The Effects of Glutamate on Fibrinolytic Proteins in the Mouse SCN

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The Effects of Glutamate on Fibrinolytic Proteins in the Mouse SCN

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Abstract

Circadian rhythms are daily rhythms in our behavior and physiology controlled by an internal clock that maintains 24-hour time through endogenous mechanisms. This clock is located in the suprachiasmatic nucleus (SCN) in the brain. Our lab investigates the mechanisms through which the phase, or timing, of circadian rhythms can be shifted. In this project there are three primary proteins being studied for their ability to modulate glutamate: brain-derived neurotrophic factor (BDNF), plasminogen, tissue plasminogen activator (tPA). Based on previous work, we believe these proteins interact with one another in order to phase-shift the SCN clock.

Light resets the SCN clock through an interactive process involving the neurotransmitter (signaling chemical), glutamate, and the growth factor, BDNF. BDNF is initially produced in an inactive form that can be converted to its active form by plasmin. Plasmin is generated from the protein plasminogen through cleavage by the protein, tPA. We have shown that tPA-dependent conversion of plasminogen to plasmin is required for glutamate to phase-shift the SCN clock. Here we are investigating whether glutamate increases tPA levels, which would increase the ability of glutamate to phase shift the SCN clock.

To test for an increase in tPA, mouse brain slices containing the SCN were either left untreated or were treated for 10 minutes with glutamate (10mM) at varying times. Tissue samples were then analyzed for the amount of tPA protein present. Our results indicate that glutamate treatment at ZT 16 (when the circadian clock is phase delayed) significantly increases the amount of tPA protein in the SCN (p<0.05). Conversely, there is not a significant change in tPA expression after glutamate treatment at either ZT 6 or
ZT 23. The increase in tPA at ZT 16 may be important for glutamate-induced resetting of the SCN circadian clock.
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Chapter I

Background and Significance

Circadian rhythms are daily rhythms in our behavior that are controlled by an internal clock that maintains 24 – hour time through different mechanisms. In addition, circadian rhythms have two distinguishing characteristics: first, as long as the environment remains stable, the circadian pattern retains the same periodicity. Second, deviations from the stable environment, such as photic stimulation at unusual times, can alter the circadian pattern (Dunlap et. al. 2004). Circadian rhythms are essential to an organism’s way of life. The circadian clock organizes essential behavioral and physiological mechanisms of an organism into a systematic schedule such that they occur at the most suitable time of day. It also coordinates these individual mechanisms to work in tandem with one another to prepare for environmental changes (Dunlap et. al., 2004).

There is an extremely long list of circadian behaviors for mammals. One example of a circadian rhythm is our sleep-wake cycle. People usually wake up around the same time every day as well as get sleepy around the same time every night, and this cycle is controlled by the circadian clock. Another prominent example is metabolism. Human metabolism has two opposing stages: the nocturnal stage and the diurnal stage. The nocturnal stage is responsible for development and repair of the body which is why people need to rest and not exert themselves during this time. At the other end of the spectrum are the diurnal processes that provide support for the energy required for functionality and mental awareness. For this reason, people exhibit better mental and physical performance during the circadian day as opposed to the circadian night. Another well-known example of a circadian rhythm is one’s core body temperature. In order to
prepare the body for sleep, one’s core temperature will drop in the evening and the
opposite will occur in the morning to initiate awakening (Dunlap et. al. 2004). However,
these circadian rhythms can become out of proper phase. Flying from one time zone to
another can cause the clock to be out of proper phase, as well as changing from one work
shift schedule to another, such as from night shift to day shift. Unfortunately, it may take
several days to adjust to this new schedule as one’s internal circadian clock adjusts by
about one hour per day. This is just one of the reasons behind our research in Dr.
Prosser’s lab: to see whether or not certain proteins can stimulate the circadian clock to
phase shift and become in proper phase.

All living organisms have internal clocks. In mammals, the location of this
internal clock is in the suprachiasmatic nucleus (SCN) in the brain. The SCN recognizes
input from the retina, which acts as a photic sensor for the clock (Dunlap et. al. 2004).
Light stimulates the retina, and signals from the retina travel down the optic nerve to
regulate the internal clock. Through these signals, the SCN oscillator maintains
synchrony with the environment (Dunlap et. al. 2004). The signals regulate the internal
clock by causing the release of glutamate, which initiates a series of intracellular
processes (Figure 1). Glutamate binds to its NMDA receptor, causing an influx in
calcium. This influx of calcium ions stimulates nitric oxide synthase, which synthesizes
nitric oxide. Nitric oxide then causes a phase advance, which occurs during the late
subjective night, by stimulating guanylate cyclase to produce cGMP. The presence of
cGMP leads to the activation of both MAPK and CREB which advance the phase of the
clock. Nitric oxide can also induce a phase delay, which occurs during the early
subjective night, by stimulating its ryanodine receptor to release calcium ions, effectively causing a phase delay.

