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13. ABSTRACT (Maximum 200 words)

Photoreceptors of the chick pineal are one of the only vertebrate intrinsic circadian oscillators amenable to cellular analysis. We characterized the electrophysiological properties of these cells using patch clamp and fura-2 recording techniques. We analyzed several types of voltage-and cGMP-activated ionic channels, and obtained evidence that cGMP-activated channels participate in phototransduction. We also examined intracellular Ca^{2+} dynamics and obtained evidence for the existence of intracellular Ca^{2+} stores and Ca^{2+} oscillations that can be mobilized by drugs and hormones that increase cAMP and promote melatonin secretion. Norepinephrine, which inhibits melatonin secretion, had no effect on Ca^{2+} dynamics. Most importantly, we discovered a new type of ionic channel, I_{LOT}, whose gating is under direct circadian control. I_{LOT} is permeable to Ca^{2+}, but its gating is not controlled by membrane potential or intracellular ligands. I_{LOT} has unusual kinetic properties, as it can stay open for seconds at a time. This feature is not seen in other types of ionic channels. I_{LOT} is *only* active in the nighttime, even in pineal cells free-running in constant-dark conditions. I_{LOT} is also present in chick retinal photoreceptors, which also contain a circadian oscillator. I_{LOT} therefore represents a logical target for pharmacological manipulation of circadian output.

14. SUBJECT TERMS

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1. Accomplishments/new findings. The goals of this research project were to characterize more fully the fundamental electrophysiological properties of photoreceptor and circadian oscillator cells of the chicken pineal gland, one of the classic model systems for the cellular analysis of circadian rhythms. In particular, we hoped to learn more about the phototransduction process, the regulation of intracellular free Ca^{2+} by the intrinsic circadian oscillator and by extrinsic neuronal inputs to the gland, and to determine if any of the intrinsic electrophysiologal properties of the pineal cells are regulated by the circadian oscillator. Nearly all of these goals were acheived. Among the many observations that we made and published, the most important result was the discovery of a completely new type of ionic channel in pineal and retinal photoreceptors that is under the direct control of the intrinsic circadian oscillator. The importance of this observation needs to be stressed, as *it is the first mechanism that can explain how intrinsic circadian oscillators can control the electrical behavior of excitable cells and photoreceptors*. The importance of these findings is indicated by the fact that our first report on these channels was published in *Nature* (D'Souza & Dryer, 1996), and by the number of lecture invitations (at other research institutions and at major international meetings) that were received as a result of this paper.

This observation, although one at the level of basic research, is of considerable importance in the field of basic circadian biology. As such it is directly relevant to the mission of the Air Force, as it suggests a potential strategy for the pharmacological modulation of the outputs of circadian oscillators. For an example, an inhibitor of this channel could alter melatonin secretion, which is a dark-adaptive signal for the retina, and is an endocrine signal indicating nightime in several other psychophysiological processes. This same channel may be an essential part of the output pathways of other circadian oscillators, including those located in the suparachiasmatic nucleus. In theory, this suggests a practical approach to a significant Air Force and civilian technology challenge, namely how to deal with cognitive and physiological problems associated with jet-lag, shift work, or any other situation where personnel are subjected to altered temporal cues, changes in light-dark cycles, or long periods of sleeplessness. Obviously this occurs in pilots and other aircraft personnel, who may have to make difficult and potentially costly decisions under conditions where the physiological status is not well suited for high-level cognitive, perceptual, and motor functioning. This situation also arises in other situations, such as when personnel work nights during the week, but try to live on a normal temporal cycle on weekends; this is common for offshore oil workers, for example, as well as with certain Navy personnel. Realizing the potential of this basic science discovery will require

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additional research on the properties and distribution of this channel, and to determine how it is regulated by the circadian oscillator.

a. Voltage-activated ionic channels and circadian regulation of intracellular free Ca^{2+} in chick pineal cells. Chick pineal cells exhhibit increases Ca^{2+} influx during the nightime, a portion of which is blocked by Ca^{2+} channel antagonists such as nifedipine. Acutely-isolated chick pineal cells express voltage-activated dihydropyridine-sensitive L-type Ca^{2+} channels that can be readily detected in whole-cell recordings (Henderson & Dryer, 1992) and fura-2 imaging experiments (D'Souza & Dryer, 1994). We have not seen daily rhythms in the amplitudes of Ca^{2+} currents in whole-cell patch clamp experiments on cultured pineal cells (D'Souza & Dryer, unpublished data). Our data are consistent with the idea that L-type Ca^{2+} channels serve as an amplification mechanism to enhance nighttime Ca^{2+} influx triggered by changes in the gating of other plasma membrane ionic channels. Evidence for this will be presented further below.

