Urokinase-type plasminogen activator (uPA) regulates the expression and function of growth-associated protein 43 (GAP-43) in the synapse

Received for publication, August 13, 2019, and in revised form, November 19, 2019 Published, Papers in Press, December 9, 2019, DOI 10.1074/jbc.RA119.010644

Paola Merino[‡], **Ariel Diaz**[‡], **Enrique R. Torre**[‡], **and Manuel Yepes**[‡§¶1]

From the [‡]*Division of Neuropharmacology and Neurologic Diseases, Yerkes National Primate Research Center, Atlanta, Georgia 30329-4208, the* [§]*Department of Neurology and Center for Neurodegenerative Disease, Emory University School of Medicine, Atlanta, Georgia 30322-0001, and the* [¶]*Department of Neurology, Veterans Affairs Medical Center, Atlanta, Georgia 30033-4004*

Edited by Enrique M. De La Cruz

Growth-associated protein 43 (GAP-43) plays a central role in the formation of presynaptic terminals, synaptic plasticity, and axonal growth and regeneration. During development, GAP-43 is found in axonal extensions of most neurons. In contrast, in the mature brain, its expression is restricted to a few presynaptic terminals and scattered axonal growth cones. Urokinase-type plasminogen activator (uPA) is a serine proteinase that, upon binding to its receptor (uPAR), catalyzes the conversion of plasminogen into plasmin and activates signaling pathways that promote cell migration, proliferation, and survival. In the developing brain, uPA induces neuritogenesis and neuronal migration. In contrast, the expression and function of uPA in the mature brain are poorly understood. However, recent evidence reveals that different forms of injury induce release of uPA and expression of uPAR in neurons and that uPA/uPAR binding triggers axonal growth and synapse formation. Here we show that binding of uPA to uPAR induces not only the mobilization of GAP-43 from the axonal shaft to the presynaptic terminal but also its activation in the axonal bouton by PKC-induced calcium-dependent phosphorylation at Ser-41 (pGAP-43). We found that this effect requires open presynaptic *N*-methyl-D-aspartate receptors but not plasmin generation. Furthermore, our work reveals that, following its activation by uPA/uPAR binding, pGAP-43 colocalizes with presynaptic vesicles and triggers their mobilization to the synaptic release site. Together, these data reveal a novel role of uPA as an activator of the synaptic vesicle cycle in cerebral cortical neurons via its ability to induce presynaptic recruitment and activation of GAP-43.

GAP-43 plays a central role in the development of reactive synaptogenesis (1), growth of presynaptic terminals (2), axonal formation and regeneration (3, 4), and induction of synaptic plasticity (5). It is expressed in neurons (4), and its abundance varies according to developmental stage. Although, during the early phases of development, GAP-43 is detected in axonal extensions of most neurons (6), in the mature brain, its expression is restricted to the presynaptic terminal of neurons of highly plastic areas such as the neocortex, hippocampus, and cerebellum (7). In contrast, multiple studies have failed to detect it in dendrites and myelinated axons (8).

During development, the expression of GAP-43 is regulated by transcriptional processes and *N*-methyl-D-aspartate (NMDA)[2] receptor–mediated posttranscriptional mechanisms (9). In contrast, in mature neurons, its abundance is controlled by mRNA stabilization by the neuron-specific RNA-binding protein HuD (10). In line with these observations, the abundance of HuD increases during synaptic plasticity and axon formation and regeneration (11, 12).

GAP-43 palmitoylation at Cys-3 and Cys-6 in the endoplasmic reticulum–Golgi intermediate compartment triggers its binding to the cytoplasmic side of the plasma membrane (13), which, in turn, is followed by its activation by PKC-β–induced phosphorylation at Ser-41 (14). This process is regulated by calmodulin. Accordingly, when the concentration of calcium is low, calmodulin binds to GAP-43 and inhibits its phosphorylation (15). In contrast, when the concentration of calcium increases during neuronal activity, calmodulin dissociates from GAP-43 (16). Furthermore, GAP-43 expression and activation can be elicited by external stimuli such as treatment with nerve growth factor and neural cell adhesion molecule–mediated fibroblast growth factor receptor activation (17).

The presynaptic terminal harbors neurotransmitter-filled synaptic vesicles grouped in three pools: the recycling pool, the reserve pool, and the readily releasable pool. However, although the vesicles of the first two are clustered and tethered to the cytoskeleton by synapsin I (18), those of the readily releasable pool are directly docked to the active zone (AZ) and available for immediate release (19). The synaptic vesicle cycle is a carefully orchestrated sequence of events whereby phos-

This work was supported in part by NINDS, National Institutes of Health Grant NS-NS091201 (to M. Y.), VA MERIT Award IO1BX003441 (to M. Y.), and American Heart Association Postdoctoral Fellowship Grant 19POST-34380009 (to A. D.). The authors declare that they have no conflicts of interest with the contents of this article. The content is solely the responsibility of the authors and does not necessarily represent the official views of the National Institutes of Health.

[1] To whom correspondence should be addressed: Division of Neuropharmacology and Neurologic Diseases, Yerkes National Primate Research Center, 954 Gatewood Rd. NE, Atlanta, GA 30329-4208. Tel.: 404-712-8358; Fax: 404-727-8070; E-mail: myepes@emory.edu.

[2] The abbreviations used are: NMDA, *N*-methyl-D-aspartate; AZ, active zone; uPA, urokinase-type plasminogen activator; uPAR, urokinase-type plasminogen activator receptor; CiliD, ciliobrevin D; NMDAR, *N*-methyl-D-aspartate receptor; NLA, neurosphere-like aggregate; SYP, synaptophysin; ERK, extracellular signal-regulated kinase; E16, embryonic day 16; ANOVA, analysis of variance; ATF, N-terminal fragment.

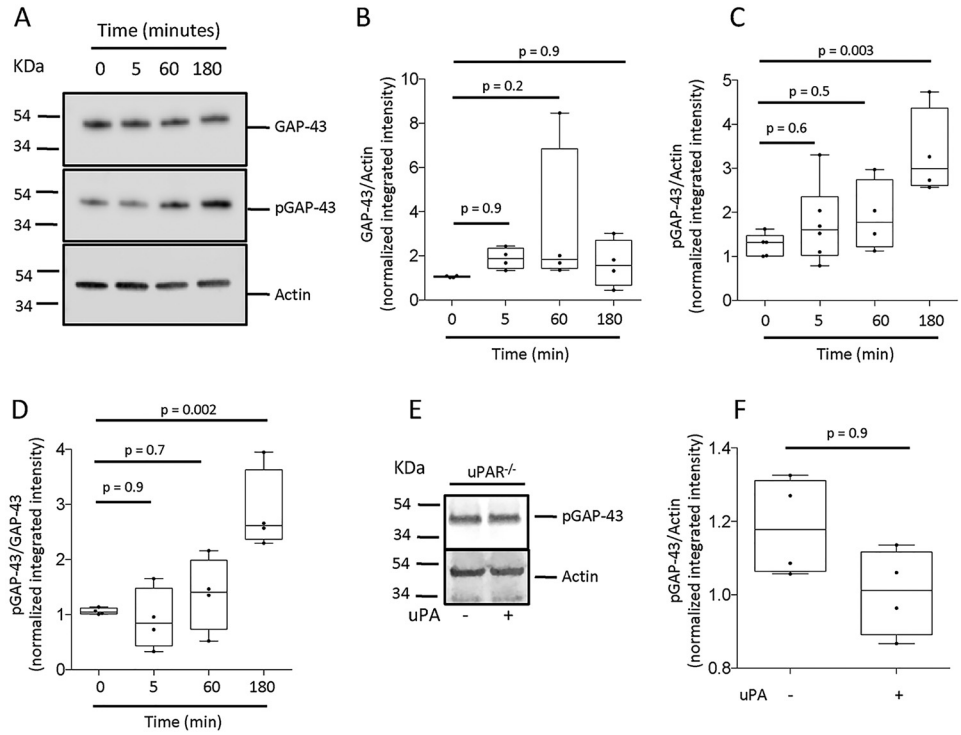

Figure 1. Effect of uPA on GAP-43 in cerebral cortical neurons. *A–D*, representative Western blot analysis (*A*) and normalized integrated intensity of the band (*B–D*) of total GAP-43 and pGAP-43 in WT cerebral cortical neurons incubated for 0–180 min with 5 nM uPA. $n = 4$/experimental condition; one-way ANOVA with Tukey's multiple comparisons test. *E* and *F*, representative Western blot analysis (*E*) and normalized integrated intensity of the band (*F*) of pGAP-43 expression in cerebral cortical neurons genetically deficient in uPAR ($uPAR^{-/-}$) treated for 3 h with 5 nM uPA or vehicle (control). $n = 4$/experimental condition; two-tailed Student's *t* test.

phorylation of synapsin I at serine 9 (pSyn-9) frees the clusters of vesicles of the reserve and recycling pools, allowing them to move to the AZ (20). When these vesicles release their content into the synaptic cleft, their membranes are reconstituted into new synaptic vesicles that are endocytosed to replenish the recycling and reserve pools. Importantly, this sequence of events is activated by phosphorylation of GAP-43 (1)

Urokinase-type plasminogen activator (uPA) is a serine proteinase that, upon binding to its receptor (uPAR), is cleaved by plasmin and other proteases to generate an active two-chain form that catalyzes the conversion of plasminogen into plasmin (21, 22) and activates cell signaling pathways by plasminogen-dependent and -independent mechanisms (23). In the developing brain, uPA is abundantly detected in neurons and oligodendrocytes (24). In contrast, its expression and function in the mature brain are poorly understood. However, recent experimental evidence indicates that the expression of uPAR increases in growth cones after axonal injury and that uPA/uPAR binding induces axonal recovery following mechanical injury *in vitro* and ischemic stroke *in vivo* (25, 26) and promotes the formation of new synaptic contacts and repair of those damaged by ischemic injury (27, 28).

In this study, we show that uPA binding to uPAR induces mobilization of GAP-43 from the axonal shaft to the presynaptic terminal and that, when in the axonal bouton, uPA triggers its activation by PKC-induced calcium-mediated phosphorylation at Ser-41. Our data indicate that the effect of uPA on GAP-43 does not require plasmin generation but, instead, that is mediated by open presynaptic NMDA receptors. Furthermore, our results reveal that, following its activation by uPA treatment, pGAP-43 colocalizes with presynaptic vesicles and that this is followed by synapsin I phosphorylation at Ser-9 with resultant mobilization of synaptic vesicles from the reserve and recycling pools to the synaptic release site. This study reveals a novel role of uPA in the brain as an activator of the synaptic vesicle cycle in cerebral cortical neurons via its ability to trigger mobilization and activation of GAP-43 in the presynaptic terminal.

