These effects prevented testing UML as a blocker of 5-HT at concentrations greater than $10^{-4}$M. At a lower concentration ($10^{-4}$M), UML did not block 5-HT induced phase shifts. Eyes treated with 5-HT ($10^{-5}$M, N=1 and $5 \times 10^{-7}$M, N=4) plus UML ($10^{-4}$M) showed advance phase shifts (+2.2 ± 1.0 h., N=5) not significantly different ($p < .08$) from eyes simultaneously treated with 5-HT ($10^{-5}$M, +1.8h., N=2; $5 \times 10^{-7}$M, +2.0 h., N=2). UML ($10^{-4}$M, N=2) did not cause phase shifts alone (+0.2 h., range -.5 to +1.0h.).

Cinanserin -HCl has proven to be an effective blocker of 5-HT induced effects in several tissues in which other 5-HT blockers are ineffective (Liebswagen, et al., 1975). Cinanserin ($10^{-4}$M, N=5) had
only a weak depressant effect on spontaneous CAP frequency (-12%, range 0-33%), but caused large decreases in the amplitude of the CAP rhythm after removal (N=4) and frequently caused the CR to become irregular as well (N=3). These effects on the rhythm often became apparent 24-48 h. after cinanserin had been removed from the bathing media. Lowering the concentration of cinanserin to 10^{-5} M (N=2) eliminated these post-treatment effects on the CR. Although several of the cinanserin-treated (10^{-4} M) eyes appeared to be shifted relative to controls, this effect may have been due to the gross changes in waveform and irregularities in the rhythm produced by cinanserin (+ 0.5 ± 2.3 h., N=4). In spite of its potent effects on the CR however, the phase shifts produced by cinanserin (10^{-4} M) plus 5-HT (5 x 10^{-7} M, + 2.9 ± 1.0 h., N=4) were not significantly different (p < .9) from the phase shifts of simultaneously run 5-HT (5 x 10^{-7} M, N=3) treated eyes (+ 2.2 ± 1.0 h.). Eyes treated with both (10^{-4} M) cinanserin and 5-HT (5 x 10^{-7} M) had low CAP frequencies, but the CRs did not become irregular as did eyes treated with (10^{-4} M) cinanserin alone. It is as yet unclear how 5-HT acts to protect the CR from the irregularities produced by cinanserin (10^{-4} M).

Lysergic acid diethylamide (LSD) and its structurally related indoles, bromo-LSD (Br-LSD) and bufotenine, have either agonistic or antagonistic actions on 5-HT receptors in a variety of tissues (see Ascher and Kehoe, 1975; and Gershon, 1977, for review). In the isolated Aplysia eye, LSD, Br-LSD and bufotenine mimicked the effects of 5-HT to varying degrees. All three compounds, bufotenine (+ 3.4 ± 1.0 h., 10^{-3} M, N=4), Br-LSD (+ 3.3, range +2.5 - + 4.0 h., 10^{-4} M, N=3), and LSD (+ 2.2 ± .7 h., 10^{-4} M, N=5) produced advance phase shifts when
applied during the late projected day phase (CT 05-11) (Table 1). In general however, these drugs were not as potent as 5-HT in reproducing the effects that 5-HT produced on the eye. For example, medium concentrations of LSD ($5 \times 10^{-6}$M, $N=2$) produced only small advances ($+0.5 \pm 1.0$ h.) and depressed spontaneous CAP activity only slightly ($-20\%$). This may be compared to 5-HT which produced large phase advances (up to $+4$ h., Fig. 3) and significant depression of CAPs ($>80\%$) at concentrations as low as $10^{-7}$M. In addition, of the three psychomimetic drugs, only bufotenine (chemically the most similar to 5-HT) produced increases in CAP peak frequencies of treated eyes one to two cycles after removal (Fig. 8). The peak CAP frequencies of eyes treated with LSD and Br-LSD were frequently depressed during subsequent cycles. Finally, none of the three drugs produced phase delays of the CR when applied during the late projected night phase at which 5-HT treatments produced phase delays (CT 20-02; LSD, $10^{-4}$M, 0.0 to $-1.0$ h., $N=2$; Br-LSD, $10^{-4}$M, +2.5 to $-1.0$h., $N=2$; bufotenine, $10^{-3}$M, +1.0 to $-0.5$ h, $N=2$). Thus, though LSD, Br-LSD and bufotenine acted as agonists of 5-HT in their ability to produce advance phase shifts of the eye CR, they may not mimic all of the effects of 5-HT on the eye.

Since many drugs, including LSD, are known to display both agonist and antagonist properties at different concentrations, eyes were treated with a lower concentration ($2 \times 10^{-7}$M) of LSD before 5-HT ($5 \times 10^{-7}$M) treatment to see if at the lower concentration, LSD might act as an antagonist of 5-HT. At this concentration ($2 \times 10^{-7}$M), LSD alone produced phase shifts in only one of four eyes ($+0.8 \pm 2.0$ h.) and had no effects on CAP activity. LSD ($2 \times 10^{-7}$M) plus 5-HT ($5 \times 10^{-7}$ M) produced phase shifts ($+2.2$, range $+4.0$ to $+1.0$ h., $N=3$) which
were not significantly different (p < .8) from phase shifts produced by simultaneously run 5-HT (5 x 10^{-7} M) controls (+ 2.2, range +3.5 to 1.0 h., N=2).

When eyes were treated with LSD (10^{-6} M) or bufotenine (10^{-3} M) plus 5-HT (5 x 10^{-7} M) no competitive antagonist effects were observed, but neither were the phase shifting effects on the eye additive (phase shifts not significantly different, p < .8, from simultaneous 5-HT, 5 x 10^{-7} M). This suggests, along with the dose response curve data, that the phase shift mechanism affected by these drugs was maximally stimulated, or that the same chemical receptor mediated phase shifts by bufotenine, LSD, and 5-HT.

III. Mechanisms of 5-HT induced phase shifts.

1) Involvement of synaptic action

The phase shifts and the PRC produced by 5-HT suggest that this putative neurotransmitter could be part of an ocular pathway which carries temporal information to the eye CO. One possibility is that 5-HT shifts the phase of the CR from the eye indirectly, by stimulating other cells to secrete transmitters which in turn act on the cell(s) containing the eye CO. To examine this possibility, eyes were placed in solutions containing low concentrations of Ca^{2+} (0.1mM = 0.01 x normal) and a high concentration of Mg^{2+} (200 mM = 4 x normal), both prior to adding 5-HT and throughout the 5-HT treatment.

Similar solutions (HiMg^{2+} - LoCa^{2+}) have been shown to block release of transmitter and secretory substances in Aplysia and other organisms (Cedar, et al., 1972; Carew, et al., 1974; Halstead and Jacklet, 1974; Harf, et al., 1976). This treatment did not block the phase shifts
caused by $10^{-5}$ M 5-HT ($\pm 2.3 \pm .8 \text{ h.}, N=5$). HiMg$^{2+}$ - LoCa$^{2+}$ solutions did, however, completely block spontaneous CAP activity both in the presence and absence of 5-HT, but had no phase shifting effects on the CR alone ($-0.2 \text{ h.}, \text{ range } 0 \text{ to } -0.5 \text{ h.}, N=3$). LoCa$^{2+}$ solutions (.1mM) with lower concentrations of Mg$^{2+}$ (125 mM) had different effects on CAP activity, causing it to increase gradually to many times (5-10x) control levels. This solution also did not block 5-HT ($10^{-5}$ M) induced phase shifting ($+2.0 \pm .8 \text{ h.}, N=5$). These results indicate that 5-HT is not acting on the rhythm by stimulating other cells to release transmitters or secretory products which in turn affect the CO. It is still possible that 5-HT acts on cells that are electrotonically coupled to the cells containing the CO. This possibility cannot be tested at this time because treatments which disrupt electrotonic junctions also produce phase shifts of the rhythm (Eskin and Corrent, 1977a; Eskin, 1977).

2) **Effects of Indoles and Dopamine**

5-HT could produce its effects on the eye by several different mechanisms. It might act as a specific transmitter of temporal information to the eye, and produce its effects by interacting with a classical membrane bound receptor. Alternatively, the effects of 5-HT might be due to non-physiological actions of the drug on the eyes. For example, 5-HT could act by interfering with a metabolic pathway which utilizes indoleamines similar to 5-HT, by competing with the uptake of another transmitter, or by acting as a "false" transmitter. In order to test if the phase shifting response of the eye to 5-HT was specific for 5-HT, eyes were treated with metabolites and precursors of 5-HT, with other putative neurotransmitters, and with indole derivatives similar in
structure to 5-HT. All treatments were applied during the late day phase (CT 05-11) at which 5-HT produced the largest advance phase shifts.

5-HIAA (10^{-5} M) is a major metabolite of 5-HT in vertebrate nerve cells and in some molluscs (though apparently not in Aplysia) but it had no significant effects on the CR or on CAP activity (+ 0.2 h., range + 1.0 to - 0.5 h., N=3). 5-hydroxytryptophan (5-HTP), the immediate anabolic precursor of 5-HT, did not produce significant phase advances (+0.75 h., range + 0.5 - 1.0 h., N=2) when given during the late subjective day (CT 05-11). Two indole analogues of 5-HT, 7-methyl tryptamine (5 x 10^{-5} M, N=2) and 5-methoxygramine (5 x 10^{-5} and 5 x 10^{-6} M, N=2) produced no phase shifts (range + 0.0 - +0.5 h.) or changes in CAP frequency. These results suggest that the mechanism through which 5-HT produces phase shifts is selective and relatively specific for 5-HT, since other 5-HT-like indoleamines do not reproduce the effects of 5-HT. Dopamine (DA) is a monoamine which fulfills some of the criteria for being a putative neurotransmitter in Aplysia (Ascher, 1972; McCaman, et al., 1973). Evidence has been published for both the presence of DA and its synthesis in the eye of Aplysia (Luborsky-Moore and Jacklet, 1978b; Loh and Jacklet, 1977; Corrent, et al., 1978). The receptors for the DA in molluscan nervous tissue will bind significant amounts of 5-HT, and both 5-HT and DA cells have shown the ability to take up both DA and 5-HT (Fuxe and Ungerstedt, 1968; Carpenter, et al., 1971; Kuhar, et al., 1972; Drummond, et al., 1978). Thus it is possible that 5-HT could produce its effects on the CR by stimulating
night phases (CT 20-02) during which 5-HT treatments delayed the CR.

