TEMPORAL CHANGES IN THE ABUNDANCE AND CELLULAR DISTRIBUTION OF GAPDH

A Senior Scholars Thesis

by

JESSICA CARTER

Submitted to the office of Undergraduate Research
Texas A&M University
In partial fulfillment of the requirements for the designation as

UNDERGRADUATE RESEARCH SCHOLAR

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Associate Dean for Undergraduate Research: Robert C. Webb

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ABSTRACT

Temporal Changed in the Abundance and Cellular Distribution of GAPDH (April 2006)

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Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) is a metabolic enzyme originally thought to be only involved in glycolysis. New studies have shown that this protein contains novel functions not previously anticipated. Furthermore, separate circadian oscillations in glycolytic processes and "clock" genes exist. It is hypothesized that GAPDH is a potential link between the glycolytic and clock gene pathways. The changes in the amount and location of GAPDH in chicken retinas, pineal glands, and brain tissues at four time points throughout the day while in constant darkness (DD) were examined. GAPDH was localized using standard immunohistochemical techniques. In the retinal tissue, GAPDH was found in a high abundance in the ganglion cells, outer plexiform layer, outer nuclear layer, inner nuclear and plexiform layer, and the photoreceptor cell layer of the retina. Additionally, a circadian rhythm in GAPDH abundance was observed in the outer plexiform layer, outer nuclear layer, inner nuclear and plexiform layer, and the photoreceptor cell layer, which all had a rhythmic expression and gradual decrease at CT12. The pineal gland and parasympathetic

terminals also displayed rhythmic expression. The parasympathetic terminal showed high abundance at CT0 and CT6, with a sharp drop at CT12. The pineal gland contained a similar temporal distribution, but with a gradual decrease to CT12. Data is still being collected on the brain sections. The results indicate that the relative abundance of the “glycolytic” protein, GAPDH, is under the control of an endogenous circadian clock.

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CHAPTER I

INTRODUCTION

Almost all organisms express circadian rhythms in a diversity of biological functions, such as sleep, hormone production, and brain activity that seem in approximately 24 hour cycles. Circadian rhythms can be entrained or reset daily by external cues, most commonly by light, and persist when organisms are maintained in constant conditions, reflecting the endogenous nature of these rhythms. In such cases, the expressed period is slightly longer or shorter than 24 hours, and is typically called a free-running rhythm. Additionally, they are unaffected by temperature when prevailing temperatures fall within the physiological range.

Regulation of the avian circadian system is hypothesized to be under control of a neuroendocrine loop. The suprachiasmic nuclei (SCN) located in the hypothalamus of the brain, along with the pineal gland, and retina are all known to be circadian oscillators that send activating and inhibitory signals to each other and work together as a whole system. In mammals, the SCN is thought to be the "master clock," while in birds the pineal gland is considered to be the pacemaker. The SCN is metabolically active at daytime and can be excited by light perceived through the retina and extraocular photoreceptors. Electrical activity and neural outputs are then sent through a multisynaptic pathway to a structure called the sympathetic superior cervical ganglia or, SCG. Through postganglionic fibers, the SCG feeds the neurotransmitter, norepinephrine directly to the pineal gland via the parasympathetic terminal. The pineal

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This thesis follows the style of *The Anatomical Record.*

gland is involved in the production and secretion of the characteristic "nighttime" hormone melatonin. The melatonin output from the pineal gland is directly inhibited by light and the norepinephrine signal from the SCG, however light but not norepinephrine can alter the phase of the pineal clock. When these inhibitions are taken away, melatonin synthesis begins followed shortly after by its secretion into the blood and cerebral spinal fluid. This signal in turn suppresses SCN activity. When melatonin levels fall and/or photoreceptors receive light input, SCN activity will increase (Cassone, 1984).

The retina plays a crucial role in the entrainment of the avian circadian system to the external light/dark cycle. Rhythmic neurotransmitter synthesis and release has been observed in the retina, but these substances are contained within the eye and not secreted into the blood (Green, 2004). These substances include, most notably, dopamine production in the amacrine cells and melatonin production in the photoreceptors. Dopamine and melatonin each antagonizes the other's production, and this antagonism helps create circadian rhythms within the retina and prepare the photoreceptor cells for the drastic changes in light intensity that occurs during a 24 hour period (Iuvone, 2000). Furthermore, they modulate the retina's interaction with the SCN via the retino-hypothalamic tract and aid in entraining the system to the environment (Cassone, 1984).

