The Role of Brain-Derived Neurotrophic Factor in Glutamate-Induced Circadian Phase Advances

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UNIVERSITY HONORS PROGRAM

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The Role of Brain-Derived Neurotrophic Factor in Glutamate-Induced Circadian Phase Advances

A Senior Project Presented for the College Scholars Program and the University Honors Program
The University of Tennessee, Knoxville

Jeffery A. Ollis II
December 2003
Abstract

Nearly all organisms exhibit circadian rhythms, physiological and behavioral patterns with a period of approximately twenty-four hours. These twenty-four hour rhythms are timed by an endogenous clock located in the suprachiasmatic nuclei (SCN) of the hypothalamus. One of the most interesting features of the circadian timing system is its ability to shift in response to light. This feature provides a mechanism for resetting the clock in order to maintain synchrony with the environmental light/dark cycle. During the early morning, light induces a phase advance in the clock, compensating for what appears to be an early onset of day. During the late night, light induces a phase delay in the clock that allows compensation for what appears to be a lengthening of daylight. During the subjective day, light has no effect on the clock since the presence of light confirms what is expected during the daytime. Previous studies have shown a circadian expression of brain-derived neurotrophic factor (BDNF), a substance known to enhance synaptic transmission and regulate neural input in other brain regions, and have provided supporting evidence for a potential role of BDNF in regulating the SCN’s sensitivity to photic input in vivo. In this study, BDNF’s role in advancing the clock was investigated in vitro through the use of K252a, a substance known to block the functional receptor for BDNF. When glutamate, the substance released onto the SCN in response to light, was applied in the early morning, large phase advances of about 3.25 hours were observed. Application of K252a with glutamate was shown to block this response, whereas application of K252a alone was shown to have no effect. These results are consistent with previous experiments conducted in vivo and continue to support a role for BDNF in regulating photic sensitivity in the SCN.
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Introduction and Rationale for Circadian Research

The 24 hour rotation of the earth on its axis results in an environmental stimulus of cyclic light and dark cues that influences the behavior of nearly all forms of life, including plants, animals, and even humans. In adapting to this cycle of stimuli, most organisms display circadian rhythms, “endogenous oscillations with a period of approximately 24 hours” (Rusak and Zucker, 1979). Although once considered a metaphysical conception (Dunlap et al, 2004), circadian rhythms are found in all eukaryotic organisms as well as many prokaryotic organisms (Takahashi, 1993) and influence various “changes in physiology and behavior, such as hormone secretion, temperature, and the sleep-waking cycle” (Hannibal, 2002). The presence of circadian rhythms allows for significant adaptation by synchronizing internal activities with fluctuations in the external environment (Rusak and Zucker, 1979).

The history of circadian research dates back to the 4th century B.C. when Androsthenes, a scribe of Alexander the Great, documented daily patterns in plant leaf and flower petal movements, but these and other such cycles were for many years dismissed as responses to environmental stimuli rather than patterns of endogenously driven mechanisms (Dunlap et al, 2004; Lamburg, 1994). Ultimately, the existence of endogenously driven circadian rhythms was substantiated and met with widespread acceptance by the 1950s, and the source of this circadian activity was identified as the suprachiasmatic nuclei (SCN) (See section on the Suprachiasmatic Nucleus).
Today, there are currently three basic questions that neurobiologists seek to answer through their investigations of the circadian system: 1.) how does the SCN generate a circadian signal, 2.) how does light adjust this signal, and 3.) how does the SCN use this signal to influence other areas of the brain? Aside from a scientific advancement of knowledge, there are also a number of medical reasons for studying circadian function. The activity of the SCN influences a variety of physiological functions, and there are several disorders in humans that are related to circadian rhythmicity that stand to be alleviated through circadian research.

The outputs of the SCN (See Figure 1) synapse primarily within the hypothalamus with additional projections to the midline thalamus and the basal forebrain (Moore, 1996). Projections to the basal forebrain and midline thalamus influence higher order functions of the brain through secondary projections (Moore, 1996) whereas secondary projections from the hypothalamus give the SCN circadian influence over various activities such as sleep-wake cycles, hormone concentration in the blood, heart rate, blood pressure, body temperature, eating and drinking, and metabolism (Moore, 1996; Refinetti, 2000). The SCN influences the activity of many parts of the body either directly or indirectly, so it is clearly evident that disruption of the circadian system can have a significant impact on the body’s function.