If, as the above results suggest, the phase shifting action of 5-HT is produced via increases in cAMP, treatments which increase cAMP levels in cells might also phase shift. Inhibitors of phosphodiesterases (PDE inhibitors), the enzymes which degrade cAMP, have been shown to increase cAMP levels in Aplysia nerve tissue as well as tissues from other organisms (Treistman and Levitan, 1976; see Weiss and Fertel, 1977) for review). PDE inhibitors have also been found to potentiate the effects of 5-HT on certain tissues in Aplysia (Cedar and Schwartz, 1972; Levitan et al., 1974; Shimahara and Tauc, 1977).

IBMX (isobutylmethylxanthine) belongs to a class of compounds (methylxanthines) which are widely used as PDE inhibitors, and is pharmacologically related to theophylline, which has been shown to increase cAMP levels in Aplysia tissue when used together with 5-HT (Cedar and Schwartz, 1972; Levitan et al., 1974). Eyes treated (CT 05-11) with IBMX (5 x 10^{-4}M) alone generated high rates of singlet CAPs (2-3x normal) during treatment, but displayed either small or no phase advances (+ 1.0 h., range 0 to + 2.0 h., N=3). Since PDE inhibitors have been shown to potentiate the effects of 5-HT on cAMP levels, eyes were treated with IBMX (5 x 10^{-4}M) plus concentrations of 5-HT (10^{-8}M) which are subthreshold for phase shifting. Although the average phase advances produced were still small (+ 1.7 h. ± 1.0, N=5) they were significantly larger (p > .98) than the phase shifts produced by 10^{-8}M 5-HT alone (+ 0.2 h., N=7), and phase shifts as
DA receptors in the eye, or by competing with DA for reuptake into cells. Both of these possibilities would have the effect of increasing the stimulation of DA receptors in the eye. To test these possibilities, eyes were treated with DA \((10^{-5} \text{M})\) for 6 h. (CT 05-11). DA did not phase shift the CR (range 0 to + 1.0 h., N=3). In addition, DA produced increases in CAP frequency which were the opposite of the effects of 5-HT. Thus it seems unlikely that 5-HT could be causing its effects in the eye by prolonging or mimicking the effect of DA. Finally, the effects of 5-HT on the CR were not blocked or mimicked by low Na\(^+\) treatments (see Ions and Ion Gradients section, below). Since low Na\(^+\) solutions should block or inhibit 5-HT (and other neurotransmitters) reuptake into cells, this provides evidence against the possibility that 5-HT is producing its effects by interfering with other transmitter reuptake systems or by being taken up into cells as a false transmitter.

3) **Ions and Ion Gradients**

Gerschenfeld and Paupardin-Tritsch (1974) have described 6 different conductance changes brought about by iontophoretic applications of 5-HT on *Aplysia* nerve cells. These changes include both increases and decreases in membrane conductance and involve the ions Na\(^+\), K\(^+\), and Cl\(^-\). Pellmar and Carpenter (1979) have described a slow, regenerative Ca\(^{2+}\) conductance change in *Aplysia* cells induced by 5-HT, and Hill and Yantorno (1979) have provided evidence that 5-HT may produce its effects on the *Aplysia* heart by changing membrane permeability to Na\(^+\). To test the possibility that similar changes in membrane permeability might be involved in 5-HT induced phase shifting,
concentrations of Na\(^+\), Cl\(^-\), or Ca\(^{2+}\) ions in the external media were lowered to levels approximately equal to or below their electrochemical equilibrium. Lowering Na\(^+\) to 7mM and Cl\(^-\) to 330 mM (LoNa\(^+\) - LoCl\(^-\) solutions) eliminated CAP activity from the eyes for the duration of treatment. LoNa\(^+\) - LoCl\(^-\) solutions did not cause phase shifts when given alone (+ 0.3 h., range + 1.0 to 0 hr., N=3), nor did they block 5-HT (10\(^{-5}\)M) induced phase shifts (+ 2.1 ± .8 h., N=4). If phase shifting involved fluxes of either Na\(^+\) or Cl\(^-\) ions due to membrane conductance changes, LoNa\(^+\) - LoCl\(^-\) should have reduced or eliminated the movement of ions and the subsequent phase shifts. Since it did not block phase shifts, such permeability changes are unlikely to be required for phase shifting by 5-HT.

The Ca\(^{2+}\) potential gradient across nerve cell membranes is quite large and changes in Ca\(^{2+}\) permeability can cause significant potential changes in cells. EGTA was added to Lo-Ca\(^{2+}\) solutions to "buffer" Ca\(^{2+}\) concentrations to levels low enough to eliminate the Ca\(^{2+}\) gradient (Portzehl, et al., 1964; Dipolo, et al., 1976). EGTA was used because Ca\(^{2+}\)-free solutions may lower the concentration of Ca\(^{2+}\) very slowly and irregularly in dense tissue. Thus, although we had already shown that LoCa\(^{2+}\)-HiMg\(^{2+}\) solutions did not block phase shifting, this result constituted insufficient evidence for determining the involvement of postsynaptic Ca\(^{2+}\) currents in phase shifting by 5-HT.

Eyes treated with EGTA-LoCa\(^{2+}\) (free Ca\(^{2+}\) = 5 x 10\(^{-9}\)M) solutions displayed high frequency CAP activity, as much as 5 to 10 x over the highest levels observed in control eyes. EGTA-LoCa\(^{2+}\) solution did not cause phase shifts (+0.5 h., range + 0.5 to =1.5 h., N=3) and did not
block phase shifting due to 5-HT (10^{-5} M) treatments (+ 2.1 ± .4 h., N=4). The phase shifts produced by EGTA-LoCa^{2+} plus 5-HT eyes were not significantly different (p < .8) than the shifts produced in simultaneously run control eyes treated only with 5-HT (10^{-5} M, +2.0 h., range + 2.5 to + 1.5 h., N=3). These results indicate that 5-HT-induced Ca^{2+} fluxes are probably not required for phase shifting, and reaffirm the fact that 5-HT does not cause phase shifts by stimulating transmitter release from other cells.

It is possible that 5-HT phase shifts the CR by producing conductance changes to K^{+} ions. 5-HT induced hyperpolarizations and depolarizations due to K^{+} permeability changes have been described in Aplysia cells by Gerschenfeld and Paupardin-Tritsch (1974). However, increasing external K^{+}, which should depolarize cells, has been shown to phase shift the CR from Aplysia eyes (Eskin, 1972). Lowering (K^{+})_{out} should also depolarize cells, due to inhibition of the Na^{+}-K^{+} pump (Hill and Yantorno, 1979; Lieberman, 1977). In preliminary experiments Lo-K^{+} treatments slowly blocked spontaneous CAP activity in the treated eyes, and caused an advance phase shift (+ 2.0 h.) in one of two treated eyes. Since either raising or lowering the K^{+} gradient around the eye cells may produce phase shifts, this method cannot be readily used to investigate the possibility that 5-HT produces phase shifts by changing K^{+} fluxes across cell membranes in the eye.

4) Involvement of cAMP

In the literature on transmitter-sensitive increases in cyclic AMP, the effects of 5-HT on Aplysia tissue are particularly well represented.
Increases in cAMP due to 5-HT have been reported in *Aplysia* heart, abdominal ganglia, connective nerves, pedal-pleural ganglia, and in muscles which control feeding movements (Higgins, 1977; Cedar and Schwartz, 1972; Levitan, et al., 1974; Weiss, et al., 1978). In addition, application of cAMP or its derivatives mimics the effects of 5-HT in these and other systems in *Aplysia*, and a 5-HT sensitive adenylate cyclase has been isolated from *Aplysia* neurons (Brunelli, et al., 1976; Shimahara and Tauc, 1977; Levitan, 1978). Since such a widespread case has been made in *Aplysia* for the involvement of cAMP in mediating 5-HT effects, the possible involvement of cAMP in the various effects of 5-HT on the eye was investigated.

Isolated eyes were treated for 6 h. during the late subjective day phase (CT 05-11) with dibutyryl-cAMP (db-cAMP) and 8-benzylthio-cAMP (8bt-cAMP), analogs of cAMP which cross cell membranes more readily and are more resistant to breakdown by phosphodiesterases than cAMP (Meyer and Miller, 1974). Dibutyryl-cAMP (2 x 10^-3 M, N=3) had no effect on CAP activity, on the CR (+ 0.3 h., range 0 to +1.0 h., N=3), or on CAP peak frequencies after it was removed. However, since exogenous db-cAMP had failed to produce effects in other systems in which cAMP is involved, (8bt-cAMP) was also tried. 8bt-cAMP (10^-3 M and 2 x 10^-3 M, N=4) depressed CAP activity (-20 to -90%), and produced phase advances of the CR (+ 2.75 ± 1.5 h., N=4) which were not significantly different from phase shifts produced by 10^-5 M 5-HT (p < .8). Increases in the CAP peaks of the rhythm were not observed after 8bt-cAMP treatments. 8bt-cAMP (2 x 10^-3 M) also produced phase delays (-2.5 h., range -2.0 to -3.0 h., N=2) when applied during the late
large as + 3.0 h. were produced by IBMX + 5HT (10^{-8} M) treatment. This result indicates that IBMX can potentiate the phase shifting effects of 5-HT on the eye, an important criteria for demonstrating that the effects of a treatment are mediated by cAMP.

