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Tissue-type Plasminogen Activator Induces Synaptic Vesicle Endocytosis in Cerebral Cortical Neurons

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Abstract

The release of the serine proteinase tissue-type plasminogen activator (tPA) from the presynaptic terminal of cerebral cortical neurons plays a central role in the development of synaptic plasticity, adaptation to metabolic stress and neuronal survival. Our earlier studies indicate that by inducing the recruitment of the cytoskeletal protein β II-spectrin and voltage-gated calcium channels to the active zone (AZ), tPA promotes Ca^{2+} -dependent translocation of synaptic vesicles (SVs) to the synaptic release site where they release their load of neurotransmitters into the synaptic cleft. Here we used a combination of *in vivo* and *in vitro* experiments to investigate whether this effect leads to depletion of SVs in the presynaptic terminal. Our data indicate that tPA promotes SVs endocytosis via a mechanism that does not require the conversion of plasminogen into plasmin. Instead, we show that tPA induces calcineurin (CaN) - mediated dynamin I dephosphorylation, which is followed by dynamin I-induced recruitment of the actin binding protein profilin II to the presynaptic membrane, and profilin II-induced F-actin formation. We report that this tPA-induced sequence of events leads to the association of newly formed SVs with F-actin clusters in the endocytic zone. In summary, the data presented here indicate that following the exocytotic release of neurotransmitters tPA activates the mechanism whereby SVs are retrieved from the presynaptic membrane and endocytosed to replenish the pool of vesicles available for a new cycle of exocytosis. Together, these results indicate that in cerebral cortical neurons tPA plays a central role coupling SVs exocytosis and endocytosis.

Keywords

Tissue-type plasminogen activator; Plasminogen; Plasmin; Synaptic Vesicles; Endocytosis

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The arrival of an electrical impulse to the presynaptic terminal of a chemical synapse induces Ca^{2+} -dependent mobilization, docking and fusion of synaptic vesicles (SVs) with an electron-dense thickening of the presynaptic membrane known as the active zone (AZ) where the exocytic release of neurotransmitters takes place (Gundelfinger et al., 2003). This sequence of events does not lead to depletion of SVs because it is coupled to the activation of a complex system of scaffolding proteins that retrieves SVs membranes from the AZ and reconstitutes them into new SVs that then are refilled with neurotransmitters and returned to the pool of vesicles available for another cycle of exocytosis (Haucke et al., 2011).

Tissue-type plasminogen activator (tPA) is a serine proteinase that upon membrane depolarization is rapidly released from the presynaptic terminal of cerebral cortical neurons (Echeverry et al., 2010). Our earlier studies indicate that tPA induces the recruitment of the cytoskeletal protein β II-spectrin to the AZ and promotes its binding to SVs, therefore bringing them in close proximity to the synaptic release site (Wu, 2015). Furthermore, tPA also increases the expression of presynaptic voltage-gated Ca^{2+} channels (VGCC), leading to Ca^{2+} -dependent phosphorylation of synapsin I, SVs translocation to the AZ and exocytic release of neurotransmitters into the synaptic cleft. However, it is unknown whether this effect leads to depletion of SVs from the presynaptic terminal, impairing the release of neurotransmitters during sustained synaptic activity, or if instead tPA also promotes their endocytic retrieval from the presynaptic membrane.

Three pathways have been identified for the endocytic recovery of synaptic vesicles from the presynaptic membrane. The first is a ~ 20 seconds slow endocytic pathway that occurs at distant places from the AZ via clathrin scaffolds (Heuser and Reese, 1973). The second, known as kiss-and-run, is a ~ 1 second mechanism that retrieves SVs by reversing their neck in the presynaptic membrane. Finally, recent studies have described a third ~50 milliseconds ultrafast endocytic retrieval pathway that takes place at the edges of the AZ (Watanabe et al., 2013).

The dephosphins are nerve terminal phosphorylated proteins essential for SV endocytosis that are rapidly dephosphorylated upon entrance of Ca^{2+} through VGCC (Cousin and Robinson, 2001). The best characterized dephosphins are the large GTPase dynamin I, the adaptor protein AP180, and the accessory proteins amphiphysin I/II, synaptojanin, epsin, eps15 and phosphatidylinositol phosphate kinase type $\text{I}\gamma$ (Clayton et al., 2007). Dynamin I was initially identified as a phosphoprotein that is rapidly dephosphorylated following membrane depolarization, and phosphorylated again upon membrane repolarization (Robinson et al., 1994). A substantial body of experimental evidence indicates that dynamin I plays a pivotal role in SV endocytosis (Smilie and Cousin, 2005). Accordingly, phosphorylated dynamin I is restricted to the cytosol and its Ca^{2+} -dependent calcineurin-mediated dephosphorylation facilitates its translocation to the plasma membrane (Liu et al., 1994) where it forms an electron dense collar that constricts and severs the neck of the clathrin-coated invaginations formed during the initial stage of SVs endocytosis, leading to the formation of a new SV that then is recycled back to the pool of SVs available for a new cycle of neurotransmitter release (Marks et al., 2001).

The recycling of SVs requires the interaction of the endocytic machinery with the actin cytoskeleton (Shupliakov et al., 2002), and although several details of this interaction are still unknown, it has been shown that the actin cytoskeleton acts as a scaffold for the return of the endocytosed SVs to the recycling pool (Sankaranarayanan et al., 2003). In agreement with these observations, actin is found in the endocytic zone, and perturbations of the actin cytoskeleton in the presynaptic terminal impair the recycling of SVs (Shupliakov et al., 2002).

The studies presented here indicate that tPA activates the mechanism of SVs endocytosis. Our data show that tPA induces calcineurin-dependent dynamin I phosphorylation, dynamin I-mediated recruitment of profilin II, and profilin II-induced F-actin assembly in the endocytic zone, and that this sequence of events leads to the retrieval of SVs from the presynaptic membrane following the exocytic release of neurotransmitters. In summary, our data indicate that the release of tPA from the presynaptic terminal of cerebral cortical neurons is a mechanism that couples SVs exocytosis with its endocytic retrieval from the presynaptic terminal.