A thorough understanding of the circadian system holds the potential for alleviating a variety of medical conditions. Statistics have shown various correlations between time of day and infant mortality immediately after birth, sudden death after abdominal surgery, and adult suicides (Refinetti, 2000). Furthermore, individuals who work in healthcare, law enforcement, or other fields that require night shifts or frequent
Figure 1: Primary and secondary projections of the SCN and the functions regulated by its output. (From Moore, 1996)
shift rotations are more likely to experience heart disease, sleep disorders, digestive disorders, and mood disturbances (Lamburg, 1994), and individuals who frequently travel across time zones will more often suffer from the sleep disruption, mood swings, gastrointestinal distress, impaired concentration, decreased cognitive ability, and lack of coordination associated with jet lag (Moore, 1996).

In addition to jet lag and work-shift syndrome, problems with adjustment of the clock (See section on entrainment) may also cause delayed sleep phase syndrome (DSPS) or advanced sleep phase syndrome (ASPS). Individuals with these disorders have difficulty going to sleep or awakening at traditional hours, either failing to fall asleep until very late at night and only awakening naturally in the late morning or afternoon or becoming sleepy during the evening and consequently awakening in the very early morning (Refinetti, 2000; Moore, 1996). Individuals suffering from these disorders show no alteration of normal sleep quality or duration if they are socially capable of altering their schedules to accommodate such atypical times for sleep, but oftentimes work or family obligations result in sleep deprivation among these patients (Refinetti, 2000).

Some blind patients may suffer from problems with light-regulated clock adjustment (See section on entrainment) if their visual damage affects the neural afferents to the SCN (Moore, 1996). At least one study has documented that some individuals may be visually blind but still capable of photic clock adjustment while others may display free-running melatonin rhythms that may become out of sync with the solar cycle (Czeisler, 1995). Individuals who are totally blind and unable to photically regulate the pacemaker report sleep disturbances and insomnia during times in which the melatonin rhythm is out of phase with the intended sleep period (Czeisler, 1995).
Aging is another problem that affects the circadian system. Decreased amplitude of the circadian rhythm as well as changes in the clock’s period are characteristic of aging (Zhang et al, 1996). Decreased amplitude has been associated with greater fragmentation of sleep, less slow-wave sleep, decreased amplitude of the body temperature rhythm, and a decrease in the amplitude of the cortisol and melatonin rhythms (Moore, 1996). Decreased amplitude of the circadian rhythm is especially pronounced in individuals with Alzheimer’s disease and is most likely the result of a decrease in the number of cells found in the SCN (Moore, 1996).

Circadian research may also benefit the medical field by improving the very timing of medical therapy. Chronopharmacology refers to the practice of administering drugs at times in which the drugs are known to be most effective and have the least side effects (Refinetti, 2000). The symptoms of various diseases, including asthma, arthritis, epilepsy, and hypertension, demonstrate circadian rhythmicity, and research has shown that the pharmacodynamics and pharmacokinetics of many drugs also display circadian rhythmicity (Refinetti, 2000).

Continued research on the SCN at the cellular and molecular level will be instrumental for gaining a comprehensive understanding of how the clock works, including how it is reset and how it influences behavior and physiology. Likewise, treatment for medical disorders will require a more solid understanding of how the SCN works at the molecular level, helping to not only further explain the causes of certain medical conditions but to improve the efficacy of our treatments through a better understanding of the rhythmic variations in our physiology.
The Suprachiasmatic Nucleus

With the acknowledgement of a circadian clock came the search to find it. Ultimately, researchers were able to conclude that the internal clock activity of mammals rests within the suprachiasmatic nucleus (SCN). There is substantial evidence of the SCN’s role in circadian rhythmicity, including experiments involving ablation of neurons in the SCN and subsequent loss of circadian rhythmicity; persistence of circadian rhythmicity of neurons in the SCN after isolation from the rest of the brain in vivo or in vitro; and adoption of donor circadian periodicity in subjects receiving transplantation of fetal SCN tissue after loss of their own rhythmicity due to SCN ablation (Moore, 1996).

The SCN are a pair of small, bilateral structures located in the anterior hypothalamus, ventrolateral to the base of the third ventricle and just dorsal to the optic chiasm. It is estimated that there are about 8000 neurons in each SCN (van den Pol, 1991), and SCN neurons are among the smallest neurons anywhere in the brain (van den Pol, 1991; Rusak and Zucker, 1979). The SCN are sensitive to various stimuli and can self-regulate its sensitivity to various stimuli to achieve the desired synchrony with environmental and physiological cues (Gillette and Mitchell, 2002).