Results

Effect of uPA on GAP-43 expression in cerebral cortical neurons

GAP-43 is crucial for axonal growth (29), formation of new synaptic contacts (30), and neuronal repair (31). Because our earlier studies indicate that uPA plays a central role in these events (25, 26, 28), we decided to study the expression of GAP-43 in whole-cell extracts prepared from WT cerebral cortical neurons incubated for 0–180 min with 5 nM uPA. Our data indicate that uPA does not cause a significant increase in the abundance of GAP-43 (Fig. 1, *A* and *B*). Because phosphorylation of GAP-43 at serine 41 (pGAP-43) leads to its activation (32), we performed similar experiments with antibodies that detect pGAP-43. We found that the abundance of pGAP-43 increased 3 h after treatment with uPA (Fig. 1, *A*, *C*, and *D*). Notably, uPA failed to increase the abundance of pGAP-43 in $uPAR^{-/-}$ cerebral cortical neurons (Fig. 1, *E* and *F*).

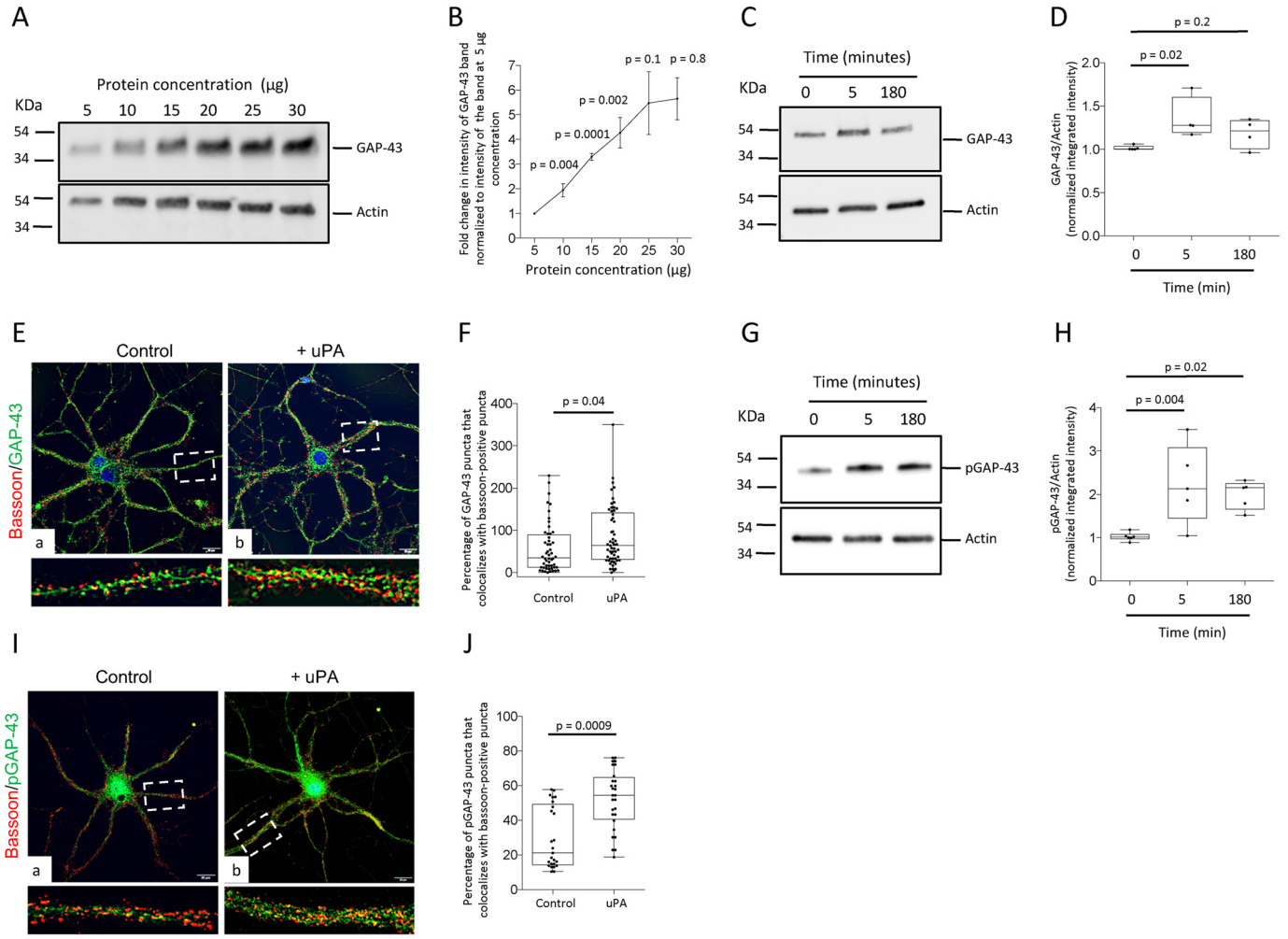

Figure 2. Effect of uPA on synaptic GAP-43. *A*, representative Western blot analysis of GAP-43 and actin expression in 5–30 μg of extracts prepared from synaptoneurosomes from WT cerebral cortical neurons. *B*, -fold change of the intensity of the band of GAP-43 expression at 10–30 μg protein concentration in relation to the intensity of the band at 5 μg concentration. Each observation was repeated four times per experimental point. *p* values were calculated with one-tailed Student's *t* test, comparing pairs of samples with consecutive concentrations (*i.e.* 10 μg *versus* 5 μg, 15 μg *versus* 10 μg, 20 μg *versus* 15 μg, 25 μg *versus* 20 μg, and 30 μg *versus* 25 μg). *C* and *D*, representative Western blot analysis (*C*) and normalized integrated intensity of the band (*D*) of GAP-43 expression in synaptoneurosomes prepared from WT cerebral cortical neurons after 0–180 min of treatment with 5 nM uPA. $n = 4$/experimental condition; one-way ANOVA with Dunnett's multiple comparisons test. *E*, representative confocal micrographs at ×60 magnification of bassoon (*red*) and GAP-43 (*green*) expression in WT cerebral cortical neurons treated for 5 min with 5 nM uPA or vehicle (control). *Bottom panels*, ×4 electronic magnification of the areas in the *dashed squares* in *a* and *b*. *F*, percentage of total GAP-43–positive puncta that colocalized with bassoon in WT cerebral cortical neurons treated as described in *C*. $n = 80$ extensions examined from four different neuronal cultures; two-tailed Student's *t* test. *G* and *H*, representative Western blot analysis (*G*) and normalized integrated intensity of the band (*H*) of pGAP-43 expression in synaptoneurosomes prepared from WT cerebral cortical neurons treated for 0–180 min with 5 nM uPA. $n = 5$–6 observations/experimental condition; one-way ANOVA with Dunnett's multiple comparisons test. *I*, representative ×60 confocal micrographs of bassoon and p-GAP-43 expression in WT cerebral cortical neurons treated as described in *E* and *F*. *Bottom panels*, ×4 electronic magnification of the areas depicted in the *dashed squares* in *a* and *b*. *J*, percentage of total pGAP-43–positive puncta that colocalized with bassoon in WT cerebral cortical neurons treated as described in *E* and *F*. $n = 60$ extensions examined from four different neuronal cultures; two-tailed Student's *t* test.

uPA increases the synaptic abundance of GAP-43 and pGAP-43 by a uPAR-dependent, plasminogen-independent mechanism

Because GAP-43 plays a central role in synaptic function (33), we decided to study its expression in synaptoneurosomes (assembled by the sealed axonal bouton and attached postsynaptic terminal) prepared from WT cerebral cortical neurons incubated for 0–180 min with 5 nM uPA. However, to assess the linearity of our observations, we first quantified the intensity of the band of GAP-43 expression in immunoblots loaded with 5–30 μg of synaptosomal protein. Our studies showed a linear relation between the protein concentration used in our studies (10 μg) and the intensity of the band of our inmmunoblots (Fig. 2, *A* and *B*). We found that, with this experimental design, uPA induced a rapid (5 min) but transient increase in the abundance of GAP-43 in the synapse (Fig. 2, *C* and *D*). These observations were corroborated by our confocal microscopy studies revealing that 5 min of uPA treatment induced colocalization of GAP-43 with the presynaptic marker bassoon (Fig. 2, *E* and *F*). We then performed similar experiments with anti-pGAP-43 antibodies. Our immunoblotting and confocal microscopy studies indicated that uPA induced a rapid and sustained increase in the abundance of pGAP-43 in the presynaptic terminal (Fig. 2, *G–J*). Furthermore, we found that this effect was independent of uPA's proteolytic activity, as it was also observed following treatment of WT neurons with uPA's