1.0. Experimental Procedures

1.1. Animals and reagents

Murine strains were 8–12 weeks/old male wild-type (Wt) C57BL/6J mice. Experiments were approved by the Institutional Animal Care & Use Committee of Emory University, Atlanta GA, following guidelines established by ARRIVE (Animal Research: Reporting *In Vivo* Experiments). Recombinant murine tPA and proteolytically inactive tPA (itPA) with an alanine for serine substitution at the active site Ser481 (S481A) were acquired from Molecular Innovations (Novi, MI). Other reagents were antibodies against synaptophysin and Tau (EMD Millipore, Billerica, MA), profilin II (Abcam, Cambridge, MA), dynamin I phosphorylated at Serine 774 and β actin (Sigma-Aldrich; St. Louis, MO); AM1-44 (Biotium Inc.; Hayward, CA), the dynamin inhibitor dynasore (Calbiochem; Billerica, MA), rhodamine-phalloidin (Life technologies; Grand Island, NY), advasep-7 and cyclosporine A (CsA; Sigma-Aldrich; St. Louis, MO). The receptor-associated protein (RAP) was a kind gift of Dr. Dudley K Strickland (University of Maryland).

1.2. Neuronal cultures and live confocal microscopy

Cerebral cortical neurons were cultured from E16–18 Wt mice as described elsewhere (Wu et al., 2012). Briefly, the cerebral cortex was dissected, transferred into Hanks' balanced salt solution containing 100 units/ml penicillin, 100 μ g/ml streptomycin and 10 mM HEPES, and incubated in trypsin containing 0.02% DNase at 37°C for 15 min. Tissue was then triturated and the supernatant was re-suspended in B27-supplemented neurobasal medium containing 2 mM L-glutamine and plated onto 0.1 mg/ml poly-L-lysine-coated wells. To study presynaptic activity 6000 neurons/cm² were plated in glass microwell dishes (MatTek Corp. Ashland, MA) with astrocytes attached to a separate glass coverslip. The day of the experiment (DIV 15–17) the layer of astrocytes was removed, the media was changed to Hibernate (Life Technologies, Grand Island, NY), and cells were continuously monitored with a live confocal microscopy (Nikon A1R) at 40 X under basal conditions during 5

minutes, followed by the addition of 4 μM of AM1-44 during 20 minutes (in preliminary experiments we found that after 20 minutes the baseline uptake of AM1-44 reaches a plateau). Then 5 nM of proteolytically active or inactive tPA and 0.5 mM of advasep-7 were added during 15 minutes. To measure the uptake of AM1-44, image sequences were transformed to stacks in ImageJ (NIH) and 350 (atPA) - 375 (itPA) areas of interest (AOI) were drawn over the presynaptic terminal on the distal 50 μm axons from 15 neurons per experimental group. The intensity of AM1-44 labeling was measured in each AOI in aligned stacks at one-minute intervals using Stack/Plot Z-axis profile. To determine the mean uptake of AM1-44, intensity values obtained in each AOI after the addition of tPA were subtracted from the background intensity value in each AOI.

1.3. Preparation of synaptoneurosomes

Synapse-enriched fractions containing the pre-synaptic terminal and the apposing post-synaptic membrane (synaptoneurosomes) were prepared according to a modification of published protocols (Rao and Steward, 1991, An et al., 2008, Weingarten et al., 2014, Wilhelm et al., 2014) from Wt cerebral cortical neurons (DIV 15-17) treated during 60 seconds with 5 nM of tPA prepared on a solution containing HBSS, B27 and 10 mM of glucose, or with HBSS, B27 and glucose alone (herein referred to as vehicle-control). Cells were homogenized and centrifuged at 2000 $\times$ g for 5 min. Pellets were discarded and the supernatants centrifuged in an SS-20 fixed angle rotor at 32000 $\times$ g for 10 min to obtain the pellet 2 (P2). Pellets were re-suspended in 400 μl of lysis buffer (LB) containing 0.25 M sucrose, 1mM EGTA and 20 mM HEPES at pH 8, layered on top of a 5 %, 9 % and 13% discontinuous Ficoll gradient (Fisher, Fair Lawn; NJ) and centrifuged at 45 000 $\times$ g for 20 min at 4°C in a TLS 55 rotor using a Beckman Optima TLX tabletop ultracentrifuge. Synaptoneurosomes were collected from the 9 % / 13 % interface.

1.4. Western blot analysis

Extracts from synaptoneurosomes prepared from Wt cerebral cortical neurons incubated 0 – 60 seconds with 5 nM of tPA, alone or in combination with either 0.08 μM of cyclosporine A (CsA) or 25 μM of the dynamin inhibitor dynasore were homogenized and protein concentration was quantified using the BCA assay. Fifteen μg were loaded per sample, separated by 4–20% linear gradient polyacrylamide gel, transferred to a PVDF membrane by semi-dry transfer system, blocked with 5% non-fat dry milk in Tris-buffered saline pH 8.0 with 0.1% Tween 20 buffer, and immunoblotted with antibodies against either dynamin phosphorylated at Serine 774 (1:1000), or profilin II (1:000), or β -actin as a loading control. Each experiment was repeated 4 – 8 times.

1.5. Immunocytochemistry and quantification of synaptophysin and AM1-44 staining

Wt cerebral cortical neurons were incubated 0 – 60 seconds with 4 μM of AM1-44 and 5 nM of tPA or a comparable volume of vehicle (control), alone or in the presence of 1 $\mu\text{g}/\text{ml}$ of CsA or 25 μM of the dynamin inhibitor dynasore. A separate set of neurons was incubated during 60 seconds with either 0 – 10 nM of tPA, or 100 nM of the receptor-associated protein (RAP), or a combination of 5 nM of tPA and 100 nM of RAP. Following treatments, cells were fixed, permeabilized with 50 $\mu\text{g}/\text{ml}$ of digitonin and labeled with anti-synaptophysin antibodies and Alexa 488-conjugated goat antibodies. Microphotographs

were obtained with a Photometric Quantix digital camera connected to an Olympus-BX51 epifluorescence microscope. Each experiment was repeated with neurons from 3 different cultures. For the studies of AM1-44/synaptophysin co-localization labeling was terminated with 3 rapid washes with Hank's balanced salt solution without $\text{Ca}^{2+}/\text{Mg}^{2+}$ followed by 15 min incubation in complete media plus 1 mM Advasep7 to reduce unspecific labeling of membranes. Cells were fixed in 4% paraformaldehyde (PFA)/sucrose for 15 min at room temperature and processed for immunocytochemistry. AM1-44 staining was detected with a fluorescein filter. To quantify AM1-44/Synaptophysin co-localization, pictures taken from the distal axons from tPA- and control-treated neurons were straightened with ImageJ (NIH) and electronically magnified 3-fold. Images were inverted and merged in Photoshop by placing a copy of the RGB image in the blue channel, and then regions where green and red channel co-localized were copied to the blue channel rendering them white. With this technique the degree of co-localization varies within a spectrum from light gray to white. In each case, the number of AM1-44/Synaptophysin-positive puncta was quantified with the cell counter of ImageJ.

