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Spatial and Temporal Characterization of Extracellular Proteases in the SCN, the Mammalian Circadian Clock

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BCMB 457: Honors Thesis

Dr. Rebecca Prosser

Spatial and Temporal Characterization of Extracellular Proteases in the SCN, the Mammalian Circadian Clock

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1.0 Introduction

1.1 Circadian Rhythms and the Suprachiasmatic Nucleus

Circadian clocks regulate physiological processes in nearly all organisms. Located within the hypothalamus, the mammalian central clock identified as the suprachiasmatic nucleus (SCN) synchronizes peripheral clocks. Lesioning the SCN eliminates circadian rhythm patterns associated with body temperature, sleep, locomotor activity and hormones [1-3]. Retinal ganglion cells signal the presence of light to the SCN via the retinohypothalamic tract (RHT) and release of the neurotransmitter, glutamate. The intracellular processes associated with glutamate signaling (outlined below) are well understood; however, the extracellular processes involved with light-induced glutamate signaling are not completely understood.

The focus of my research is on a group of extracellular proteins best known for their role in the vasculature to regulate fibrinolysis: tissue-type plasminogen activator (tPA), plasminogen, and plasminogen activator inhibitor-1 (PAI-1). Experiments have determined that these proteins also function in SCN to modulate photic regulation of the circadian clock. Brain-derived neurotrophic factor (BDNF) is required for light/glutamate to reset the clock; it is activated by plasmin which is produced from plasminogen by tPA [4]. Glutamate-induced phase shifts are blocked by PAI-1, which stays active through binding with vitronectin, (VN) [4]. However, the precise spatial and temporal characteristics of these proteins' activities and interactions have yet to be determined. Our goal is to answer questions about the mechanisms through which these proteins regulate glutamate signaling in the SCN. This includes spatial location of tPA and PAI-1 in the SCN and how they change over the circadian cycle.

1.2 Importance of Fibrinolytic Proteins in Clinical Areas

The roles of tPA, PAI-1 and plasminogen in fibrinolysis are well known. Fibrinolysis, the body's ability to degrade fibrin, is an integrated part of hemostasis. Overactivity causes bleeding and underactivity causes thrombosis. Abnormal structural variants and abnormal levels of tPA, PAI-1 and plasminogen give rise to bleeding or to thrombosis [5]. The biological control of tPA-mediated fibrinolysis is both cellular and humoral, indicating its physiological importance [6]. The cellular regulation involves synthesis of tPA and PAI-1 as well as their release/uptake. The humoral regulation includes the tPA/PAI-1 reaction, the fibrin-stimulated plasminogen activation and the plasmin degradation of fibrin [6]. More recently these proteins have been shown to affect normal brain functions, including neural remodeling in early development, stress-induced anxiety in the amygdala, and learning behaviors [7-10]. tPA's activity in the brain is most prominent in the hippocampus and the hypothalamus[11]. However, less is known about the roles these extracellular proteases play in the SCN.

1.3 Tissue-type Plasminogen Activator (tPA) & Plasmin

Tissue-type plasminogen activator (tPA) is an extracellular protease that cleaves plasminogen into the active form plasmin. This is a crucial step in the cascade of events shown in Figure 1. In the vasculature this helps regulate fibrinolysis, while in the brain it appears to regulate NMDA/glutamate signaling (12, 13). There is accumulating evidence that tPA/plasmin are involved in SCN physiology.

The SCN is anatomically and functionally organized into two subdivisions: a core that lies adjacent to the optic chiasm and a shell that surrounds the core in its dorsomedial portion [14]. Plasminogen expression is prominent in the dorsomedial shell portion of the SCN [14]. tPA has been shown to localize at vasopressin-containing neurons within the SCN, where high levels of plasminogen have been found [15, 16]. Due to the actions of tPA on plasminogen, it is expected to be located in the same region of the brain as plasminogen. tPA is often found in extracellular milieu, as a soluble protein, or bound to extracellular matrix (ECM) or cell-surface receptors; its activity depends on its location and partners with which it interacts. For example, tPA can bind to the ECM proteins fibrin [17, 18] and laminin [19, 20] or to annexin II [21-23] and α-enolase [23, 24]located on cell membranes. tPA is inhibited directly or indirectly by several proteins but we will focus on its inhibition by plasminogen activator inhibitor (PAI-1); PAI-1 stability is enhanced when it is bound to vitronection (VN) as described in Section 1.4. It has been determined that tPA mRNA levels are expressed rhythmically in the adult SCN, with a peak in late day [25].