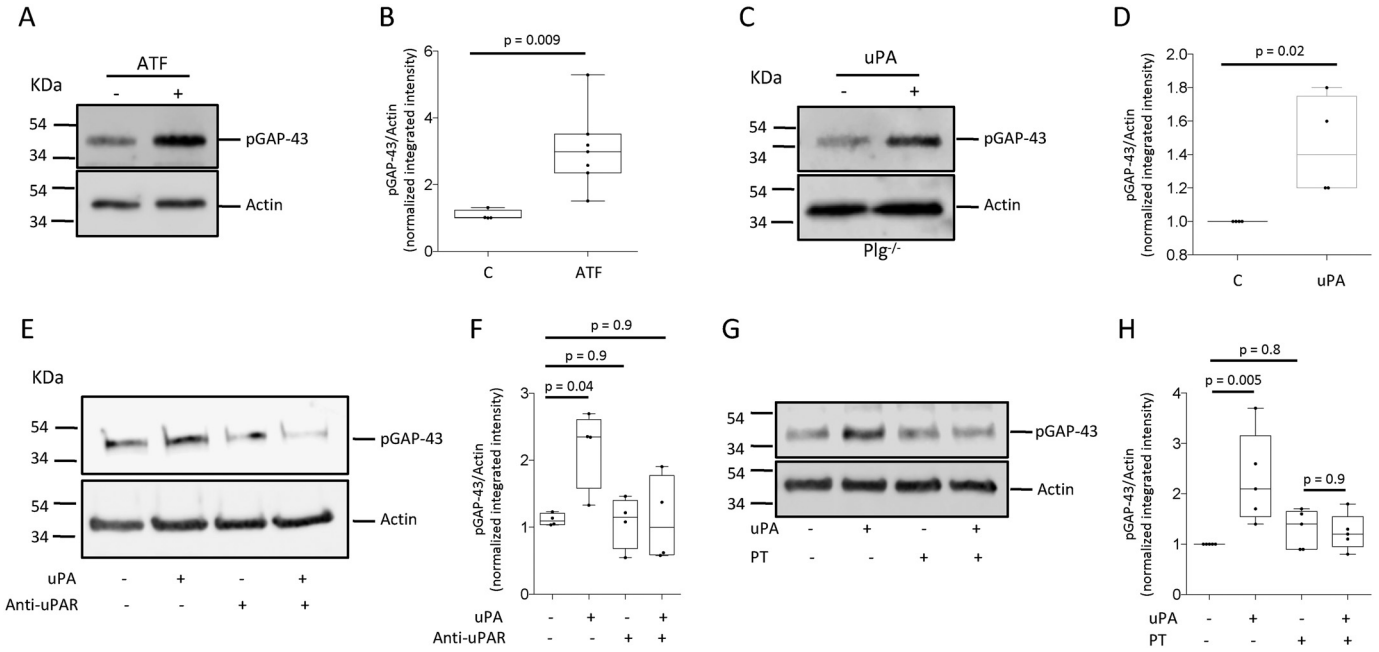

Figure 3. uPA induces GAP-43 phosphorylation by a uPAR/FPR2-mediated plasminogen-independent mechanism. *A* and *B*, representative Western blot analysis (*A*) and normalized integrated intensity of the band (*B*) of pGAP-43 expression in synaptoneurosomes from WT cerebral cortical neurons treated for 5 min with 5 nM uPA's N-terminal fragment (*ATF*; devoid of proteolytic activity) or vehicle (*C*, control). $n = 4$ observations/group; two-tailed Student's *t* test. *C* and *D*, representative Western blot analysis (*C*) and normalized integrated intensity of the band (*D*) of pGAP-43 expression in synaptoneurosomes prepared from $Plg^{-/-}$ cerebral cortical neurons treated for 5 min with 5 nM uPA or vehicle (control). $n = 4$ observations/group; two-tailed Student's *t* test. *E* and *F*, representative Western blot analysis (*E*) and normalized integrated intensity of the band (*F*) of pGAP-43 expression in synaptoneurosomes prepared from WT cerebral cortical neurons treated with uPA alone or in the presence of 4 μg/ml anti-uPAR-blocking antibodies. $n = 4$ observations/experimental condition; one-way ANOVA with Tukey's multiple comparisons test. *G* and *H*, representative Western blot analysis (*G*) and normalized integrated intensity of the band (*H*) of pGAP-43 expression in synaptoneurosomes prepared from WT cerebral cortical neurons treated with uPA alone or in the presence of 0.5 μg/ml pertussis toxin (*PT*). $n = 5$ observations/experimental condition; one-way ANOVA with Tukey's multiple comparisons test.

N-terminal fragment (ATF; devoid of proteolytic activity; Fig. 3, *A* and *B*) and $Plg^{-/-}$ neurons with uPA (Fig. 3, *C* and *D*) and requires its binding to uPAR, as it is blocked by cotreatment with anti-uPAR antibodies (Fig. 3, *E* and *F*). Because *N*-formyl peptide receptor 2 (FPR2) is an essential coreceptor for uPAR signaling (34), we performed similar experiments in the presence of pertussis toxin. Our data indicated that FPR2 acted as a uPAR coreceptor for uPA-induced GAP-43 phosphorylation (Fig. 3, *G* and *H*).

uPA induces vesicular transport and phosphorylation of GAP-43 in the synapse

Because our results indicated that uPA treatment triggered a transient increase in the abundance of GAP-43 in extracts from synaptoneurosomes (Fig. 2*A*) but not from whole-cell extracts (Fig. 1*A*), we postulated that uPA induces mobilization of GAP-43 from the axonal shaft to the synapse. To test this hypothesis, we studied the expression of GAP-43 in synaptoneurosomes prepared from WT cerebral cortical neurons treated with 5 nM of uPA alone or in the presence of 25 μM ciliobrevin D (CiliD), an inhibitor of axonal vesicular transport (35). We found that CiliD abrogates the effect of uPA on the abundance of GAP-43 in the synapse (Fig. 4, *A* and *B*). These data suggested that the observed effect of uPA on the expression of pGAP-43 may be a consequence of a greater availability of GAP-43 in the synapse, caused by increased vesicular transport from the axonal shaft triggered by uPA, instead of a direct effect of uPA on phosphorylation of GAP-43. Surprisingly, our finding that CiliD does not abrogate the effect of uPA on GAP-43 phosphorylation in the synapse indicates that this is not the case and, instead, that uPA induces not only vesicular transport of GAP-43 from the axonal shaft but also its phosphorylation in the synapse (Fig. 4, *A* and *C*). Furthermore, we found that this effect was mediated by PKC and calcium, as it was abrogated by treatment with the PKC inhibitor chelerythrine chloride (Fig. 4, *D* and *E*) and the cell-permeant calcium chelator BAPTA-AM (Fig. 4, *F* and *G*).

Presynaptic NMDA receptors mediate the effect of uPA on pGAP-43

Because the levels of GAP-43 during development are in part regulated by NMDA-activated transcriptional mechanisms (9), and because our data indicate that calcium mediates the effect of uPA on GAP-43 phosphorylation (Fig. 4, *F* and *G*), we decided to study the expression of pGAP-43 in extracts from synaptoneurosomes prepared from WT cerebral cortical neurons incubated for 5 min with 5 nM uPA alone or in the presence of 10 μM MK-801 (to block open synaptic NMDA receptors). Remarkably, these experiments showed that open synaptic NMDARs are required for uPA to induce GAP-43 phosphorylation in the synapse (Fig. 5, *A* and *B*). Because GAP-43 is found in the presynaptic terminal (4), it is possible that uPA induces entry of calcium into the dendritic spine via NMDARs and that the ensuing raise in the intracellular concentration of calcium in the postsynaptic terminal triggers a transsynaptic signal that induces phosphorylation of GAP-43 in the axonal bouton.

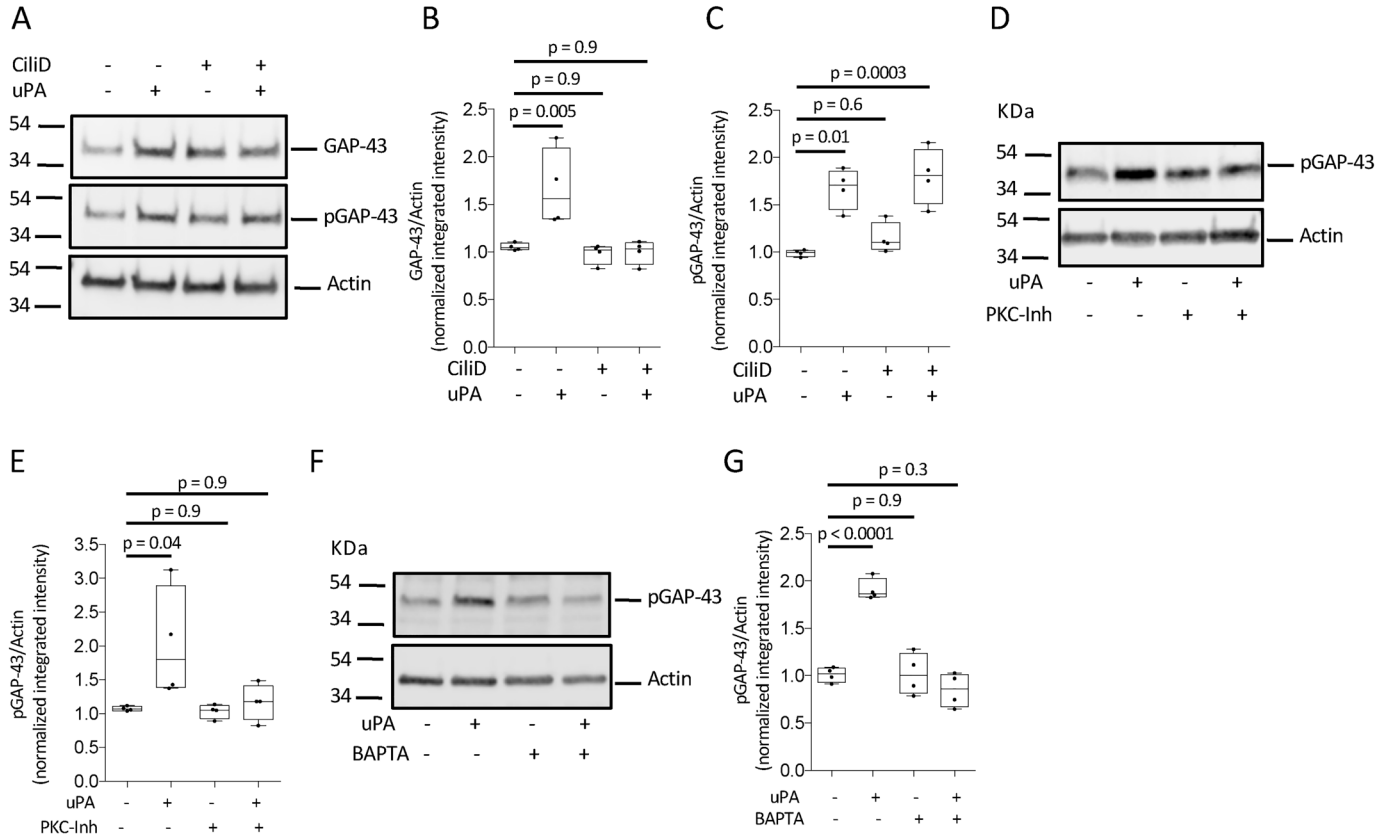

Figure 4. uPA induces calcium-dependent PKC-mediated phosphorylation of GAP-43 in the synapse. *A–C*, representative Western blot analysis (*A*) and normalized integrated intensity of the band (*B* and *C*) of GAP-43 and p-GAP-43 expression in synaptoneurosomes prepared from WT cerebral cortical neurons treated for 5 min with uPA alone or in the presence of 25 μM CiliD. $n = 4$ observations/experimental group; one-way ANOVA with Dunnett's multiple comparisons test. *D* and *E*, representative Western blot analysis (*D*) and normalized integrated intensity of the band (*E*) of pGAP-43 expression in synaptoneurosomes prepared from WT cerebral cortical neurons treated with uPA or vehicle (control), alone or in the presence of 10 μM PKC inhibitor (*PKC-Inh*; chelerythrine chloride). $n = 4$ observations/experimental condition; one-way ANOVA with Tukey's multiple comparisons test. *F* and *G*, representative Western blot analysis (*F*) and normalized integrated intensity of the brand (*G*) of pGAP-43 expression in synaptoneurosomes prepared from WT cerebral cortical neurons treated with uPA alone or in the presence of 30 μM BAPTA-AM. $n = 4$/experimental condition; one-way ANOVA with Tukey's correction test.