Each SCN is composed of two subdivisions: core and shell (Moore et al, 2002). In rats, the shell contains about 57% of the total neurons in the SCN, and neurons in the core comprise the remaining 43% (Moore et al, 2002). Innervation of the SCN from the retinohypothalamic tract (RHT) terminates predominately in the core (Moore et al, 2002) (See Figure 2). A projection from the intergeniculate leaflet (IGL) via the geniculohypothalamic tract (GHT) and a serotonin projection from the midbrain raphe nuclei also terminate in the core of the SCN (Moore et al, 2002). The shell receives
Figure 2: Afferents to the core and shell of the SCN. (From Dunlap et al, 2004)
unnecessary for photic entrainment suggests the existence of additional photoreceptors (Ruby et al, 2002). Melanopsin has been suggested as the most likely photopigment used for entrainment of the circadian pacemaker, but recent experiments with mice have used knockout genes to show that melanopsin is not necessary for entrainment (Ruby et al, 2002). However, phase and period responses were reduced in these knockout mice, so while not essential, melanopsin does appear to play a significant role in contributing photic information to the circadian pacemaker (Ruby et al, 2002).

*The Circadian Rhythm and Entrainment*

Subjects maintained in an environment with no external timing cues demonstrate a pattern of circadian activity that approximates 24 hours (Moore, 1996). This means that the circadian clock persists in a free-running state, a state in which an endogenous rhythm with inherent periodicity persists without environmental cues. This rhythm can be visualized based on the activity of the subject, such as wheel running in mice for example, by plotting this activity over a twenty-four hour period. Cells of the SCN continue to fire in a circadian pattern for at least three days *in vitro*, so electrical recordings can also be used to visualize the circadian rhythm by plotting the frequency of action potential firing in SCN cells over time. In this case, such a plot (See Figure 3) will demonstrate the classic sinusoidal oscillation of circadian rhythmicity with peak activity near mid-subjective day and diminished activity during the subjective night (Gillette, 1991).

Because circadian pacemakers generate their rhythms endogenously, the SCN must be capable of resetting itself regularly in order to compensate for variations in the
Figure 3: Schematic of the circadian rhythm depicting the firing rate of neurons in the SCN of an entrained mouse housed in alternating 12 hour periods of light and darkness (12:12 LD). The lights-on period occurs at zeitgeber time 0 (ZT 0; zeitgeber means “time giver”; ZT begins at lights-on in the entrained cycle and repeats after 24 hours) and lights off occurring at ZT 12. The dark horizontal bar between ZT 12 and ZT 24 signifies the lights-off period (i.e. the subjective night). Peak firing activity occurs near midday (ZT 6) with firing activity lessened during the subjective night.
amount of daylight throughout the year, discrepancies between the period of the endogenous clock and the period of the earth’s rotation, or differences in environmental cues resulting from travel across time zones. This process of adjusting the clock is called entrainment and is achieved primarily through the environmental stimulus of light (Moore, 1996). This resetting of the clock in response to a stimulus such as light can be visualized by plotting the SCN neuronal firing rate in vitro over time (See Figure 4).

In order to relay a clock-adjusting light stimulus to the brain, the RHT releases the neurotransmitter glutamate (Gillette and Mitchell, 2002). In vivo experiments with light pulses and in vitro experiments with glutamate application have both demonstrated biphasic responses in the circadian rhythm when given during the subjective night (Gillette and Mitchell, 2002). When given during the early night, a delay in behavioral activity or peak neuronal firing activity in the SCN is observed, whereas an advance of activity is observed when the light/glutamate stimulus is given during the late night (Gillette and Mitchell, 2002). Intuitively, these observations make sense because a light stimulus during the early night would indicate a delay of sunset, and a light stimulus just before the lights-on period would be indicative of an early onset of daylight.