Papaverine is a PDE inhibitor which has been shown to be a more potent inhibitor than the methylxanthines in some tissues, and has been shown to raise cAMP levels in Aplysia ganglia (Treistman and Levitan, 1976). When applied to isolated eyes during the late projected day (CT 05-11), papaverine (2 x 10^{-4} M), produced phase advances (+ 2.2 h., range 2.0 - 2.5 h., N=3). Papaverine also slightly depressed the spontaneous CAP activity during treatment (-18 to -26% but did not cause increased peak CAP frequencies after removal. Eyes treated at CT 05-11 with papaverine (2 x 10^{-4} M) plus 10^{-8} M 5-HT showed full size phase advances (+ 2.75, range 2.5 to + 3.0 h., N=2). When eyes were treated during the late projected night (CT 17-23 to 19-01, 3 x 10^{-4} M) small delay phase shifts (- 1.9 h., range - 1.5 to - 2.0 h., N=3) were observed. The response of the rhythm to papaverine during the late night phases was somewhat erratic, and only 40% of eyes treated with lower concentrations of papaverine (2 x 10^{-4} M) had delayed CRs (-0.1 h., range - 2.0 - + 1.0 h., N=5). A higher concentration of papaverine (5 x 10^{-4} M) severely depressed the amplitude of the rhythm and caused multiple CAP peaks and irregular waveforms.

5) 5-HT Effects on the Light Response of Eyes

Unlike the depressant effects of 5-HT on spontaneous CAP activity, 5-HT stimulated the response of isolated eyes to light. The response
of the *Aplysia* eye to light, as measured extracellularly from the optic nerve, can be divided into two parts based on the temporal organization of the response (Jacklet, 1969b and 1979; Audesirk, 1973). The early or phasic response is comprised of low amplitude impulses superimposed on and following a slow, low amplitude electroretinogram (ERG). These small impulses grade up to CAPs with time to form the tonic light response of the eye. Focal ERG potentials were also recorded directly from the eyes using sharpened platinum wire electrodes. The ERG recorded in this way was comprised of three alternating potential changes (Fig. 9a). The earliest potential change was used as the basis for measurements since it was most likely to represent a response of only receptor cells. This assumption is supported by comparisons of intracellular and extracellular light responses (Jacklet, 1969).

Over 82% (14 of 17) of the eyes treated with 5-HT (10^{-5} to 5 \times 10^{-7} M) exhibited an increase in the amplitude of the initial wave of the ERG elicited by a light pulse (avg. +24%, range -30 to +100%, Fig. 9c and 9d). The increased amplitude declined slowly and after 2 h. only nine (of 17) eyes were exhibiting responses above their pre-5-HT levels. All three potential changes of the ERG were increased by 5-HT, although the degree and time courses of the increase differed for each. Control rinses with BFSW rarely produced significant (> 5%) increases in the ERG (3 of 13 eyes) during the first light pulse after the solution exchange (avg. +3%, range +16% to -11%). These increases were short lived, and no significant increases in ERGs were observed during the second light pulse (16 min.) after BFSW.
exchange. Significant decreases in ERG (-5 to -11%) were detected after control rinses in two of the 13 eyes. These ERGs remained at their new lower values, and thus, may have been due to small position shifts of the eye or electrode during the rinse.

The response of the ERGs to 5-HT, and the ERGs themselves, were quite complex. The response to 5-HT varied depending on electrode position and possibly the phase of the CR. Ten of the ten eyes treated with 5-HT during the projected day and early projected night (CT 05 to CT 19) showed initial increases in the ERG amplitude after treatment. However, three of the seven eyes treated during the late projected night (CT 20-24) showed decreased ERG amplitudes after 5-HT treatment. Although these data are suggestive of a rhythmic variation in the sensitivity of the receptor cells to 5-HT, this possibility was not systematically investigated as part of this study. The small number of eyes used and the possible involvement of other factors such as electrode placement make it impossible to determine from these data if these differences at different phases are significant.

The position of the recording electrode affected the shape of the ERG wave and the response to 5-HT. Recordings from electrodes placed parallel to the surface of the eye into the thinnest part of the retina near the "cornea", produced regularly shaped triphasic potentials (Fig. 9a). ERGs recorded from the base of the eye, close to the origin of the optic nerve, often had more complex wave forms, possibly due to the contribution of different cell types (Fig. 9b). In five (of 17) eyes, the ERG was recorded simultaneously by two electrodes placed in different areas of the eye. In all 5 eyes, the electrode placed in
the thicker part of the retina (closer to the base of the eye) recorded the largest increase in ERG after 5-HT treatments. It is not known at this time how much of the variation in the ERG results is due to differences in the placement of the recording electrodes.

The tonic response of the eye to light, recorded from the optic nerve, was also sensitive to 5-HT. 5-HT treatments (10^-4 - 5 x 10^-7 M) decreased (N=2) or eliminated (N=2) the normally silent period between the early and the tonic responses to light. Treated eyes also showed increased frequency of CAPs relative to control eyes (mean increase, +105%, range +27 to +168%). The grouping of CAPs differed, with untreated eyes spiking in repeating "bursts" of CAPs throughout most of the light pulse while treated eyes "beat" with high frequency single CAPs in no discernable pattern. Since the effects of 5-HT were investigated at only two phases, the late night (CT 18-24, N=2) and late day (CT 05-11, N=2), no attempt was made to test if the sensitivity of the tonic light response to 5-HT varied with phase. No significant differences in phase shifts were noted between eyes which received both 5-HT and light pulses and control eyes which received only 6 h. light pulses, at either of the two phases tested (CT 18-24, N=2 and CT 05-11, N=2).
DISCUSSION

The data presented in this thesis have led to the formation of two testable hypotheses, and have suggested some potential mechanisms to explain 5-HT induced phase shifting in the Aplysia eye. The hypotheses are:

1) 5-HT performs a neurotransmitter role in the eye.
2) 5-HT acts to transmit temporal information to the eye.

These hypotheses and the experimental results which support each of them will be discussed in turn. Finally, some mechanisms for how 5-HT might produce its effects on the Aplysia eye will be discussed.

I. Neurotransmitter aspects of 5-HT

Numerous lists of criteria have been proposed to aid in determining if a substance acts as a neurotransmitter in a particular tissue (Paton, 1958; Florey, 1961; Werman, 1966; Dudel, 1968). 5-HT has been tested to see if it fulfills some of these criteria in the Aplysia eye, and additional data, consistent with a neurotransmitter function for 5-HT, have been presented. The data from the above tests and the supportive evidence can be summarized by six results.

1) 5-HT is found in the eye in appropriately high concentrations (50 ng/mg protein) to be a likely candidate for a neurotransmitter.
2) Depolarizing stimuli reduce the 5-HT content of the eyes, suggesting that 5-HT is present in a releasable form.
3) The cells of the eye can synthesize 5-HT from tryptophan, a conversion requiring specific enzymes presumably found only in cells which in vivo synthesize and release 5-HT.
4) Mimicking the effects of 5-HT on the CR requires a strict, but not absolute chemical structural specificity, possibly the result of a receptor binding requirement. Thus some chemically similar indoles, LSD, Br-LSD and bufotenine, mimic some of the effects of 5-HT on the CR, while other slightly different indoles, 5-hydroxytryptophan, tryptamine, 5-methoxygramine, and 7-methyltryptamine, do not.

5) 5-HT produces phase shifts and inhibits neural activity (CAPs) at low concentrations (10^{-7}M).

6) The effects of 5-HT on the eye are decreased or eliminated at high concentrations (>10^{-3}M). This suggests that the target tissues for 5-HT desensitize when overstimulated, a characteristic of neurotransmitter receptors.

5-HT concentration in the eye

A prerequisite for suggesting that a substance performs a neurotransmitter role in a tissue is the evidence that the tissue contains the putative neurotransmitter. We have reported that the mean amount of 5-HT/eye is about 50 ng/mg protein. This concentration of 5-HT is about average for various pieces of Aplysia nervous tissue. The amount of 5-HT in the eye is greater than the concentration of 5-HT found in the buccal or pleural ganglia, and similar to the amounts of 5-HT measured in the abdominal and cerebral ganglia (Carpenter, et al., 1971; and McCaman, et al., 1973). The cerebral ganglia contains two giant serotonin containing cells (GSCs) which meet nearly all of the criteria for using 5-HT as a neurotransmitter. Thus, there is good evidence that 5-HT acts as a neurotransmitter in Aplysia (Eisenstadt, et al., 1973; Gerschenfeld, et al., 1978; Weiss, et al., 1978).
The above data suggest that 5-HT is being synthesized, sequestered, and utilized by the cells or the processes of cells located in the Aplysia eye. The relatively large concentration of 5-HT found in eyes implies that the 5-HT containing cells of the eye are either numerous or very active in synthesizing and storing 5-HT, and that 5-HT probably performs a functional role in the eye. The cells of the eye which synthesize, store, and release 5-HT have not yet been identified. It is possible that any or all of the classes of cells in the eye could contain, synthesize, or release 5-HT, including pigmented support cells or glia. However, cytochemical studies on the eye which selectively localized monoamine neurotransmitters (5-HT, DA, norepinepherine) as electron-dense chromium precipitates, demonstrated that only secondary cells (neurones) and synaptic terminals in the neuropil of the eye appeared to contain the chromium reaction products (Luborsky-Moore and Jacklet, 1977). This result indicates that 5-HT (and possibly other specific catecholamine neurotransmitters) is largely, if not totally, located in the secondary neurones and synaptic processes in the eye.