However, it is also possible that uPA increases entry of calcium directly into the axonal bouton via presynaptic NMDARs, which are known to play a central role in the regulation of synaptic function (36).

To test this hypothesis, we studied the expression of pGAP-43 in presynaptic terminals isolated from the cerebral cortex of WT mice, as described under "Experimental procedures," and treated them for 5 min with 5 nM uPA alone or in combination with 10 μM MK-801. The purity of these preparations (*i.e.* the absence of components of the postsynaptic terminal) was demonstrated by their immunoreactivity to GAP-43 (present only in the axonal bouton) and syntaxin-I (which delineates the presynaptic membrane) but not to PSD-95 (a postsynaptic marker; Fig. 5*C*). Our data indicated that open presynaptic NMDARs mediated the effect of uPA on GAP-43 phosphorylation in the axonal terminal (Fig. 5, *D* and *E*). To determine whether NMDA receptors mediate uPA-induced activation of other signaling pathways, we studied the expression of ERK 1/2 phosphorylated at Thr-202/Tyr-204 in synaptoneurosomes prepared from WT cerebral cortical neurons treated for 30 min with uPA alone or in the presence of MK-801. We found that uPA induced ERK 1/2 activation and that this effect was also mediated by open synaptic NMDA receptors (Fig. 5, *F* and *G*). To further characterize uPA-induced GAP-43 phosphorylation in the presynaptic terminal, we performed immunocytochemical staining with anti-pGAP-43 antibodies and phalloidin (to detect actin-rich presynaptic terminals) in the axonal mantle of neurosphere-like aggregates (NLAs; these preparations are surrounded by an axonal mantle devoid of dendrites) prepared with WT cerebral cortical neurons as described under "Experimental procedures" and depicted in Fig. 5*H*, following 5 min of treatment with 5 nM uPA or vehicle (control). These studies revealed that uPA has a direct effect on the phosphorylation of GAP-43 in the presynaptic terminal of cerebral cortical neurons (Fig. 5, *I* and *J*).

UPA-induced GAP-43 activation triggers recruitment of synaptic vesicles to the active zone

Because GAP-43 plays a role in the regulation of the synaptic vesicle cycle (1), we used confocal microscopy to quantify the number of pGAP-43/synaptophysin (SYP; a protein bound to the membrane of synaptic vesicles)–positive puncta in extensions of WT cerebral cortical neurons treated for 5 min with 5 nM uPA or vehicle (control). Our studies indicated that uPA induced colocalization of pGAP-43 with synaptic vesicles, as denoted by an increase in the number of pGAP-43/SYP–positive puncta in uPA-treated neurons (Fig. 6, *A* and *B*).

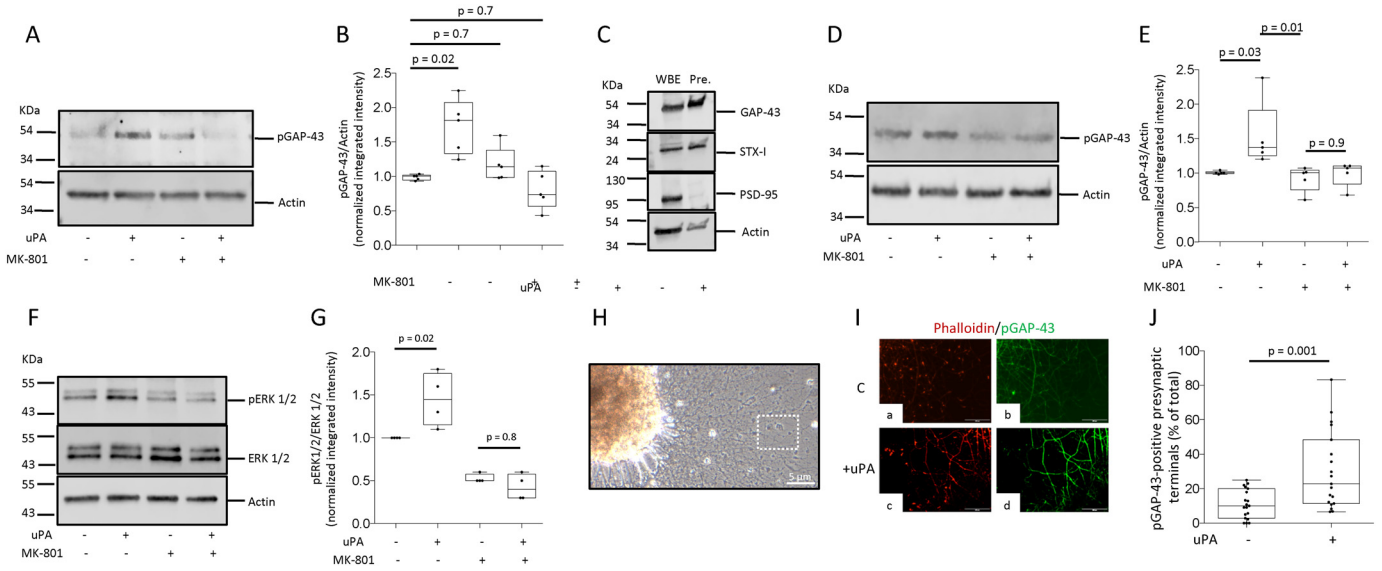

Figure 5. Presynaptic NMDA receptors mediate the effect of uPA on synaptic GAP-43. *A* and *B*, representative Western blot analysis (*A*) and normalized integrated intensity of the band (*B*) of pGAP-43 expression in synaptoneurosomes prepared from WT cerebral cortical neurons treated for 5 min with 5 nM uPA or vehicle (control) alone or in the presence of 10 μM MK-801. $n = 4$ observations/experimental group; one-way ANOVA with Tukey's multiple comparisons test. *C*, representative Western blot analysis of GAP-43, syntaxin I (*STX-I*), PSD-95, and actin expression in extracts prepared from whole-brain extracts (*WBE*) and presynaptic terminals (*Pre.*). *D* and *E*, representative Western blot analysis (*D*) and normalized integrated intensity of the band (*E*) of pGAP-43 expression in extracts prepared from presynaptic terminals from WT cerebral cortical neurons incubated for 5 min with uPA or vehicle (control) alone or in the presence of MK-801. $n = 4$ observations/group; one-way ANOVA with Tukey's correction. *F* and *G*, representative Western blot analysis (*F*) and normalized integrated intensity of the band (*G*) of pERK 1/2 and total ERK 1/2 expression in synaptoneurosomes prepared from WT cerebral cortical neurons incubated for 30 min with uPA alone or in the presence of MK-801. $n = 4$ in *G*; one-way ANOVA with Tukey's correction. *H*, representative micrograph of an NLA and its surrounding axonal mantle, prepared from WT neurons. The *dashed square* denotes the area where the observations were made. *I*, representative micrographs at ×40 magnification of phalloidin (*red*) and pGAP-43 (*green*) expression in the area demarcated by the *dashed square* in *F* of NLAs treated for 5 min with uPA or vehicle (control). *J*, percentage of presynaptic terminals that express pGAP-43 in the axonal mantle of 19 NLAs treated with uPA or vehicle (control) as described in *G*. Each point in *H* is the average of three micrographs per NLA for a total of 57 micrographs in 19 NLAs per experimental condition; two-tailed Student's *t* test.

pSyn-9 is followed by mobilization of synaptic vesicles from the reserve and recycling pools to the AZ (20). Then, to investigate whether, besides inducing colocalization of pGAP-43 with synaptic vesicles (Fig. 6, *A* and *B*), uPA also triggers their mobilization to the synaptic release site, we studied the expression of pSyn-9 in synaptoneurosomes prepared from WT cerebral cortical neurons treated for 5 min with 5 nM uPA alone or in combination with 10 μM MK-801. Our data showed that uPA induced synapsin I phosphorylation and that this effect was mediated by open synaptic NMDARs (Fig. 6, *C* and *D*). To determine whether this leads to mobilization of synaptic vesicles to the AZ, we studied the expression of SYP in synaptic membranes isolated from WT cerebral cortical neurons treated with uPA or vehicle (control). Because SYP is attached to presynaptic vesicles, its abundance in synaptic membranes denotes synaptic vesicles attached to the synaptic release site. These experiments revealed that uPA-induced GAP-43 phosphorylation was followed by mobilization of synaptic vesicles to the AZ (Fig. 6, *E* and *F*).

Following their fusion to the AZ, synaptic vesicles are reconstituted before returning to the reserve and recycling pools (37). Because, during this process, these vesicles take up dyes added to the synaptic cleft (38), their intensity is a marker of the abundance of synaptic vesicles docked to the AZ (39). To corroborate the observed effect of uPA on the mobilization of synaptic vesicles to the AZ, we measured the uptake of AM1-44 dye from synaptic vesicles from WT cerebral cortical neurons incubated with AM1-44 alone (control) or in combination with either 45 mM KCl (known to trigger mobilization of synaptic vesicles to the AZ; this was an internal control for our experimental system) or 5 nM uPA. Our data showed that uPA-induced colocalization of pGAP-43 and SYP was followed by mobilization of synaptic vesicles to the AZ at levels comparable with those observed following KCl treatment (Fig. 6, *G* and *H*).