The gating of voltage-activated Ca^{2+} channels can be indirectly influenced by changes in membrane potential induced by the opening or closing of other types of ionic channels. We have found that cultured chick pineal cells also express delayed rectifier K^+ currents that can be blocked by tetraethylammonium (TEA) (Henderson & Dryer, 1992). Some pineal cells also express Ca^{2+}-activated K^+ channels and many express a rectifying cationic current similar to the I_H expressed in retinal photoreceptors. However, we have not observed daily rhythms in the mean amplitudes and densities of any of those currents (D'Souza & Dryer, in preparation).

Chick pineal cells contain intracellular Ca^{2+} stores that can be mobilized by application of inhibitors such as thapsigargin, which block reuptake into intracellular stores (D'Souza & Dryer, 1994). The purpose of these stores is unknown, but it should be noted that the fill state of these stores is coupled to Ca^{2+} influx pathways in the plasma membrane. Thus, depletion of intracellular stores activates a Ca^{2+} influx pathway in the plasma membrane that presumably allows for rapid refilling of the stores under normal physiological conditions. In addition, the intracellular store equilibrate rapidly with the cytosol, with the result being that blockade of Ca^{2+} uptake into the intracellular stores leads to rapid depletion of these stores (D'Souza & Dryer, 1994).

A subpopulation of chick pineal cells (10-20%) exhibit spontaneous oscillations in the intracellular free Ca^{2+} concentration at any given point in time (D'Souza & Dryer, 1994). These oscillations appear to depend primarily on influx from the extracellular compartment. Our initial observations suggest that these do not exhibit a circadian rhythm, but this may reflect the use of the indicator dye fura-2, which emits light in the

visible range, and which may alter system it is trying to measure. We have shown that agents that increase intracellular cAMP (such as forskolin, 8-Br-cAMP, VIP) also increase intracellular free Ca^{2+} (D'Souza & Dryer, 1994). The mechanism of this effect is unknown, but it indicates a positive feedback relaitonship between these two messengers, as Ca^{2+} is known to increase cAMP in pineal and retinal photoreceptors.

b. Cyclic GMP-activated cationic channels are expressed in chick pineal cells. These channels are an essential component of phototransduction cascades in rods and cones. Two different type of cGMP-activated channels can be detected in inside-out patches excised from acutely-isolated chick pineal cells (Dryer & Henderson, 1991, 1993). The predominant form found in pineal cells can be activated by cGMP (ED_{50} = 35 μM, Hill slope = 2.8) but not by cAMP at concentrations as high as 500 μM. The unitary conductance of these channels is 22 pS at -60 mV, and there is some rectification in the current-voltage relationship in symmetrical NaCl. These channels exhibit rapid voltage-dependent blockade in the presence of millimolar concentrations of divalent cations, especially Mg^{2+}, applied to either side of the patch membrane. The second form of channel is similar except that it has a slightly higher unitary conductance (Dryer & Henderson, 1993). The density of cGMP-activated channels in pineal cells is much lower than in rod or cone outer segments, as determined by electrophysiology (Dryer & Henderson, 1991). Supraphysiological concentrations of cyclic AMP can cause some activation of the cGMP-gated channels. Many of the openings under these conditions are to subconductance states, and the results are consistent with the idea thatsingly liganded channels open to subconductane states. Application of phosphodiesterase inhibitors causes activation of cGMP-activated channels within 1-3 min in intact acutely-isolated chick pineal cells under visible illumination (D'Souza & Dryer, 1995). This activity is immediately suppressed by patch excision. By contrast, application of forskolin for up to 30 min does not stimulate cGMP-activated channels. This suggests that phototransduction mechanisms, at least for the acute inhibition of melatonin secretion, are similar to those in retina. The presence of cGMP-activated channels and other essential components of a "retinal" phototransduction cascade suggests a mechanism for daily rhythms in intracellular Ca^{2+} in chick pineal cells maintained on light-dark cycles as well as for the acute inhibitory effects of light on melatonin secretion. However, we have not seen any evidence for rhythms in the gating of cGMP-activated channels in chick pineal cells free-running under constant dark conditions (D'Souza & Dryer, unpublished data). This suggests that some other mechanism is responsible for the free-running circadian

rhythms in Ca^{2+}-influx. Evidence that I_{LOT} represents such a mechanism is discussed below.

