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Temporal Changes in the Levels of Gamma-Aminobutyric Acid in the Brain of the Cockroach, Leucophaea Maderae

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TEMPORAL CHANGES IN THE LEVELS OF GAMMA-AMINOBUTYRIC ACID IN THE BRAIN OF THE COCKROACH, *LEUCOPHAEA MADERAE*

A Thesis

Presented to

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In Partial Fulfillment of

the Requirements for the Degree

Master of Science

by

Jonathan Christopher Newton

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TEMPORAL CHANGES IN THE LEVELS OF GAMMA-AMINOBUTYRIC ACID IN THE BRAIN OF THE COCKROACH, *LEUCOPHAEA MADERAE*
ACKNOWLEDGMENTS

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I wish to dedicate this thesis to my parents, David and Terry Newton, who have made this achievement possible through their continued love and support.
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Figure 2. Representative chromatogram of a methanol blank that was derivatized and passed through the HPLC-Fluorometric system. The excitation wavelength was 355 nm and the emission wavelength was 440 nm. 

Figure 3. Representative chromatogram of a GABA and AVA standard. The GABA concentration was 0.5 μg/mL and the AVA concentration was 4.5 μg/mL. 

Figure 4. Standard curve (with standard error bars) used to determine the amount of GABA in brain tissue according to the samples' GABA/AVA peak height ratio. A linear regression performed on the data yielded the equation: y=211.224x - 9.694. 

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Circadian rhythms have been shown to exist in all studied species and play an important role in their survival. The cockroach is an excellent model organism in which to study circadian systems because of the wealth of background information regarding both its anatomy and circadian system. Clock activity measured electrophysiologically in the cockroach system is 180 degrees out of phase with locomotor activity, with the maximum neural output occurring in the morning, twelve hours prior to the onset of locomotor activity. This inverse relationship of locomotor and neural activity suggests that the clock output may be inhibitory. In vertebrates, the suprachiasmatic nuclei (SCN) is the site of the biological clock, and it has been shown that gamma-aminobutyric acid (GABA) plays an important role in the vertebrate circadian system. The importance of GABA in the vertebrate SCN and its presence in the brain of the cockroach made the inhibitory neurotransmitter GABA a prime candidate for related studies.
To determine if GABA was expressed according to a daily rhythm, a brain extract was collected from individual cockroaches at different times of the day and the levels of GABA were determined using HPLC coupled with fluorometric detection. O-Phthalaldehyde was used to make fluorescent derivatives of GABA and the surrogate internal standard, AVA, that could be detected by the system. The levels of GABA determined ranged from $530 \pm 49$ ng/brain at 1800 h, the time when locomotor activity is initiated and neural output from the isolated clock is low (Page 1989), to $793 \pm 50$ ng/brain at 1200 h, the time of maximum neural output from the clock measured electrophysiologically. These GABA levels and the times at which they occur suggest that the clock may be inhibitory in its action. However, an ANOVA performed on the data did not show a significant difference between the GABA levels at the 95% confidence level, but did at the 84% confidence level ($P \leq 0.162$). The sample set was small ($n=3$ for each time period), and it is possible that with a larger sample set a significant difference at the 95% confidence level could be detected.
INTRODUCTION

For an individual's survival, it is important that the individual appropriately adapt to the constantly but predictably changing environment. The development of the circadian clock has allowed for the individual to meet the challenges of the environment with suitable changes in behavior and physiological parameters. The mechanism of this clock is not well known. Previous studies have shown that the onset of locomotor activity occurs at the same time that neuronal output from the clock is lowest (Page 1989). This finding suggests that the clock may act in an inhibitory manner, i.e., the animal is active until the clocks inhibitory output causes the animal to greatly reduce its level of motor activity. The inhibitory neurotransmitter GABA has been shown to be the primary neurotransmitter involved in the vertebrate circadian system (Moore and Speh 1993), and it is the primary inhibitory neurotransmitter in invertebrates (Sloley and McKenna 1993). It is possible that GABA may be mediating the activity of the cockroach circadian clock.