5-HT depletion

The ability of depolarizing stimuli (high K⁺ solutions) to deplete the 5-HT in the Aplysia eye suggests that 5-HT is present in the eye in a releasable form. These results do not distinguish between the possibilities of depolarization induced release, and depolarization induced degradation of 5-HT. Since transmitter release is dependent on external Ca²⁺, while Hi-K⁺ induced breakdown of 5-HT is not likely to require external Ca²⁺, lowering the Ca²⁺ concentration in the bathing solution should provide evidence for distinguishing between Hi-K⁺-induced release
or breakdown of 5-HT.

Synthesis of 5-HT

Evidence has been presented in this thesis that Aplysia eyes are capable of synthesizing significant amounts of 5-HT from its precursor amino acid, tryptophan. While several studies have been published on 5-HT synthesis in Aplysia and closely related gastropod molluscs, it is difficult to compare the results of these studies because of great differences in the techniques which were used to measure synthesis (Eisenstadt, et al., 1973; Liebswar, et al., 1975; Cottrell and Powell, 1971). However, if the 5-HT content of the different tissues is used as a gross measure of the 5-HT synthetic capacities of the tissues, then some comparisons can be attempted. It should first be noted that comparisons based on the proposition that 5-HT content is correlated with 5-HT synthesis are subject to several criticisms. First, tissues which contain 5-HT are not necessarily capable of synthesizing it (i.e., some cells in Aplysia, Brownstein, et al., 1974; and sympathetic axons in the pineal gland, and platelets). Second, the rate of turnover of 5-HT in different cells with similar 5-HT concentration may vary greatly depending on cell activity and the density of 5-HT release sites. Nevertheless, the comparison of different studies is desirable because it allows the utilization of older, better characterized systems as models to determine if our hypothesis, in this case that the eye synthesizes 5-HT for use as a transmitter, is reasonable.

The giant serotonin containing cells (GSCs) in the Aplysia cerebral ganglia are ideal models for comparison because they fulfill most of the criteria required to prove that 5-HT is acting as a neurotransmitter.
The GSCs contain large amounts of 5-HT, and can take it up, package it in vesicles, transport it, release it when stimulated or depolarized with high K⁺, and can synthesize 5-HT from tryptophan (Weinreich, et al., 1973; Goldman and Schwartz, 1974; Gerschenfeld, et al., 1978; Eisenstadt, et al., 1973). The rate of synthesis of 5-HT by the GSCs is about 5 pg/cell/h, which represents the new synthesis of about 0.4% of the 5-HT in the cell per hour. The Aplysia eye synthesizes 5-HT at somewhat lower rates, 1 to 2.5 pg/h, but this represents a greater proportion of newly synthesized 5-HT, about .4% to 1% of the total 5-HT in the eye per hour. The slightly higher rates (per amount of 5-HT) in the eye, may be contributed to by any of a number of differences between the two preparations. For example, the rates of spontaneous nerve activity in the two tissues are different (the eye is spontaneously active, the GSCs are not), the eye is probably composed of a number of small 5-HT cells while the GSC is a single large cell, and unlike the GSCs, the eyes were partially depleted of 5-HT prior to measuring synthesis. Nevertheless, it is interesting that despite the differences in the techniques used, the rates of 5-HT synthesis in the GSCs and the eyes are remarkably similar. Some discrepancies do exist, however, between the data obtained from the synthesis studies on serotonergic Aplysia cells and the eye data. After injecting radioactive tryptophan, Eisenstadt, et al. (1973) found that about 1-2% of the radioactivity recovered from the GSCs ran with standard 5-HT, and Liebswar, et al., (1975) reported that 8% of radioactivity recovered from RB cells in the abdominal ganglion had chemical properties similar to 5-HT. In the eye only about .2 to .4% of the radioactivity recovered had properties identical with
native 5-HT. This difference is probably directly attributable to the different methods used. Both Eisenstadt, et al. (1973) and Liebwar, et al. (1974) examined large identified neurons into which they directly injected labeled tryptophan. The 5-HT containing cells in the Aplysia eye, however, have not yet been identified, and the labeled tryptophan was added to the bathing media. Thus all the cells, 5-HT synthesizing or not, had an equal likelihood of taking up the tryptophan and incorporating it into polypeptides, or metabolizing it in myriad ways. In addition, nonspecific binding of tryptophan to a variety of tissues is much less likely to be a problem if the amino acid is injected into specific cells rather than bathing the entire tissue with it. Much higher intracellular concentrations can be obtained instantly by injection than can be achieved during even long incubations. This point is particularly important because the rate of synthesis of 5-HT seems to show a linear dependence on intracellular concentration (Eisenstadt, et al., 1973). Finally, the uptake of tryptophan may be a crucial factor in controlling 5-HT synthesis, since identified cells injected with tryptophan converted a far greater proportion of it to 5-HT than did identical cells which took up tryptophan from the media (Eisenstadt, et al., 1973).

When the dissimilar methods used in the above studies are taken into account, I feel that the differences between 5-HT synthesis in identified Aplysia neurons and in the Aplysia eye are far less significant than the similarities.

In the 5-HT synthesis experiments presented in this thesis tryptophan was chosen as the substrate because the enzyme which converts
tryptophan to 5-HP, tryptophan hydroxylase, confers specificity for 5-HT synthesis on serotonergic neurons and controls the rate limiting step in the synthesis pathway (Kuhar, et al., 1972; Goldman and Schwartz, 1974; Eisenstadt, et al., 1973). The other enzyme in the pathway, aromatic amino acid decarboxylase (AAD), is found in many tissues not thought to normally synthesize or contain 5-HT (Weinreich, 1973). This explains why many cells can convert 5-hydroxy-tryptophan to 5-HT, but only cells which naturally utilize 5-HT are capable of converting tryptophan to 5-HT (see Gershon, 1977, for review). Thus, the formation of significant amounts of $^3$H-5-HT from $^3$H-tryptophan by the isolated eye is evidence that cells or nerve terminals in the eye are serotonergic.

Parachlorophenylalanine (p-CPA) which inhibited 5-HT synthesis in Aplysia eye tissue, has been shown to be an effective inhibitor of 5-HT synthesis in a variety of mammalian tissues, as well as in ganglia of the gastropod mollusc Helix pomatia (Sanders, et al., 1977; Osborne and Neuhoff, 1974). Studies have shown that the major site of action of p-CPA is on the enzyme tryptophan hydroxylase (Jequier, et al., 1967; Koe and Weissman, 1966). Osborne and Neuhoff (1974) found that p-CPA ($10^{-3}$M) inhibited 5-HT formation from tryptophan by over 70% after 3 h. If 5-HT synthesis in p-CPA treated eyes is estimated to be just below the levels of detectability then inhibition would range from about 50% after four hours to as much as 90% after 22 h. Unlike the reported action of p-CPA in Helix ganglia, however, this drug also caused a significant decrease (50-75%) in the total amounts of radiolabeled material recovered from the eyes (Osborne and Neuhoff, 1974). This suggests that p-CPA may have had an inhibitory effect on the uptake of tryptophan as well as on the formation of 5-HT and that the reduction in the amount
of 5-HT synthesized may have been due to blockage of tryptophan uptake. Parachlorophenylalanine inhibition of tryptophan uptake has been reported in other tissues (Gal, et al., 1970; Knapp and Mandell, 1973).

**5-HT agonists and receptors**

Conventional models of neurotransmitter action require that the transmitter effect be produced by binding the transmitter to a structurally specific receptor on the post-synaptic cell. While the specific binding of the neurotransmitter and receptor is manifested in a low Kd (high affinity) for the reaction, the receptor is generally not so specific as to exclude binding of molecules closely related structurally to the neurotransmitter. Thus, for most putative neurotransmitters, structurally similar compounds can be found to act as agonists or antagonists. Binding to the receptor may either mimic the transmitter's effects (agonist), or produce no effect other than to block the binding of neurotransmitter (antagonists). Eleven different drugs, chemically and structurally related to 5-HT, were tested on the eye. Of these, three—bufotenine, LSD, and Br-LSD—acted as partial agonists of 5-HT. None of the compounds tested blocked the effects of 5-HT. In addition, neither dopamine nor acetylcholine, both putative neurotransmitters found in *Aplysia*, produced 5-HT-like effects on the eye (Eskin, 1977, and unpublished results).

While the fact that only three of the drugs tested had significant effects might be taken as an indication of the specificity of the receptor, the question might be asked why no antagonist effects were observed. Gerschenfeld and Paupardin-Tritsch (1974a, b) have described six effects
of 5-HT on *Aplysia* nerves cells which can be distinguished on the basis of the conductance changes involved and the sensitivity of the effects to 5-HT blockers. Two of the 5-HT effects, both involving conductance decreases, were not blocked by any of the antagonists used. It is quite possible that the phase shifting effects of 5-HT in the *Aplysia* eye are mediated by 5-HT receptors similar to those mediating the nerve cell conductance decreases described by Gerschenfeld and Paupardin-Tritsch (1974a, b).

Although LSD, Br-LSD, and bufotenine mimicked the ability of 5-HT to depress CAP frequency and produce advance phase shifts. Preliminary experiments provided no evidence that any of the three drugs could cause delay phase shifts. Each of the drugs was tested twice at a single phase during the late projected night (CT 20–02) at a high concentration (10⁻³M to 10⁻⁴M).

