Investigating subcellular localization of tPA and PAI-1 in the mammalian circadian clock

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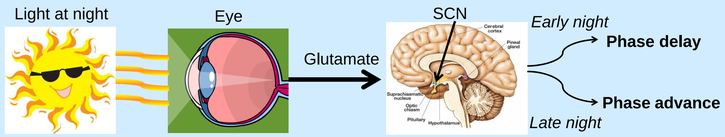
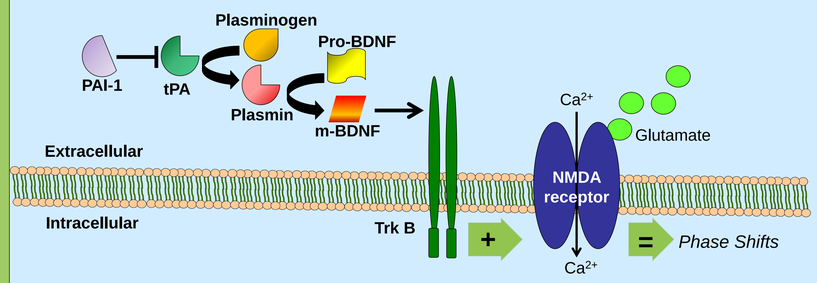
Introduction

Circadian rhythms are 24-hour cycles in physiological function found in most organisms. In mammals, the **suprachiasmatic nucleus (SCN)** of the hypothalamus is the circadian rhythm's **master pacemaker.** The SCN functions to continuously maintain the 24-hour cadence of the internal circadian clock and can adapt to external light stimuli in order to synchronize the animal's circadian rhythm to the environment. Light is a Zeitgeber, German for "time-giver," that mediates entrainment by inducing shifts in the clock phase. Notably, **phase shifts only occur at night, but the mechanisms gating phase shifting remain poorly understood.**



Light stimulates the retina which signals retinal ganglion cells to secrete the neurotransmitter, glutamate, onto SCN cells in the brain. This causes a phase shift. Light during the early subjective night leads to a phase delay while light during the late subjective night leads to a phase advance. However, light during the day does not cause a phase shift.

Extracellular proteases and neurotrophins are known to contribute to gating phase shifts. In particular, **tissue type plasminogen activator (tPA)** and mature brain-derived neurotrophic factor (m-BDNF) are necessary for the SCN to shift phase.[5] **Plasminogen activator inhibitor (PAI-1)** inhibits tPA.



Glutamate from the retinohypothalamic tract activates NMDA receptors in the SCN every time light is detected; however, phase shifts can only occur when mBDNF can activate the Trk B receptor. PAI-1 inhibits tPA which activates plasminogen into plasmin which converts pro-BDNF into m-BDNF.

tPA is a serine protease that converts inactive plasminogen, into active plasmin.[7] Outside the cell, it can cleave portions of transmembrane proteins such as LRP-1 and NMDA receptors to modify intracellular functions and activate signal transduction. tPA is also known to be involved in long-term potentiation in the hippocampus which is associated with memory formation.[2,7] In blood vessels, it also functions in thrombolysis.[7] In the extracellular matrix, tPA can modify the ECM which is necessary for rearranging cell connections such as synapses.[7,8]

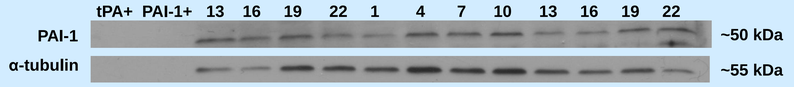
Total tPA levels in SCN brain slices have been shown to express 24-hour rhythms with lower levels during the day and higher levels at night.[6] **However, tPA can be found inside the cell, the extracellular matrix, and the extracellular space serving different functions depending on its location.**

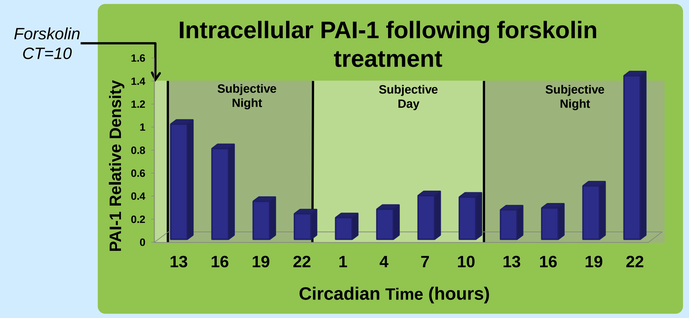
→ **Our aim is to determine the temporal localization of tPA and PAI-1 in the SCN**. This will yield evidence of the function tPA is performing during phase shifts to further elucidate what mechanisms gate them. We utilize the SCN 2.2 cell culture to investigate potential rhythms.