c. Experimental designs for studying circadian regulation of ionic channels in chick pineal photoreceptors. The free-running circadian rhythm in melatonin secretion from chick pineal cells persists for at least 10 cycles in dissociated cell culture under constant dark conditions if they are maintained at 39°-40°. Rhythms dampen after 3-4 cycles at 37°. Here we will summarize certain important details of experimental design before discussing the results. Similar considerations also apply to studies of cultured chick retinal cells.

Chick pineal glands are obtained from chicks 1-2 days post-hatch (P1-2), dissociated, and cells are plated onto poly-D-lysine-coated glass coverslips. For studies of circadian regulation, cells are maintained at 40° in CO_2 incubators equipped with cool fluorescent lights and timers for entrainment *in vitro*. Cells are entrained to a 12 hr:12 hr light-dark cycle for four days, at which point they are switched to constant darkness at the expected lights out. With this experimental design, it is possible to record from cells at different phases of the light-dark cycle, *or* to record from cells at various circadian times after they have been switched to constant dark conditions. *This enables us to determine if a rhythm is driven by the light-dark cycle or by the intrinsic circadian oscillator.* Experiments performed during normal 12 hr:12 hr light-dark cycles are made on the fourth day *in vitro*. In most of our studies to date, patch clamp recordings have been made under visible illumination during the light phase at zeitgieber time 4-5 (ZT4-6) or under infrared illumination during the dark phase (ZT16-18). Recordings from free-running cells have been made under infrared illumination on the *second* day of constant darkness (daytime, circadian time 4-6 -- CT4-6; and nighttime, CT16-18).

Patch clamp recordings are made using standard methods at room temperature (21-23°). Electrodes are coated with Sylgard™ 184 resin (Dow Corning) and fire-polished. The recording apparatus includes an inverted stage microscope equipped with Hoffman modulation contrast optics housed in a room that can be kept dark. Cells are protected by second layer of light shielding achieved by tightly covering the Faraday cage and all microscope light sources with heavy black cloth. Infrared illumination is obtained by passing a 790 nm cutoff filter into the light path of the microscope. An infrared-sensitive videocamera and a videomonitor allow for electrode placement in the absence of visible light. Unfortunately, it is not possible to make recordings from a single cell that last long enough to reliably observe circadian changes in channel gating. Therefore, it is necessary to sample populations of cells at different circadian times. In the case of I_{LOT}, we monitor a single cell for 10 min before deciding whether or not an active channel is

present. Data are stored on videotape for later off-line digitization and analysis with PCLAMP *v* 6.03 software (Axon Instruments).

d. I_{LOT}, a non-selective cationic channel under direct circadian control in chick pineal photoreceptors. This is the most important discovery in the course of our investigations to date. I_{LOT} is a non-selective cationic channel that we have found to be under circadian control in cultured chick pineal photoreceptors. We have also detected this channel in cultured chick retinal photoreceptors where preliminary results suggest that it is also rhythmic (see below). More detailed descriptions of the behavior of these channels can be found in our *Nature* paper (D'Souza & Dryer, 1996). *To the best of our knowledge, I_{LOT} represents the first case of a plasma membrane ionic that is regulated by a vertebrate intrinsic circadian oscillator. As such it provides a mechanism whereby the molecular circadian oscillator, which is comprised of a series of transcription factors and other nuclear/cytosolic mediators, can alter the electrical behavior of excitable cells at the plasma membrane. This is a major finding.* I_{LOT} is spontaneously active in cell-attached patch recordings made during the nighttime and can be easily identified because of the very long open-time of the unitary currents in pineal cells and in retinal cells. *The gating of these channels is not voltage-dependent* and is not altered by application of negative or positive pressure to the recording pipette. I_{LOT} channels occasionally stay open without apparent interruptions for seconds at a time. These long-lived events account for a considerable fraction of the total integral current accumulated over a period of minutes. Bursts of I_{LOT} openings are often separated by quiescent periods of several seconds. I_{LOT} can also be detected during the nighttime in cell-attached patches when recording pipettes are filled with 80 mM $CaCl_2$ in the absence of other cations. Although unitary current amplitudes and open-channel noise are reduced under those conditions, the characteristic long open-times still allow for unambiguous identification of these channels. *This indicates that I_{LOT} channels have a finite permeability to Ca^{2+}, but the precise Na^+:K^+:Ca^{2+} permeability ratios are unknown. It will be important to determine these ratios, as they can provide considerable insight into the amount of Ca^{2+} influx that can occur upon activation of an I_{LOT} channel and thus to the physiological functions of these channels.*