The simplest explanation for the lack of effect is that 5-HT and its agonists exhibit different potencies in their effects on the eye. There is ample evidence for this suggestion based on comparison of the effects of low concentrations of 5-HT and LSD. Since different doses of a phase shifting treatment may yield not only different amplitude phase shifts but also a different shaped phase response curve (PRC), the PRCs of LSD, Br-LSD and bufotenine are likely to be different from the PRC of 5-HT. Thus, the PRCs for all these drugs could have both delay and advance phase shifts, but the phases at which 5-HT produces delays could be different from the phase during which LSD produces delays. This possibility can be tested by giving LSD, Br-LSD, or bufotenine treatments at different phases and forming a complete PRC for these drugs. A second possible explanation is that the 5-HT receptors
which mediate advance phase shifts are different from the receptors which mediate delay phase shifts. Thus while the phase advance receptor is sensitive to LSD, Br-LSD, and bufotenine, the receptor which mediates delays is not. An analogy can be drawn with the two types of 5-HT receptors in mammalian brain. Bufotenine, LSD, and Br-LSD are powerful agonists of 5-HT when applied to the pre-synaptic 5-HT receptors in the midbrain raphe nuclei, but they have a much lower potency in mimicking 5-HT effects on postsynaptic receptors in other brain nuclei (Haigler and Aghajanian, 1977).

Effects of low 5-HT concentrations

Production of effects by low concentrations of a putative neurotransmitter can be regarded as evidence of binding to a high affinity receptor, and thus, as evidence consistent with a physiological role for the neurotransmitter. Alternatively, if results can only be elicited by high concentrations of the drug, non-specific effects of the putative transmitter (e.g., cross-reacting with other transmitter receptors) must be suspected. Although some other molluscan tissues, notably the heart, are more sensitive than the eye to 5-HT, the concentrations of 5-HT (10⁻⁷M) which caused phase shifts and inhibited CAPs in the Aplysia eye compare favorably to 5-HT doses required to produce effects in most molluscan nerve and muscle tissue (Trembley, et al., 1976; Brunelli, et al., 1976; Levitan and Drummond, 1979). The dose-response curve for 5-HT stimulation of cAMP synthesis in Aplysia buccal muscle is remarkably similar to the dose-response curve for 5-HT induced phase shifting (Weiss, et al., 1978). In addition, the threshold concentration of 5-HT for potentiation of Aplysia buccal muscle contraction
is approximately $10^{-8}$M, while the threshold concentration of 5-HT required to produce phase shifts occurs between $10^{-8}$M and $10^{-7}$M (Weiss, et al., 1978; Corrent, et al., 1978). This is an important comparison because $10^{-8}$M is the threshold concentration of bath applied 5-HT which can mimic the effects of an identified 5-HT synapse in Aplysia.

Desensitization

Specific desensitization is a characteristic of some hormone and neurotransmitter target tissues, and is attributable to overstimulation of the transmitter receptors by high concentrations or repeated application of the transmitter. Desensitization of 5-HT induced responses in Aplysia nerve tissue and Mercenaria heart have been reported after treatment with high concentrations ($>10^{-5}$M) of 5-HT (Gerschenfeld and Stpehani, 1966; Gerschenfeld and Paupardin-Tritsch, 1974; Higgins, 1978). The report of Mercenaria heart is particularly interesting since it describes the desensitization of a 5-HT induced stimulation of adenyl cyclase, and since both desensitization and stimulation of adenylate cyclase may be properties of 5-HT induced phase shifting for 5-HT in Aplysia eye. Thus, the elimination of advance phase shifts by high concentrations ($>10^{-3}$M) of 5-HT may be due to desensitization of a 5-HT neurotransmitter receptor in the eye. It has been noted that the effects of 5-HT on the CR appear to desensitize at 5-HT concentrations ($2 \times 10^{-3}$M) much higher than the concentrations of 5-HT which caused desensitization in other Aplysia tissue ($10^{-5}$ to $5 \times 10^{-5}$M). The reasons for these differences are, at present, not known, although it suggests that either different 5-HT receptors are involved than those described for other Aplysia tissue or a different process of desensitization is
occurring.

Two other possibilities exist to explain the lack of phase shifting by 2 x 10^{-3}M 5-HT. The first is that the desensitization produced by 5-HT may not be "specific" desensitization due to the alteration of the 5-HT receptor to a non-reactive state, but instead may be due to a non-specific effect of the high 5-HT concentration, for example, stimulation of a different amine receptor. The second possibility is that the effect of high concentrations of 5-HT is not due to desensitization at all, but rather is part of a bimodal effect, with 5-HT producing advances at low concentrations and delays at high concentrations. Several drugs, including bufotenine, LSD, and 5-HT, have been observed to have biphasic effects on invertebrate tissue, when used at different concentrations (Nathanson and Greengard, 1974; Gerschenfeld and Paupardin-Tritsch, 1974a; Abrahams, et al., 1976; Florey and Rathmayer, 1978). This possibility can be tested by using even larger concentrations of 5-HT to see if delay phase shifts are produced.

II. 5-HT as a transmitter of circadian information to the CO of the Aplysia eye.

Evidence has been presented in this thesis that 5-HT acts to transmit circadian information to the CO in the Aplysia eye. The evidence can be summarized as follows:

1) The phase response curve (PRC) of 6 h 5-HT pulses has both advance and delay portions and 5-HT fulfills the criteria for being an entraining agent.

2) 5-HT is found in high concentrations in the Aplysia eye and optic nerve and meets other criteria for being a transmitter in the eye.
(see Discussion, Part I).

3) 5-HT appears to be acting either directly on the CO cell(s) or on cells electrotonically coupled to the CO, since conditions which should inhibit chemical release (HiMg$^{2+}$-LoCa$^{2+}$) do not block phase shifting by 5-HT.

Thus, 5-HT has some effects on the CR from the Aplysia eye (the production of a PRC with advances and delays, with a maximum phase shift of about 3-4 h) which are similar to the effects of light, an established natural entraining agent. Along with the evidence that 5-HT is found in the eye and fulfills other criteria for being a transmitter in the eye (see Discussion, Part I), these data suggest that 5-HT is acting as part of a physiological pathway to transmit circadian information to the CO in the eye. However, a caveat concerning the role of 5-HT in transmitting circadian information in the eye must be raised here. Release of 5-HT, or any other chemical transmitter does not appear to be necessary for phase shifting by light in the isolated eye. This observation is supported by two results.

1) Under conditions which should inhibit chemical release, light pulses phase shift the CR from the isolated eye (Eskin, 1977); and
2) the PRC generated by 5-HT treatments is shifted in time relative to a PRC for light pulses on the eye, and relative to the light-PRC of most diurnal animals. These results will be discussed in turn.

The first point raises the question of why a chemical transmitter of circadian information is needed, if the eye is fully competent to assimilate phase shifting information without one. The answer to this is that there is considerable evidence which implies that the eye CO re-
ceives light:dark (L:D) information from extraocular sources as well as directly (Eskin, 1971; Block, et al., 1974; Block and Page, 1977; Frichard and Lickey, 1978). In addition, at least three COs exist in *Aplysia*, and circadian information may be transmitted among these pacemakers to insure coherent CRs and internal synchronization (Strumwasser, 1967; Lickey, et al., 1976; Hudson and Lickey, 1977). One potential pathway by which such phase shifting information might reach the eye is via the efferent fibers of the optic nerve. Activity in these fibers mimics an effect of 5-HT by decreasing CAP activity from the eye (Eskin, 1971; Luborsky-Moore and Jacklet, 1976a). In addition, the optic nerve contains significant amounts of 5-HT, although it is not yet known if the 5-HT is located in the efferent fibers, the afferent fibers, or both. Another potential pathway for transmission of circadian information to the eye involves 5-HT release into the blood vessels which terminate around the eye. There are thus a number of potential sources of circadian information which might utilize 5-HT as a transmitter in the eye (Fig. 1).

Although transmitter release is not necessary for phase shifting by light, it is still possible that 5-HT could have been released by a parallel pathway and contributed to light induced phase shifting at phases other than the phase tested (CT 18-00, Eskin, 1977). That is, blocking the 5-HT contribution to a light-produced phase shift might only cause an obvious difference if the experiment was performed at phases during which 5-HT produced large phase shifts. Recent experiments (Eskin, Corrent, unpublished) indicate that transmitter release does not appear to contribute to light induced phase shifting of the
isolated eye even at phases (CT 05-11) at which 5-HT treatments alone produce large phase shifts. These results do not rule out the possibility that light pulses could cause a release of 5-HT by an extraocular pathway. Thus 5-HT might transmit L:D information received by extraocular receptors and carried to the eyes, or L:D information received by one eye and passed on either to the contralateral eye or back to the same eye, via a circuit involving the optic nerve and passage of the information through the cerebral or other ganglia. The existence of extraocular photoreceptors transmitting circadian information has been demonstrated by the ability of eyeless Aplysia to entrain to an L:D cycle (Block and Lickey, 1973). An extraocular pathway to the eyes which requires an intact optic nerve for phase shifting by red light pulses has also been demonstrated (Block, et al., 1974). Thus, it is not unlikely that 5-HT is transmitting L:D information to the eye, it is just unlikely that 5-HT is involved in an entirely intraocular pathway for light-induced phase shifting.

Another possibility is that 5-HT may be released by a pathway stimulated by light pulses and contained within the eye, but the release of 5-HT is delayed for some period after the light pulse. At first, this appears to be a reasonable suggestion since the phases during which light and 5-HT produce similar phase shifts differ by about 6-12 h. A light pulse which would produce a phase advance in the eye CR would be given during the late projected night. For this light pulse to stimulate the release of 5-HT which would then produce or add to an advance phase shift, the 5-HT would have to be released during the late projected day, at least 6 h after the light pulse. This possibility does not
seem unlikely, however, since light induced phase shifts are expressed almost immediately after the light pulse, while most 5-HT induced phase shifts require 24-48 h. to be fully expressed. Since release of 5-HT would have to be delayed at least 6 h. after the end of the light pulse to act at the correct phase and the 5-HT effects on the CR could require another 24 h. to be expressed, the difference in the appearance of the phase shifts produced by light and 5-HT suggest that delayed 5-HT release is not involved in the mechanism of light induced phase shifting in the isolated eye.

