Tissue-type plasminogen activator is a modulator of the synaptic vesicle cycle

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The presynaptic terminal of chemical synapses neurotransmitters are stored in synaptic vesicles of ~40 nm of diameter that participate in a cycle that permits their repeated use during sustained synaptic activity. Synaptic vesicles are distributed in three groups known as readily releasable, recycling, and reserve pools (Rizzoli and Betz, 2004). The synaptic vesicles of the readily releasable pool are docked to an electron dense thickening in the presynaptic membrane, known as the active zone, where exocytosis takes place. In contrast, synaptic vesicles from the recycling and reserve pools are not docked to the active zone and release their load of neurotransmitters through translational processes that are not dependent on the active zone. The readily releasable pool is rapidly depleted following membrane depolarization. Hence, the mobilization of synaptic vesicles from the recycling and reserve pools to replenish the readily releasable pool is of pivotal importance to maintain neurotransmitter release during sustained synaptic activity.

To study the expression of tPA in the presynaptic terminal, we prepared synaptoneurosomes from wild-type cerebral cortical neurons and subjected them to sucrose density fractionation to isolate the synapse assembled by the presynaptic membrane, synaptic vesicles docked to the active zone, and the attached post-synaptic density of the post-synaptic membrane. Then each gradient fraction was immunoblotted with antibodies against tPA, synaptophysin (an integral transmembrane protein found in synaptic vesicles), syntaxin 1 (a transmembrane protein found in the presynaptic plasma membrane), and post-synaptic protein-95 (detects the post-synaptic density). Our data indicate that in the presynaptic terminal tPA is stored outside the active zone and in line with these observations, electron microscopy studies detected tPA-containing vesicles either intermixed with small clear-core vesicles, or in direct contact with the presynaptic membrane, but always outside the active zone. This finding is of significant importance because it indicates that tPA is released at extra-synaptic sites and suggest that as it has been described for presynaptic neuromodulators released outside the active zone, tPA may also be able to regulate synaptic function in a large number of neurons, even those located at a long-distance within the brain. More importantly, because the presynaptic release of these neuromodulators is proportional to the magnitude of the stimulus, it is plausible to postulate that the presynaptic release of tPA is a mechanism that matches the intensity of the depolarizing stimulus with a proportional release of excitatory neurotransmitters.

To study whether the release of tPA has an effect on the presynaptic terminal we performed mass spectrometry in preparations from the active zone isolated from wild-type cerebral cortical neurons previously treated with tPA. Interestingly, we found that tPA increases the abundance of the cytoskeletal protein βII-spectrin in the active zone. Additionally, our Western blot analyses not only confirmed these observations but also indicated that this effect does not require tPA's ability to catalyze the conversion of plasminogen into plasmin.

Spectrin, a cytoskeletal protein critical for membrane structural integrity, is a heterotrimer composed of two α and two β subunits (Bennett and Lambert, 1991). It was first discovered in erythrocytes where it plays a pivotal role maintaining the shape and flexibility of the cell membrane by forming complexes with actin and other intracellular proteins. Subsequent studies indicated that one member of the spectrin family, βII-spectrin, is found in the presynaptic terminal (Phillips et al., 2001) where it plays an important role in synaptic transmission (Sikorski et al., 2000). Our studies indicate that tPA induces the recruitment of βII-spectrin to the active zone of cerebral cortical neurons, and that by doing so it also increases the size of the synaptic release site.

