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 poorly understood.

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 TrkB receptor. PAI-1 inhibits tPA which activates plasminogen into plasmin which converts pro-BDNF into m-BDNF.

Extraocular photoreceptors 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. tPA activates plasminogen into plasmin which converts pro-BDNF into m-BDNF.

IPA is a serine protease that converts inactive plasminogen, into active plasmin. 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. IPA is also known to be involved in long-term potentiation in the hippocampus which is associated with memory formation. In blood vessels, II also functions in thrombosis. In the extracellular matrix, IPA can modify the ECM which is necessary for reannealing cell connections such as synapses.

Total IPA levels in SCN brain slices have been shown to express 24-hour rhythms with lower levels during the day and higher levels at night. However, IPA 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 IPA and PAI-1 in the SCN. This will yield evidence of the function IPA 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. Their individual circadian clocks can be synchronized with chemical treatments of either forskolin or serum. 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 extracellular matrix, and media fractions while brain slices cannot.

An important concept for this project is Circadian Time (CT). CT 1-12 is the subjective day while CT 13-24 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 13.

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 24 hours and stored at -80°C until protein extraction.

Immunoblotting

All proteins were precipitated from the cell fraction using TCA extraction. Trichloroacetic acid is negatively charged and 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 IPA, PAI-1, and α-tubulin. Antibodies included anti-IPA 70 kDa mouse monoclonal (H278B) ab26374 1,500 from Abcam, anti-PAI-1 48 kDa [135] sc-6979 1,500 from Santa Cruz, and anti-α-tubulin at 55 kDa [B-5-1-2] sc-29946 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 lead control, α-tubulin. Values were normalized to CT 13.

References