1.6. Quantification of F-actin expression, actin clusters length and F-actin / AM1-44 intensity and colocalization

F-actin expression was detected with phalloidin in Wt cerebral cortical neurons treated during 60 seconds with 5 nM of tPA or with a comparable volume of vehicle (control), alone or in the presence of 4 μM of AM1-44. Micrographs of the distal axons were taken at 60 X magnification and a heat map was created in Photoshop from individual gray images with a gradient from black to white assigned to each 50 gray value units. To measure the length and intensity of F-actin and AM1-44 in each actin cluster pictures were magnified 400 %, straightened in ImageJ and then each F-actin cluster was straightened again to measure its length and the intensity of phalloidin and AM1-44 at 0.02 μm intervals.

1.7. Statistical analysis

Statistical analysis was performed with two-tailed *t* test and one-way ANOVA with Greenhouse-Geisser correction, as appropriate. *p*-values of < 0.05 were deemed as significant.

2.0. Results

2.1. tPA induces synaptic vesicle endocytosis by a plasminogen-independent mechanism

Our previous studies indicate that the release of tPA from the presynaptic terminal of cerebral cortical neurons induces the mobilization of SVs to the AZ and the subsequent release of their load of neurotransmitters into the synaptic cleft (Wu, 2015). To investigate whether tPA also has an effect on SVs endocytosis we used continuous confocal live-cell imaging to monitor the uptake of the fluorescent styryl dye AM1-44 by the presynaptic terminal of Wt cerebral cortical neurons under baseline conditions (Fig 1A & B) and following incubation with 5 nM of tPA or vehicle (control; Fig 1A & C). We found that tPA induces the rapid uptake of AM1-44 (Fig 1C). To investigate whether this effect requires the conversion of plasminogen into plasmin, we performed similar observations after 5 minutes of incubation with 5 nM of either proteolytically active or inactive tPA (atPA and itPA,

respectively). Our data indicate that tPA induces SVs endocytosis via a plasminogen-independent mechanism (Fig 1D).

To further characterize these observations and to exclude the uptake of AM1-44 by presynaptic organelles other than SVs, Wt cerebral cortical neurons were incubated with AM1-44 and treated 0 – 60 seconds with 5 nM of tPA or a comparable volume of vehicle (control). After 0, 5, 15 or 60 seconds of treatment cells were immunostained with antibodies against synaptophysin (SYP; an integral membrane protein found in SVs), and the number of SYP-positive (both AM1-44-positive and -negative) and SYP/AM1-44-positive puncta in the distal axons was quantified as described in the Experimental Procedures section. Our data indicate that while tPA does not have an effect on the abundance of SVs, as denoted by an unchanged number of SYP-positive puncta (AM1-44-positive and -negative; Fig 1E), it induces their endocytosis, as shown by an increase in the number of AM1-44-containing SVs (SYP/AM1-44-positive puncta) from 5.5 $\pm$ 0.57 in untreated neurons to 8.1 $\pm$ 0.45, 9.49 $\pm$ 0.61 and 10.84 $\pm$ 0.46 following 5, 15 and 60 seconds of treatment with tPA, respectively (Fig 1F & G). To investigate whether the observed effect of tPA on AM1-44 uptake was dose-dependent, we performed similar observations following 60 seconds of incubation with 0 – 10 nM of tPA. We found that the uptake of AM1-44 increased with 1 nM of tPA and was maximal with a dose of 5 nM (Fig 1H). Our previous studies indicate that the low-density lipoprotein receptor-related protein-1 (LRP1) mediates several effects of tPA in the brain (Yepes et al., 2003). Thus we decided to investigate the effect of treatment with the receptor-associated protein (RAP), an antagonist of members of the LDL receptor family, on tPA-induced AM1-44 uptake by synaptic vesicles. Our data indicate that that a member of the LDL receptor family mediates tPA-induced endocytosis of synaptic vesicles (Fig 1I).

2.2. Calcineurin mediates tPA-induced SVs endocytosis

Because activation of the Ca²⁺/calmodulin-dependent protein phosphatase 2B ((calcineurin (CaN)) is required for SVs endocytosis (Marks and McMahon, 1998), we performed similar observations in neurons treated 60 seconds with tPA, alone or in combination with cyclosporine A (CsA), an inhibitor of CaN activity. Our data indicate that the number of AM1-44-containing SVs in the distal axons (SYP/AM1-44-positive puncta) decreases from 14.35 $\pm$ 0.39 in neurons treated with tPA alone to 7.92 $\pm$ 0.58 in neurons treated with tPA in the presence of CsA (Fig 2A & B; $p < 0.001$). Importantly, neither tPA nor CsA had an effect on AM1-44 uptake by non-synaptic vesicles. Accordingly, the percentage of SYP-negative / AM1-44-positive synaptic vesicles in each experimental condition remained unchanged (4.1 % in controls, 4.4 % in tPA-treated neurons, 3.9 % in cells treated with tPA and CsA, and 4.2 % in neurons incubated with CsA alone. $p = \text{Ns}$, one-way ANOVA).