As mentioned, the product of tPA cleavage of plasminogen is plasmin. Plasmin has well documented roles in fibrinolysis [26], wound healing [27], and ECM degradation [27]. In the hippocampus, plasmin converts the inactive pro-BDNF to the active form, mBDNF, which is necessary to enhance the cellular processes associated with long term potentiation (LTP), which underlies learning [20]. Our research suggests that tPA and plasmin function in a similar manner in the SCN. α_2-antiplasmin (an endogenous inhibitor of plasmin) inhibits glutamate-induced phase shifts and decreases the conversion of inactive to active BDNF in the SCN [4]. Exogenous mBDNF (200 ng/mL) applied 30 minutes prior to co-application of glutamate and α_2-antiplasmin fully restores the glutamate-induced phase shifts [4]. This phase shift cannot be accomplished without the presence of glutamate; mBDNF on its own has no effect on the phase shift [4]. These data support the model shown in Figure 1; our working model is that plasmin cleaves the inactive form of BDNF, pro-BDNF, into the active form, m-BDNF. m-BDNF activates the TrkB receptor, which in turn enhances NMDA channel activity. This ultimately allows glutamate to induce clock phase shifts.

1.4 Plasminogen Activator Inhibitor-1 (PAI-1) & Vitronectin

PAI-1 is a protease that acts primarily to inhibit tPA activity. PAI-1 binds to a glycoprotein called vitronectin (VN); this interaction increases PAI-1's stability 10-fold. Similar to tPA, PAI-1 mRNA levels are expressed rhythmically

in the adult SCN. PAI-1 mRNA levels peak approximately 6 hours after peak levels of tPA mRNA [28]; PAI-1 mRNA levels are modulated by BDNF [29]. In previous studies, PAI-1 effects in the SCN were well documented. Glutamate (1mM) applied to brain slices in the early subjective night for 10 minutes delayed the SCN neuronal activity rhythm by 2-3 hours [4]. Pretreating the brain slices with PAI-1 for 30 minutes prior to glutamate application blocked these glutamate-induced phase-shifts from occurring [4]. This inhibition is dependent on PAI-1 dosage and can be overcome by excess tPA application [4].

VN (62 kD glycoprotein) stabilizes PAI-1 by opposing PAI-1's tendency to relax to an inactive form [30-33]. It is necessary for PAI-1 actions in the SCN[4]. Experiments have shown that PAI-1 is not capable of blocking glutamate-induced phase shifts in brain slices prepared from VN knockout mice; PAI-1 could not inhibit these phase shifts without its cofactor, VN [4]. VN stabilizes PAI-1 via extensive interactions and also localizes PAI-1 to specific extracellular locations. Notably, VN is expressed in neural tissues during development and following trauma [34-40]. Microarray studies have found that VN mRNA is expressed in the SCN but is not rhythmic [41].

1.5 Proposal & Hypothesis

We believe that the fibrinolytic proteins described above affect the circadian clock in part through their ability to activate BDNF. If this is the case, then these proteins should be localized extracellularly, possibly associated with the extracellular matrix. Our goal is to assess the spatial location of the above mentioned proteins and how they change over the circadian cycle. SCN2.2 cells are a good model system in which to study these proteins. This is an immortalized cell line derived from rat SCN. Its distinguishing characteristic is the ability to generate circadian rhythms similar to those of the SCN *in vivo* [42]. Thus, these cells are not only capable of measuring time (like a clock) but also of regulating the timing of other events (as a pacemaker). Their pace-making ability has been determined *in vivo*. The behavioral activity of intact rats is highest at night, while SCN-lesioned rats exhibit arrhythmicity. After transplantation of SCN2.2 cells into the hypothalamus, the circadian rhythm of wheel-running behavior was restored within 4-10 days [42].