I_{LOT} can also be detected in inside-out patches from pineal cells excised into Ca^{2+}-free external salines. Spontaneous activity is not significantly changed by patch excision (D'Souza & Dryer, 1996). The unitary slope conductance of I_{LOT} under these recording conditions is 40 pS and there is very little rectification in the current-voltage relationship. I_{LOT} channels reverse at close to 0 mV under conditions where the calculated chloride

equilibrium potential (E_{Cl}) is -59 mV. *These results indicate that I_{LOT} is a non-selective cationic channel whose gating is not dependent upon continued exposure to soluble cytosolic messengers.* Consistent with this, patch excision followed by bath application of 1-10 μM Ca^{2+} does not cause activation of I_{LOT} in quiescent inside-out patches. Thus, I_{LOT} *gating is not inhibited by cytosolic messengers.* Active I_{LOT} channels are not blocked 1 mM $MgCl_2$. We do not know if patch excision alters the gating of I_{LOT} channels in cultured retinal cells.

Active I_{LOT} channels are readily detected in cultured chick pineal cells with normal recording pipettes during the night, when melatonin secretion is highest, but are almost never observed during the day. This is seen in cells maintained on a 12 hr:12 hr light-dark cycle, as well as in cells free-running in constant darkness for 2 day (see Table 1, D'Souza & Dryer, 1996). These results indicate that the gating of I_{LOT} is under direct circadian control. Moreover, I_{LOT} gating detected during the night is not inhibited by 100-lux pulses of visible light applied for up to 10 min. Therefore, I_{LOT} channels are not components of a fast phototransduction cascade.

e. The molecular mechanisms that control the gating of I_{LOT} are unknown, but the nighttime appearance of active I_{LOT} channels requires protein synthesis. I_{LOT} provides a mechanism for coupling of the electrophysiological properties of a photoreceptor cell to the output of its clock. However, the usual factors that regulate ion channel gating do not affect I_{LOT} gating. Thus, gating of I_{LOT} is not voltage-, stretch- or Ca^{2+}-dependent and does not require continued contact with soluble cytosolic messengers. Moreover, I_{LOT} is not activated in quiescent cells by patch excision, application of 10 μM melatonin, acute activation of adenylate cyclase, depletion of internal Ca^{2+} stores, or application of NO donors such as sodium nitroprusside. *However, the nighttime appearance of I_{LOT} requires protein synthesis (D'Souza & Dryer, 1997). This is true in free-running pineal cells as well as in cells maintained on 12 hr:12 hr light-dark cycles.* In these experiments, chick pineal cells were treated with the translational inhibitor anisomycin (1 μM) at CT10 and ZT10. In control experiments, we found that I_{LOT} channels are not active at the time of anisomycin treatment (0/50 cells at in both cases). Active I_{LOT} channels were observed at the normal frequency in control cells at CT18 (16/47 cells) and ZT18 (9/45 cells) but were *not* observed in anisomycin-treated cells at CT18 (0/56 cells) or ZT19 (0/85 cells). These results are highly statistically significant ($P<.0005$, G-test). Thus, protein synthesis is required for the normal nighttime expression of functional I_{LOT} channels, even in the presence of light-dark cycles, but it is not clear *what* proteins are being synthesized.