2.3. TPA induces endocytosis of synaptic vesicles via dynamin I dephosphorylation

Dephosphins are nerve terminal proteins that upon dephosphorylation by CaN activate the synaptic endocytic machinery (Cousin and Robinson, 2001). Because the dephosphin dynamin I is required for synaptic vesicle endocytosis (Ferguson et al., 2007) we studied the expression of dynamin I phosphorylated at serine 774 in the cytosol of synaptoneurosomes prepared from Wt cerebral cortical neurons treated 0 – 60 seconds with 5 nM of tPA. Our

data indicate not only that tPA induces dynamin I dephosphorylation (Fig 3A – C) but also that this effect is mediated by calcineurin (Fig 3D & E). More importantly, we found that inhibition of dynamin's GTPase activity with dynasore attenuates tPA-induced SVs endocytosis as denoted by a decrease in the number of AM1-44-containing SVs (SYP/AM1-44-positive puncta) in the distal axons of Wt neurons from 11.98 $\pm$ 0.4 in neurons treated with tPA alone to 8.75 $\pm$ 0.31 in neurons treated with tPA in the presence of dynasore (Fig 3F).

2.4. Effect of tPA on F-actin assemblage in the endocytic zone

The formation of F-actin in the endocytic zone provides a scaffold for the return of SVs retrieved from the presynaptic membrane to the reserve and recycling pools (Sankaranarayanan et al., 2003). Because the actin monomer-binding protein profilin II is a ligand for dynamin I (Gareus et al., 2006, Pilo Boyl et al., 2007) that promotes the formation of F-actin filaments (Pollard and Borisy, 2003), we decided to study the expression of profilin II in membrane extracts from synaptoneurosomes prepared from Wt cerebral cortical neurons treated with tPA, alone or in the presence of the dynamin inhibitor dynasore. Our data indicate that tPA induces dynamin I-mediated recruitment of profilin II to the presynaptic membrane (Fig 4A – B).

Then we studied the expression of F-actin in the distal axons of Wt cerebral cortical neurons treated with 5 nM of tPA or a comparable volume of vehicle (control). We found that while most of the F-actin in vehicle (control)-treated cells is found in the axonal shaft (Fig 4C, panels a & b), treatment with tPA increases its expression in the axonal bouton (Fig 4C, panels c & d); and remarkably, this effect was accompanied by enlargement of the presynaptic terminal. To better characterize and quantify these findings we used Alexa 599-conjugated phalloidin to detect F-actin in Wt neurons treated with 5 nM of tPA or vehicle (control) in the presence of AM1-44. F-actin fluorescence was quantified every 0.02 μ m in optically magnified and straightened images from actin clusters located in the distal axons. Surprisingly, we found that compared to control-treated cells, F-actin intensity is decreased in several areas of the actin clusters from tPA-treated neurons (red in Fig 4D). However, these differences disappeared when the intensity values from all the examined points were pooled together (Fig 4E). We reasoned that an increase in the length of actin clusters in tPA-treated cells could compensate for the decrease in F-actin intensity within these clusters when all measurements were analyzed together. In agreement with this hypothesis, we found that F-actin clusters were larger in tPA-treated neurons than in control cells (0.33 $\pm$ 0.04 vs 0.48 $\pm$ 0.05 μ m). Importantly, this effect was abrogated by dynamin inhibition (0.29 $\pm$ 0.02 nm; Fig 4F).

2.5. TPA promotes the association of SVs with F-actin clusters

Although together our data indicate that tPA increases the length of F-actin clusters by promoting the recruitment of G-actin monomers to their periphery via dynamin I - mediated recruitment of profilin II, it does not explain the low intensity of F-actin fluorescence detected in several areas of actin clusters from tPA-treated neurons (Fig 4C). Because actin serves as a scaffold for the recovery of SVs in the endocytic zone (Sankaranarayanan et al., 2003) we postulated that the lower intensity of F-actin in these areas was due to their

occupancy by endocytosed SVs. To test this hypothesis we measured the intensity of AM1-44 in the same actin clusters from tPA- and control-treated neurons examined in Fig 4C. We found that the areas with low F-actin intensity in tPA-treated neurons corresponded to zones with increased AM1-44 fluorescence (Fig 5A), and further analysis indicated that the valleys of F-actin intensity in tPA-treated neurons (Fig 4C) correspond to peaks of high AM1-44 fluorescence (Fig 5B). Furthermore, when all values were analyzed together, the average intensity of AM1-44 fluorescence associated with F-actin clusters was significantly higher in tPA-treated neurons than in control cells (Fig 5C). In agreement with these observations, the number of AM1-44 puncta per actin cluster increased from 4.2 ± 0.34 in control cells to 7.30 ± 0.58 in tPA-treated neurons (Fig 5D). Because an increase in the number of AM1-44-containing SVs could be just the consequence of the enlargement of each actin cluster, we decided to obtain a ratio of AM1-44-positive puncta/length of actin cluster. Our data indicate that tPA increases both the size of each actin cluster and the number of AM1-44-containing SVs associated with them (Fig 5E).

3.0. Discussion

The serine proteinase tissue-type plasminogen activator (tPA) is abundantly expressed in neurons (Samson and Medcalf, 2006), and its rapid release from the presynaptic terminal promotes the development of synaptic plasticity (Qian et al., 1993, Seeds et al., 1995, Muller and Griesinger, 1998, Pawlak et al., 2003), neuronal survival (Echeverry et al., 2010, Haile et al., 2012, Wu et al., 2013a), adaptation to metabolic stress (Wu et al., 2012, Wu et al., 2013b), and neuroglial coupling (An et al., 2014). TPA is found in the presynaptic terminal of cerebral cortical neurons, either intermixed with clear-core SVs of the reserve and recycling pools, or in direct contact with the presynaptic membrane (Wu, 2015). Our earlier studies indicate that tPA has a direct effect on the presynaptic terminal, promoting the mobilization of SVs from the reserve and recycling pools to the AZ and the release of excitatory neurotransmitters into the synaptic cleft. These observations not only agree with subsequent studies by a different group of investigators reporting the expression of tPA in a well defined subset of pyramidal glutamatergic neurons (Louessard et al., 2015), but also suggest that in the presynaptic terminal of cerebral cortical neurons tPA plays a central role in the activation and modulation of the synaptic vesicle cycle. Together with our previous studies on the effect of tPA on the post-synaptic compartment (Wu et al., 2012, An et al., 2014), the data presented here suggest a role for tPA as an integrator of pre- and postsynaptic activity (Yepes, 2015).