The full mechanism through which light acts on the SCN in order to cause a phase shift is unknown, but a number of pathways are thought to be involved. In response to light, the retinal afferents to the SCN release glutamate, which acts on SCN neurons by activating N-methyl-D-aspartate (NMDA) receptors (Ding et al, 1994). The rise in intracellular Ca$^{2+}$ concentration through activated NMDA receptors causes stimulation of nitric oxide synthase (NOS) (Ding et al, 1994), which releases nitric oxide (NO) when it
Figure 4: Schematic of the circadian rhythm depicting the advance in peak firing rate of neurons in the SCN in response to an early morning glutamate stimulus (glutamate is released onto the SCN in response to light). The dashed curve represents the normal circadian rhythm of a mouse housed in 12:12 LD, whereas the solid curve represents the phase-advanced rhythm in response to a glutamate stimulus. The horizontal dark bar represents “lights-off” in the colony, and the vertical, dashed line represents time of peak firing activity under control conditions. The vertical, shaded bar represents time of light stimulus.
converts L-arginine to L-citrulline. The downstream events in this signal transduction pathway are less clear, but it is believed that production of NO induces phosphorylation of the Ca$^{2+}$/cAMP response element binding protein (CREB) (Ding et al, 1997) and activation of Ca$^{2+}$/cAMP response element (CRE)-mediated transcription (Obrietan et al, 1999).

Experiments have revealed a circadian oscillation in CRE-mediated gene expression in dark-adapted animals and have shown CRE-mediated transcription triggered in response to photic stimulation during the subjective night (Obrietan et al, 1999). This pattern of CREB activation correlates with the induction of many immediate early genes that have suspected involvement in phase shifting, and many of these genes have activity-sensitive CRE promoter elements that are regulated by CREB (Obrietan et al, 1999; Deisseroth et al, 2003). Taken together, there is increasing evidence to support the idea that light activates gene expression through the CREB/CRE-transcriptional pathway in order to phase shift the clock (Obrietan et al, 1999; Deisseroth et al, 2003).

While the specific pathway leading to CREB phosphorylation is not known, the differing effects of light/glutamate stimuli between early and late night suggests that different pathways may be involved. The increase in Ca$^{2+}$ concentration could activate Ca$^{2+}$/calmodulin-dependent protein kinase (CaM kinase), which has been shown to play a role in P-CREB formation in hippocampal neurons (Ding et al, 1997; Deisseroth et al, 2003). A recent experiment showed that disruption of the p42/44 mitogen-activated protein kinase (MAPK) pathway blocks clock entrainment, and this pathway was reported to function downstream of the CaM kinase pathway (Butcher et al, 2002). The production of NO from NOS activation could also lead to activation of guanylate cyclase

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(GC), production of cyclic GMP (cGMP), and subsequent activation of protein kinase G (PKG) (Ding et al, 1997). Because light/glutamate-induced phase advances have been blocked by a specific PKG inhibitor (Weber et al, 1995), this NO/GC/cGMP/PKG pathway is likely activated during the late night. However, other pathways may still be activated during this timeframe, particularly in light of the fact that the amplitude of the light/glutamate-induced phase shift is smaller than that of the phase shift induced by cGMP/PKG alone (Gillette and Mitchell, 2003).

During the early night, experiments have shown that blocking the cGMP/PKG pathway has no effect on glutamate-induced phase delays, so the NO/GC/cGMP/PKG pathway involved in glutamate-induced phase advances is not involved in glutamate-induced phase delays. (Ding et al, 1998). Additional experiments revealed glutamate-like phase delays through activation of ryanodine receptors (RyR) whereas inhibition of RyRs blocked glutamate-induced phase delays (Ding et al, 1998). The specific pathway involved between glutamate and RyRs is unclear, but nitric oxide as an intermediary could stimulate the ADP ribosyl cyclase pathway and lead to activation of RyRs through nitric oxide-mediated polynitrosylation (Ding et al, 1998).

Regardless of the specific pathways involved, the ability of the SCN to gate its sensitivity to light is an instrumental feature of the circadian system. Identification of the mechanism by which the SCN gates its sensitivity to photic input is an important step toward understanding the process of entrainment. Recently, attention has been directed toward a specific growth factor as a potential candidate for regulating the SCN’s sensitivity to light, and this substance will be the central focus of my research in this project.
Brain-Derived Neurotrophic Factor

The mechanism through which the SCN gates its sensitivity to photic input is of central importance to understanding circadian function. Recently, a regulatory role in pacemaker sensitivity to light has been suggested for brain-derived neurotrophic factor (BDNF), a secretory protein that belongs to the neurotrophin family and preferentially binds the tyrosine kinase B receptor (TrkB) (Lu 2003). Part of the evidence for this hypothesis has come from the realization that BDNF plays critical roles in synaptic transmission and plasticity in other areas of the brain (See Lu, 2003 for review). These roles include facilitation of long-term potentiation in the hippocampus and visual cortex, modulation of dendritic and axonal growth in the brain, and regulation of synaptic transmission in glutamatergic, and GABAergic synapses, all of which support a role for BDNF in modulation of synaptic structures and function (Lu, 2003).