Discussion

GAP-43 is a phosphoprotein that, during early development, is abundantly found in neuronal extensions (40), where its activity is pivotal for formation of presynaptic terminals, axonal growth, and cytoskeletal reorganization (29). In contrast, expression of GAP-43 in the mature brain is restricted to scattered presynaptic terminals and axonal growth cones of highly plastic areas (41), where it has been shown to play a central role in formation of axonal boutons (31) and development of synaptic plasticity and neurorepair (42, 43). Interestingly, there are many parallels between the expression and function of uPA and GAP-43 in the developing and mature brain. Indeed, like GAP-43, uPA is also abundantly found in the developing brain (44–46), where it promotes neuritogenesis and neuronal migration (47). In contrast, its expression and function in mature neurons are not fully understood. However, our earlier work indicates that release of neuronal uPA and expression of uPAR in axonal growth cones increases following mechanical injury *in vitro* and ischemic injury *in vivo* and that, as described for GAP-43, uPA binding to uPAR induces not only formation of new axons and synaptic contacts under physiological conditions *in vitro* but also repair of those damaged by different forms of injury (25–27). Remarkably, despite these and several other similitudes, to

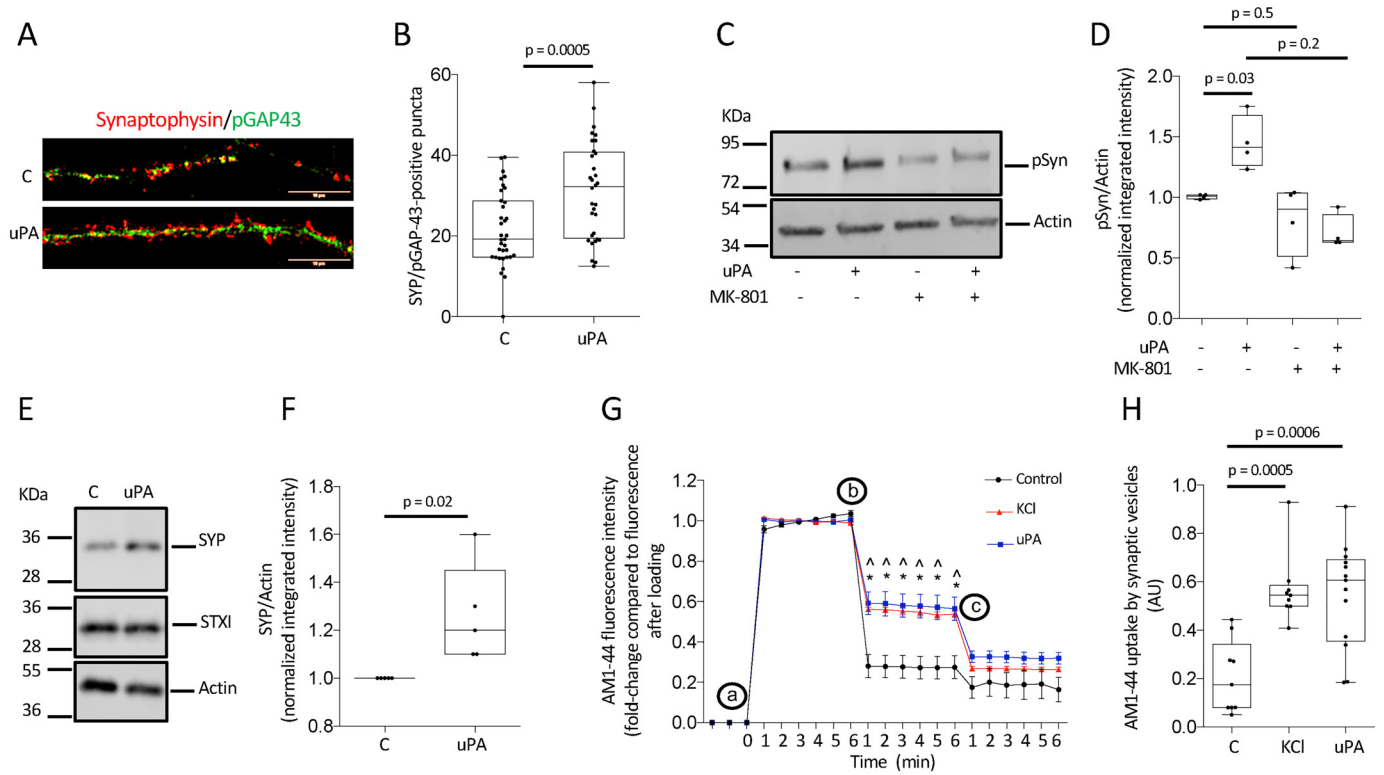

Figure 6. uPA induces mobilization of synaptic vesicles to the active zone. *A*, representative ×60 confocal micrographs of pGAP-43 (*green*) and synaptophysin (*red*) expression in WT cerebral cortical neurons incubated for 30 min with 5 nM uPA or a comparable volume of vehicle (*C*, control). *B*, mean number of pGAP-43/synaptophysin–positive puncta in 36 extensions from WT cerebral cortical neurons from three different cultures treated as described in *A*; two-tailed Student's *t* test. *C* and *D*, representative Western blot analysis (*C*) and normalized integrated intensity of the band (*D*) of pSyn-9 in extracts prepared from WT cerebral cortical neurons treated for 30 min with 5 nM uPA alone or in the presence of 10 μM MK-801. $n = 4$ observations/experimental group; one-way ANOVA with Tukey's multiple comparisons test. *E*, synaptoneurosomes were prepared from the cerebral cortex of WT mice, treated for 5 min with 5 nM uPA or vehicle (control), exposed to osmotic lysis, and placed on a linear 0.3–1.5 M sucrose gradient. The band containing synaptic membranes was immunoblotted with anti-SYP antibodies (denotes synaptic vesicles attached to the presynaptic membrane). *F*, normalized integrated intensity of SYP associated with synaptic membranes (denotes synaptic vesicles attached to the active zone) in WT synaptoneurosomes treated as described in *E*. $n = 6$ observations/experimental group; two-tailed Student's *t* test. *G* and *H*, WT cerebral cortical neurons were incubated for 2 min with 5 μM AM1-44, alone (control) or in combination with either 45 mM KCl or 5 nM uPA (*a*). Cells were then washed and treated 5 min later with 5 μM ADVASEP-7 to reduce background and induce release of membrane-bound AM1-44 (*b*). Fluorescence intensity at this point corresponds to AM1-44 loaded into synaptic vesicles (depicted in *H*). Five min later, cells were treated with 45 nM KCl to induce release of AM1-44 incorporated into synaptic vesicles (*c*). Values in *H* correspond to the -fold-change in fluorescence intensity before the second dose of KCl (*c*) compared with the intensity before ADVASEP-7 treatment (*b*). Each experiment was repeated 27 times. * and , $p <$ 0.05 when control values at each time point were compared with the fluorescence intensity in KCl- and uPA-treated cells. Two-way ANOVA with Tukey's multiple comparisons test (*G* and *H*).

date it is unknown whether there is a functional link between uPA and GAP-43. The data presented here indicate that uPA regulates the expression and function of GAP-43 in mature synapses and that uPA-induced uPA/FPR2-mediated GAP-43 phosphorylation leads to activation of the synaptic vesicle cycle in cerebral cortical neurons.

Despite the fact that the abundance of GAP-43 protein decreases during late developmental stages, specific areas of the mature brain maintain high levels of GAP-43 mRNA (49), which is stabilized by HuD, a neuron-specific RNA-binding protein (10, 50) that also stabilizes the mRNA of several components of the plasminogen activation system (51). Importantly, nerve regeneration and synaptic plasticity increase HuD expression and translation of GAP-43 mRNA in axons and growth cones (52). Furthermore, GAP-43 mRNA translation is also induced by treatment with nerve growth factor (17) and NMDA receptor and neural cell adhesion molecule–mediated fibroblast growth factor receptor activation (9, 17, 53). Following GAP-43 mRNA translation, GAP-43 protein translocates from the cell body to growth cones using the fast axonal vesicular transport machinery (13). Our data show that uPA induces a transient increase in GAP-43 in the synapse, and that this effect is abrogated by inhibition of axonal vesicular transport indicates that uPA triggers mobilization of GAP-43 from the axonal shaft to the synapse. However, the mechanism(s) whereby uPA regulates axonal vesicular transport of GAP-43 are still unclear.

GAP-43 is activated by PKC-β–induced calcium-mediated phosphorylation at Ser-41 (54). Our experiments with whole-cell extracts revealed that, although uPA induces GAP-43 phosphorylation after 5 min of treatment, this effect reaches statistical significance only following 3 h of incubation. In contrast, our immunoblotting with synaptic extracts showed a transient but statistically significant increase in pGAP-43 expression in the synapse 5 min after uPA treatment. An explanation for these results is that GAP-43 phosphorylation occurs in the synapse and, therefore, this effect is less apparent in whole-cell extracts containing other extrasynaptic proteins. Importantly, our observation that vesicular axonal transport inhibition blocks the uPA-induced increase in GAP-43 in the synapse but

not uPA-induced GAP-43 phosphorylation indicates that uPA induces mobilization of GAP-43 from the axonal shaft to the synapse and that this is followed by uPA-induced GAP-43 activation by phosphorylation at Ser-41. However, we cannot rule out the possibility that GAP-43 is phosphorylated in the axonal shaft before its synaptic translocation.

Our experiments revealed that, although the effect of uPA on GAP-43 is not mediated by plasmin, it requires its binding to uPAR. However, GAP-43 is bound to the cytoplasmic surface of the plasma membrane, and uPAR is a glycosylphosphatidylinositol-anchored receptor that has neither transmembrane nor cytoplasmic domains. Therefore, uPAR needs a coreceptor to induce axonal mobilization and synaptic phosphorylation of GAP-43 (25–27, 55). Thus, although our previous work has shown that different integrins and low-density lipoprotein receptor–related protein 1 (LRP1) are coreceptors that mediate the effect of uPA/uPAR binding on axons and dendrites, our finding that uPA-induced GAP-43 mobilization and activation requires open synaptic NMDA receptors suggests that they may act as co-receptors for uPAR. Our data agree with reports indicating that NMDA receptor activation triggers GAP-43 phosphorylation (56) and that other plasminogen activators (*i.e.* tissue-type plasminogen activator) interact with NMDA receptors (57). Furthermore, our results show that NMDA receptors also mediate the effect of uPA on activation of other cell signaling pathways (*i.e.* ERK 1/2).

Because GAP-43 is found in the presynaptic terminal, the observation that synaptic NMDA receptors mediate the effect of uPA on GAP-43 mobilization and phosphorylation suggests a model in which the interaction of uPA with NMDA receptors located in the postsynaptic terminal induces activation of a messenger in the dendritic spine that then would diffuse transsynaptically to induce mobilization and phosphorylation of GAP-43 in the axonal bouton. However, our work with presynaptic extracts indicated that the effect of uPA is mediated by presynaptic NMDA receptors, which, by allowing entry of calcium in the axonal bouton, trigger a sequence of events that leads to an increase in the abundance of GAP-43 and its activation in the synapse. These observations were corroborated by our immunocytochemical studies with NLAs surrounded by a mantle with axons and presynaptic terminals but not dendrites or dendritic spines. These data are of significant relevance because presynaptic NMDA receptors have a central role in development of presynaptic plasticity and neurotransmission (36). This is the first report of a functional interaction between uPA and these receptors. However, our data do not rule out the existence of a coreceptor such as LRP-1 or an integrin that mediates the observed functional interaction between uPAR and NMDA receptors.