There are at least three different classes of mechanisms whereby the circadian oscillator could plausibly control I_{LOT} gating: 1) Gating of plasma membrane I_{LOT} channels may be controlled by covalent modification, possibly by direct phosphorylation/dephosphorylation of the channel molecules or closely associated auxiliary subunits. According to this scheme, the biosynthesis of regulatory kinases, phosphatases, or other protein modifying enzymes is under direct circadian control; 2) I_{LOT} gating may be regulated by other substances whose biosynthesis is under circadian control and dependent on protein synthesis. Moreover, we propose that these substances remain associated with the plasma membrane during patch excision. Examples of such substances could include lipids or G-proteins; 3) I_{LOT} channel molecules or a portion thereof may only be present in the plasma membrane during the nighttime. Biosynthesis and/or plasma membrane insertion of the channel is under circadian control and dependent on protein synthesis. These theories are not mutually exclusive, and one could certainly conceive of other schemes of varying complexity. In this regard, we have data suggesting that the nighttime appearance of active I_{LOT} channels is blocked by brefeldin-A (BFA). BFA is a drug that blocks processing of membrane proteins and secreted proteins in the Golgi apparatus. This result suggests that the protein whose synthesis is required is a membrane protein, and it may be I_{LOT} itself (D'Souza & Dryer, preliminary results). In other words, this result is consistent with the third hypothesis listed above.

f. Evidence for multiple populations of chick pineal cells and additional similarities with retinal photoreceptors. We have obtained data indicating existence of multiple histochemically and morphologically distinct cell types in our cultures. Summarizing briefly, we have been able to show that *a subpopulation of chick pineal cells both in culture and in frozen sections contain oil droplets, a hallmark of cones in the chicken retina. These same pineal cells express other cone markers.* Photoreceptor oil droplets can be stained with lipophilic dyes such as Oil . The 3 day post-hatch chick pineal gland has a follicular structure in which presumptive photoreceptive cells (based on the expression of "retinal" proteins and a 9+0 cililum) are located around the follicular lumen, as well as around the external margins of the follicles. This is also where the cells with oil droplets are found. In cell culture, these oil droplets can be easily observed in small (7-9 μ m) pineal cells under phase-contrast, Nomarsky or Hoffman optics. *Note that I_{LOT} channels and cGMP channels are only found in cells with oil droplets*, and are not seen in larger (11-15 μm) cells that lack oil droplets. The oil droplets are not heavily pigmented. It should also be noted that most, but not all of the small chick pineal cells that contain oil droplets are also labeled with peanut hemaglutinin (PHA), a lectin that selectively labels

subpopulations of cones in many vertebrate retinas including the chick. Chick pineal cells that lack oil droplets are never labeled with PHA. We have obtained robust PHA labeling in cultured pineal and retinal cells as well as in frozen sections of the whole chick pineal. In frozen sections, labeling is observed around the follicular lumen and around the margins of the follicles but is never observed in adjacent brain areas. We see similar PHA labeling restricted to the photoreceptor layer in frozen sections of the retina and in cultured retinal cells. As with pineal cells, PHA labeling is only observed in cultured retinal cells containing oil droplets. These results underscore that *chick pineal cells are simply another class of photoreceptor with many fundamental similarities to retinal photoreceptors, especially cones. They certainly represent as valid a model system as any invertebrate photoreceptor, especially for studies of circadian regulation.*