Here we show that tPA-induced exocytosis of SVs does not lead to their depletion in the axonal bouton because tPA also triggers the endocytic retrieval from the presynaptic membrane of SVs that have already released their load of neurotransmitters into the synaptic cleft. The data presented here indicate that in cerebral cortical neurons tPA plays an essential role coupling SVs exocytosis to their endocytic retrieval, thus making possible the uninterrupted release of neurotransmitters during sustained neuronal activity.

The endocytosis of SVs is a highly plastic process that follows different pathways. In the brain the best characterized is clathrin-mediated endocytosis, whereby SVs are generated directly from clathrin-coated buds formed in the presynaptic membrane. Activation of a

second pathway, known as “bulk endocytosis”, is observed after a strong excitotoxic burst and induces the formation of SVs from endocytic vacuoles by an as yet unknown mechanism. The third pathway is called “kiss and run” and is characterized by rapid closure of a fusion pore without collapse of the vesicle (Saheki and De Camilli, 2012). Additionally, recent work identified a fourth ultrafast endocytic pathway that takes place at the edges of the AZ (Watanabe et al., 2013).

Clathrin-mediated endocytosis begins with a nucleation step in which a heterotetramer composed of α , β 2, μ 2, and σ 2 adaptins (AP2) recruits the dephosphin AP180 and clathrin to nucleation points near to the site of exocytosis leading to the formation of a complex that builds the vesicle coat. This is followed by an invagination stage in which the dephosphin amphiphysin links clathrin and the AP2 complex to the cytosolic phosphoprotein dynamin I, and a fission step in which dynamin I constricts and severs the neck of the invaginated vesicles, generating a new SV that rapidly loses its clathrin coat. The data presented here indicate that tPA has a direct effect on the fission step by inducing the dephosphorylation of dynamin I and its recruitment to the presynaptic membrane.

The family of dynamin-like proteins in mammals is composed of three isoforms with diverse expression patterns: dynamin I, found mostly in the presynaptic terminal, dynamin II, ubiquitously expressed, and dynamin III, detected in dendritic spines and testis (Urrutia et al., 1997). Dynamin I has four well defined domains: N-terminal GTPase, plekstrin homology (PH), GTPase effector, and C-terminal proline-rich domains (Smillie and Cousin, 2005). The phosphorylation status of dynamin I regulates its localization within the nerve terminal. Accordingly, the raise in intracellular Ca^{+2} induced by membrane depolarization leads to dynamin I dephosphorylation that then is recruited from the cytosol to the presynaptic membrane where it forms collars around the neck of the nascent clathrin-coated invaginations. Then its GTPase activity generates the mechanical force needed for SV fission. Our previous studies indicate that tPA induces the recruitment of voltage-gated calcium channels to the presynaptic terminal (Wu, 2015). Here we show that this leads to calcineurin-mediated dynamin I dephosphorylation. The functional significance of these findings is underscored by the demonstration that inhibition of either calcineurin or dynamin I abrogates tPA - induced SVs endocytosis. However, because it has been recently described that dynasore, the dynamin I inhibitor used in these studies, also disrupts lipid raft organization in the presynaptic membrane by a proposed effect on labile cholesterol (Preta et al., 2015), an alternative explanation for our data is that changes in the composition of cholesterol in the presynaptic membrane mediate the effect of tPA on SVs endocytosis. We believe that this possibility is highly unlikely given our finding that tPA induces calcineurin-mediated dynamin I dephosphorylation, which is the first step of dynamin I recruitment to the presynaptic membrane.

A substantial body of experimental evidence indicates a link between clathrin-mediated endocytosis and actin formation. Accordingly, studies with synapses of the giant axon of the lamprey have shown that actin perturbation in the presynaptic terminal leads to inhibition of SVs endocytosis (Shupliakov et al., 2002). In contrast, other groups have reported that pharmacological disruption of actin does not affect SVs in the mammalian synapse and, instead, that the main role of the actin cytoskeleton is to act as a scaffold for the return of the

endocytosed SVs to the recycling pool (Sankaranarayanan et al., 2003). In agreement with the latter observations, we found that tPA induces F-actin expression and its association with endocytosed SVs.

Profilin is an actin-binding protein that plays a key role in actin polymerization. It binds to actin monomers, acting as a nucleotide exchange factor and accelerating actin filament growth (Pantaloni and Carlier, 1993). Profilin II is abundantly expressed in the brain whereas profilin I is ubiquitously expressed in all tissues. Because dynamin I interacts with profilin (Witke et al., 1998) and localizes together with actin at actin-rich regions (McNiven et al., 2000), profilin II is the best candidate to link tPA-induced recruitment of dynamin I to the presynaptic membrane with tPA-induced F-actin formation. According to this model, by recruiting dynamin I to the presynaptic membrane tPA also induces profilin II-mediated F-actin assembly. This hypothesis is supported by our data indicating that tPA induces the recruitment of profilin II to the presynaptic membrane and that this effect, as well as tPA-induced F-actin formation, are abrogated by dynamin I inhibition.

4.0 Conclusions

Based on these observations and our earlier studies indicating that tPA induces SVs exocytosis (Wu, 2015), here we propose a model whereby the release of tPA from the presynaptic terminal of cerebral cortical neurons couples neuronal depolarization with the activation of the SV cycle. Accordingly, tPA-induced recruitment of VGCC to the presynaptic terminal not only activates SVs exocytosis, but also promotes the recovery of SVs from the presynaptic terminal and its return to replenish the pool of vesicles available for a new exocytotic cycle. These data indicate that tPA is an activator of the synaptic vesicle cycle in cerebral cortical neurons.