Synapsin I is a phosphoprotein that, by cross-linking synaptic vesicles, prevents their mobilization from the reserve and recycling pools to the AZ. However, synapsin I phosphorylation at Ser-9 leads to its dissociation from synaptic vesicles, freeing them to move to the AZ (58). Our data indicate that pGAP-43 colocalizes with synaptic vesicles in uPA-treated neurons and that this is followed by synapsin I phosphorylation at Ser-9 and mobilization of synaptic vesicles to the AZ. These observations agree with reports from other groups indicating that, following its association with synaptic vesicles (59, 60), pGAP-43 activates the synaptic vesicle cycle and induces release of neurotransmitters (1). Importantly, our results indicate that these events require entry of calcium through open presynaptic NMDA receptors.

In summary, the results presented here indicate a novel role for uPA where its binding to uPAR triggers mobilization of GAP-43 from the axonal shaft to the synapse and subsequent PKC-mediated activation by phosphorylation at Ser-41. We found that this effect does not require plasmin generation but is mediated by open presynaptic NMDA receptors. Finally, our data reveal that mobilization and activation of GAP-43 induced by uPA lead to activation of the synaptic vesicle cycle. These results have significant implications for our understanding of the mechanisms of presynaptic plasticity and synaptic repair induced by uPA/uPAR binding.

Experimental procedures

Reagents

Recombinant murine uPA and uPA N-terminal fragments (ATFs) were purchased from Molecular Innovations (Novi, MI). Other reagents were ciliobrevin D (EMD Millipore, Burlington, MA); chelerythrine chloride, BAPTA-AM, and MK-801 (Tocris, Minneapolis, MN); AM1-44 and ADVASEP-7 (Biotium, Freemont, CA); phalloidin rhodamine and Hoechst (Thermo Fisher, Waltham, MA); and pertussis toxin (Millipore Sigma, Burlington, MA). We also used antibodies against GAP-43 (Novus Biologicals, Centennial, CO); GAP-43 phosphorylated at serine 41 (pGAP-43, GenTex (Irvine, CA) and Abcam (Cambridge, MA)); actin, synaptophysin, and syntaxin I (Millipore); bassoon (Enzo, Farmingdale, NY); uPAR (R&D Systems, Minneapolis, MN); and synapsin I phosphorylated at serine 9 (pSynI), postsynaptic density protein 95 (PSD-95), pERK 1/2, and total ERK 1/2 (Cell Signaling, Danvers, MA). Secondary antibodies for immunoblotting were IRDye 800 mouse, IRDye 800 rabbit, IRDye 680 mouse, and IRDye 680 rabbit (all purchased from LI-COR, Lincoln, NE) and goat anti-rabbit Alexa Fluor 488 (1:500) and donkey anti-mouse Alexa Fluor 594 (1:500), obtained from Invitrogen.

Neuronal cultures and preparation of NLAs

Cerebral cortical neurons were cultured from E16–E18 WT, $Plg^{-/-}$, and $uPAR^{-/-}$ mice as described elsewhere (61). Briefly, the cerebral cortex was dissected, transferred into Hanks' balanced salt solution containing 100 units/ml penicillin, 100 μg/ml streptomycin, and 10 mm HEPES and incubated in trypsin containing 0.02% DNase at 37 °C for 15 min. Tissue was triturated, and the supernatant was resuspended in GS21-supplemented Neurobasal medium containing 2 mM L-glutamine and plated onto 0.1 mg/ml poly-L-lysine–coated wells. NLAs were prepared as described elsewhere (62) with few modifications. Briefly, dissociated cortical neurons cultured from E17–E18 WT embryos, as described above, were allowed to aggregate for 48 h as hanging drops (4000 cells/drop) containing 0.2% Methocel and 10% horse serum in Neurobasal/B27 medium and plated on glass coverslips pretreated with a combination of poly-L-lysine and 10 μg/ml fibronectin in the presence of a coculture of WT astrocytes and 10 μM AraC to prevent glial

proliferation on the coverslip. Experiments were performed 7 days later, when NLAs were surrounded by a dense mantle of axons.

Preparation of synaptoneurosomes

Synapse-enriched fractions containing the sealed presynaptic terminal and the attached postsynaptic membrane (synaptoneurosomes) were prepared according to a modification of published protocols (63–66). Briefly, cells were homogenized and centrifuged at 2000 × *g* for 5 min. Pellets were discarded, and the supernatants were centrifuged at 32,000 × *g* for 10 min. Pellets were resuspended and layered on top of a 5%, 9%, and 13% discontinuous Ficoll (Fisher, Fair Lawn. NJ) gradient and centrifuged at 25,000 × *g* for 25 min at 4 °C in a TLS 55 rotor using a Beckman Optima TLX tabletop ultracentrifuge. Synaptoneurosomes were collected from the 5%/9% and 9%/13% interfaces and then centrifuged at 35,000 rpm for 10 min. The pellet was resuspended in radioimmune precipitation assay buffer and used for Western blot analysis.

Isolation of membrane-bound synaptic vesicles

Synaptoneurosomes were prepared as described above from the frontal cortex of WT mice and resuspended in 500 μl of Neurobasal/B27 medium pre-equilibrated at 37 C° and 5% CO_2 and incubated for 5 min with 5 nM uPA or a comparable volume of vehicle (control), followed by addition of 2 ml of 0.25 M sucrose/EGTA (pH 8.1) buffer and centrifugation at 80,000 × *g* for 10 min. Osmotic lysis was accomplished by resuspension in 10 mM HEPES, followed by homogenization and centrifugation at 100,000 × *g* for 30 min. The membrane pellet was resuspended in 200 μl of 0.25 M sucrose/EGTA (pH 8.1). Proteins were measured, and 200 μg of membrane pellets was overlaid on top of a 0.3%-1.2% sucrose gradient, followed by 90 min of centrifugation at 93,000 × *g*. The band containing synaptic elements was identified as described in our previous work (39, 67) and centrifuged for 20 min at 100,000 × *g*. Membrane-bound synaptic vesicles were resuspended in 0.5% SDS and used for immunoblotting.

Isolation of presynaptic terminals

Presynaptic terminals were isolated from the murine cerebral cortex as described elsewhere (48). Briefly, each cortex was homogenized in 1 ml of buffer containing 0.32 M sucrose and 1 mM EDTA and centrifuged for 15 min at 1600 × *g*. The supernatant was removed, loaded onto a 0.75, 1, and 2 M discontinuous sucrose gradient, and centrifuged for 40 min at 242,000 × *g*. At this point, three bands were observed, containing the presynaptic terminal, cellular organelles, and membranes. The fraction containing presynaptic terminals was recovered and centrifuged at 242,000 × *g* during 10 min, and the pellet was resuspended in 50 μl of a solution containing 0.32 M sucrose and 1 mM EDTA. The purity of these preparations was confirmed by their immunoreactivity to antibodies against GAP-43 (present only in the presynaptic terminal) and syntaxin I (delineates the presynaptic membrane) but not PSD-95 (detects the postsynaptic terminal; Fig. 4*C*).

Western blot analysis

To study the effect of uPA on GAP-43 and pGAP43 expression in whole-cell extracts, WT and $uPAR^{-/-}$ cerebral cortical neurons were incubated for 0–180 min with 5 nM murine uPA. To study the effect of uPA on expression of GAP-43 and pGAP-43 in the synapse, synaptoneurosomes were prepared from either WT or $Plg^{-/-}$ cerebral cortical neurons treated for 0–180 min with 5 nM uPA, for 5 min with 5 nM ATF, or for 5 min with 5 nM uPA alone or in the presence of 4 μg/ml anti-uPAR-blocking antibodies. To determine whether FPR2 is a coreceptor for uPAR in this system, synaptoneurosomes were prepared from WT cerebral cortical neurons incubated for 30 min with 0.5 μg/ml pertussis toxin or a comparable volume of vehicle (control), followed by 5 min of treatment with 5 nM uPA or vehicle (control). To study the role of axonal vesicular transport, PKC, calcium, and open synaptic NMDARs in the effect of uPA on GAP-43 and pGAP-43, synaptoneurosomes were prepared from WT cerebral cortical neurons incubated for 30 min with either 25 μM CiliD (an inhibitor of vesicular transport), 30 μM BAPTA-AM (a cell-permeant calcium chelator), 10 μM or chelerythrine chloride (a PKC inhibitor), or 10 μM MK-801, followed by 5 min of treatment with 5 nM uPA or a comparable volume of vehicle (control). To study the effect of uPA on GAP-43 in the axonal bouton, presynaptic terminals were isolated from the cerebral cortex of WT mice and treated for 5 min with 10 μM MK-801 or vehicle (control), followed by 5 min of incubation with 5 nM uPA. To investigate the effect of uPA on synapsin I phosphorylation, WT cerebral cortical neurons were incubated for 30 min with 10 μM MK-801 or vehicle (control), followed by 5 min of treatment with 5 nM uPA. Each experiment was repeated as described in the corresponding figure legend. Protein concentration was quantified in whole-cell extracts and synaptoneurosomes with a BCA assay. 10 μg was loaded per sample, separated in a 4%–20% precast linear gradient polyacrylamide gel (Bio-Rad), transferred to a PVDF membrane using a semidry transfer system, blocked with 5% nonfat dry milk in Tris-buffered saline (pH 8.0) with 0.1% Tween 20 buffer, and immunoblotted with antibodies against GAP-43 (1:2000), pGAP-43 (1:2000), pERK 1/2 (1:1000), total ERK 1/2 (1:1000), actin (1:5000), syntaxin I (1:2000), PSD-95 (1:1000), or synapsin I phosphorylated at serine 9 (1:2000). To assess the linearity of GAP-43 signal in our immunoblots, we performed similar experiments with antibodies against GAP-43 and actin in gels loaded with 5–30 μg of synaptosomal protein. Membranes were developed in a LI-COR Odyssey imaging system. Densitometry analysis was performed in each band using Image Studio (LI-COR). All values were normalized to actin.

Immunocytochemistry

WT cerebral cortical neurons and NLAs were incubated for 5 min with 5 nM uPA or a comparable volume of vehicle (control). Cells and NLAs were fixed with 4% paraformaldehyde, washed three times in Tris-buffered saline, and incubated for 30 min in a blocking solution containing 1 ml of 0.2 mM glycine, 20 μl/ml casein, and 5 μl/ml donkey serum. Then samples were kept overnight in a solution containing antibodies against either bassoon (1:100) and GAP-43 (1:5000), pGAP-43 (1:5000), or syn-

aptophysin (1:250) and pGAP-43 (1:5000). Secondary antibodies were goat anti-rabbit Alexa Fluor 488 (1:500) and donkey anti-mouse Alexa Fluor 594 (1:500). NLAs were incubated for 30 min with Rhodamine-594–conjugated phalloidin (1:1000). The number of bassoon/GAP-43– and synaptophysin/GAP-43–positive puncta was quantified in neuronal extensions with ImageJ without plugins in pictures taken with a Fluoview FV10i confocal laser-scanning microscope (Olympus) at ×40 magnification. Images were processed using 2D deconvolution with five iterations incorporated in the CellSens Dimension Olympus software. The number of phalloidin/GAP-43-positive puncta was quantified in the axonal mantle of NLAs in images taken with an Olympus microscope (IX83) and a DP80 camera at ×40 magnification with ImageJ without plugins.