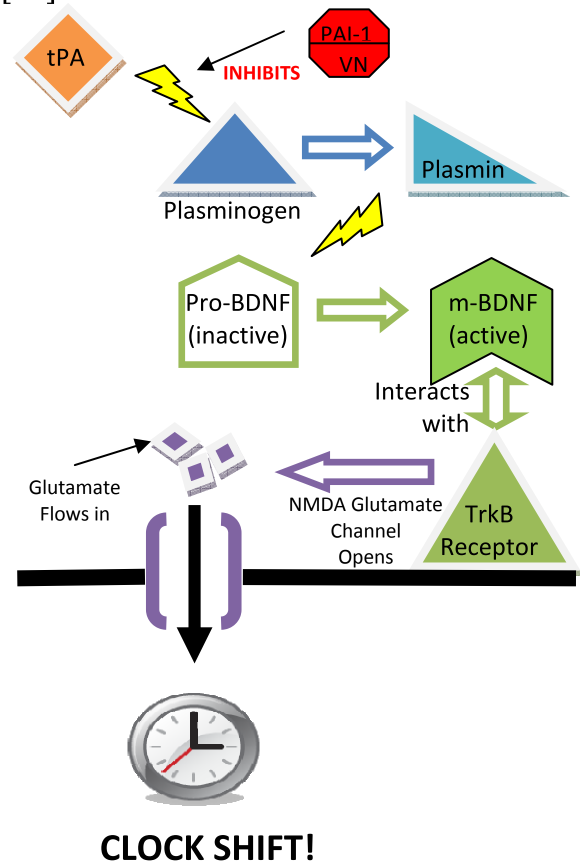


Figure 1. The extracellular protease, tPA, cleaves plasminogen into plasmin. Plasmin turns the inactive form of BDNF, pro-BDNF, into the active form, m-BDNF. m-BDNF interacts with the TrkB Receptor on the cell membrane, opening up the glutamate NMDA channels. Glutamate now freely flows in the cell and causes a clock shift. As shown, tPA activity is inhibited by the PAI-1/VN complex.

2.0 Methods

2.1 Cell Culture Procedure

Frozen, immortalized SCN 2.2 cells were thawed out and grown in laminin-coated flasks at 37°C in Minimal Essential Media (Gibco) supplemented with 10% FBS (Fetal Bovine Serum), 2% glucose, 1% glutamine and 1% gentamicin until >60% confluence. Before cells were harvested, samples of media were collected and stored at -80°C. Following media collection, cells were rinsed with 6 ml of DPBS (Dulbecco PBS). Cells were then lifted and lysed in 1 ml of 0.2M ammonium hydroxide after a ten minute incubation. Cell extracts were collected and immediately stored at -80°C.

For time-course samples, prior to harvesting, cells were treated with forskolin (FSK) dissolved in dimethyl sulfoxide to synchronize the cells (denoted as circadian time 0) or with vehicle. Both FSK- or vehicle-treated samples were collected at 3 hour increments for up to 27 hours subsequent to treatment.

2.2 Protein Extraction Procedure

To assess protein expression, rat SCN2.2 cells were isolated into three fractions (media, ECM and cells) and then frozen at -80°C for later Western blot assay. These fractions were supplied by former lab postdoctoral, Dr. Beth Cantwell.

Trichloroacetic acid (TCA) was used to precipitate proteins (from cell fractions). Our protocol has been slightly modified from the classical TCA procedures in that we do not incubate our proteins on ice or at -20°C for any amount of time [43, 43]. The TCA/acetone protein extraction procedure was chosen to precipitate the proteins because it has been proven to produce highly purified tPA with a high yield [45]. After adding ten percent volume of ice-cold 100% TCA to each sample, samples were centrifuged at 4°C for 30 minutes at 17,000 RPM, and then the supernatant was discarded. 250 µl of ice-cold 1% TCA in acetone was added to wash the pellet. Samples were then vortexed, centrifuged at 4°C for 15 minutes at 17,000 RPM and the supernatant was removed. After washing twice with 1% TCA, the pellet was further washed with 250 µl of ice-cold acetone, centrifuged at 4°C for 5 minutes at 17,000 RPM and the supernatant was removed. The samples were left to dry overnight.

The pellets were resuspended in 1X loading buffer containing sodium dodecyl sulfate (SDS) and dithiothreitol, followed by a series of boiling and mixing steps. Each boiling step was 5 minutes and at 100°C, and samples are stored in -20°C or immediately used for western blotting.

2.3 Western Blots

SDS-Page and Immunoblotting

TCA-extracted samples were boiled for 5 minutes and loaded into 10% SDS-polyacrylamide gels. Proteins in the samples were separated by size and transferred onto nitrocellulose by electrophoresis in Tris-Glycine buffer. Membranes were then rinsed with PBST (1X phosphate buffered saline with 0.05% Tween-20) and blocked in PBST with

10% milk. Primary antibodies were applied for 2 hours at room temperature or overnight at -4°C, rinsed, and incubated with horseradish peroxidase (HRP)-conjugated secondary antibodies for 1 hour. The primary antibody used was rabbit-anti-mouse (Molecular Innovations) in a concentration of 1:1000 with 2% milk and the secondary antibody was horse-anti-mouse IgG (Vector Laboratories) in a concentration of 1:2000 with 2% milk. Visualization was performed using Supersignal West Pico (Thermoscientific), a chemiluminescent substrate cleaved by HRP.

3.0 Results

Cell extracts and media prepared by TCA extraction were subjected to SDS-PAGE followed by western blotting with antibodies for tPA as shown in Figure 2. The tPA antibody gave immunoreactive bands at approximately 62 kD, corresponding to the expected weight. At this point, our positive control (purified PA protein) did not give a strong signal, but we later confirmed the band corresponded with the native protein. As shown in Figure 2, the cell extract replicates do not show up consistently. However, the media extracts have much higher levels of protein.