The **SCN 2.2 cell culture** is composed of immortalized, undifferentiated fetal rat SCN cells that exhibit characteristics of both neurons and glia.[1] Their individual circadian clocks can be synchronized with chemical treatments of either **forskolin** or **serum.**[1] They maintain circadian protein expression rhythms comparable to those of the SCN in vivo. The culture is important because it can be **reliably separated into cell, extracellular matrix, and media fractions** while brain slices cannot.

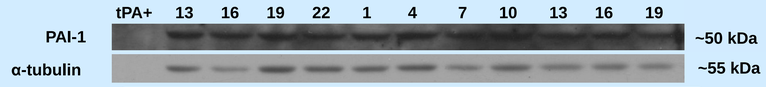
An important concept for this project is **Circadian Time (CT)**, based on the internal circadian clock.
CT 0-12 is the subjective day while CT 12-24 is the subjective night. For the forskolin treatment, as soon as the cells are synchronized, they begin the 24 hour cycle in late subjective day at CT 10.[3]

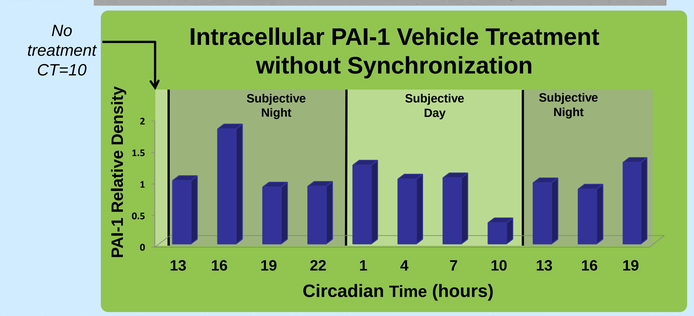
PAI-1 is present within the cell fraction and exhibits a potential rhythm in expression





Relative abundance of PAI-1 throughout the 36-hour time course. There appears to be a noticeable rhythm in PAI-1 inside the cell after forskolin synchronization. Previous work in our lab has shown that PAI-1 presence in brain slices peaks during the early night.[5] n=1





Relative abundance of PAI-1 throughout the 36-hour time course. [Hour 36 was an outlier]. There does not appear to be any noticeable rhythm of PAI-1 inside the SCN cells with vehicle control, which is to be expected.[1] n=1

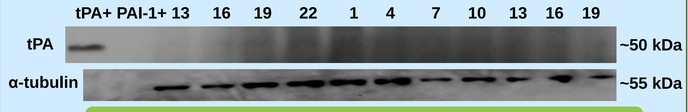
Methods

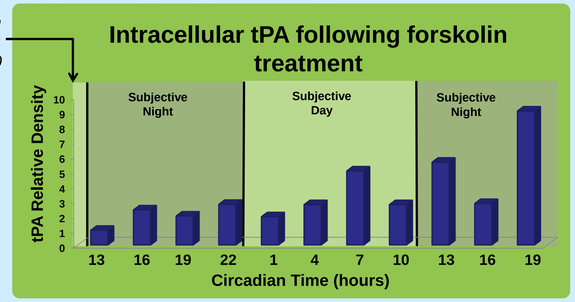
Sample Preparation
SCN 2.2 cells were synchronized with forskolin, serum, or vehicle control. Media, extracellular matrix, and cell fraction samples were collected every three hours for 36 hours and stored at -80 ºC until protein extraction.

Immunoblotting
All proteins were precipitated from the cell fraction using TCA extraction.[4] Trichloroacetic acid hydrogenates negatively charged proteins so they become insoluble. Proteins were separated on a 10% acrylamide SDS page gel using electrophoresis and then transferred to a PVDF membrane. Immunoblotting was used to visualize tPA, PAI-1, and α-tubulin. Antibodies included anti-tPA 70 kDa mouse monoclonal [H27B6] ab28374 1:500 from Abcam, anti- PAI-1 48 kDa [H135] sc-8979 1:500 from Santa Cruz, and anti- α-tubulin at 55 kDa [B-5-1-2]: sc-23948 1:500 also from Santa Cruz.

Analysis
We scanned Western Blot x-ray films into the computer. ImageJ from the NIH was used to analyze the pixel density of each protein band. Then we used Microsoft Excel to compare the amount of detected luminescence for each protein in each lane to that of the load control, α-tubulin. Values were normalized to CT 13.

tPA is present inside the cell fraction





Relative abundance of tPA inside the cell throughout the 36-hour time course. [Hour 36 is an outlier]. tPA appears to increase over the time course; however, no circadian rhythms are apparent. n=1

Conclusions

- tPA and PAI-1 are expressed in the **cell fraction** of SCN 2.2 cells.
- Preliminary results suggest a potential rhythm in PAI-1 **intracellular** expression following synchronization but are inconclusive for tPA.

Future Studies

- Replicate experiments in the cell fraction.
- Investigate the temporal expression patterns of tPA and PAI-1 in the media and extracellular matrix fractions.
- Expand timecourse to examine continuity of potential rhythms.

References

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