Quantification of AM1-44 uptake

WT cerebral cortical neurons were incubated for 2 min with Tyrode's buffer containing 125 mM NaCl, 2 mM KCl, 2 mM $CaCl_2$, 2 mM $MgCl_2$, 25 mM HEPES, 30 mM D-glucose, and 5 μM AM1-44 alone (control) or in combination with either 45 mM KCl (in this case, the NaCl concentration was 82 mM) or 5 nM uPA. Cells were then washed. 5 min later, 5 μM ADVASEP-7 was added to reduce background and induce release of membrane-bound AM1-44. Five minutes later, cells were treated with 45 nM KCl to induce release of AM1-44 incorporated into synaptic vesicles. Quantification was made with images taken with an Olympus microscope (IX83) and a DP80 camera at ×20 magnification.

Statistical analysis

Statistical analysis was performed with one- or two-way ANOVA with corrections described in each figure legend, as appropriate. $p < 0.05$ was considered significant.

Author contributions—P. M. and M. Y., conceptualization; P. M. formal analysis; P. M., A. D., and E. R. T., methodology; P. M., writing-original draft; A. D., investigation; M. Y., supervision; M. Y., funding acquisition; M. Y., project administration; M. Y., writing-review and editing.

References

1. Neve, R. L., Coopersmith, R., McPhie, D. L., Santeufemio, C., Pratt, K. G., Murphy, C. J., and Lynn, S. D. (1998) The neuronal growth-associated protein GAP-43 interacts with rabaptin-5 and participates in endocytosis. *J. Neurosci.* **18,** 7757–7767 CrossRef Medline
2. Lin, L. H., Bock, S., Carpenter, K., Rose, M., and Norden, J. J. (1992) Synthesis and transport of GAP-43 in entorhinal cortex neurons and perforant pathway during lesion-induced sprouting and reactive synaptogenesis. *Brain Res.* **14,** 147–153 CrossRef Medline
3. Snipes, G. J., Chan, S. Y., McGuire, C. B., Costello, B. R., Norden, J. J., Freeman, J. A., and Routtenberg, A. (1987) Evidence for the coidentification of GAP-43, a growth-associated protein, and F1, a plasticity-associated protein. *J. Neurosci.* **7,** 4066–4075 CrossRef Medline
4. Benowitz, L. I., and Routtenberg, A. (1997) GAP-43: an intrinsic determinant of neuronal development and plasticity. *Trends Neurosci.* **20,** 84–91 CrossRef Medline
5. Son, H., Davis, P. J., and Carpenter, D. O. (1997) Time course and involvement of protein kinase C-mediated phosphorylation of F1/GAP-43 in area CA3 after mossy fiber stimulation. *Cell. Mol. Neurobiol.* **17,** 171–194 CrossRef Medline
6. Chao, H. M., Spencer, R. L., Sakai, R. R., and McEwen, B. S. (1992) The expression of growth-associated protein GAP-43 mRNA in the rat hippocampus in response to adrenalectomy and aging. *Mol. Cell. Neurosci.* **3,** 529–535 CrossRef Medline
7. Casoli, T., Stefano, G. D., Fattoretti, P., Solazzi, M., Delfino, A., Biagini, G., and Bertoni-Freddari, C. (2003) GAP-43 mRNA detection by *in situ* hybridization, direct and indirect *in situ* RT-PCR in hippocampal and cerebellar tissue sections of adult rat brain. *Micron* **34,** 415–422 CrossRef Medline
8. Ramakers, G. J., Oestreicher, A. B., Wolters, P. S., van Leeuwen, F. W., De Graan, P. N., and Gispen, W. H. (1991) Developmental changes in B-50 (GAP-43) in primary cultures of cerebral cortex: B-50 immunolocalization, axonal elongation rate and growth cone morphology. *Int. J. Dev. Neurosci.* **9,** 215–230 CrossRef Medline
9. Cantallops, I., and Routtenberg, A. (1999) Activity-dependent regulation of axonal growth: posttranscriptional control of the GAP-43 gene by the NMDA receptor in developing hippocampus. *J. Neurobiol.* **1,** 208–220 CrossRef Medline
10. Sanna, M. D., Quattrone, A., Mello, T., Ghelardini, C., and Galeotti, N. (2014) The RNA-binding protein HuD promotes spinal GAP43 overexpression in antiretroviral-induced neuropathy. *Exp. Neurol.* **261,** 343–353 CrossRef Medline
11. Deschênes-Furry, J., Mousavi, K., Bolognani, F., Neve, R. L., Parks, R. J., Perrone-Bizzozero, N. I., and Jasmin, B. J. (2007) The RNA-binding protein HuD binds acetylcholinesterase mRNA in neurons and regulates its expression after axotomy. *J. Neurosci.* **27,** 665–675 CrossRef Medline
12. Perrone-Bizzozero, N., and Bolognani, F. (2002) Role of HuD and other RNA-binding proteins in neural development and plasticity. *J. Neurosci. Res.* **68,** 121–126 CrossRef Medline
13. Denny, J. B. (2006) Molecular mechanisms, biological actions, and neuropharmacology of the growth-associated protein GAP-43. *Curr. Neuropharmacol.* **4,** 293–304 CrossRef Medline
14. Sheu, F. S., Marais, R. M., Parker, P. J., Bazan, N. G., and Routtenberg, A. (1990) Neuron-specific protein F1/GAP-43 shows substrate specificity for the β subtype of protein kinase C. *Biochem. Biophys. Res. Commun.* **171,** 1236–1243 CrossRef Medline
15. Chan, S. Y., Murakami, K., and Routtenberg, A. (1986) Phosphoprotein F1: purification and characterization of a brain kinase C substrate related to plasticity. *J. Neurosci.* **6,** 3618–3627 CrossRef Medline
16. Kumar, V., Chichili, V. P., Zhong, L., Tang, X., Velazquez-Campoy, A., Sheu, F. S., Seetharaman, J., Gerges, N. Z., and Sivaraman, J. (2013) Structural basis for the interaction of unstructured neuron specific substrates neuromodulin and neurogranin with Calmodulin. *Sci. Rep.* **3,** 1392 CrossRef Medline
17. Irwin, N., Chao, S., Goritchenko, L., Horiuchi, A., Greengard, P., Nairn, A. C., and Benowitz, L. I. (2002) Nerve growth factor controls GAP-43 mRNA stability via the phosphoprotein ARPP-19. *Proc. Natl. Acad. Sci. U.S.A.* **99,** 12427–12431 CrossRef Medline
18. Chi, P., Greengard, P., and Ryan, T. A. (2001) Synapsin dispersion and reclustering during synaptic activity. *Nat. Neurosci.* **4,** 1187–1193 CrossRef Medline
19. Rizzoli, S. O., and Betz, W. J. (2004) The structural organization of the readily releasable pool of synaptic vesicles. *Science* **303,** 2037–2039 CrossRef Medline
20. Bonanomi, D., Menegon, A., Miccio, A., Ferrari, G., Corradi, A., Kao, H. T., Benfenati, F., and Valtorta, F. (2005) Phosphorylation of synapsin I by cAMP-dependent protein kinase controls synaptic vesicle dynamics in developing neurons. *J. Neurosci.* **25,** 7299–7308 CrossRef Medline
21. Lijnen, H. R., Van Hoef, B., and Collen, D. (1987) Activation with plasmin of tow-chain urokinase-type plasminogen activator derived from single-chain urokinase-type plasminogen activator by treatment with thrombin. *Eur. J. Biochem.* **169,** 359–364 CrossRef Medline
22. Lijnen, H. R., Zamarron, C., Blaber, M., Winkler, M. E., and Collen, D. (1986) Activation of plasminogen by pro-urokinase: I: mechanism. *J. Biol. Chem.* **261,** 1253–1258 Medline
23. Smith, H. W., and Marshall, C. J. (2010) Regulation of cell signalling by uPAR. *Nat. Rev. Mol. Cell Biol.* **11,** 23–36 CrossRef Medline