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Glossary

tPA	tissue-type plasminogen activator
AZ	active zone
SV	synaptic vesicles
NMDA	N-methyl-D-Aspartate

8.0. References

- An J, Haile WB, Wu F, Torre E, Yepes M. Tissue-type plasminogen activator mediates neuroglial coupling in the central nervous system. *Neuroscience*. 2014; 257:41–48. [PubMed: 24200922]
- An JJ, Gharami K, Liao GY, Woo NH, Lau AG, Vanevski F, Torre ER, Jones KR, Feng Y, Lu B, Xu B. Distinct role of long 3' UTR BDNF mRNA in spine morphology and synaptic plasticity in hippocampal neurons. *Cell*. 2008; 134:175–187. [PubMed: 18614020]

- Clayton EL, Evans GJ, Cousin MA. Activity-dependent control of bulk endocytosis by protein dephosphorylation in central nerve terminals. *The Journal of physiology*. 2007; 585:687–691. [PubMed: 17584836]
- Cousin MA, Robinson PJ. The dephosphins: dephosphorylation by calcineurin triggers synaptic vesicle endocytosis. *Trends in neurosciences*. 2001; 24:659–665. [PubMed: 11672811]
- Echeverry R, Wu J, Haile WB, Guzman J, Yepes M. Tissue-type plasminogen activator is a neuroprotectant in the mouse hippocampus. *JClinInvest*. 2010; 120:2194–2205.
- Ferguson SM, Brasnjo G, Hayashi M, Wolfel M, Collesi C, Giovedi S, Raimondi A, Gong LW, Ariel P, Paradise S, O'Toole E, Flavell R, Cremona O, Miesenbock G, Ryan TA, De Camilli P. A selective activity-dependent requirement for dynamin 1 in synaptic vesicle endocytosis. *Science*. 2007; 316:570–574. [PubMed: 17463283]
- Gareus R, Di Nardo A, Rybin V, Witke W. Mouse profilin 2 regulates endocytosis and competes with SH3 ligand binding to dynamin 1. *The Journal of biological chemistry*. 2006; 281:2803–2811. [PubMed: 16319076]
- Gundelfinger ED, Kessels MM, Qualmann B. Temporal and spatial coordination of exocytosis and endocytosis. *Nature reviews Molecular cell biology*. 2003; 4:127–139. [PubMed: 12563290]
- Haile WB, Wu J, Echeverry R, Wu F, An J, Yepes M. Tissue-type plasminogen activator has a neuroprotective effect in the ischemic brain mediated by neuronal TNF-alpha. *JCerebBlood Flow Metab*. 2012; 32:57–69.
- Haucke V, Neher E, Sigrist SJ. Protein scaffolds in the coupling of synaptic exocytosis and endocytosis. *Nature reviews Neuroscience*. 2011; 12:127–138. [PubMed: 21304549]
- Heuser JE, Reese TS. Evidence for recycling of synaptic vesicle membrane during transmitter release at the frog neuromuscular junction. *The Journal of cell biology*. 1973; 57:315–344. [PubMed: 4348786]
- Liu JP, Powell KA, Sudhof TC, Robinson PJ. Dynamin I is a Ca(2+)-sensitive phospholipid-binding protein with very high affinity for protein kinase C. *The Journal of biological chemistry*. 1994; 269:21043–21050. [PubMed: 8063723]
- Louessard M, Lacroix A, Martineau M, Mondielli G, Montagne A, Lesept F, Lambolez B, Cauli B, Mothet JP, Vivien D, Maubert E. Tissue Plasminogen Activator Expression Is Restricted to Subsets of Excitatory Pyramidal Glutamatergic Neurons. *Molecular neurobiology*. 2015
- Marks B, McMahon HT. Calcium triggers calcineurin-dependent synaptic vesicle recycling in mammalian nerve terminals. *Current biology : CB*. 1998; 8:740–749. [PubMed: 9651678]
- Marks B, Stowell MH, Vallis Y, Mills IG, Gibson A, Hopkins CR, McMahon HT. GTPase activity of dynamin and resulting conformation change are essential for endocytosis. *Nature*. 2001; 410:231–235. [PubMed: 11242086]
- McNiven MA, Cao H, Pitts KR, Yoon Y. The dynamin family of mechanoenzymes: pinching in new places. *Trends in biochemical sciences*. 2000; 25:115–120. [PubMed: 10694881]
- Muller CM, Griesinger CB. Tissue plasminogen activator mediates reverse occlusion plasticity in visual cortex. *NatNeurosci*. 1998; 1:47–53.
- Pantaloni D, Carlier MF. How profilin promotes actin filament assembly in the presence of thymosin beta 4. *Cell*. 1993; 75:1007–1014. [PubMed: 8252614]
- Pawlak R, Magarinos AM, Melchor J, McEwen B, Strickland S. Tissue plasminogen activator in the amygdala is critical for stress-induced anxiety-like behavior. *NatNeurosci*. 2003; 6:168–174.
- Pilo Boyl P, Di Nardo A, Mulle C, Sassoe-Pognetto M, Panzanelli P, Mele A, Kneussel M, Costantini V, Perlas E, Massimi M, Vara H, Giustetto M, Witke W. Profilin2 contributes to synaptic vesicle exocytosis, neuronal excitability, and novelty-seeking behavior. *The EMBO journal*. 2007; 26:2991–3002. [PubMed: 17541406]
- Pollard TD, Borisy GG. Cellular motility driven by assembly and disassembly of actin filaments. *Cell*. 2003; 112:453–465. [PubMed: 12600310]
- Preta G, Cronin JG, Sheldon IM. Dynasore - not just a dynamin inhibitor. *Cell communication and signaling : CCS*. 2015; 13:24. [PubMed: 25889964]
- Qian Z, Gilbert ME, Colicos MA, Kandel ER, Kuhl D. Tissue-plasminogen activator is induced as an immediate-early gene during seizure, kindling and long-term potentiation. *Nature*. 1993; 361:453–457. [PubMed: 8429885]