With regard to the fact that the PRCs of 6 h. and 1 h. light pulses and 6 h. 5-HT pulses are shifted in time relative to each other, two points should be considered. The first point concerns whether the differences between the 5-HT and light PRCs have any significance. If evidence can be found which suggests that the differences are significant, the second point to consider is whether the PRC differences can be explained in terms of different entrainment pathways for light and 5-HT.

It seems reasonable to suggest that the effects produced by a 6 h. pulse of (10^{-5}M) 5-HT could be quite different than the effects of 5-HT released physiologically by an \textit{in vivo} entrainment pathway. Some results [a PRC of 6 h. pulses of 5 \times 10^{-5}M 5-HT, Corrent, unpublished; and phase shifts due to 3.0 h. 5-HT (5 \times 10^{-7}M) treatments, Eskin and Kay, unpublished] indicate that changes in the length or concentration of 5-HT pulses could produce somewhat different PRCs. Nevertheless, under none of the conditions tried has 5-HT been found to produce advance phase shifts at times when light pulses produce advances. Thus, there do appear to be some inherent differences between the effects of 5-HT and light on the
CR at any given phase, which suggests that 5-HT is probably not involved in contributing to light-induced phase shifting in the isolated eye.

The differences in the PRCs of 5-HT and light, however, could easily be reconciled by assuming delayed release of 5-HT by an extraocular pathway utilizing 5-HT to transmit L:D information. The delayed release of 5-HT would produce phase shifts complementary in sign (advance:delay) with the phase shifts produced by light. Alternatively, if 5-HT is used to transmit circadian information from other COs in *Aplysia*, the phase of 5-HT release could be timed to coincide with the necessary correction, and the PRC of 5-HT would not be expected to bear any relation to the PRC of light.

III. Potential Cellular Mechanism of 5-HT Induced Phase Shifting:

Non-Physiological Mechanisms of 5-HT Activity

It has been proposed in this thesis that 5-HT acts as a neurotransmitter in the eye and transmits circadian information to the CO of the eye. The possibility that 5-HT might be producing phase shifts by some non-physiological mechanism has also been investigated, and the evidence against such a role will be discussed.

5-HT causes phase shifts of the CR at low concentrations (10^{-7}M). This indicates that the system on which 5-HT is acting has a high affinity for 5-HT, and that 5-HT may be producing effects by binding to a high affinity receptor. The existence of such a 5-HT receptor in the eye would support the idea that 5-HT performs a physiological role in phase shifting. The fact that 5-HT precursors, metabolites, and several chemically related compounds do not phase shift, while three structurally related indoleamines (LSD, Br-LSD and bufotenine) do phase shift,
is evidence that the effect of 5-HT is structurally specific. Structural specificity is also a characteristic of physiological receptors. Two other putative neurotransmitters which are found in Aplysia, dopamine (DA) and acetylcholine (ACh), have been applied to the eye to test if they produce phase shifts (Eskin, 1977a and unpublished results; Corrent et al., 1978). Neither of these drugs caused significant phase shifts. This provides support for the idea that phase shifting is a specific effect of 5-HT in the Aplysia eye.

5-HT and Changes in Membrane Permeability

The classical model of neurotransmitter action requires that the transmitter substance bind to a specific receptor site on the post synaptic membrane and subsequently alter the ionic conductance of the post synaptic cell. At least seven and possibly more 5-HT induced membrane potential changes have been described in Aplysia cells, involving permeability changes to Na\(^+\), K\(^+\), Cl\(^-\), Ca\(^{2+}\) (Gerschenfeld and Paupardin-Tritsch 1974a; Pellmar and Carpenter, 1979). The ion manipulation experiments described in this thesis have provided evidence against the involvement of conductance changes to Na\(^+\), Cl\(^-\), and Ca\(^{2+}\) as a mechanism of 5-HT induced phase shifts. Conductance increases or decreases to K\(^+\) remain possibilities. However, tryptamine, which blocked 5-HT induced increases in K\(^+\) conductance, but not the conductance decreases described by Gerschenfeld and Paupardin-Tritsch (1974a), did not block 5-HT induced phase shifts. Thus, if an increase in K\(^+\) conductance due to 5-HT is involved in phase shifting, the 5-HT receptor mediating the effect has different properties from the receptor mediating the K\(^+\) conductance increases described by Gerschenfeld and Paupardin-Tritsch (1974a). It is
not possible to determine, without using intracellular techniques, if 5-HT is producing increases or decreases in membranes resistance. Even if such experiments were done, the results would not be very helpful in determining the processes involved in phase shifting unless the 5-HT effects on the cells producing the CR were examined. Since these cells are, as yet, unknown, this is not possible at present. Though not attempted, it may be possible to examine the involvement of a permeability increase to K⁺ in 5-HT phase shifting, though some obstacles do exist. Either raising or lowering the external K⁺ concentration appears to be capable of producing phase shifts (Eskin, 1972, and Corrent, unpublished results). However, to lower the K⁺ equilibrium potential to near the resting potential (from -50 to -70 mv, depending on the Aplysia cell) it should only be necessary to raise (K⁺)ₜₒᵤₜ to about twice its normal concentration. This assumes that the increase in (K⁺)ₜₒᵤₜ has little effect on the resting membrane potential or on (K⁺)ᵢₙ, which both appear to be reasonable assumptions (Lieberman, 1977; Gorman and Marmor, 1970). Since 20 mM (K⁺)ₜₒᵤₜ (2 x normal) is probably below the level required for HiK⁺-induced phase shifts (Eskin, 1974, and unpublished results), and since the experiment can be run at a phase during which HiK⁺ produces either small or no phase shifts, phase shifting by HiK⁺ may not be a problem in testing the involvement of K⁺ conductance changes in 5-HT phase shifting.

The possibility that 5-HT may cause phase shifts by decreasing membrane conductance to K⁺ is supported by the results of Gerschenfeld and Paupardin-Tritsch (1974a, b). They have described two 5-HT responses in Helix and Aplysia neurons which involved decreases in the membrane
conductance to K\textsuperscript{+} and for which no antagonists were found. Although 5-HT antagonists effective in blocking the 5-HT induced conductance increases described by Gerschenfeld and Paupardin-Tritsch (1974a) were tried on the eye along with other 5-HT blockers found to be effective in Aplysia, none of the drugs used were effective antagonists of the phase shifts produced by 5-HT. This suggests that the receptors mediating the conductance decreases described by Gerschenfeld and Paupardin-Tritsch (1974a) (designated \( \alpha \) and \( \beta \) receptors) may be similar or identical to the 5-HT receptors mediating phase shifting since both are insensitive to inhibition by common 5-HT antagonists. Alternatively, it may be that a conductance change is not a necessary step in 5-HT induced phase shifting. Workers investigating 5-HT effects in a number of different systems have suggested that 5-HT is not acting via some classical transmitter-receptor mechanism, but by other mechanisms not involving conductance changes of the plasma membrane. In the ABRM muscle of Mytilus (Twarog, 1968) and the lobster neuromuscular junction (Glusman and Kravitz, 1978) evidence has been presented that 5-HT may act to release Ca\textsuperscript{2+} from intracellular stores. Cedar and Schwartz (1972) reported that 5-HT treatments stimulated increases in cAMP in Aplysia nerve tissue even when all permeant ions were removed from the external solution. This result implies that most normal transmitter induced conductance changes were not required for 5-HT induced cAMP increases, although it does not rule out the possibility that K\textsuperscript{+} fluxes or changes in membrane potential were involved. It has also been suggested that 5-HT increases secretion from blowfly salivary glands by an intracellular action (Rasmussen, personal communication). Thus, in some systems it
has been suggested that classical plasma membrane permeability changes may not be necessary for 5-HT induced effects, and such a mechanism for the 5-HT effects on the eye has not been disproven.

Finally, it may be that the 5-HT effects on membrane conductance take a considerable amount of time to be expressed, and by altering ionic conditions only during the period of 5-HT treatment, the actual period of the conductance change is missed. Such an explanation gains plausibility in light of the observations that 5-HT and cAMP-stimulated increases in the phosphorylation of specific protein bands only appeared consistently 15-22 h. after the beginning of treatment (Levitan, et al., 1974). If, as in the model proposed by Greengard (1976), cAMP modulates slow changes in membrane conductance by stimulating the phosphorylation of membrane proteins, then the significant accumulation of such proteins, and the concomitant changes in membrane conductance, could require considerably longer than six hours to become apparent.

**cAMP As a Mediator of 5-HT Induced Phase Shifts**

Although 8bt-cAMP and papaverine have been shown to produce phase shifts of the CR from the eye, there is no direct evidence to connect these effects with phase shifting by 5-HT. The indirect evidence that cAMP may mediate 5-HT's effects on the CR is strongly suggestive, however. Both 5-HT and 8bt-cAMP produced about 3 h. advances when applied during a late projected day phase (CT 05-11) and 2 to 3 h. delays when given during the late projected night. 8bt-cAMP also mimicked the depressant effects of 5-HT on spontaneous CAP activity during treatment. Papaverine, a phosphodiesterase inhibitor, also produced advance and delay phase shifts during the phases at which 5-HT and 8bt-cAMP were
effective. This drug has been shown to elevate cyclic nucleotide
levels by blocking the enzymatic degradation of both cAMP and cGMP. Al-
though the effects of papaverine were somewhat less potent and more
variable than either 5-HT or 8bt-cAMP, this is not an unexpected result
in view of papaverine's relatively indirect and non-specific action on
cyclic nucleotides. The above data provides support for the idea that
5-HT produces its effects on the eye via changes in the levels of cAMP.
In a preliminary study, groups of five eyes treated with 5-HT(10\(^{-5}\)M)
showed increased levels (57% over controls) of cAMP after incubation for
1 h., but no increase was seen in eyes incubated for 5 h. Since signif-
icant phase shifts do not appear to occur in eyes treated with 5-HT for
less than three hours, it is difficult to relate the initial increase
in cAMP to a phase shifting process. However, a clue to interpreting
the cAMP results may come from the results of skeleton 5-HT pulse exper-
iments. The preliminary skeleton 5-HT data indicate that the phases of
onset and termination of stimulation may be more important than the
absolute duration of the pulse. Thus, it may be that the crucial changes
in cAMP come at the onset of 5-HT treatment and 6 h. later at the termi-
nation. Alternatively, the crucial changes in 5-HT stimulated cAMP may
occur only in a few required cells, and this increase may be too small
to be easily detected. These possibilities will be investigated. The
possibility that cAMP mediates the effects of 5-HT on the eye is support-
ed by the number of studies in which 5-HT effects on Aplysia nerve tis-
sue are associated with increases in cAMP, potentiated by PDE inhibitors,
or mimicked by cAMP and its analogs (Weiss, et al., 1978; Brunelli, et al.,
1976; Shimahara and Tauc, 1977; Levitan, et al., 1974; Cedar and Schwartz,
1972; Higgins, 1977). In addition, a 5-HT stimulated adenylate cyclase has been isolated from Aplysia neuronal cell bodies (Levitan, 1978). Direct evidence of 5-HT stimulated cAMP increases in the eye must now be sought, along with evidence that the interactions of 5-HT and cAMP in the eye meet other criteria for demonstrating cAMP mediation of transmitter effects (Beam and Greengard, 1975).