Expression of BDNF and its cognate receptor, TrkB, was recently identified in the SCN of adult rats (Liang et al, 1998b), and both BDNF and BDNF mRNA levels were recently found to oscillate in a circadian pattern, increasing during the early subjective night, peaking near the mid subjective night, and tapering off to minimal levels throughout the subjective day (Liang et al, 1998a). These findings support the idea that BDNF could gate the response of the SCN to photic input since its concentration is maximal during the subjective night when light/glutamate is capable of producing a shift in the circadian curve but is minimal during the subjective day when stimulation by light/glutamate has no effect (Liang et al, 1998a).

Recently, BDNF was shown to stimulate nuclear CREB phosphorylation in the hippocampus through stimulation of Ca\(^{2+}\)/calmodulin-dependent protein kinase 2
(CaMKII), subsequent downstream activation of the p38 subfamily of mitogen-activated protein kinases (MAPK), and then downstream activation of MAPK-activated protein kinase 2 (MAPKAPK-2) (Blanquet et al, 2003). Because CREB phosphorylation is involved in phase-shifting the circadian clock, BDNF’s role in CREB phosphorylation in the hippocampus further supports a possible role for BDNF in gating photic input in the SCN.

Another study has shown that BDNF can rapidly enhance synaptic transmission by promoting phosphorylation and dephosphorylation of NMDA receptor subunits and thereby increasing or decreasing their likelihood of being open (Lin et al, 1999). As described above, NMDA receptors open in response to glutamate activation in the SCN and allow influx of Ca$^{2+}$. Therefore, this activity of BDNF could have a significant impact on the SCN’s response to glutamate and provide another potential mechanism for gating photic sensitivity.

In order to better substantiate a role for BDNF in photic gating, a series of experiments were performed in vivo to determine if K252a, a substance known to block tyrosine kinase receptors, could eliminate light-induced phase shifts of the wheel running activity in rats (Liang et al, 2000). These experiments utilized an implanted cannula to allow infusion of K252a into the SCN. Multiple trials were conducted in which rats were exposed to thirty minute light pulses at either zeitgeber time 14 (ZT 14; zeitgeber means “time giver” and zeitgeber time begins at ZT 0 with the onset of the lights-on period in the colony and continues for twenty four hours before repeating) or ZT 22, times known to induce maximal phase delays and phase advances respectively, in the presence and absence of K252a. The results of these trials were compared with the results of trials
without light stimulation in which K252a was injected at ZT 14 and ZT 22 to ensure that the blocker itself did not cause any unintended effects. These trials showed that infusion of K252a blocks light-induced phase advances and phase delays. Another experiment was conducted in which the inability of light to induce a phase shift during the subjective day was confirmed through a light stimulus at ZT 6. Then, infusion of BDNF was given prior to a light pulse at ZT 6. BDNF administration was shown to enable large light-induced phase advances during the subjective day.

The results of this study led the researchers to conclude that BDNF modulates the receptivity of the circadian pacemaker to photic input. This was based on the observations that blocking the functional BDNF receptor prevented light-induced phase shifts, while application of BDNF enabled light to induce phase shifts when it ordinarily would not. However, this series of experiments were conducted in vivo, and certain difficulties can arise in controlling for all variables in experiments of this type. Therefore, I have set out to replicate these experiments in vitro in order to isolate the SCN and its surrounding region in a better controlled environment and provide a more definitive analysis of the role of BDNF in photic entrainment. The focus of this study will be limited to an analysis of the role of BDNF in light/glutamate-induced phase advances.
Data Acquisition and Analysis

Data collection began at ZT 0 of day two, approximately two hours after drug treatments commenced (see below). During these experiments, a microelectrode filled with 3M saline solution was used to record from individual neurons for five-minute periods, and the activity of four to seven neurons per hour was recorded. Data were collected and analyzed with a DataWave system (Longmont, CO). Later, these data were analyzed to visibly identify and remove background noise picked up by the microelectrode. Then, the clean recordings were used to determine the firing rates of the individual neurons. Two hour running averages were then calculated with standard error and plotted on a graph in order to identify the time of peak firing activity. This peak time was visually estimated to the nearest quarter hour based on the symmetry of the curve. This information was then used to identify phase-shifts of the circadian curve and thereby assess the effects of the experimental treatments.