For most organisms to function efficiently and to be competitive in the natural world, the importance of the time of day becomes pronounced—in that an organism can meet the predictable challenges of a cyclic environment with the appropriate physiological and behavioral changes. One possible means whereby organisms can
achieve this effect is through an internal biological clock that is reset by cyclical environmental cues, such as light/dark, or temperature cycles. There are two important characteristics that are typical of such biological clocks (Page 1990). First, the clocks are internally regulated by the organism—with a period of approximately 24 hours, but never exactly 24 hours, that persist in the absence of environmental stimulation. Second, the clocks can be reset, or entrained, by external stimuli (i.e., the onset of light at dawn). This entrainment allows the environment to set the endogenous clock such that it is concordant with the environmental time (i.e., the time of day).

Circadian rhythms have a periodicity of approximately (but not exactly) 24 hours and are inherent to the organism, but can be reset by environmental cues. Circadian rhythms are different from daily rhythms. Daily rhythms are exactly 24 hours in period length and can be generated by physical or environmental cycles. When the environmental entraining stimulus that causes a daily rhythm, such as the regular onset of light, is removed, the rhythm may disappear because it is not controlled by the organism's internal clock but by the external stimulus. On the other hand, a circadian rhythm persists in the absence of an environmental entraining stimulus. However, without the entraining stimulus, the rhythm will not be reset and the onset of the activity will be determined by the periodicity of the internal clock. This lack of entrainment by the environment will result in a change in the time of day when the onset of the activity occurs. When the animal is not exposed to the entraining stimulus, the rhythm is said to be free-running.
Circadian rhythms have been observed in all metazoans studied, including cockroaches (Page 1977, 1981, 1982), hamsters (Kornhauser et al. 1990), *Drosophila melanogaster* and humans (Cote and Brody 1986). Circadian rhythms have also been identified in plants and unicellular eukaryotes, such as the dinoflagellate alga *Gonyaulax polydra* (Ronneberg and Morse 1993).

Several physiological parameters exhibit circadian rhythms in cockroaches: locomotor activity (Nishiitsutsuji-Uwo and Pittendrigh 1968, Lukat and Webber 1989), electroretinogram amplitude (Wills et al. 1985), and ommatidial structure (Ferrell and Reitcheck 1993). The anatomical position of the clock, or pacemaker, in cockroaches was first determined by Nishiitsutsuji-Uwo and Pittendrigh (1968), demonstrating that the cells governing the circadian locomotor activity of cockroaches are housed in the optic lobes. Later, it was shown that the cockroach has two independent but interacting pacemakers (Page et al. 1977, Page 1981), one in each of the optic lobes (Page 1982). When light reaches the compound eye of the cockroach, information is transmitted via the optic nerve to the optic lobe (which houses the circadian pacemaker) in the brain. Depending on the time of day that light impacts the eye, the pacemaker can be reset to varying degrees. Each pacemaker has three possible output pathways: one returning to the eye via the optic nerve (controlling eye sensitivity and eye morphology (Ferrell and Reitcheck 1993)), another to the pacemaker in the contralateral optic lobe (and in this way the pacemakers are mutually coupled), and the third to the drive system for locomotor activity (Page 1990) (Fig. 1).
Figure 1. Diagram of the possible output pathways of each pacemaker in the cockroach brain: one returning to the eye via the optic nerves (controlling eye sensitivity and eye morphology), another to the pacemaker in the contralateral optic lobe (and in this way the pacemakers are mutually coupled), and the third to the drive system for locomotor activity.
Clock activity measured electrophysiologically is 180 degrees out of phase with locomotor activity; the maximum number of action potentials were measured in the morning approximately 12 hours earlier than the onset of locomotor activity (Page 1989). This finding led to the postulation that the clock is inhibitory in its mode of action. Gamma-aminobutyric acid (GABA) is a major inhibitory neurotransmitter in invertebrates (Sloley and McKenna 1993) and vertebrates (Moore and Speh 1993).

In vertebrates, GABA is the principal neurotransmitter of both the suprachiasmatic nuclei and the intergeniculate leaflet of the lateral geniculate complex, the two major components of the circadian timing system. The suprachiasmatic nuclei interact with the areas that they innervate through inhibitory control due to the action of GABA (Moore and Speh 1993). Due to GABA's importance as a transmitter in vertebrates and invertebrates and its role in the vertebrate circadian system, further investigation regarding possible regulatory activities in the cockroach circadian system is needed.