- Rao A, Steward O. Evidence that protein constituents of postsynaptic membrane specializations are locally synthesized: analysis of proteins synthesized within synaptosomes. *The Journal of neuroscience : the official journal of the Society for Neuroscience*. 1991; 11:2881–2895. [PubMed: 1880554]
- Robinson PJ, Liu JP, Powell KA, Fykse EM, Sudhof TC. Phosphorylation of dynamin I and synaptic-vesicle recycling. *Trends in neurosciences*. 1994; 17:348–353. [PubMed: 7526507]
- Saheki Y, De Camilli P. Synaptic vesicle endocytosis. *Cold Spring Harbor perspectives in biology*. 2012; 4:a005645. [PubMed: 22763746]
- Samson AL, Medcalf RL. Tissue-type plasminogen activator: a multifaceted modulator of neurotransmission and synaptic plasticity. *Neuron*. 2006; 50:673–678. [PubMed: 16731507]
- Sankaranarayanan S, Atluri PP, Ryan TA. Actin has a molecular scaffolding, not propulsive, role in presynaptic function. *Nature neuroscience*. 2003; 6:127–135. [PubMed: 12536209]
- Seeds NW, Williams BL, Bickford PC. Tissue plasminogen activator induction in Purkinje neurons after cerebellar motor learning. *Science*. 1995; 270:1992–1994. [PubMed: 8533091]
- Shupliakov O, Bloom O, Gustafsson JS, Kjaerulff O, Low P, Tomilin N, Pieribone VA, Greengard P, Brodin L. Impaired recycling of synaptic vesicles after acute perturbation of the presynaptic actin cytoskeleton. *Proceedings of the National Academy of Sciences of the United States of America*. 2002; 99:14476–14481. [PubMed: 12381791]
- Smillie KJ, Cousin MA. Dynamin I phosphorylation and the control of synaptic vesicle endocytosis. *Biochemical Society symposium*. 2005:87–97. [PubMed: 15649133]
- Urrutia R, Henley JR, Cook T, McNiven MA. The dynamins: redundant or distinct functions for an expanding family of related GTPases? *Proceedings of the National Academy of Sciences of the United States of America*. 1997; 94:377–384. [PubMed: 9012790]
- Watanabe S, Rost BR, Camacho-Perez M, Davis MW, Sohl-Kielczynski B, Rosenmund C, Jorgensen EM. Ultrafast endocytosis at mouse hippocampal synapses. *Nature*. 2013; 504:242–247. [PubMed: 24305055]
- Weingarten J, Lassek M, Mueller BF, Rohmer M, Lunger I, Baeumlisberger D, Dudek S, Gogesch P, Karas M, Volkandt W. The proteome of the presynaptic active zone from mouse brain. *Molecular and cellular neurosciences*. 2014; 59:106–118. [PubMed: 24534009]
- Wilhelm BG, Mandad S, Truckenbrodt S, Krohnert K, Schafer C, Rammner B, Koo SJ, Classen GA, Krauss M, Haucke V, Urlaub H, Rizzoli SO. Composition of isolated synaptic boutons reveals the amounts of vesicle trafficking proteins. *Science*. 2014; 344:1023–1028. [PubMed: 24876496]
- Witte W, Podtelejnikov AV, Di Nardo A, Sutherland JD, Gurniak CB, Dotti C, Mann M. In mouse brain profilin I and profilin II associate with regulators of the endocytic pathway and actin assembly. *The EMBO journal*. 1998; 17:967–976. [PubMed: 9463375]
- Wu F, Echeverry R, Wu J, An J, Haile WB, Cooper DS, Catano M, Yepes M. Tissue-type plasminogen activator protects neurons from excitotoxin-induced cell death via activation of the ERK1/2-CREB-ATF3 signaling pathway. *Molecular and cellular neurosciences*. 2013a; 52:9–19. [PubMed: 23063501]
- Wu F, Nicholson AD, Haile WB, Torre E, An J, Chen C, Lee AK, Duong DM, Dammer EB, Seyfried NT, Tong FC, Votaw JR, Yepes M. Tissue-type plasminogen activator mediates neuronal detection and adaptation to metabolic stress. *Journal of cerebral blood flow and metabolism : official journal of the International Society of Cerebral Blood Flow and Metabolism*. 2013b; 33:1761–1769.
- Wu F, Torre E, Cuellar-Giraldo D, Cheng L, Yi H, Bichler EK, Garcia PS, Yepes M. Tissue-Type Plasminogen Activator Triggers the Synaptic Vesicle Cycle in Cerebral Cortical Neurons. *J Cereb Blood Flow Metab* 2015. 2015 Dec; 35(12):1966–76.
- Wu F, Wu J, Nicholson AD, Echeverry R, Haile WB, Catano M, An J, Lee AK, Duong D, Dammer EB, Seyfried NT, Tong FC, Votaw JR, Medcalf RL, Yepes M. Tissue-type plasminogen activator regulates the neuronal uptake of glucose in the ischemic brain. *The Journal of neuroscience : the official journal of the Society for Neuroscience*. 2012; 32:9848–9858. [PubMed: 22815500]
- Yepes M. Tissue-type plasminogen activator is a neuroprotectant in the central nervous system. *Frontiers in cellular neuroscience*. 2015; 9:304. [PubMed: 26347605]

Yepes M, Sandkvist M, Moore EG, Bugge TH, Strickland DK, Lawrence DA. Tissue-type plasminogen activator induces opening of the blood-brain barrier via the LDL receptor-related protein. *JClinInvest*. 2003; 112:1533–1540.

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Highlights

- The effect of tPA on synaptic vesicle (SV) endocytosis was examined.
- TPA induces SV endocytosis in cerebral cortical neurons by a mechanism that does not require the conversion of plasminogen into plasmin
- TPA induces dephosphorylation and membrane recruitment of dynamin I.
- TPA promotes F-actin formation and the association of newly formed SVs with F-actin clusters in the endocytic zone.