Experimental Protocol

Control Experiments:

Five trials of experiments were initially conducted to establish the normal pattern of circadian activity in the SCN of mice housed under 12:12 LD conditions. During these trials, extracellular recording of individual neurons in the SCN began at ZT 0. Five-minute recordings were obtained from four to seven cells each hour until ZT 10. While this timeframe did not allow for monitoring the entire twenty-four hour rhythm, this ten hour period allowed me to accurately determine the time of peak firing activity of
neurons and allowed for accurate visualization of any phase shifting of peak activity resulting from experimental treatments.

**Glutamate Treatment:**

Four trials of experiments were conducted using exogenous glutamate application at ZT 22. To apply the glutamate, the perfusion was stopped, and the normal medium was removed from the dish and replaced with a 1mM solution of glutamate dissolved in EBSS. After ten minutes, this solution was removed, and normal perfusion of EBSS was resumed.

**K252a Treatment:**

Four trials of experiments were conducted using exogenous application of K252a, a substance known to block the TrkB receptor, the functional receptor for BDNF. At five minutes before ZT 22, the perfusion was stopped, and the normal medium was removed from the dish. The dish was then filled with a 1μM solution of K252a dissolved in EBSS. K252a was added five minutes before ZT 22 so that all receptors for BDNF would be blocked at the time of glutamate application (ZT 22). Although glutamate was not used in this series of control experiments, K252a application began five minutes early in order to remain consistent with subsequent experimental trials. After twenty minutes, this solution was removed and normal perfusion of EBSS was resumed. K252a was left in the dish five minutes beyond the time of intended removal of glutamate in order to protect against the effects of any lingering glutamate in the dish that could have potentially induced a phase-shift once receptors for BDNF were no longer blocked.
Again, K252a application was prolonged in this series of control experiments in order to remain consistent with K252a application in the subsequent experimental trials.

**Glutamate + K252a Treatment:**

Four trials of experiments were conducted with application of K252a and glutamate. At five minutes before ZT 22, the perfusion was stopped, and the normal medium in the dish was replaced with a 1μM solution of K252a dissolved in EBSS. At ZT 22, this solution was removed from the dish and replaced with a 1μM solution of K252a dissolved in a 1mM solution of glutamate and EBSS. After ten minutes, this solution was removed and once again replaced with a 1μM solution of K252a dissolved in EBSS. After five minutes, this solution was again removed, and normal perfusion of EBSS was resumed.

**DMSO Trials:**

As purchased, K252a was dissolved in DMSO. Because DMSO was also present in solutions containing this blocker, two experiments were conducted with a 1mM solution of DMSO dissolved in EBSS. At five minutes before ZT 22, perfusion was stopped, and the DMSO solution replaced the normal EBSS in the dish. After twenty minutes, this solution was removed from the dish, and normal perfusion of EBSS was resumed.
Statistical Analysis

The statistical significance of the times of peak neuronal firing activity were assessed using Student’s t-test, and the level of significance was set at $P < 0.05$. 
Chapter 3

Results

Experiments \((n = 5)\) conducted with untreated brain slices revealed a circadian curve of neural firing activity peaking near midday. The mean time of peak activity for untreated slices was \(ZT \ 5.95 +/- 0.47\) hours (See Figure 5). This is consistent with previous experiments for this lab (Prosser, 2003). When slices were treated with the \(1mM\) glutamate solution at \(ZT \ 22\), the mean time of peak firing activity in the SCN was \(ZT \ 2.44 +/- 0.18\) (See Figure 6). This translates to a statistically significant phase advance of approximately 3.5 hours \((p < 0.05)\). This phase shift is similar to that shown in previous \textit{in vitro} experiments using SCN brain slices from rats (Ding et al, 1997).