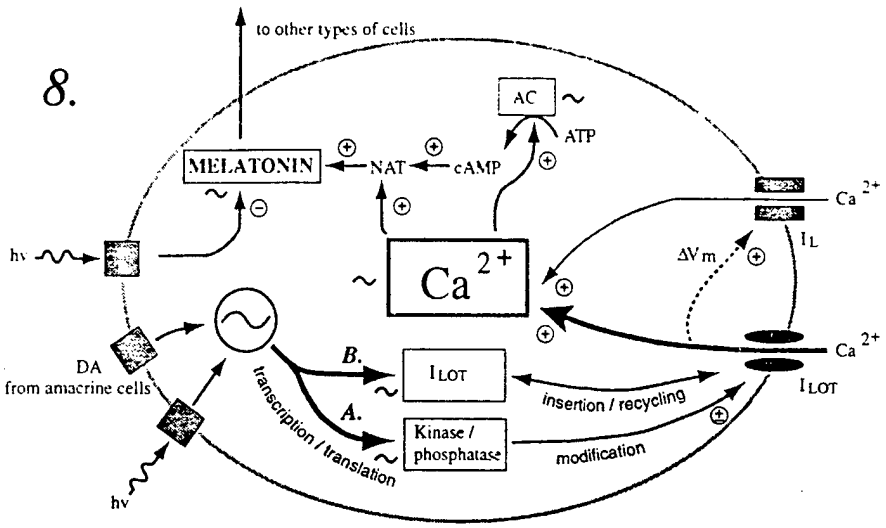
g. I_{LOT} can be detected in cultured chick retinal photoreceptors. As mentioned above, several groups, including ourselves, have looked for and found "retinal" proteins (opsins, arrestin, recoverin, transducin, cGMP-activated channels, etc.) in chick pineal photoreceptors. We have recently taken the same road in the other direction, and have been able to detect I_{LOT} in cultured chick retinal photoreceptors. In order to do this, we dissociate retinal relatively late in embryonic development, at embryonic days 18-19 (E18-19) and record after 4-7 d *in vitro*. The reason for doing this is that robust free-running circadian rhythms in retinal melatonin secretion are not detected until E20 *in vivo* and reach maximum amplitude at P3. The culture conditions are the same as for pineal cells except that the medium contains 40 ng/ml ciliary neurotrophic factor (CNTF) which we find improves the survival and health of the photoreceptors. With this culture system, we can maintain presumptive cone photoreceptors (easily identified by their prominent oil droplets and PHA staining) for up to two weeks *in vitro*. Survival rates drop dramatically if cells are dissociated later than E19.

We have detected I_{LOT} channels in 8 out of 14 cell-attached patches on cultured retinal photoreceptors at ZT16-20 (nighttime) (Reiser & Dryer, in preparation). *These channels carry current into the cell, have very long open-time kinetics indistinguishable from those of I_{LOT} channels of pineal cells, and their gating is not voltage-dependent. However, I_{LOT} channels have been seen in only 1 out of 12 retinal cells at ZT6-10 (daytime). These data, while preliminary, suggest that I_{LOT} gating is rhythmic in retinal cells, at least when they are maintained on 12 hr:12 hr light-dark cycles in vitro.* In the most widely used methods for culturing chick retinal photoreceptors, retinas are dissociated at embryonic day 6-8 (E6-8) and kept *in vitro* for several days. Under these culture conditions, one can detect free-running circadian rhythms in iodopsin gene

expression and robust cAMP-stimulation of melatonin secretion, but *free-running* circadian rhythms in melatonin secretion are not observed (J. Takahashi, personal communication). Some essential mechanism for coupling circadian clock output to melatonin secretion is apparently missing at early developmental stages. If the working model described below is correct, I_{LOT} would be a plausible candidate.

h. A working model of I_{LOT} function in circadian oscillators of retinal and pineal photoreceptors. Some of the possible schemes for I_{LOT} regulation were discussed above. This model is deliberately simplified so as to restrict attention to the physiological role of I_{LOT} in the context of melatonin secretion. We propose that similar processes take place in chick pineal and retinal photoreceptors. The model is comprised of a single cell containing an intrinsic circadian oscillator that can be entrained by light (and also by dopamine in the case of retinal photoreceptors). The most proximal outputs of the oscillator are assumed to be the transcriptional and/or translational control of a relatively small number of genes. In the diagram, the transcription of various protein kinases/phosphatases and I_{LOT} are shown as alternative clock outputs (*A.* and *B.* respectively), and we do not conceive of these alternatives as mutually or all inclusive. Upon translation, newly synthesized I_{LOT} channels pass through the Golgi apparatus and are inserted into the plasma membrane. This is depicted as a reversible process, as it is possible that the I_{LOT} rhythm reflects cycling of the channels in and out of the plasma membrane. In addition, we propose that the clock-regulated kinases and/or phosphatases cause direct modification of the I_{LOT} channels, thereby regulating their gating. For reasons of graphical simplicity, the diagram implies that these as cytosolic enzymes, but they could also be membrane bound and/or the endpoint of a complex biochemical cascade. We propose that activation of I_{LOT} results in substantial and physiologically significant Ca^{2+} influx. In addition, we propose that I_{LOT} activation causes membrane depolarization leading to activation of L-type Ca^{2+} channels, which serves to amplify the signal by causing additional Ca^{2+} influx. The resulting increase in intracellular free Ca^{2+} causes a *direct* increase in NAT activity, as well as a secondary cAMP-mediated increase in NAT. The increase in NAT activity is responsible for enhanced nighttime melatonin secretion. Light causes acute inhibition of melatonin secretion (probably in part due to inhibition of cGMP-activated channels, but this is not included in the figure). This preliminary model is supported by some data from our own and other laboratories as described above. For example, we know that the nighttime expression of I_{LOT} requires the synthesis of *some* protein or proteins, as it is blocked by anisomycin. We know that I_{LOT} channels are permeable to Ca^{2+} ions, although the available data do not allow us to make any statements as to the *amount* of Ca^{2+} influx that