Circadian rhythms are also molecularly regulated through negative feedback loops of canonical clock genes. Clock gene expression is localized mainly in the SCN (Lowrey, 2000) when the transcription factors, CLOCK and BMAL1 heterodimerize and activate transcription of *Per* and *Cry* genes. After translation and phosporylation, these

proteins form a complex and directly inhibit CLOCK/BMAL1, thereby ceasing their own transcription. This negative feedback loop results in the rhythmic expression of *Per* and *Cry* genes their and protein products (Iuvone, 2000). SCN, pineal, and retina activities are all thought to be regulated by rhythmic expression of these genes. In the retina and pineal gland, it is believed that these transcription factors function to regulate rhythmic expression of arylalkylamine N-acetyltransferase (AANAT) which is a key enzyme in melatonin production (Green, 2004).

Glyceraldehyde 3-Phosphate Dehydrogenase (GAPDH) is a metabolic enzyme originally thought to be involved only in glycolysis. It is an important and unique enzyme in the pathway because it is the first step that produces ATP through substrate-level phosphorylation and is the only enzyme in the pathway to reduce NAD+ to NADH. New studies have shown that this protein contains novel functions not previously anticipated and that it can also translocate into the nucleus. These new functions include the regulation of histone gene translation, telomere structure, nuclear membrane fusion and recognition of fraudulent DNA nucleotides (Sirover, 2005). Multiple studies have shown GAPDH binding to single and double stranded DNA, containing helicase activity (Nagy, 2000), and being involved in transcription (Ronai, 1999). In particular, the NAD+ binding site and redox state of the protein has shown specific import. Through competition studies (Ronai, 1999) and using mutated GAPDH protein (Nagy, 2000) it has been suggested that the NAD+ site actually binds to DNA. Consequently, it is thought that GAPDH's ability to bind to DNA can be regulated by the ratios of NAD+/NADH and the redox status of the cell (Arutyunova, 2003). Interestingly, the

ratio of NAD+/NADH has been shown to help regulate circadian rhythms (Rutter, 2001). In relation to circadian rhythms, GAPDH mRNA has been found to be expressed rhythmically in chick retinas (Bailey, 2004). It has been speculated that GAPDH can be used in the transcriptome regulating some clock and clock controlled genes, and could therefore serve as a link between metabolic pathways and molecular circadian rhythms. To explore this idea, the rhythmycity of GAPDH protein in the major circadian oscillators must be examined. In unpublished work previously done by the Cassone lab, GAPDH levels were examined in animals during a light-dark cycle, and were shown to be rhythmic in both pineal and retinal tissues. The current experiment is a continuation of that, and was conducted to observe if rhythmic expression of GAPDH persist in chicken circadian oscillators when maintained in constant conditions, thereby determining if they are a component of endogenous circadian rhythmcity.

CHAPTER II

METHODS

Chickens where entrained to a 12 hour light, 12 hour dark cycle for one week then moved to constant darkness, DD. After 3 days, chicks were sacrificed at one of four time points in six hour intervals: circadian time (CT) 0, 6, 12 and 18. CT 0 corresponds to the beginning of the subjective day and CT 12 the beginning of subjective night. Animals were euthanized with CO2 and transcardially perfused with heparinized phosphate buffered saline (PBS) followed by perfusion with 4% paraformaldehyde. The brains, eyes and pineals were removed and post-fixed in 4% paraformaldehyde for 3 more days. Tissues were then infused with PBS and infiltrated with 30% sucrose solution. Retinas, pineals, and brains were frozen sectioned using a cryostat after embedding in a cryomatrix. Retinas and pineals were sectioned at 15µm and brains were at 25µm. Tissues were thaw mounted directly onto warmed gelatin coated slides.

Immunohistochemistry was used to visualize GAPDH in the retina and pineal samples. Sections were washed with PBS and then incubated in phosphate buffered saline/goat serum/triton X-100 (PBSGT) for 30 minutes. This step blocks any non-specific binding sites and reduces background. The samples are then incubated with polyclonal rabbit primary antibodies (AbCam) diluted in PBSGT for one day at 4C. Sections are washed in PBS and then incubated in biotinylated goat anti-rabbit secondary antibody (Jackson Labs) diluted in PBSGT overnight. After washing in PBS the sections are incubated in avidin-biotin (ABC) complex (Vector Labs) for 2 hours at

room temperature, washed in PBS, and then preincubated in diaminobenzidine (DAB) for 5 minutes. Afterwards, 35 µl of 30% hydrogen peroxide was added with the DAB, mixed, and then incubated until the desired contrast is obtained. Staining was intensified with 1% cobalt chloride. Sections were then washed one final time with water, dehydrated in an ethanol series, cleaned with xylene, mounted and coversliped with Permount.