Each band shown in Figures 2, 3, 4 and 5 lies at approximately 62-kD, which matched the positive control as shown in Figure 3 and Figure 5 and is consistent with previous studies [4].

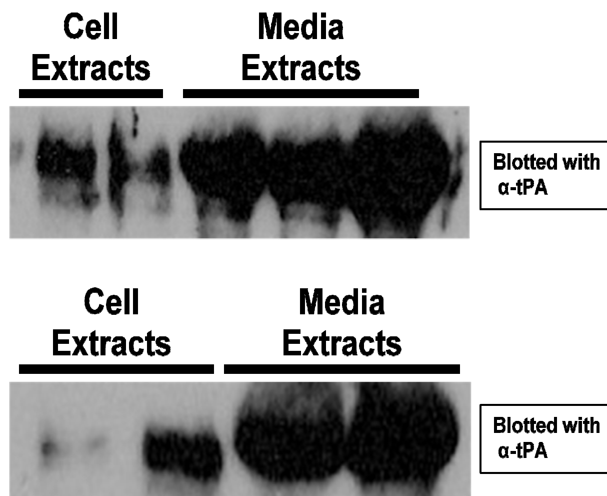
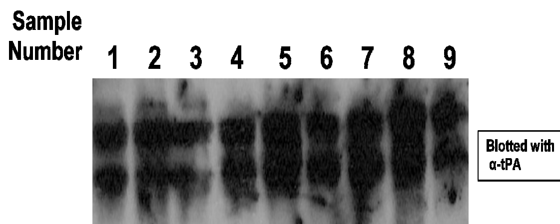


Figure 2. Cell extract and media extract replicates extracted as described in the Methods section. The media extract samples have significantly higher levels of protein than the cell extracts.

Vehicle-treated cell extract samples are shown alongside PAI-1 and tPA positive controls in Figure 3. This immunoblot shows weak expression of tPA protein positive control. The PAI-1 positive control shows cross-reactivity to the rabbit polyclonal, anti-mouse tPA antibody. Later blots attempted to address these re-occurring issues. In many of our cell extract experiments, including those in Figure 3, we attempted to blot for actin, which is a structural protein that should act to normalize the amount of target protein in order to control between groups. However, we were not successful as no reactivity appeared even in the extended (20 minutes) exposures.

Figure 3. Vehicle-treated cell extract samples extracted as described in the Methods section shown alongside PAI-1 and tPA positive controls.

The vehicle treated cells act as a control for the forskolin treated cells. Forskolin activates adenylate cyclase [46] and is known to synchronize SCN cells *in vitro* [47]. Figure 4 shows two western blots of time-course samples that had been collected from circadian time (CT) 3 to CT27. Figure 4A shows a vehicle-synchronized blot compared to Figure 4B showing a forskolin-synchronized blot. In Figure 4B, expression at CT6 and CT9 were particularly high.



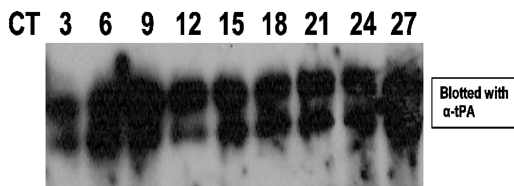


Figure 4. **(A)** Vehicle-treated samples extracted as described in the Methods Section. **(B)** Forskolin-treated, synchronized time-course samples extracted as described in the Methods Section. Cell samples were collected at Circadian Time (CT) 3-27 at 3 hour increments.

While the cell extract samples shown in Figure 5 below are not showing a consistent amount of protein, the PAI-1 and tPA positive controls have a much stronger presence even when loading a small amount. This is due to using new stock products.

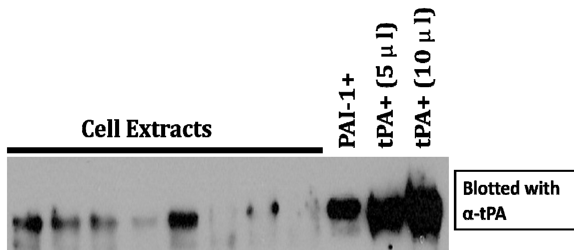


Figure 5. Vehicle-treated cell extracts, extracted as described in the Methods section. Also shown are PAI-1 and two tPA positive controls, loaded with different amounts of protein (5µl and 10µl). Both tPA positive controls were loaded at a concentration of 10µg/µl.

4.0 Discussion

The extracellular processes that regulate glutamate signaling in the SCN (and therefore its light-induced circadian activity) are largely unknown. From our previous study involving PAI-1/VN and the tPA/plasmin proteolytic cascade, the regulation of mBDNF production by these proteins is a critical factor for the SCN clock to respond to light/glutamate signaling [4]. Prior studies have shown that tPA is released into the extracellular space, shifting the balance of proBDNF secreted from dendrites to mature BDNF [48]. Moreover, a recent report showed that BDNF, tPA and plasmin protein are packaged in dense core granules and transported into dendrites [49].