Figure 1. Series of intracellular processes regulating the SCN clock.

Photic resetting of the mammalian circadian clock in the suprachiasmatic nucleus (SCN) involves the release of glutamate, which activates NMDA receptors. We, and others, have found that brain-derived neurotrophic factor (BDNF) is also necessary for glutamate to initiate its phase shift (Liang, et al.; Michel; Mou). BDNF activation of its Trk B receptor can lead to phosphorylation of the NMDA receptor, which enhances its activity. Recently we began investigating mechanisms regulating BDNF availability. Jiang et. al (2000) showed that BDNF levels in the SCN are high at night and low in the
day. However, these data did not take into account the conversion of inactive (pro)BDNF into active BDNF (mBDNF). Figure 2 illustrates how this conversion of BDNF occurs.

**Figure 2** In the hippocampus, tissue plasminogen activator (tPA) activates BDNF through converting plasminogen to plasmin, which converts proBDNF to mature BDNF, which is required for learning and memory (L-LTP). tPA is inhibited by plasminogen activator inhibitor-1 (PAI-1), while vitronectin binds to and enhances PAI-1 activity. In the SCN mBDNF enhances glutamate-induced phase resetting.

BDNF is normally secreted in its inactive form, and then converted to mBDNF thru the extracellular protease, plasmin (Pang et al, 2004). Plasmin is produced by enzymatic cleavage of plasminogen by tissue plasminogen activator (tPA). In previous studies, plasmin, plasminogen, and tPA have been shown to be expressed in the hippocampus.
where they participate in the production of long-term potentiation (LTP). Several experiments were conducted that support tPA playing a role in LTP, most likely by converting proBNDF to mBDNF: tPA expression is increased during LTP generation, axonal terminals and neuronal growth cones release tPA, and tPA is released into the extracellular space in the hippocampus via neuronal membrane depolarization (Pang et al., 2004). Furthermore, only a few extracellular proteases are capable of activating proBDNF, and plasmin is one of those proteases. The order of the proteins in the process was determined through treatment of knock-out slices. Treatment of tPA \(-/-\) slices with tPA in one trial and plasmin in another restored LTP. However, in plasminogen \(-/-\) slices, tPA treatment was not able to restore LTP. Similar results were found with treatment of tPA to BDNF\(^{+/-}\) slices. These findings indicate that tPA is active before plasmin and BDNF. Finally, the order of plasmin, plasminogen, and BDNF was determined. mBDNF application to brain slices that were plasminogen \(-/-\) restored LTP function. However, plasmin was not able to restore LTP function when applied to BDNF\(^{+/-}\) brain slices. These results indicate that the pathway was as described above (Pang et al., 2004).

We have shown that tPA, plasminogen and related extracellular proteins are expressed in the SCN and participate in glutamate-induced phase resetting (Mou et al.). Treating SCN-containing brain slices with glutamate during the early evening resets the circadian clock. However, this resetting of the clock is inhibited by plasminogen activator inhibitor-1 (PAI-1) which acts as a tPA inhibitor. Furthermore, \(\alpha_2\)-antiplasmin, which inhibits plasmin activity, also inhibits glutamate-induced phase-shifting of the circadian clock, but BDNF application restores glutamate-induced phase shifts. Together, these results show that these proteases are expressed in the SCN, as well as in the
hippocampus, and are involved in converting proBDNF to mBDNF (Mou, et. al., unpublished).

In addition to the fibrinolytic proteins modulating glutamate-induced signaling, it is possible that glutamate in turn modulates their levels of expression. To begin to explore this possibility, in these experiments we investigated whether glutamate affects tPA protein expression in the SCN in vitro.
Chapter II
MATERIALS AND METHOD

Experimental Setup

A Hatton-style chamber was used to maintain the brain slices before treatment. The dish contains a center compartment that holds the slices, and an outer compartment that functions as a water bath. A constant perfusion of oxygenated (95% O2/5% CO2) Earle’s Balanced Salt Solution (EBSS; Sigma) supplemented with glucose and bicarbonate (pH 7.4) was circulated through the center compartment to sustain the slices. The solution was maintained at 37°C.

Brain Slice Preparation

Coronal brain slices (500 µm) containing the SCN were prepared from C57BL/J6 mice (Prosser, 2003). The mice were housed in an environment with a cycle of twelve hours of light followed by twelve hours of darkness (12:12 LD) with “lights on” defined as zeitgeber (ZT) 0 and “lights off” occurring at ZT 12. All mice were maintained in this light/dark cycle for at least one week before being used for these experiments in order to allow sufficient time for entrainment. Brain slices containing the SCN were prepared and placed in the brain slice chamber.