Although 8bt-cAMP and papaverine (a PDE inhibitor) mimicked the effects of 5-HT on the CR, IBMX (a different class of PDE inhibitor) produced only weak effects on the CR when used alone, and dibutyryl cAMP, a cAMP analog, produced no significant effects. These results may not conflict with those obtained with 8bt-cAMP and papaverine for the following reasons. IBMX and other drugs in the xanthine family are widely used as PDE inhibitors, but evidence exists that some of their effects may be due to properties unrelated to their abilities to inhibit PDE. In addition, in many tissues inhibition of PDE by xanthine analogs was much less potent than the inhibition produced by papaverine and its derivatives (see Weiss and Fertel, 1977, for review). It is possible therefore that IBMX was either less effective than papaverine as a PDE inhibitor in the eye, or that other actions of IBMX competed with or reduced the effects of increasing cAMP on the CR. It is important to note, however, that IBMX treatments significantly increased the effects of subthreshold concentrations of 5-HT on the CR. The demonstration that inhibiting the rate of breakdown of cyclic nucleotides potentiates the effects of a treatment is considered to be one criteria for demonstrating that the treatment produces its effects by increasing cyclic nucleotide levels (Beam and Greengard, 1975).
Dibutyryl cAMP (db-cAMP) produced no significant effects in the eye, but slow and negative results from db-cAMP treatments have been reported previously in *Aplysia* tissue in which cAMP is believed to mediate activity. Triestman and Levitan (1976) reported that db-cAMP had no effect on cell bursting rhythms in *Aplysia* abdominal ganglia, while 8bt-cAMP, papaverine, and IBMX altered the bursting pattern. Db-cAMP was reported to increase phosphorylation of proteins in *Aplysia* ganglia, but only after incubations of 15 h. or longer. In addition, it has been suggested that while db-cAMP probably crosses membranes more quickly than cAMP and is more resistant to breakdown, it is also less effective intracellularly than cAMP and may even competitively inhibit cAMP activity (Berridge, 1970). Thus, plausible explanations exist for the lack of effect of db-cAMP on the *Aplysia* eye.

Cyclic AMP has been reported to mediate changes in membrane permeability, intracellular Ca\(^{2+}\) concentration, protein synthesis, and cellular metabolism and energy utilization (Greengard, 1976; Berridge and Lipke, 1978; and see Makman, 1977, for review). This list of potential cAMP mediated actions is matched by an equally broad list of cellular mechanisms which might be involved in phase shifting in the *Aplysia* eye. Procaine, mephenesin, and high K\(^{+}\) treatments (all phase shift the CR from the eye) all have effects on membranes or membrane potentials (Eskin, 1971; 1977). Manganese, and the Ca\(^{2+}\) ionophore A23187 (both phase shift *Aplysia* eye CR) have primary effects on intracellular Ca\(^{2+}\) concentration, and 2,4-dinitrophenol and Na-cyanide (both phase shift *Aplysia* eye CR) affect both energy utilization and metabolism (Eskin and Corrent, 1977). Unlike 5-HT, however, none of the non-
physiological treatments described above produce phase shifts or a PRC which closely resembles the phase shifts produced by cAMP derivatives. It is possible that some of these drugs are more limited in the scope of their effects than 5-HT and cAMP, and thus produce PRCs with only delay phase shifts (Mn²⁺, A23187, 2,4-DNP, NaCN). Other of these treatments may alter or bypass normal regulatory mechanisms in the cell and thus produce much larger phase shifts than 5-HT and cAMP (mephenesin, high K⁺, protein synthesis inhibitors). It will be interesting to discover if any of the mechanisms involved in phase shifting by the above treatments are also involved in the phase shifting effects of cAMP on the CR from the Aplysia eye.

5-HT effects on CAP activity

5-HT, in addition to its ability to cause phase shifts, also depressed spontaneous CAP activity during treatment, and often caused increases in the amplitude of CAP peaks as much as 48 h. after it had been rinsed out. These results suggest that 5-HT has both long and short term effects on the cells of the eye, and support the idea that 5-HT has a physiological role in the eye. It is not known at this time if either or both of these effects of 5-HT are due to 5-HT activity on synaptic receptors. Such actions of 5-HT are plausible, however, since in Aplysia ganglion cells, 5-HT has been shown to depress cellular excitability, and stimulation of a 5-HT containing neuron in Aplysia has been reported to inhibit some post-synaptic cells (Gerschenfeld and Paupardin-Tritsch, 1974; Weiss, et al., 1978).

The connection between the effects of 5-HT on CAP activity and on the CR are not clear. While some evidence seems to indicate that both
types of effects have similar sensitivities to 5-HT, most available evidence indicates that the 5-HT inhibition of CAP activity, and 5-HT induced increases in the amplitude of the CR, are not necessary steps in phase shifting by 5-HT. There are five groups of results which support this statement. First, the magnitude of the CAP effects is usually not correlated with the magnitude of phase shifts. Second, other drugs which do not produce these effects on CAPs or produce them only weakly, do cause phase shifts of similar magnitude to phase shifts caused by 5-HT. Third, the CAP effects of 5-HT can be masked or eliminated by treatments which do not block phase shifting. Fourth, 5-HT produces CAP effects at concentrations which do not block phase shifting. Fifth, several treatments which inhibit spiking don't phase shift. These results are evidence that 5-HT is not acting on the CO by altering CAP activity and they are in good agreement with data obtained from a number of different treatments on the eye in which effects on CAPs were not correlated with phase shifts (Eskin, 1977; Eskin and Corrent, 1977).

Nevertheless, some interesting parallels can be drawn between the phase shifting effects of 5-HT and both of the effects of 5-HT on CAP frequency. At some 5-HT concentrations, though not all, there is a weak correlation between the size of the phase-shift produced and the increase in the amplitude of the circadian oscillation of CAPs. Bufo-tenine, which produced larger phase shifts than LSD, also caused increases in the peaks of the CAP rhythm and strongly depressed spontaneous CAP activity, while LSD had much weaker effects in both areas. 8 bt-cAMP, which produced phase shifts of the rhythm similar to 5-HT phase shifts, also mimicked 5-HT's depression of CAP activity, while
db-cAMP, which did not significantly phase shift the CR, had no effects on CAP activity. Short 5-HT pulses (2-3.5 h.) which did not phase shift, also did not cause amplification of CAP peaks, while longer 5-HT pulses (8-12 h.) produced both phase shifts and increased CAP peaks. All of the above results suggest that the receptors (or processes) which mediate both the long and short term effects of 5-HT on CAPs seem to be similar (in their sensitivities to 5-HT agonists and cAMP analogues) to the receptors (or processes) which mediate phase shifting by 5-HT. However, although the receptors (or processes) mediating the different effects of 5-HT on the eye are similar, the data indicates that the changes in spontaneous CAP activity and increases in the amplitude of the circadian oscillation (of CAP frequency) caused by 5-HT are not necessary steps for phase shifting.

**Light Response**

5-HT increases both the ERG and the tonic responses of the *Aplysia* eye to light. While this indicates that 5-HT can change the sensitivity of the eye to light, and thus suggests a means by which 5-HT could affect the CR, the bearing of these results on phase shifting by 5-HT (in DD) is not clear. However, the data do support a physiological role for 5-HT in the eye and suggest that the receptor cells of the eye may be the target cells for 5-HT effects. This possibility is intriguing because one model of the circadian system in the eye places the CO in the receptor cells (Eskin, 1977a). We caution, however, that there is no data to suggest that other cell types may not be involved in the effects which 5-HT produces in the eyes.
Conclusions

The cellular processes upon which 5-HT and cAMP act to produce phase shifts are, as yet, unknown. Nevertheless the phase shifting effects of these drugs represent a major step in the attempts to elucidate both the physiological mechanisms involved in entrainment and the cellular processes involved in the generation of circadian rhythms. If the physiological processes, which when altered change the time keeping properties of the CO, can be elucidated, we will then have a pathway which must eventually lead us to the time keeping mechanism itself. The results presented in this thesis are particularly significant because they may represent steps in a physiological entrainment pathway. 5-HT is the first endogenous substance to produce phase shifts in Aplysia and one of the few natural substances known to produce effects on the CR from any system (Gwinner, 1974; Daan, et al., 1975; Morin, et al., 1977; Tureck, et al., 1976; Andrews, 1968).

Though much work remains to be done, it is hoped that the results presented in this thesis will provide a significant impetus to research into the mechanisms of entrainment and the physiological nature of circadian systems.