In the SCN, plasminogen expression is prominent in the dorsomedial shell [14]. Moreover, tPA has been shown to localize at vasopressin-containing neurons, where high levels of plasminogen have been found [15, 16]. However, these studies did not address the critical issue of the cellular localization of these proteins, which we hypothesize to be located extracellularly.

Our results suggest that tPA is expressed in SCN neurons and, importantly, it is present in the media. Secretion of tPA into the media is consistent with its proteolytic activities shown in previous studies, although these studies are not in the SCN. Therefore, these results greatly strengthen our hypothesis that these proteins act extracellularly to regulate glutamate-induced phase shifts.

In order to further confirm our hypothesis, we need to determine whether the tPA/plasmin and PAI-1 expression patterns in the media change over 24 hours. Our data from FSK-synchronized cells appear to indicate there are higher levels of tPA in the media during mid-subjective day (CT3-9). Unfortunately, these data are not conclusive because the samples must be normalized to actin to confirm equal amounts of protein were loaded in each lane. We are now focusing our work on trouble-shooting the actin immunoblots.

Another concern is that the tPA antibody reacted with the PAI-1 positive controls. This cross-reactivity could be due to a problem that occurred during the antibody production. In addition, in Figure 4, rather than having one specific band in each blot, there were two distinctive bands. In other blots, the bands were not concise but rather expansive. This could be due to the tPA antibody binding non-specifically to other proteins. Alternatively, tPA is known to be cleaved into slightly smaller products, and so these bands could represent those cleavage products. In addition, different isoforms of tPA have been reported, and some of these are differentially glycosylated [50, 51].

Until 2006, protein precipitation of rat brain proteins had utilized the TCA/acetone procedure [52]. Classical TCA/acetone extraction methods have been determined to be very effective as a precipitant [53], instantly eliminating proteolytic and other modifying enzymatic activity [54]. However, a disadvantage of TCA precipitated protein is that it is difficult to redissolve, and loss of protein may occur [52, 55]. This may explain the inconsistency of the tPA bands

in our western blots as well as why actin did not show up as it was expected to.

Studies concerning the localization and abundance of tPA, plasmin and PAI-1 in the brain are scarce. The few studies that do investigate their presence in the brain are limited to the hippocampus, cerebral cortex and hypothalamus. An abundant and localized expression of tPA is observed in the bed nucleus of the stria terminalis (BST) in the hypothalamus; restraint stress and injection of corticotopin-releasing factor cause tPA to be released into the extracellular space, promoting neuronal activation [56]. The function of this release however is not known.

Of particular interest in my studies is the reported interaction of tPA with the NMDA (N-methyl D-aspartate) receptor. tPA has been determined to act directly with the NMDA receptor via subunit cleavage [57] as well as indirectly via its effects on BDNF. It has been determined that when cortical neurons are depolarized, they release tPA that interacts with and cleaves the NR1 subunit of the NMDA receptor [57]. Thus, tPA potentiates signaling mediated by glutamatergic receptors by modifying the properties of the NMDA receptor [57]. The cleavage interaction between the subunit and tPA can be prevented by addition of PAI-1 [57]. In addition, tPA treatment selectively potentiates the NMDA-induced increase in intracellular Ca^{+2} concentrations, which is pertinent to the activation of the NMDA receptor [57].

In the hippocampus, NMDA receptors play a crucial role in long term potentiation (LTP), which is the mechanism for the production of long term memory. tPA has profound effects on the late phase of LTP (L-LTP). An inhibitor of tPA blocks L-LTP, whereas extracellular application of tPA enhances L-LTP induced by a single tetatus, which by itself induces early LTP (E-LTP) [58]. Induction of L-LTP in turn enhances the expression of tPA in the hippocampus [59]. tPA can also be secreted from neuronal growth cones and axonal terminals [60], and neuronal membrane depolarization also induces secretion of tPA into the extracellular space in the hippocampus in a Ca^{+2} dependent manner [61]. The inactive zymogen, plasminogen (which is converted to plasmin via tPA) mRNA and protein in the hippocampus are exclusively expressed in neurons and primarily in the apical dendrites of pyramidal cells [62]. Not only do tPA knockout mice exhibit severe impairment of L-LTP, so did plasmin knockout mice [12]. Since it has been determined that a major function of the tPA/plasmin system is to convert proBDNF to mBDNF at hippocampal synapses, it has also been determined that such conversion is critical and necessary for the expression of L-LTP [12]. As already discussed, we have determined the glutamate-induced phase shifts require this conversion in the SCN as well.