Retinal slides were viewed using a Zeiss Axiophot microscope, while the pineal slides were viewed using an Olympus microscope. Digital images were then taken at 10x magnification for the retina and 4x and 10x magnification of the pineal. Relative staining density measurements of the images for both the pineal (4x magnification images were used) and retina were taken using Image J freeware (NIH) for objective measurements. Density measurements for the retinal samples were taken from each of the different cell layers in that tissue. Separate measurements were obtained for the photoreceptors, outer nulear layer, outer plexiform layer, inner nuclear and inner plexiform layer, and ganglion cell layer. In the pineal tissue, separate density measures were taken for the actual pineal body and the parasympathetic terminal, which directly feeds from the SCG. Time did not permit the performance of immunohistochemistry and analysis of the brain sections.

CHAPTER III

RESULTS

Visual observation of the retinal tissue showed GAPDH in high abundance in all cell layers and at all time points. Relative staining densities were normalized and graphed using Excel (Fig. 1). The data appears to be rhythmic with a gradual decrease in abundance at CT12, and higher concentrations during the subjective day.

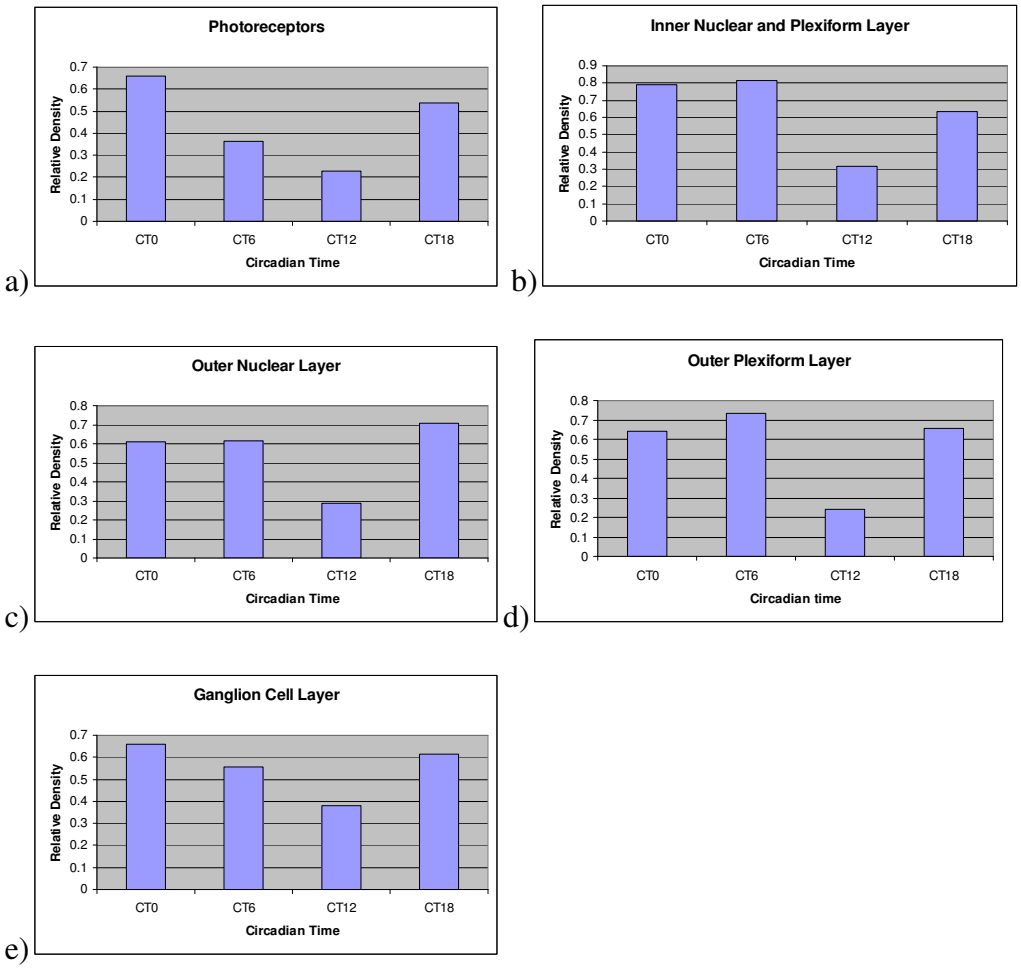
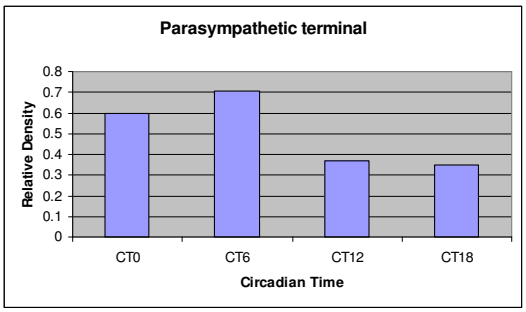


Fig. 1: Mean density of GAPDH immunoreactivity of retinal layers in conditions of constant darkness. The vertical values represent relative staining density, where staining density corresponds with GAPDH abundance. Data was taken at 6 hour intervals and shows a gradual decrease at CT12 for all cell layers.