A neurotransmitter can be described as a chemical that is secreted from a pre-synaptic neuron end terminal and crosses the synaptic cleft to be recognized by a specific receptor on the surface of an adjacent post-synaptic neuron. The transmitter either excites or inhibits the adjacent target cell (Axelrod 1974). Excitatory neurotransmitters stimulate or excite the post-synaptic cells causing neural transmission along that neuron, and inhibitory neurotransmitters (such as GABA) inhibit the cells from transmitting neural impulses.
GABA is a very ubiquitous molecule found in almost every class of living organism, not only animals. It has the role of a neurotransmitter in both vertebrates (Moore and Speh 1993) and invertebrates (Pfeiffer-Linn and Glantz 1989). It is synthesized by the decarboxylation of the amino acid and excitatory neurotransmitter L-glutamic acid by the enzyme glutamic acid decarboxylase. The relationship between GABA and L-glutamic acid may be of significant importance regarding the balance between excitation and inhibition in the brain (Davidson 1976).

GABA has inhibitory effects on both the central and peripheral nervous systems of vertebrates and invertebrates (Pfeiffer-Linn and Glantz 1991). It also has pronounced effects on heart rhythm and hind gut (Wagner et al 1991). Large quantities of GABA have been found in the optic lobe of the crayfish _Procambarus clarkii_ (Garcia and Arechiga 1986). Because the biological clock of the cockroach is housed inside the optic lobe (Page 1982), these findings encouraged the determination of brain levels of GABA in the cockroach brain, examining the possibility of a role for GABA in the circadian system. In vertebrates, as shown in rats (Moore and Speh 1993), GABA is the principal neurotransmitter of the circadian system.

Originally one type of GABA receptor had been identified in the cockroach, _Periplaneta americana_, which resembled the vertebrate GABA_A receptor (Sattelle 1990), however differences in distribution and mechanisms of this receptor compared to the vertebrate receptor exist (Wafford and Sattelle 1986). It has since been reported that two GABA receptors exist in this cockroach _species_, the second receptor resembling the GABA_B receptor of vertebrates (Hue
The insect receptors are linked to a chloride ion channel (Shimahara et al. 1987, Hue 1991) and GABA acts upon post-synaptic cells by hyperpolarizing or depolarizing the cells (Pfeiffer-Linn and Glantz 1990, Hue 1991) by increasing chloride ion conductance across the membrane. GABA is also antagonistic to acetyl-choline in tangential neurons and dimming fibers of crayfish (Pfeiffer-Linn and Glantz 1991).

The levels of GABA in the cockroach system have been examined to varying degrees. In the hemolymph and subesophageal ganglion of Leucophaea maderae relative levels of GABA were found to be less than 0.3% of the total amino acid content (Wagner et al. 1991). To my knowledge no study regarding the concentration of GABA in the cockroach brain has been conducted.

GABA was the focus of this study because it seemed a likely source for involvement in the transmission of the clock's inhibitory impulses—either directly or indirectly—because of its previously determined importance in the vertebrate circadian system and because of its being the primary inhibitory neurotransmitter in invertebrates. In addition, because the activity of the neurons in cultured optic lobes is highest during the subjective day (Page 1987), and the activity of the animal is greatest during the subjective night, it is possible that the clock is 180 degrees out of phase with locomotor activity. Considering this latter possibility, the clock may act to control the expression of overt rhythms through inhibition (i.e., the clock inhibits activity) (Page 1989).
MATERIALS AND METHODS

Animals: Adult male cockroaches (*L. maderae*), obtained from a rearing colony, were kept in an environmental chamber at 25 ± 2°C under alternating 12 h light and 12 h dark cycles, with the onset of light at 600 h. The lights in the incubation chambers were 100 watt bulbs, held at a constant height. The animals were supplied with food (i.e., puppy chow brand dog food) and water ad libitum. Representative cockroaches were placed in running wheels equipped with switches wired to recorders for monitoring of the animals' locomotor activity. These running wheels were equipped with two magnets mounted on the base that, when rotated by the running animal, would close a magnetic switch coupled to an Esterline-Angus event recorder. A dash mark was printed on the moving recorder paper with every 180° turn of the wheel. Using this system the locomotor activity patterns of the representative animals were determined (Fig. 1).