5.0 Conclusion

We hypothesized that the extracellular actions of tPA/plasmin regulate mBDNF production which modulates how glutamate signaling affects SCN rhythmic activity. This led us to believe that the expression, localization, and activity levels of these proteins are regulated in the SCN. Our results suggest that tPA is expressed in SCN neurons and present in the media. However, we have yet to determine the

pattern of tPA/plasmin and PAI-1 expression in the media due to difficulties in normalizing actin to the protein expression levels and the possibility of non-specific binding of the tPA antibody to other proteins. Future studies will initially focus on fine-tuning the actin immunoblots and then will turn to producing consistent protein expression patterns.

Literature Cited

[1] Moore RY, Eichler VB. Loss of a circadian adrenal corticosterone rhythm following suprachiasmatic lesions in the rat. *Brain Res* 1972; 42:201-206.

[2] Stephan FK, Zucker I. Rat drinking rhythms: Central visual pathways and endocrine factors mediating responsiveness to environmental illumination. *Physiol Behav* 1972; 8:315-326.

[3] Refinetti R, Kaufman CM, Menaker M. Complete suprachiasmatic lesions eliminate circadian rhythmicity of body temperature and locomotor activity in golden hamsters. *J Comp Physiol* 1994; 175:223-232.

[4] Mou X, Peterson CB, Prosser RA. Tissue-type plasminogen activator-plasmin-BDNF modulate glutamate-induced phase-shifts of the mouse suprachiasmatic circadian clock *in vitro. Eur J Neurosci* 2009; 30:1451-1460.

[5] Akiyama H, Kawamata T, Dedhar S, McGeer PL. Immunohistochemical localization of vitronectin, its receptor and beta-3 integrin in Alzheimer brain tissue. *J Neuroimmunol* 1991; 32:19-28.

[6] Ranby M, Brandstrom A. Biological control of tissue plasminogen activator mediated fibrinolysis. *Enzyme* 1988; 40 (2-3):130-143.

[7] Zhang Y, Pothakos K, Tsirka S-A. Extracellular proteases: biological and behavioral roles in the mammalian central nervous system. *Curr Topics Devel Biol* 2005; 66:161-188.

[8] Viviven D, Buisson A. Serine protease inhibitors: novel therapeutic targets for stroke. *J Cereb Blood Flow Met* 2000; 20:755-764.

[9] Matys T, Pawlak R, Matys E, Pavlides C, McEwen BS, Strickland S. Tissue plasminogen activator promotes the effects of corticotropic-releasing factor on the amygdala and anxiety-like behavior. *PNAS* 2004; 101:16345-16350.

[10] Horwood JM, Ripley TL, Stephens DN. DRL performance in mice with deletion of tPA, uPA or PAI-1 genes. *Beh Pharmacol* 2001; 12:487-496.

[11] Sappino A-P, Madani R, Huarte J, Belin D, Kiss JZ, Wohlwend A *et al.* Extracellular proteolysis in the adult murine brain. *J Clin Invest* 1993; 92:679-685.

[12] Pang, PT, Ten JK, Zaitsev E, Woo NT, Sakata K, Zhen S *et al*. Cleavage of proBDNF by tPA/plasmin is essential for long-term hippocampal plasticity. *Science* 2004; 306:487-491.

[13] Samson AL, Nevin ST, Croucher D, Neigo B, Daniel PB, Weiss TW *et al*. Tissue-type plasminogen activator requires a co-receptor to enhance NMDA receptor function. *J Neurochem* 2008; 107:1091-1101.

[14] Taniguchi Yuki, *et al*. Localization of plasminogen in mouse hippocampus, cerebral cortex, and hypothalamus. *Cell Tissue Res* 2011; 343:303-317.

[15] Miyata S., Nakatani Y, Hayashi N, Nakashima T. Matrix-degrading enzymes tissue plasminogen activator and matrix metalloprotease-3 in the hypothalamo-neurohypophysial system. *Brain Res* 2005; 1058:1-9.

[16] Imamura, Y., Morita S., Nakatani Y, Okada K., Ueshima S., Matsuo O., Miyata S. Tissue plasminogen activator and plasminogen are critical for osmotic homeostasis by regulating vasopressin secretion. *J Neuroscience* 2010; 88;1995-2006.

[17] Myohanen H, Vaheri A. Regulation and interactions in the activation of cell-associated plasminogen. *Cell Mol Life Sci* 2004; 61: 2840-2858.

[18] Salonen E-M, Saksela O, Vartio T, Vaheri A, Nielson LS, Zeuthen J. Plasminogen and tissue-type plasminogen activator bind to immobilized fibronectin. *J Biol Chem* 1985; 260:12302-12307.