The parasympathetic terminals were stained considerably darker than the pineal at all time points. Its highest relative density at CT0 and CT6 with a sharp decrease at CT 12 (Fig. 2). The pineal gland proper showed lighter staining than the parasympathetic terminal, but still demonstrated a high abundance of immunoreactivity. It had high concentrations at CT0 and CT6, with a gradual drop at CT 12 (Fig. 2).

a)



b)

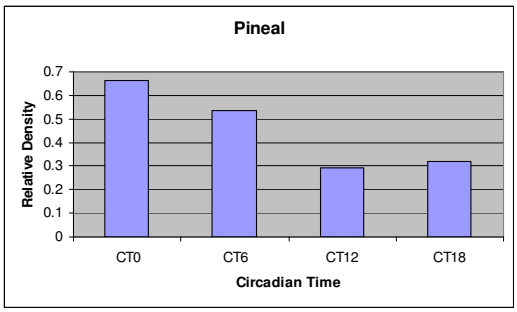


Fig. 2: Mean density of GAPDH immunoreactivity of the parasympathetic terminal and pineal gland in conditions of constant darkness. The vertical values represent relative staining density, where staining density corresponds with GAPDH abundance. Data was taken at 6 hour intervals and shows a gradual decrease at CT12 for the parasympathetic terminal and the pineal gland.

CHAPTER IV

CONCLUSION

Previous work examining GAPDH rhythmcity in LD was performed on chicken retinas and pineal glands. Retinal immunoreactivity showed low expression at all time points with the exception of ZT6, which had extremely high concentrations of GAPDH. This was not observed in the current study of chicken retina in DD. This could be due, in part, to the fact that the previous study measured levels taken from the retina as a whole, rather than of individual cell layers as in this study. More likely, however, these differences are a reflection of the masking effects of light, where GAPDH abundance is influenced acutely by the lighting regime. The fact that rhythmic expression of GAPDH is maintained in the absence of these external cues suggests that the rhythm is endogenously generated. It is very interesting to observe that with no external cues, a drop in levels at CT 12, the beginning of subjective night, occurs in all cell layers. This is the time point where retinal tissue would become less metabolically active in preparation for the night, the time when AANAT is highly expressed and melatonin is produced in the photoreceptor cells.

The parasympathetic terminals exhibited high concentrations of GAPDH during the subjective day and a sharp and dramatic drop at CT12, again with no external cues present. The SCG is involved in secreting norepinephrine on the pineal via this structure and its metabolic activity is consistent with the phasing of this norepinephrine cue in the literature. The pineal gland, on the other hand, showed a gradual decrease at CT12; this might possibly be a reflection of the pineal's reaction to the inhibitory effects of the

SCG. In the aforementioned LD study GAPDH expressed a bimodal distribution of abundance in the pineal, but they did not measure the staining density of the parasympathetic terminal separately. Also differences in phase due to free running rhythms in DD could explain the discrepancies in the broadness of the peaks between the current and the LD experiment. The expression pattern observed in the pineal exposed to constant conditions raises and interesting question: if the pineal gland synthesizes and secretes melatonin at night why is this not reflected in GAPDH abundance. It could be explained that the initial steps in the melatonin biosynthesis pathway occur during the day, and that these precursors could require more metabolic activity to complete than the conversion to melatonin does.

Evidence has previously demonstrated GAPDH's ability to enter the nucleus, bind to DNA, and be involved in transcription. In addition, these activities have been shown to be influenced by the redox status of the cell and NAD+ levels. Our current data now shows that GAPDH protein abundance is expressed rhythmically in both LD and DD in the circadian oscillators of the chicken pineal and retinas. This information serves support for the contention that GAPDH may be used in the transcriptome of some clock controlled genes, and potentially provides a link between metabolic pathways of the organism and its circadian rhythm. In particular, the ability of GAPDH to influence NAD+/NADH ratios may implicate several clock genes in this role. A continuation of immunocytochemistry and subsequent analysis is expected to be preformed on the brain sections already collected in this experiment. If rhythmicity is observed, specifically in the hypothalamic region where the SCN is located, it would add even more evidence to

support this hypothesis given the SCN's pivotal role in synchronizing and coordinating the avian circadian system.

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