Synapsin I is a member of the synapsin family of phosphoproteins that plays a central role in clustering synaptic vesicles of the reserve pool. Accordingly, synapsin I is associated with the cytoplasmic surface of synaptic vesicles where it serves as a linker with other synaptic vesicles and the actin cytoskeleton of the presynaptic terminal. However, following membrane depolarization synapsin I is phosphorylated in a Ca^{2+}-dependent manner, leading to its dissociation from synaptic vesicles, which are then free to translocate to the active zone to release their content of neurotransmitters into the synaptic cleft (Chi et al., 2001). βII-spectrin has a synapsin I-binding site (Sikorski et al., 1991); therefore it is plausible to postulate that tPA not only induces the recruitment of βII-spectrin to the active zone but also promotes its binding to synapsin I-expressing synaptic vesicles. This hypothesis was corroborated by our immunoprecipitation studies with synapse-enriched fractions from cerebral cortical neurons treated with tPA. In summary, our data suggest a model where the presynaptic release of tPA induces the recruitment of βII-spectrin to the active zone and by doing so not only enlarges its size but also promotes βII-spectrin binding to synaptic vesicles.
Glutamate (pink circles)-containing synaptic vesicles are distributed in three groups known as readily releasable (light yellow ovals), recycling (not depicted in the figure), and reserve (dark yellow ovals) pools. The synaptic vesicles of the readily releasable pool are docked to an electron dense thickening of the presynaptic membrane, known as the active zone (orange triangles), where exocytosis of glutamate takes place. In contrast, synaptic vesicles from de reserve pool are clustered away from the active zone by homodimers of synapsin I (pink lines). (B) The release of tPA (red circles) from the presynaptic terminal leads to calcium-mediated phosphorylation of synapsin I at serine 9 releasing it from its binding to the surface of presynaptic calcium channels (green triangles), where exocytosis of glutamate takes place. (C) The resultant influx of calcium into the presynaptic terminal leads to calcium-mediated phosphorylation of synapsin I at serine 9 releasing it from its binding to the surface of presynaptic calcium channels (green triangles). (C) The resultant influx of calcium into the presynaptic terminal leads to calcium-mediated phosphorylation of synapsin I at serine 9 releasing it from its binding to the surface of presynaptic calcium channels (green triangles).

**Figure 1** Tissue-type plasminogen activator activates the synaptic vesicle cycle.

(A) Glutamate (pink circles)-containing synaptic vesicles are distributed in three groups known as readily releasable (light yellow ovals), recycling (not depicted in the figure), and reserve (dark yellow ovals) pools. The synaptic vesicles of the readily releasable pool are docked to an electron dense thickening of the presynaptic membrane, known as the active zone (orange triangles), where exocytosis of glutamate takes place. In contrast, synaptic vesicles from de reserve pool are clustered away from the active zone by homodimers of synapsin I (pink lines). (B) The release of tPA (red circles) following membrane depolarization leads to recruitment of the cytoskeletal protein βII-spectrin to the active zone, enlarging its size and population of presynaptic calcium channels (green triangles). (C) The resultant influx of calcium into the presynaptic terminal leads to calcium-mediated phosphorylation of synapsin I at serine 9 releasing it from its binding to the surface of synaptic vesicles of the reserve pool, which are free to translocate to the active zone to release their load of glutamate.

Under resting conditions synapsin I clusters synaptic vesicles and tethers them to the cytoskeleton. However, during synaptic activity synapsin I phosphorylation at Serine 9 leads to its dissociation from synaptic vesicles of the reserve pool freeing them to move to the active zone. Interestingly, phosphorylation of the enzyme glycogen synthase kinase-3β at Serine 9 reduces its activity (Grimes and Jope, 2001) and this has been proposed to induce the clustering of synapsin I in the presynaptic terminal and the development of synaptic plasticity (Zhu et al., 2007). Data from our laboratory indicate that tPA induces the rapid phosphorylation of synapsin I at Serine 9 and our electrophysiology studies demonstrate that this is followed by an increase in the frequency of miniature excitatory postsynaptic currents, a postsynaptic marker of the quantal release of neurotransmitters from the presynaptic terminal.

In summary, the available data indicate that tPA plays a central role as a regulator of synaptic function. According to this model, the release of tPA from cerebral cortical neurons has a neuromodulatory effect on presynaptic function by inducing the anatomical and biochemical changes in the synaptic release site required for the release of excitatory neurotransmitters (Figure 1). Further studies are needed to understand whether this effect requires a receptor for tPA on the surface of the presynaptic terminal.

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