8.



A working model of I_{LOT} function in photoreceptors. The model proposes that I_{LOT} plays a central role in coupling melatonin secretion to the output of the intrinsic circadian oscillator. Rhythmic changes in I_{LOT} gating require protein synthesis. Two possibilities are shown here. In ***A***, the clock regulates the expression of kinases and/or phosphatases that in turn regulate I_{LOT} gating by direct modification of the plasma membrane channels. In ***B***, the clock regulates the synthesis of I_{LOT} channels directly. These newly synthesized channels then pass through the Golgi apparatus and are inserted into the plasma membrane at the beginning of the nighttime. They are removed from the plasma membrane at the end of the night. Here we propose that the majority of the nighttime Ca^{2+} influx is through I_{LOT} channels. However, activation of I_{LOT} channels also causes membrane depolarization leading to activation of L-type Ca^{2+} channels which amplify the signal by allowing for some additional Ca^{2+} influx. We also show that the circadian oscillator is entrained by light, as well as by dopamine in the case of retinal photoreceptors. Light also causes acute suppression of melatonin secretion, possibly by inhibiting cGMP-activated channels not shown in the diagram. Additional details are in the text.

could be expected at any particular membrane potential or external Ca^{2+} concentration. I_{LOT} is a non-selective cationic channel and its activation will cause some membrane depolarization. Therefore I_{LOT} activation could also lead to L-type Ca^{2+} channel activation.

This model is focused on control of melatonin secretion simply because that is the best studied biochemical output of photoreceptor circadian oscillators. *But it should be emphasized that I_{LOT} may be important (perhaps even more important) for other physiological processes in retinal photoreceptors.* For example, I_{LOT} mediated Ca^{2+} influx into the synaptic terminals of photoreceptors could potentially have significant effects on the release of neurotransmitter onto second-order neurons. It is already known that cGMP-activated channels can directly regulate exocytosis in salamander cones. I_{LOT} could have a similar function regardless of whether or not it proves to be under circadian control in retinal cells. I_{LOT} could also affect photoreceptor physiology independent of effects on Ca^{2+} influx by simply short circuiting the membrane, thereby altering the efficacy of gap junctional transmission.

2. Personnel supported.

Dr. Stuart E. Dryer, Associate Professor, Department of Biological Science, Florida State University, Tallahassee, FL. (Currently Professor of Biology and Biochemistry, University of Houston, Houston, TX).

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Michelle Reiser, Technician. (Currently technician Department of Psychology, Florida State University, Tallahassee, FL.)

Dori Henderson, Technician. (Currently Ph.D. candidate, University of Minnesota Medical School, St. Paul, MN.)

3. Relevant refereed publications since start of grant period.

D'Souza, T. & Dryer, S. E. (1997). Elevated nighttime activity of chick pineal I_{LOT} channels requires protein synthesis. ***Biological Signals*** **in press**.

D'Souza, T. & Dryer, S. E. (1996). A novel cationic channel regulated by a vertebrate intrinsic circadian oscillator. ***Nature*** **382**: 165-167.

Reiser, M.A., D'Souza, T. & Dryer, S. E. (1996). Effects of caffeine and 3-isobutyl-1-methylxanthine on voltage-activated potassium currents in vertebrate neurones and secretory cells. ***British Journal of Pharmacology*****118:** 2145-2151.

D'Souza, T. & Dryer, S. E. (1995). Effects of phosphodiesterase inhibitors and forskolin on cyclic GMP-activated channels in intact isolated cells of the chick pineal gland. ***Neurochemistry International*** **27**: 527-533.