The tissue preparation and assay procedures used were modified from Sunol *et al.* 1988.

Tissue Preparation: Every three hours during one 24 h period, three cockroaches were removed from an environmental chamber and kept under the same photoperiodic conditions as the environmental chamber (i.e., light or dark). Each cockroach was flash-frozen at -70°C in liquid petroleum ether and then transferred
from the liquid petroleum ether to a freezer. The brains were extracted by first removing the cuticle between the large compound eyes, then severing the optic nerves to each eye and the circumesophageal connectives. The frozen brain was removed and placed in a 250 μL homogenizer along with 200 μL methanol containing 22.5 mg/mL delta-aminovaleric acid (i.e., the surrogate standard; AVA). The tissue was homogenized for 30 sec and the resulting homogenate was transferred to a microfuge tube and centrifuged at 50,000g for 20 min at 4°C. The supernatant was collected in an insulin syringe and filtered with a 0.45 μm syringe filter. 10 μL of the sample was diluted 1:5 with methanol to achieve a final concentration of 4.5 mg/mL AVA. The samples were stored at -70°C until derivatization and injection into the HPLC. The rest of the process was carried out at 4°C.

Derivatization procedure: 10 μL of sample or standard was mixed with 50 μL of 0.4 M borate buffer, pH 10.5, (Lindroth and Mopper 1979) and 100 μL of OPA solution (4 mL methanol, 4 mg o- phthalaldehyde from Sigma Chemical Co., 40 μL ethanethiol from Aldrich Chemical Co.) was added to initiate the reaction. The solution was mixed on a vortex mixer for 30 sec. The reaction was stopped after exactly 90 sec with the addition of 100 μL of glacial acetic acid.

Standard Curve: The standard curve was generated as follows: Individual stocks of 10 mg/mL GABA and AVA were made in methanol (Sigma Chemical Co.). In separate microfuge tubes, 45 μL AVA was mixed with 2 μL, 5 μL, or 10 μL of the GABA stock and made to 100 μL volume total with methanol. For these standards, GABA concentrations were 0.2, 0.5, and 1.0 mg/mL, respectively, and
AVA was constant at 4.5 mg/mL for all three. These standards were derivatized and ran individually through the detection system. The respective chromatograms for each standard were analyzed to determine the peak heights for GABA and AVA. The respective peak heights were measured by drawing a tangent from the baseline of one side of the peak to the baseline of the other side of the peak. The ratio of GABA peak height to AVA peak height was determined and the calibration curve prepared by plotting known GABA concentrations versus GABA/AVA peak height ratios. All points were obtained in triplicate. A linear regression analysis was performed on the points and the resulting equation was used to calculate the amount of GABA found in the brain extracts.

Chromatographic and Fluorometric Equipment: The HPLC detection system was comprised of a Varian 5000 Liquid Chromatograph and a Shimadzu RF-540 Spectrofluorophotometer with a Shimadzu Data Recorder DR-3. The column used was a 5 μm reverse phase column (Spheri 5, 220 mm x 4.6 mm C-18 from Pierce). The mobile phase was 45% acetonitrile and 55% KH₂PO₄ (pH 2.8, 0.1 M). The excitation and emission wavelengths used were 355 nm and 440 nm, respectively, (Sunol et al 1988, and corroborated in our lab). The system was run isocratic with a flow rate of 1.5 mL/min. A 25 μL injection loop was used for all injections.
RESULTS

The animals housed in the environmental chamber were entrained according to a 12L/12D rhythm with the onset of lights at 600 h. According to the activity chart, the animals showed an onset of activity at 1800 h, exactly 180° (12 h) out of pace with the onset of light.