Figure 1. tPA induces synaptic vesicle endocytosis

A. Representative micrographs of continuous confocal live-cell imaging monitoring of AM1-44 uptake by a presynaptic terminal of a Wt cerebral cortical neuron following 0, 1 and 2 minutes of treatment with 5 nM of tPA. Arrows in b & c point to an area (red) of AM1-44 uptake. Panel d corresponds to an electronic magnification of the area indicated in panel c. Magnification 60 X. **B.** Mean baseline uptake of AM1-44 in 375 presynaptic terminals located in the distal 50 μ m of axons from 20 Wt cerebral cortical neurons. Lines denote SEM. *, **, and ***: $p < 0.0001$ compared to AM1-44 uptake after 0 and 5 minutes of incubation (two-tailed unpaired t -test). Ns: non significant. **C.** Mean AM1-44 uptake following 0 – 5 minutes of treatment with 5 nM of tPA in the 375 presynaptic terminals examined under baseline conditions in panel B. Values correspond to the difference between AM1-44 intensity in each examined presynaptic terminal at each time point minus background AM1-44 intensity in the same terminal before treatment with tPA. Lines denote SEM. *: $p < 0.001$ when intensity at each time-point is compared to its own background intensity (two-tailed unpaired t test). **D.** Mean AM1-44 loading into 300 presynaptic

terminals from 15 Wt cerebral cortical neurons treated during 5 minutes with 5 nM of proteolytically active (atPA) or inactive tPA (itPA). Values correspond to the difference between AM1-44 intensity after 5 minutes of treatment with tPA minus the average background intensity before the beginning of the experiment. Lines denote SEM. *: $p = 0.002$ and **: $p = 0.004$ compared to baseline intensity for each group of cells (two-tailed unpaired t test). Ns: non-significant when mean AM1-44 loading by SVs of neurons treated with atPA was compared to loading by SVs of neurons treated with itPA. **E.** Quantification of synaptophysin (SYP) - positive puncta in the distal 50 μm of axons of Wt cerebral cortical neurons treated 0 – 60 seconds with 5 nM of tPA. $n = 75$ axons examined per time point. Ns: non-significant (one-way ANOVA). Lines denote SEM. **F.** Representative micrographs of SYP expression (red in b & d) and AM1-44 uptake (white in a & c and green/yellow in b & d) in a distal axon of Wt cerebral cortical neurons following 60 seconds of incubation with vehicle (control; a & b) or 5 nM of tPA (c & d). Magnification 40 X. **G.** Number of SYP/AM1-44- positive puncta in the distal 50 μm of axons from Wt cerebral cortical neurons treated 0 – 60 seconds with either 5 nM of tPA or a comparable volume of vehicle (control). * $p = 0.004$, ** $p < 0.001$ and *** $p < 0.0001$ compared to neurons incubated with vehicle (control) during 5, 15 or 60 seconds, respectively (one-way ANOVA). $n = 75$ axons examined per time-point. Lines denote SEM. **H & I.** Mean number of SY-AM1-44-positive puncta in the distal segment of 50 axons of Wt cerebral cortical neurons incubated during 60 seconds with 0 – 10 nM of tPA (H) or 60 seconds with either 5 nM of tPA, or a combination of tPA and 100 nM of RAP, or with RAP alone (I). Lines denote SEM. Ns: non-significant.

Figure 2. Calcineurin mediates tPA-induced synaptic vesicle endocytosis
A. Representative micrographs of synaptophysin (SYP) expression (red in b, d, f & h) and AM1-44 uptake (white in a, c, e & g; green/yellow in b, d, f & h) in the distal axon of Wt cerebral cortical neurons treated during 60 seconds with either vehicle (control; a & b), or 5 nM of tPA (c & d), or a combination of 5 nM of tPA and 0.08 μM of cyclosporine A (CsA; e & f), or 0.08 μM of CsA alone (g & h). **B.** Quantification of SYP/AM1-44 - positive puncta in the distal 50 μm of axons from Wt cerebral neurons treated 60 seconds with 5 nM of tPA, alone or in combination with 0.08 μM of CsA. Lines denote SEM. *: $p < 0.001$ compared to untreated cells. **: $p < 0.001$ compared to neurons treated with tPA in absence of CsA (one-way ANOVA). $n = 250$ presynaptic terminals from 20 different neurons examined per condition.

Figure 3. TPA induces endocytosis of synaptic vesicles via dynamin I dephosphorylation

A. Representative micrographs of dynamin I phosphorylated at Ser 774 (p-Dyn) in the distal axon of Wt cerebral cortical neurons treated with vehicle (control) or 5 nM of tPA. **B – E.** Representative Western blot analysis of p-Dyn in cytosolic extracts from synaptoneurosomes from Wt cerebral cortical neurons treated 0 – 60 seconds with 5 nM of tPA (**B** & **C**), or 60 seconds with tPA either alone or in combination 0.08 μM of cyclosporine A (CsA; **D** & **E**). * in **C**: $p < 0.001$ compared to untreated cells. * in **E** $p < 0.001$ compared to cells incubated in the absence of tPA and CsA. **F.** Quantification of SYP/AM1-44 - positive puncta in the distal 50 μm of axons from Wt cerebral cortical neurons treated during 60 seconds with 5 nM of tPA, alone ($n = 265$ presynaptic terminals) or in combination with 25 μM of the dynamin inhibitor dynasore ($n = 250$ presynaptic terminals), or with dynasore alone ($n = 230$ presynaptic terminals). Lines denote SEM. * $p < 0.001$ compared to untreated cells and with cells treated with tPA in the presence of dynasore (one-way ANOVA).

Figure 4. Effect of tPA on the cytoskeleton of the endocytic zone

A & B. Representative Western blot analysis of profilin II expression in membrane extracts from synaptoneurosomes prepared from Wt cerebral cortical neurons treated 0 – 60 seconds with 5 nM of tPA, alone or in combination with 25 μ M of the dynamin inhibitor dynasore. * $p < 0.0001$ compared to other experimental groups (one-way ANOVA). **C.** Representative heat maps of F-actin fluorescence in the distal axons of Wt cerebral cortical neurons treated 60 seconds with vehicle (control; a) or 5 nM of tPA (c). b & d correspond to electronic magnifications of the areas denoted by the arrows in a & c, respectively. **D & E.** Mean intensity of phalloidin fluorescence measured every 0.02 μ m in optically magnified and straightened F-actin clusters from Wt cerebral cortical neurons treated during 60 seconds with 5 nM of tPA (red; $n = 443$ clusters) or vehicle (control; blue; $n = 373$ clusters). Data were pooled together to obtain an average F-Actin fluorescence intensity in each experimental group (D). Ns: non- significant. Lines denote SEM. **F.** Mean length of the F-actin clusters examined in C. A sub-group of neurons was treated with tPA in combination with 25 μ M of the dynamin inhibitor dynasore ($n = 280$ clusters examined). Lines denote SEM. * $p = 0.01$ compared to untreated cells and 0.003 compared to neurons treated with a combination of tPA and dynasore (one-way ANOVA).