Experimental Protocol

Experiments were done at three different times: ZT 16, ZT 23, and ZT 6. At ZT 16, the circadian clock is known to be phase delayed by glutamate. At ZT 23, the clock is known to be phase advanced by glutamate. Finally, at ZT 6, the circadian clock does not
phase shift in response to glutamate. These three time periods were chosen in order to determine the different ways glutamate might affect the levels of tPA depending on the time of the treatment.

At the designated times, perfusion was stopped. Half of the slices were removed and left untreated. These slices were designated as the control samples. For the treated samples, the normal medium was replaced with medium containing 10 mM glutamate. After 10 minutes, slices were placed in extraction buffer and frozen for later protein analysis (Western blot). Tissue samples were analyzed for the amount of tPA protein present. This was done by loading a control sample and a glutamate-treated sample into two different lanes in a 15% SDS page gel alongside a sample of pure tPA protein. After electrophoresis to separate the proteins according to their weight, the proteins were transferred to a nitrocellulose membrane and treated with tPA primary and secondary antibodies. A film of this membrane was developed showing the individual protein bands. After tPA analysis, the membrane was stripped of chemicals and re-probed for actin following the same procedure. The data were then expressed as a ratio of tPA/actin.

**Data Analysis**

After the film was developed showing the individual tPA and actin protein bands, the density of each band was quantified using the UN-SCAN-IT computer program. The values for tPA and actin from each protein sample were then expressed as a ratio, which normalizes the data within each sample. The tPA/actin ratio for the control sample of an individual experiment was then set to 1, and the tPA/actin ratio for the glutamate-treated sample for that same experiment was adjusted accordingly. This normalized the data
across experiments to eliminate differences based on the amount of protein loaded, the length of film exposure, etc. These normalized values were then averaged across experiments to yield the results displayed below (mean ± SEM).
Chapter III

Results

As seen in Figure 2, glutamate treatment at ZT 16 significantly increases the amount of tPA protein in the SCN (p<0.05). Mean tPA levels in the glutamate-treated samples increased about 2.5 fold as compared to control levels. Conversely, there was not a significant change at either ZT 6 (Figure 4) and ZT 23 (Figure 5). While there appears to be a trend toward a decrease in tPA in response to glutamate at ZT 6, the change was not significant (p = 0.156).

![Figure 3. Glutamate increases tPA protein expression at ZT 16. Shown are the mean=SEM levels of tPA expression in the SCN tissue (relative to actin levels) that was untreated or treated for 10 minutes with 1mM glutamate. [* p < 0.05 vs. control]
Figure 4. Glutamate treatment at ZT 6 did not change in the amount of tPA protein expressed in the SCN. See figure 3 for details.
Figure 5. At ZT 23, there was not a significant change in the amount of tPA protein present in the glutamate-treated samples. See figure 3 for details.
Chapter IV
Discussion and Future Directions

The results of this experiment are consistent with the hypothesis that glutamate increases tPA levels in the SCN at ZT 16. Whether this increase in tPA is part of the phase shifting process has yet to be determined. An increase in tPA could lead to increased conversion of plasminogen to plasmin, which in turn could increase the conversion of proBDNF to mBDNF.

On the other hand, there was no change in tPA in response to glutamate at ZT 23. This indicates that an increase in tPA is not mandatory for all glutamate-induced phase shifts. However, as discussed before, the circadian clock is phase advanced and phase delayed through different intracellular processes. These differences in intracellular processes may account for the difference in the effect of glutamate on tPA levels at ZT 16 versus ZT 23. At ZT 6, there is no change in tPA in response to glutamate. This was expected because the circadian clock does not phase shift in response to glutamate during this time.

Currently, experiments are being done to investigate the effects glutamate has on plasminogen activator inhibitor-1 (PAI-1). As shown in Figure 2, PAI-1 inhibits tPA, which prevents the conversion of proBDNF to mBDNF. Furthermore, tPA and PAI-1 mRNA are expressed rhythmically in the SCN (Panda et al., 2002; Menger et al., 2005). If, in response to glutamate, the amount of PAI-1 is decreased at ZT 16, this could further increase the activity of tPA at this time. Therefore, it would offer further insight into how the circadian clock is phase shifted.
In conclusion, at ZT 16, increased levels of tPA could increase levels of BDNF, aiding it in its enhancement of glutamate’s ability to phase shift the circadian clock.
Chapter V

References