The derivatized brain and standard samples were run over an HPLC system to separate out the components, and the chromatograms were generated by a spectrofluorometric detection system. A methanol blank (methanol that underwent the derivatization process without either AVA or GABA) showed that there were no fluorescence peaks after 3.5 min (Fig. 2). The retention times on the column for GABA and AVA were 5 and 6.5 min, respectively, with no overlapping of the peaks (Fig. 3).

A standard curve of 3 points was generated using 3 different GABA/AVA standard concentration stock solutions; 0.2/4.5 mg/mL, 0.5/4.5 mg/mL, 1.0/4.5 mg/mL. Each standard (Fig. 2) was run in triplicate and the relative peak height ratios were determined from the chromatograms. A linear regression analysis was performed on the data and yielded the equation $y = 211.224x - 9.694$ ($R = 0.99913$), where $y = \text{GABA/AVA peak height ratio}$ and $x = \text{ng GABA per brain}$ (Fig. 4).
Figure 2. Representative chromatogram of a methanol blank that was derivatized and passed through the HPLC-Fluorometric system. The excitation wavelength was 355 nm and the emission wavelength was 440 nm.
Figure 3. Representative chromatogram of a GABA and AVA standard. The GABA concentration was 0.5 μg/mL and the AVA concentration was 4.5 μg/mL.
Figure 4. Standard curve (with standard error bars) used to determine the amount of GABA in brain tissue according to the samples' GABA/AVA peak height ratio. A linear regression of the data yielded the equation: \( y = 211.224x - 9.694 \)
Figure 5. Chromatogram of a brain sample removed at 600 h spiked with the internal standard AVA.
Table 1. Mean GABA levels (ng GABA/Brain) ± one standard deviation about the mean in the cockroach brain determined in three individuals at three hour intervals throughout a 24-hour time period.
<table>
<thead>
<tr>
<th>Time</th>
<th>( N )</th>
<th>( \text{ng GABA/Brain} )</th>
<th>( \text{S.D.} )</th>
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<tr>
<td>300h</td>
<td>3</td>
<td>630 ± 110</td>
<td></td>
</tr>
<tr>
<td>600h</td>
<td>3</td>
<td>703 ± 200</td>
<td></td>
</tr>
<tr>
<td>900h</td>
<td>3</td>
<td>569 ± 155</td>
<td></td>
</tr>
<tr>
<td>1200h</td>
<td>3</td>
<td>793 ± 87</td>
<td></td>
</tr>
<tr>
<td>1500h</td>
<td>3</td>
<td>637 ± 41</td>
<td></td>
</tr>
<tr>
<td>1800h</td>
<td>3</td>
<td>530 ± 85</td>
<td></td>
</tr>
<tr>
<td>2100h</td>
<td>3</td>
<td>667 ± 137</td>
<td></td>
</tr>
<tr>
<td>2400h</td>
<td>3</td>
<td>667 ± 55</td>
<td></td>
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Figure 6. Mean GABA levels ± one standard deviation in the cockroach brain at three hour intervals throughout a 24 h time period.
GABA levels in brains obtained from animals were determined in triplicate for each time examined (Fig. 5). The levels of GABA present were determined from the ratios of GABA/AVA peak heights using the above equation. The levels of GABA in the brain ranged from $530 \pm 49$ ng/brain at 1800 h to $793 \pm 50$ ng/brain at 1200 h with a mean of 650 ng/brain and standard deviation of 80 (Table 1, Fig. 6).

An analysis of variance was conducted on these data, producing an F-ratio of 1.776 and a $P < 0.162$. From the ANOVA it can be said that at the 95% confidence level there is no significant difference between the brain levels of GABA. However, there is a significant temporal difference between the levels at the 84% confidence level. Six of the eight observations ranged from 668 ng per brain to 530 ng. The level of GABA is highest at 1200 h with $793 \pm 50$ ng per brain and lowest at 1800 h (offset of light) at $530 \pm 49$ ng per brain.
DISCUSSION

This quantification is the first for the levels of GABA in the cockroach (*L. maderae*) brain. The levels recorded had a mean value of 650 ng per brain and standard deviation of 80 ng. The GABA level in the *L. maderae* corresponds with the levels of GABA found in the crayfish brain (Garcia and Arechiga 1986).