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Potential sources of circadian information which may be transmitted to the circadian pacemaker (CP) cell(s) in the eye by 5-HT. Information may reach the CP cells via the efferent fibers in the optic nerve which directly or indirectly release 5-HT or 5-HT may be carried in blood vessels which terminate around the eye. The location of the CP cells is not known, but they are apparently either directly receptive to light (receptor cells) or connected to the receptor cells via electrotonic junctions (secondary cells).
Transmission of Information

Potential Sources of Circadian Timing Information

- receptor cell
- secondary cell
- 5-HT
- optic nerve
- circadian pacemaker
- blood vessel
- extra-ocular photoreceptors
- other circadian pacemakers
- eye receptor cells
Advance phase shift of the CR of optic nerve impulses from the isolated *Aplysia* eye, produced by a six h. treatment of $1 \times 10^{-5} \text{M} 5$-HT. The crosshatched bar represents the time and duration of treatment. Eyes were in constant dark throughout the experiment. The open bars represent the projected light portion of the L:D (12:12) schedule to which the intact animal was entrained.
Fig. 3  Dose-response curve for 6 h. 5-HT treatments. The lower threshold for the phase shifting response is between $10^{-8}$ and $10^{-7}$M. At 5-HT concentrations of $2 \times 10^{-3}$M no net phase shifts are produced. The vertical bars represent the range of results for a given group of treatments.
Advance phase shift produced by $10^{-7}$ M 5-HT. Note that there is no increase in the amplitude of the CAP rhythm recorded after treatments with $10^{-7}$ M 5-HT (as contrasted to Figs. 2, 7, 8) but the phase shifts produced are not significantly smaller than the phase shifts produced by higher 5-HT concentrations.
Fig. 5  Phase response curve for 6 h. 5-HT treatments (10^{-5} M). The horizontal bars represent the mean phase shift at a given phase and span the treatment period. The vertical bars represent the range of results for a given group of treatments.
Fig. 6  Identification by high-voltage paper electrophoresis of newly synthesized tritium-labeled 5-HT from a group of ten eyes. The eyes were incubated in $^{3}$H-tryptophan ($10^{-5}$M), for 24 h., then run on high voltage electrophoresis in 2 separate pH buffers (pH 1.9 and 4.7), with pure $^{14}$C-5-HT. The peaks of native 5-HT ($^{14}$C) and synthesized 5-HT ($^{3}$H) overlap one another precisely.
Fig. 7  Change in waveform of rhythm caused by 5-HT treatment. Note that the amplitude of the CR from the 5-HT treated eye is considerably larger than that of the control eye.
Fig. 8  Advance phase shift produced by a six h. treatment of $10^{-3} \text{M}$ bufotenine. The conditions of the experiment were identical to those in Fig. 1. Note the increased amplitude of the rhythm from the treated eye relative to control. This feature is common in eyes phase shifted by 5-HT treatments.
Fig. 9 Extracellular ERG recordings from eyes exposed to a 6.5s pulse of white light. The arrows indicate the onset of light. Figures 3A and 8C are recordings from control eyes. Figure 8B is a recording taken from the neuropile area at the base of the eye (near the attachment of the optic nerve) after 5-HT (5 x 10^{-7}M) treatment. Figure 8D shows the increase in ERG amplitude produced in the same eye as 8C, 24 min. after treating the eye with 5-HT (5 x 10^{-7}M).
FIG. 9

a) before 5-HT
b) after 5-HT
c) before 5-HT
d) increase: 40%

3 sec
Fig. 10  Possible cellular mechanisms for experimental treatments. This is a highly generalized representation of the cellular processes which may be affected by the treatments used in this these project. The diagram represents a pre-synaptic 5-HT containing neuron (upper page) and a post-synaptic target cell (lower page). This diagram is not meant to imply proof of any of the proposed mechanisms, but merely to aid the reader in localizing the most probable effects of the treatment.
1] 5-HT STORAGE
5-HT measured by GC-MS

2] TRYPTOPHAN UPTAKE
p-CPA; LoNa+; LoK+

3] 5-HT SYNTHESIS
p-CPA

4] DEPOLARIZATION/DEPLETION
HiK+; HiMg2+-LoCa2+

5] 5-HT REUPTAKE
LoNa+; Fluoxetine

6] 5-HT RECEPTOR (Phase Shifting)
5-HT; Bufotenine; Br-LSD; LSD

7] 5-HT RECEPTOR (CAP effects)
5-HT; Tryptamine; Methysergid
LSD; Br-LSD; Bufotenine

8] RECEPTORS FOR OTHER PUTATIVE NEUROTRANSMITTERS
DA; ACh

9] TRANSMITTER INDUCED CONDUCTANCE CHANGES
LoNa+; LoK+; LoCa2+-EGTA
LoCl-

10] cAMP ANALOGS
8-benzylthio-cAMP;
8-dibuteryl-cAMP?

11] PHOSPHODIESTERASE (PDE) INHIBITORS
Papaverine; IBMX

5-HT CONTAINING CELL
(Pre-synaptic cell)

5-HT TARGET CELL
(Post-synaptic cell)
Table 1  All of these drugs have been previously used to inhibit 5-HT produced effects on *Aplysia* cells. These drugs were used in six h. treatments over the concentration ranges shown at phase CT 05-11. The drugs were applied alone to test for phase shifting effects, and also were added to the eyes both before and during 5-HT treatments to test for the ability to block 5-HT induced phase shifting. The advance phase shifting effects of 5-HT on the eye are mimicked by LSD, Brom-LSD, and Bufotenine. However, at least in the case of LSD, the threshold concentration of the drug necessary to achieve an effect on the CR from the eye is at least a magnitude greater than the threshold concentration of 5-HT. None of the drugs tried had a significant antagonist effect.
<table>
<thead>
<tr>
<th>TREATMENT</th>
<th>CONCENTRATION</th>
<th>BLOCK 5-HT PHASE SHIFT?</th>
<th>PRODUCE ADVANCE PHASE SHIFTS ON CR FROM EYE</th>
<th>INHIBIT SPONTANEOUS CAP ACTIVITY</th>
</tr>
</thead>
<tbody>
<tr>
<td>1. LSD</td>
<td>$10^{-4} M$</td>
<td>NO</td>
<td>YES</td>
<td>YES</td>
</tr>
<tr>
<td></td>
<td>$2 \times 10^{-7} M$</td>
<td>NO</td>
<td>YES</td>
<td>NO</td>
</tr>
<tr>
<td>2. Br-LSD</td>
<td>$10^{-4} M$</td>
<td>NOT TRIED</td>
<td>YES</td>
<td>YES</td>
</tr>
<tr>
<td>3. Bufotenine</td>
<td>$10^{-3} M$</td>
<td>NO</td>
<td>YES</td>
<td>YES</td>
</tr>
<tr>
<td></td>
<td>$10^{-4} M$</td>
<td>NO</td>
<td>YES</td>
<td>YES</td>
</tr>
<tr>
<td>4. Tryptamine</td>
<td>$2 \times 10^{-4}$ to $5 \times 10^{-5} M$</td>
<td>NO</td>
<td>NO</td>
<td>1/2</td>
</tr>
<tr>
<td>5. D-Tubocurarine</td>
<td>$10^{-3}$ to $10^{-4} M$</td>
<td>NO</td>
<td>NO</td>
<td>NO</td>
</tr>
<tr>
<td>6. *Methysergide (UML)</td>
<td>$10^{-3}$ to $10^{-4} M$</td>
<td>NO</td>
<td>NO</td>
<td>1/2</td>
</tr>
<tr>
<td>7. *Cinanserin</td>
<td>$10^{-4}$ to $10^{-5} M$</td>
<td>NO</td>
<td>NO</td>
<td>&lt;1/4</td>
</tr>
<tr>
<td>8. 5-HT</td>
<td>$2 \times 10^{-3}$ to $10^{-9} M$</td>
<td>At high concentrations (10^{-3} M) 5-HT does not produce significant phase shifts</td>
<td>YES</td>
<td>YES</td>
</tr>
</tbody>
</table>

* At high concentrations caused irregularities in the CR.
Table 2  The ionic environment surrounding the eyes was changed to investigate the effects of reducing ionic gradients on the 5-HT induced phase shifts. If 5-HT is acting by increasing the membrane permeability of the circadian pacemaker (CP) cell(s) to $\text{Ca}^{2+}$, $\text{Na}^+$, or $\text{Cl}^-$, reducing the electrochemical gradients of these ions should reduce the size of the 5-HT induced phase shifts. Increases in the membrane permeability of these ions do not appear to be involved in the 5-HT phase shifting. Lowering $\text{Ca}^{2+}$, and raising $\text{Mg}^{2+}$, should reduce or block release of synaptic transmitters. From the results of these manipulations, it appears that 5-HT is acting either directly on the CP cells or on cells electrically coupled to them.
### Table 2

**EFFECTS OF CHANGING THE IONIC ENVIRONMENT ON 5-HT INDUCED PHASE SHIFTS**

<table>
<thead>
<tr>
<th>TREATMENT</th>
<th>BLOCK 5-HT PHASE SHIFT</th>
<th>CAUSE PHASE SHIFT</th>
</tr>
</thead>
<tbody>
<tr>
<td>1. Low Ca$^{2+}$ w/EGTA (Free Ca$^{2+}$ &lt; $10^{-8}$)</td>
<td>NO</td>
<td>NO</td>
</tr>
<tr>
<td>2. Low Ca$^{2+}$ (.1mM)-High Mg$^{2+}$ (200mM)</td>
<td>NO</td>
<td>NO</td>
</tr>
<tr>
<td>3. Low Na$^+$ (7mM) Low Cl$^-$ (287mM) (NaCl replaced w/mannitol)</td>
<td>NO</td>
<td>NO</td>
</tr>
</tbody>
</table>