[19] Salonen E-M, Zitting A, Vaheri A. Laminin interacts with plasminogen and its tissue-type activator. *FEBS Letters* 1984; 172:29-32.

[20] Goldfinger LE, Jiang L, Hopkinson SB, Stack MS, Jones JCR. Spatial regulation and activity modulation of plasmin by high affinity binding to the G domain of the α_3 subunit of laminin-5. *J Biol Chem* 2000; 275:34887-34893.

[21] Hajjar KA, Jacovina AT, Chacko J. An endothelial cell receptor for plasminogen/tissue plasminogen activator. Identity with annexin II. *J Biol Chem* 1994; 269:21191-21197.

[22] cesarman GM, Guevara CA, Hajjar KA. An endothelial cell receptor for

plasminogen/tissue plasminogen activator (tPA). II. Annexin II-mediated enhancement of t-PA-dependent plasminogen activation. *J Biol Chem* 1994:21198-21203.

[23] Roda O, Chiva C, Espuna G, Gabius H-J, Real FX, Navarro P *et al*. A proteomic approach to the identification of new tPA receptors in pancreatic cancer cells. *Proteomics* 2006; 6:S36-S41.

[24] Lopez-Alemany R, Suelves M, Munoz-Canoves P. Plasmin generation dependent on alpha-enolase-type plasminogen receptor is required for myogenesis. *Thromb Haemost* 2003; 90:724-733.

[25] Menger GJ, Lu K, Thomas T, Cassone VM, Earnest DJ. Circadian profiling of the transcriptome in immortalized rat SCN cells. *Physiol Genomics* 2005; 21:370-381.

[26] Urano, S, Metzger A, Castellino FJ. Plasmin-mediated fibrinolysis by variant recombinant tissue plasminogen activators. *Proc. Natl. Acad. Sci* 1989; 86:2568-2571.

[27] Singer A, Clark R. Mechanisms of Disease: Cutaneous Wound Healing. *The New England Journal of Medicine* 1999;738-746.

[28] Panda S, Antoch MP, Miller BH, Su AI, Schook AB, Straume M *et al*. Coordinated transcription of key pathways in the mouse by the circadian clock. *Cell* 2002; 109:307-320.

[29] Fiumelli H, Jabaudon D, Magistretti PJ, Martin JL. BDNF stimulates expression, activity and release of tissue-type plasminogen activator in mouse cortical neurons. *Eur J Neurosci* 1999; 11:1639-1646.

[30] Mottonen J, Strand A, Symersky J, Sweet RM, DAnley DE, Geoghean KF *et al*. Structural basis of latency in plasminogen activator inhibitor-1. *Nature* 1992; 355:270-273.

[31] Stout TJ, Graham H, Buckley DI, Matthews DJ. Structures of active and latent PAI-1: a possible stabilizing role for chloride ions. *Biochemistry* 2000; 39:8460-8469.

[32] Zhou A, Carrell RW, Hungton JA. The serpin inhibitory mechanism is critically dependent on the length of the reactive center loop. *J Biol Chem* 2001; 276:27541-27547.

[33] Arroyo De Prada N, Schroeck F, Sinner EK, Muehlenweg B, Twellmeyer J, Sperl S *et al*. Interaction of plasminogen activator inhibitor-1 (PAI-1) with vitronectin. *Eur J Biochem* 2002; 269:184-192.

[34] Gladson CL, Cheresh DA. Glioblastoma expression of vitronectin and the alpha v beta 3 integrin. Adhesion mechanism for transformed glial cells. *J Clin Invest* 1991; 88:1924-1932.

[35] Pons S, Trejo JL, Matinex-Morales JK, Marti E. Vitronectin regulates sonic hedgehog activity during cerebellum development through CREB phosphorylation. *Development* 2001; 128:1481-1492.

[36] Niquet J, Gillian A, Ben-Ari Y, Represa A. Reactive glial cells express a vitronectin-like protein in the hippocampus of epileptic rats. *Glia* 1996; 16:359-367.

[37] Vasilyev DV, Barish ME. Regulation of an inactivating potassium current (I_A) by the extracellular matrix protein vitronectin in embryonic mouse hippocampal neurons. J *Physiol* 2003; 547:859-871.

[38] Vasilyev DV, Barish ME. Regulation of the hyperpolarization-activated cationic current I_h in mouse hippocampal pyramidal neurons by vitronectin, a component of extracellular matrix. *J Physiol* 2004; 560: 659-675.

[39] Walker DH, McGeer PL. Vitronectin expression in purkinje cells in the human cerebellum. *Neurosci Lett* 1998; 251:109-112.

[40] Gladson CL, Wilcox JN, Sanders L, Gillespie GY, Cheresh DA. Cerebral micoenvironment influences expression of the vitronectin gene in astrocytic tumors. *J Cell Sci* 1995; 108:947-956.