Experiments conducted with brain slices treated with \(1\muM\) K252a solution at \(ZT \ 22\) revealed no statistically significant phase shift of the circadian curve. Peak firing activity was still observed near midday with a mean peak firing time of \(ZT \ 5.94 +/- 0.07\) (See Figure 7). Experiments conducted with brain slices treated with both glutamate and K252a revealed a mean peak firing time of \(ZT \ 5.88 +/- 0.36\) (See Figure 8). This shift is not statistically different from controls, but it is statistically different from the time of peak activity seen after glutamate application (See Figure 9). Experiments conducted with brain slices treated with DMSO also demonstrated a midday peak of neural firing activity (Figure not shown). The mean peak firing rate was \(ZT \ 6.13 +/- 0.18\), and this is not statistically different from controls.
Figure 5: *In vitro* circadian rhythm of neuronal activity. Dots represent two-hour running averages +/- SEM, and the dark horizontal bars between ZT 12 and ZT 24 represent the “lights off” period in the mouse’s entrained cycle. Recordings revealed a mean time of peak activity for untreated slices of ZT 5.95 +/- 0.47 hours.
Figure 6: Circadian rhythm in neuronal activity recorded after ten minute glutamate treatment at ZT 22. Shown are two-hour running averages +/- SEM with peak firing activity occurring at ZT 2.44 +/- 0.18. The horizontal bars represent "lights-off" in the colony, and the vertical bar represents time of drug treatment. The dashed vertical line represents time of peak activity in control experiments.
Figure 7: Two-hour running averages of neuronal firing activity in the SCN in response to K252a treatment at ZT 22. Peak activity occurs at ZT 5.94 +/- 0.07. Horizontal bars represent "lights-off" in the colony, and the vertical bar represents time of drug treatment. The dashed vertical line represents time of peak firing activity in control experiments.
Figure 8: Two-hour running averages of neuronal firing activity in the SCN in response to Glutamate + K252a treatment at ZT 22. Peak activity occurs at ZT 5.88 +/- 0.36. Horizontal bars represent “lights-off” in the colony, and the vertical bar represents time of drug treatment. The dashed vertical line represents time of peak firing activity in control experiments.
Glutamate phase advances blocked by TrkB antagonist K252a in vitro

Figure 9: Mean phase advances in response to specified drug treatments at ZT 22. Glutamate induced a statistically significant average phase advance of 3.5 hours. Slices treated with glutamate + K252a showed no statistically significant phase shift relative to controls, but these experiments did reveal a statistically significant phase shift relative to experiments conducted with glutamate treatments.
Chapter 4
Discussion and Future Directions

The results of this experiment are consistent with the hypothesis that brain-derived neurotrophic factor (BDNF) is involved in glutamate-induced circadian phase advances. The results of this experiment also provide the first in vitro confirmation of previous findings from in vivo experiments. Given that the role of BDNF in circadian phase shifting determined in this experiment is consistent with the results of in vivo experiments on advance shifting, one can reasonably anticipate that further in vitro experiments will continue to show that blocking the trkB receptor with K252a will block glutamate-induced phase delays as well. However, a number of additional experiments are necessary to support the conclusion that BDNF binding is a necessary event for all glutamate-induced phase shifting to occur.

First, the experiments done in this study need to be repeated at CT 14. Data from these experiments are necessary to support BDNF's role in glutamate-induced phase delays. Results from previous experiments in vivo have supported a role for BDNF in glutamate-induced phase delays, and replication of the experiments in this study at ZT 14 are expected to further support this role in vitro.

Second, we need to determine the effects of daytime glutamate application in the presence of BDNF in vitro. In previous experiments, application of BDNF enabled phase shifting to occur in response to a light stimulus at ZT 6 in vivo. Therefore, further studies must be conducted with daytime application of BDNF at ZT 6 in order to determine if the
presence of this growth factor during the subjective day can enable a phase shift to occur with daytime application of glutamate in vitro.

Third, the inhibitory effects of K252a need to be replicated with a more specific receptor tyrosine kinase inhibitor, such as AG879. Data from these experiments will further substantiate the conclusion that K252a is blocking the glutamate-induced phase shifts by blocking the TrkB receptor rather than inhibiting the activity of other tyrosine kinases.

Should these experiments confirm a role for BDNF/TrkB receptor activation in photic phase shifts, it will be important to determine exactly what BDNF is doing to achieve this effect. Data from other systems has shown that BDNF activates intracellular processes such as phosphorylation of NMDA receptor subunits and activation of CREB, both of which could potentially provide mechanisms for gating photic sensitivity, so further experiments will be necessary to determine if these and other processes occur in cells of the SCN.
References:


Gillette, Martha U. (1985) Preparation of Brain Slices from the Suprachiasmatic Nuclei of Rat can Reset the Circadian Clock J. Physiol. 369:55P.