D'Souza, T. & Dryer, S. E. (1994). Intracellular free Ca^{2+} in dissociated cells of the chick pineal gland: Regulation by membrane depolarization, second messengers, and neuromodulators, and evidence for release of intracellular Ca^{2+} stores. ***Brain Research*** **656**: 85-94.

Dryer, S. E. & Henderson, D. (1993). Cyclic GMP-activated channels of the chick pineal gland: Effects of divalent cations, pH, and cyclic AMP. ***Journal of Comparative Physiology A*** **172:** 271-279.

One other article is in preparation, and two non-refereed review articles are in press.

4. Interactions/transitions.

a. Invited lectures at international conferences and seminars given at other institutions.

Gordon Conference on Chronobiology, New London, NH, August 12, 1997.

FASEB Summer Research Conference, Biology and Chemistry of Vision, Keystone, CO, July 24, 1997.

Endocrine Society, Minneapolis, MN, June 14, 1997.

First Asia-Pacific Pineal Conference, Hamamatsu, Japan, March 29, 1997.

Japan Physiological Society, Hamamatsu Japan, March 28, 1997.

Department of Physiology, SUNY Health Sciences Center, Syracuse, NY, March 7, 1997.

Department of Pharmacology, Emory University School of Medicine, Atlanta, GA, Feb. 4, 1997

Division of Chronobiology, Massachusetts General Hospital, Boston, MA, May 24, 1996.

National Institute of Child Health Development, National Institutes of Health, Bethesda, MD, May 21, 1996.

Department of Psychiatry, St. Elizabeth's Hospital, Boston, MA, April 8, 1996.

Gordon Conference on Pineal Cell Biology, Ventura, CA, February 6, 1996.

NIH Conference on Pineal Signal Transduction, Airlie House, Warrenton VA, September 6, 1995.

Department of Anatomy, Johann Wolfgang Goethe-Universitat, Frankfurt Germany, August 23, 1995.

KFA Institut fur Biologische Informationfsverabeitung, Julich, Germany, August 21, 1995.

International Eye-Pineal Relationships Symposium, Lodz, Poland, September 23, 1994.

b. Contributed presentations (posters) at meetings.

D'Souza, T. & Dryer, S. E. (1996). Are there morphologically and electrophysiologically distinct cell types in the chick pineal gland? Twenty- sixth Annual Meeting of the Society for Neuroscience, Washington, DC.

Dryer, S. E., Drake, M., D'Souza, T. & Raucher, S. (1995). Caffeine causes direct blockade of delayed rectifier K^+ currents in vertebrate neurons and secretory cells. Twenty-fifth Annual Meeting of the Society for Neuroscience, San Diego, CA.

D'Souza, T. & Dryer, S. E. (1995). Spontaneously active cationic chanels in acutely isolated chick pineal cells. Twenty-fifth Annual Meeting of the Society for Neuroscience, San Diego, CA.

D'Souza, T. & Dryer, S. E. (1994). Regulation of intracellular Ca^{2+} dynamics in dissociated cells of the chick pineal gland. Twenty-fourth Annual Meeting of the Society for Neuroscience, Miami, FL.

D'Souza, T. & Dryer, S. E. (1994). Effects of phosphodiesterase inhibitors and forskolin on cyclic GMP-activated channels in intact isolated cells of the chick pineal gland. International Eye-Pineal Relationships Symposium, Lodz, Poland. September 1994.

c. Consultative activities.

I continue to serve as a consultant for the NSF Center for Biological Timing at the University of Virginia, Charlottesville. In the course of this, I have helped investigators there set up patch clamp recording stations, to interpret electrophysiological data, and to assist in design of electrophysiological experiments. To date I have not performed these activities for DoD, but due to my new proximity to Air Force laboratories in San Antonio TX, this may occur in the future.

d. Transitions.

I am unaware of transitions resulting from these results. However, it is possible that this could occur in the future.

5. New discoveries, inventions, or patent disclosures.

Discovery of the ILOT channel, the first known example of an ionic channel under the direct control of a vertebrate intrinsic circadian oscillator.

6. Honors/Awards

Florida State University Developing Scholar Award for distinction acheivedd in basic research, 1995-6.