[41] Menger, GJ, Lu K, et al. Circadian profiling of the transcriptome in immortalized rat SCN cells. *Physiol Genomics* 2005; 21 (3):370-381.

[42] Earnest D, Lang F, Ratcliff M, Cassone V. Immortal Time: Circadian Clock Properties of Rat Suprachiasmatic Cell Lines. *Science* 1999; 29: 693-695.

[43] Isaacson, T., Damasceno, C., Saravanan, R., He, Y., catala, C., Saladie, M., Rose, J. Sample extraction techniques for enhanced proteomic analysis of plant tissues. *Nature Protocols* 2006; 1: 769-774.

[44] Carpentier, S.C., Witters, E., Laukens, K., Deckers, P., Swennen, R., Panis, B. Preparation of protein extracts from recalcitrant plant tissues: An evaluation of different methods for two-dimensional gel electrophoresis analysis. *Proteomics* 2005; 5:2497-2507.

[45] Soeda, S., Kakiki, M., Shimeno, H., Nagakuma, A. Rapid and high-yeild purification of porcine heart tissue-type plasminogen activator by heparin-sepharose chromatography. *Life Sciences* 1986; 39:1317-1324.

[46] Laurenza, A., et al. Forskolin: a specic stimulator of adenylyl cyclase or a diterpene with multiple sites of action? *Trends Pharmacol Sci* (1989); 10:442-447.

[47] Oklejewicz, M., Destici, E., Tamanini, F, et al. Phase Resetting of the Mammalian Circadian Clock by DNA Damage. *Current Biology* (2008); 18:286-291.

[48] Nagappan, G., Zaitsev E., Santorov Jr., V.V., Yang, J., Hempstead B.L., Lu, B. Control of extracellular cleavage of ProBDNF by high frequency neuronal activity. *Proc. Natl. Acad. Sci U.S.A.* 2009; 106:1267-1272.

[49] Lochner, J.E., Spangler, E., Chavarha, M., Jacobs, C., McAllister, K., Schuttner, L.C., Scalettar, B.A. Efficient copackaging and cotransport yields postsynaptic colocalization of neuromodulators associated with synaptic plasticity. *Dev. Neurobiology* 2008; 68:1243-1256.

[50] Harris, .J., Leonard, C.K., Guzzetta, A.W., Spellman, M.W. Tissue plasminogen activator has an O-linked fucose attached to threonine-61 in the epidermal growth factor domain. *Biochemistry* 30:2311-2314.

[51] Pennica, D., Holmes, W.E., Kohr, W.J., Harkins, R.N., Vehar, G.A., Ward, C.A., Bennett, W.F., Yelverton, E., Seeburg, P.H., Heyneker, H.L., Goeddel, D.V., Collen, D. Cloning and expression of human tissue-type plasminogen activator cDNA in E. coli. *Nature* 1983; 301:214-221.

[52] Hirano, M., Rakwal, R., Shibato, J., Agrawal, G.K., Jwa, N., Iwahashi, H., Masuo, Y. New Protein Extraction/Solubilization Protocol for Gel-based Proteomics of rat (Female) Whole Brain and Brain Regions. *Molecules and Cells* 2006; 22:119-125.

[53] Damerval, C., Devienne, D., Zivy, M., Theillement, H. *Electrophoresis* 1986; 7:52-54.

[54] Wu., F.S., Wang, M.Y. *Anal. Biochem*. 1984; 139:100-103.

[55] Nandakumar, M.P., Shen, J., Raman, B., Marten, M.R. *J Proetome Res.* 2003; 2:89-93.

[56] Matys T., Pawlak, R., Strickland, S. Tissue plasminogen activator in the bed nucleus of stria terminals regulates acoustic startle. *Neuroscience* 2005; 135:715-722.

[57] Nicole, O., Docagne, F., Ali, C., Margail, I., Carmeliet P., MacKenzie, E.T., Vivien, D., Busson, A. The proteolytic activity of tissue-plasminogen activator enhances NMDA receptor-mediated signaling. *Nat Med*, 2001; 7:59-64.

[58] Baranes, D *et al. Neuron*, 1998; 21:813.

[59] Qian, Z., Gilbert, M.E., Colicos, M.A., Kandel, E.R., Kuhl, D., *Nature* 1993; 361:453.

[60] Krystosek, A., Seeds, N.W. *Science* 1981; 213:1532.

[61] Gualandris, A., Jones. T.E., Strickland, S., Tsirka, S.E. *J. Neuroscience*, 1996; 16:2220.

[62] Tsirka, S.E., Rogove, A.D., Bugge, T.H., Degen, J.L., Strickland, S. *J. Neuroscience*, 1997; 17:543.