The animals in the experimental chamber were exposed to a 12L/12D rhythm with the onset of light at 600 h, and their onset of activity was at 1800 h, showing that the animals were entrained by the environmental light/dark cycle since the light cycle was the only variable in the system. Because of this observed entrainment, it was assumed that the time at which the animals were removed from the cages corresponds to their respective time in the circadian cycle.

The chromatograms show that the GABA and AVA peaks do not overlap with anything that is native to the derivatization process nor with each other, allowing us to utilize the real peak heights measured from the chromatograms in our calculations. However, a sample of both the AVA and GABA samples will need to be analysed via mass spectrophotometry to ensure their purity. The time between the two peaks of interest was constant for all the samples used in this study. Fluctuations in the retention times were commonly due to old buffer, incorrect derivatization procedures, or degradation of the HPLC column.
Throughout the day, GABA levels vary greatly—with the low and high extremes at 1800 h and 1200 h, respectively. The 1800 h time point correspond to both the offset of light and the onset of locomotor activity, exactly 180 degrees out of phase with the onset of the entraining stimulus, light. The maximal GABA expression occurs at 1200 h, just 6 h before the onset of locomotor activity and the time of minimal GABA expression. The ANOVA analysis of the data showed that the levels of GABA determined for each of the eight times of day were not significantly different, consequently a daily rhythm in the brain levels of GABA was not detected. However the presented data are from a small sample set for each time of day. A larger sample set would possibly detect the presence of a daily rhythm of cockroach brain GABA levels and increase confidence in the mean levels at the various times of day.

The possibility that the clock may be inhibitory due to its being almost 180 degrees out of phase with activity (Page 1989) made GABA a good candidate for exhibiting a daily or circadian rhythm. Although there was no statistically significant daily rhythm at the 95% level with the small sample set, a larger sample set may show a daily rhythm at this confidence level. There was a significant difference at the 84% level which suggests that there is a low amplitude daily rhythm that might be detected with a larger sample set. If this rhythm is circadian, it will strongly suggest that GABA is involved in the circadian system, either as the primary effector mechanism of the clock or as a secondary effector of the clock. The observation that highest levels of GABA occurred during the daytime
supports the idea that the clock output is inhibitory and greatest during the day in this cockroach species.

If a rhythm can be found using a larger sample size, GABA could be a useful tool in further studies of the cockroach circadian system. *In situ* hybridization techniques could possibly be one tool by which to examine the GABAergic neuronal pathways in the cockroach brain. Staining with monoclonal antibodies to GABA or GABA receptors would allow for mapping of the GABAergic neurons in the brain and the optic lobe by showing the cells with GABA receptors. Since GABA may not be released by the actual cells of the clock, it would be important to examine the entire brain, especially the efferent neurons from the optic lobe. Mapping the brain would be of great interest because it would show all neuronal pathways that involve GABA, including those GABAergic pathways which are active when the GABA levels are high (time of maximum inhibition) but inactive when the levels are low (time of low inhibition). From these neuronal pathways one could possibly determine which cells of the brain constitute the circadian clock and when these cells are active.

Considerable research remains to be conducted before a clear understanding of the cockroach circadian system is achieved. The use of a substance that is expressed on a circadian basis as a tool would provide a new avenue to advance our understanding of the cockroach circadian system. Because of GABA’s importance in vertebrate circadian systems (Moore and Speh 1993), and its high levels in the optic lobe of the crayfish (Garcia and Arechiga 1986, Pfeiffer-Linn and Glantz 1991), it was a likely candidate for
examination. If future studies support these data showing that there are no significant differences between the levels of GABA at various times during the day, then another neurotransmitter or substance, such as octopamine, will have to be examined for circadian rhythmicity and its possible applications as a tool to further study the clock system of the cockroach.
LITERATURE CITED


Sloley, B.D., K.F. McKenna. 1993. 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP), and gamma-vinyl-gamma-aminobutyric acid (gamma-vinyl GABA) alter neurotransmitter concentrations in the nervous tissue of the goldfish (*Carassius auratus*) but not the cockroach (*Periplaneta americana*). *Neurochem Int* 22, 2 197-203.