24. Dent, M. A., Sumi, Y., Morris, R. J., and Seeley, P. J. (1993) Urokinase-type plasminogen activator expression by neurons and oligodendrocytes during process outgrowth in developing rat brain. *Eur. J. Neurosci.* **5,** 633–647 CrossRef Medline
25. Merino, P., Diaz, A., Jeanneret, V., Wu, F., Torre, E., Cheng, L., and Yepes, M. (2017) Urokinase-type plasminogen activator (uPA) binding to the uPA receptor (uPAR) promotes axonal regeneration in the central nervous system. *J. Biol. Chem.* **292,** 2741–2753 Medline
26. Merino, P., Diaz, A., Manrique, L. G., Cheng, L., and Yepes, M. (2018) Urokinase-type plasminogen activator (uPA) promotes ezrin-mediated reorganization of the synaptic cytoskeleton in the ischemic brain. *J. Biol. Chem.* **293,** 9234–9247 CrossRef Medline
27. Diaz, A., Merino, P., Manrique, L. G., Cheng, L., and Yepes, M. (2019) Urokinase-type plasminogen activator (uPA) protects the tripartite synapse in the ischemic brain via ezrin-mediated formation of peripheral astrocytic processes. *J. Cereb. Blood Flow Metab.* **39,** 2157–2171 Medline
28. Diaz, A., Merino, P., Manrique, L. G., Ospina, J. P., Cheng, L., Wu, F., Jeanneret, V., and Yepes, M. (2017) A cross-talk between neuronal urokinase-type plasminogen activator (uPA) and astrocytic uPA receptor (uPAR) promotes astrocytic activation and synaptic recovery in the ischemic brain. *J. Neurosci.* **37,** 10310–10322 CrossRef Medline
29. Apel, E. D., and Storm, D. R. (1992) Functional domains of neuromodulin (GAP-43). *Perspect. Dev. Neurobiol.* **1,** 3–11 Medline
30. Aigner, L., Arber, S., Kapfhammer, J. P., Laux, T., Schneider, C., Botteri, F., Brenner, H. R., and Caroni, P. (1995) Overexpression of the neural growth-associated protein GAP-43 induces nerve sprouting in the adult nervous system of transgenic mice. *Cell* **83,** 269–278 CrossRef Medline
31. Fishman, M. C., and Valenzuela, D. (1991) GAP-43 and neuronal remodeling. *Prog. Brain Res.* **89,** 89–95 CrossRef Medline
32. Dokas, L. A., Pisano, M. R., and Han, Y. F. (1991) Selective phosphorylation and dephosphorylation of the protein B-50. *Prog. Brain Res.* **89,** 27–36 CrossRef Medline
33. Holahan, M. R. (2017) A shift from a pivotal to supporting role for the growth-associated protein (GAP-43) in the coordination of axonal structural and functional plasticity. *Front. Cell. Neurosci.* **11,** 266 CrossRef Medline
34. Gilder, A. S., Wang, L., Natali, L., Karimi-Mostowfi, N., Brifault, C., and Gonias, S. L. (2016) Pertussis toxin is a robust and selective inhibitor of high grade glioma cell migration and invasion. *PLoS ONE* **11,** e0168418 CrossRef Medline
35. Sainath, R., and Gallo, G. (2015) The dynein inhibitor ciliobrevin D inhibits the bidirectional transport of organelles along sensory axons and impairs NGF-mediated regulation of growth cones and axon branches. *Dev. Neurobiol.* **75,** 757–777 CrossRef Medline
36. Banerjee, A., Larsen, R. S., Philpot, B. D., and Paulsen, O. (2016) Roles of presynaptic NMDA receptors in neurotransmission and plasticity. *Trends Neurosci.* **39,** 26–39 CrossRef Medline
37. Haucke, V., Neher, E., and Sigrist, S. J. (2011) Protein scaffolds in the coupling of synaptic exocytosis and endocytosis. *Nat. Rev. Neurosci.* **12,** 127–138 CrossRef Medline
38. Yepes, M., Wu, F., Torre, E., Cuellar-Giraldo, D., Jia, D., and Cheng, L. (2016) Tissue-type plasminogen activator induces synaptic vesicle endocytosis in cerebral cortical neurons. *Neuroscience* **319,** 69–78 CrossRef Medline
39. Wu, F., Torre, E., Cuellar-Giraldo, D., Cheng, L., Yi, H., Bichler, E. K., García, P. S., and Yepes, M. (2015) Tissue-type plasminogen activator triggers the synaptic vesicle cycle in cerebral cortical neurons. *J. Cereb. Blood Flow Metab.* **35,** 1966–1976 CrossRef Medline
40. Van Lookeren Campagne, M., Oestreicher, A. B., Van Bergen en Henegouwen, P. M., and Gispen, W. H. (1990) Ultrastructural double localization of B-50/GAP43 and synaptophysin (p38) in the neonatal and adult rat hippocampus. *J. Neurocytol.* **19,** 948–961 CrossRef Medline
41. Baumgärtel, K., and Mansuy, I. M. (2012) Neural functions of calcineurin in synaptic plasticity and memory. *Learn. Mem.* **19,** 375–384 CrossRef Medline
42. Kaneda, M., Nagashima, M., Mawatari, K., Nunome, T., Muramoto, K., Sugitani, K., and Kato, S. (2010) Growth-associated protein43 (GAP43) is a biochemical marker for the whole period of fish optic nerve regeneration. *Adv. Exp. Med. Biol.* **664,** 97–104 CrossRef Medline
43. Benowitz, L. I., Perrone-Bizzozero, N. I., Neve, R. L., and Rodriguez, W. (1990) GAP-43 as a marker for structural plasticity in the mature CNS. *Prog. Brain Res.* **86,** 309–320 CrossRef Medline
44. Semina, E. V., Rubina, K. A., Stepanova, V. V., and Tkachuk, V. A. (2016) Participation of urokinase receptor and its endogenous ligands in brain development and formation of cognitive functions. *Ross Fiziol Zh Im I M Sechenova* **102,** 881–903 Medline
45. Semina, E., Rubina, K., Sysoeva, V., Rysenkova, K., Klimovich, P., Plekhanova, O., and Tkachuk, V. (2016) Urokinase and urokinase receptor participate in regulation of neuronal migration, axon growth and branching. *Eur. J. Cell Biol.* **95,** 295–310 CrossRef Medline
46. Archinti, M., Britto, M., Eden, G., Furlan, F., Murphy, R., and Degryse, B. (2011) The urokinase receptor in the central nervous system. *CNS Neurol. Disord. Drug Targets* **10,** 271–294 CrossRef Medline
47. Lino, N., Fiore, L., Rapacioli, M., Teruel, L., Flores, V., Scicolone, G., and Sánchez, V. (2014) uPA-uPAR molecular complex is involved in cell signaling during neuronal migration and neuritogenesis. *Dev. Dyn.* **243,** 676–689 CrossRef Medline
48. Pfenninger, K. H., Ellis, L., Johnson, M. P., Friedman, L. B., and Somlo, S. (1983) Nerve growth cones isolated from fetal rat brain: subcellular fractionation and characterization. *Cell* **35,** 573–584 CrossRef Medline
49. Bendotti, C., Servadio, A., and Samanin, R. (1991) Distribution of GAP-43 mRNA in the brain stem of adult rats as evidenced by in situ hybridization: localization within monoaminergic neurons. *J. Neurosci.* **11,** 600–607 CrossRef Medline
50. Pascale, A., Gusev, P. A., Amadio, M., Dottorini, T., Govoni, S., Alkon, D. L., and Quattrone, A. (2004) Increase of the RNA-binding protein HuD and posttranscriptional up-regulation of the GAP-43 gene during spatial memory. *Proc. Natl. Acad. Sci. U.S.A.* **101,** 1217–1222 CrossRef Medline
51. Cuadrado, A., Navarro-Yubero, C., Furneaux, H., Kinter, J., Sonderegger, P., and Muñoz, A. (2002) HuD binds to three AU-rich sequences in the 3′-UTR of neuroserpin mRNA and promotes the accumulation of neuroserpin mRNA and protein. *Nucleic Acids Res.* **30,** 2202–2211 CrossRef Medline
52. Fallini, C., Donlin-Asp, P. G., Rouanet, J. P., Bassell, G. J., and Rossoll, W. (2016) Deficiency of the survival of motor neuron protein impairs mRNA localization and local translation in the growth cone of motor neurons. *J. Neurosci.* **36,** 3811–3820 CrossRef Medline
53. McNamara, R. K., and Routtenberg, A. (1995) NMDA receptor blockade prevents kainate induction of protein F1/GAP-43 mRNA in hippocampal granule cells and subsequent mossy fiber sprouting in the rat. *Brain Res. Mol. Brain Res.* **33,** 22–28 CrossRef Medline
54. Deloulme, J. C., Janet, T., Au, D., Storm, D. R., Sensenbrenner, M., and Baudier, J. (1990) Neuromodulin (GAP43): a neuronal protein kinase C substrate is also present in 0-2A glial cell lineage: characterization of neuromodulin in secondary cultures of oligodendrocytes and comparison with the neuronal antigen. *J. Cell Biol.* **111,** 1559–1569 CrossRef Medline
55. Wu, F., Catano, M., Echeverry, R., Torre, E., Haile, W. B., An, J., Chen, C., Cheng, L., Nicholson, A., Tong, F. C., Park, J., and Yepes, M. (2014) Urokinase-type plasminogen activator promotes dendritic spine recovery and improves neurological outcome following ischemic stroke. *J. Neurosci.* **34,** 14219–14232 CrossRef Medline
56. Leu, B., Koch, E., and Schmidt, J. T. (2010) GAP43 phosphorylation is critical for growth and branching of retinotectal arbors in zebrafish. *Dev. Neurobiol.* **70,** 897–911 Medline
57. Nicole, O., Docagne, F., Ali, C., Margaill, I., Carmeliet, P., MacKenzie, E. T., Vivien, D., and Buisson, A. (2001) The proteolytic activity of tissue-plasminogen activator enhances NMDA receptor-mediated signaling. *Nat. Med.* **7,** 59–64 CrossRef Medline
58. Huttner, W. B., and Greengard, P. (1979) Multiple phosphorylation sites in protein I and their differential regulation by cyclic AMP and calcium. *Proc. Natl. Acad. Sci. U.S.A.* **76,** 5402–5406 CrossRef Medline
59. Verkade, P., Verkleij, A. J., Annaert, W. G., Gispen, W. H., and Oestreicher, A. B. (1996) Ultrastructural localization of B-50/growth-associated protein-43 to anterogradely transported synaptophysin-positive and

calcitonin gene-related peptide-negative vesicles in the regenerating rat sciatic nerve. *Neuroscience* **71,** 489–505 CrossRef Medline

60. Verkade, P., Verkleij, A. J., Gispen, W. H., and Oestreicher, A. B. (1996) Ultrastructural evidence for the lack of co-transport of B-50/GAP-43 and calmodulin in myelinated axons of the regenerating rat sciatic nerve. *J. Neurocytol.* **25,** 583–595 CrossRef Medline
61. Echeverry, R., Wu, J., Haile, W. B., Guzman, J., and Yepes, M. (2010) Tissue-type plasminogen activator is a neuroprotectant in the mouse hippocampus. *J. Clin. Invest.* **120,** 2194–2205 CrossRef Medline
62. Torre, E. R., Gutekunst, C. A., and Gross, R. E. (2010) Expression by midbrain dopamine neurons of Sema3A and 3F receptors is associated with chemorepulsion *in vitro* but a mild *in vivo* phenotype. *Mol. Cell. Neurosci.* **44,** 135–153 CrossRef Medline
63. Rao, A., and Steward, O. (1991) Evidence that protein constituents of postsynaptic membrane specializations are locally synthesized: analysis of proteins synthesized within synaptosomes. *J. Neurosci.* **11,** 2881–2895 CrossRef Medline
64. Weingarten, J., Lassek, M., Mueller, B. F., Rohmer, M., Lunger, I., Baeumlisberger, D., Dudek, S., Gogesch, P., Karas, M., and Volknandt, W. (2014) The proteome of the presynaptic active zone from mouse brain. *Mol. Cell. Neurosci.* **59,** 106–118 CrossRef Medline
65. Wilhelm, B. G., Mandad, S., Truckenbrodt, S., Kröhnert, K., Schäfer, C., Rammner, B., Koo, S. J., Claßen, G. A., Krauss, M., Haucke, V., Urlaub, H., and Rizzoli, S. O. (2014) Composition of isolated synaptic boutons reveals the amounts of vesicle trafficking proteins. *Science* **344,** 1023–1028 CrossRef Medline
66. An, J. J., Gharami, K., Liao, G. Y., Woo, N. H., Lau, A. G., Vanevski, F., Torre, E. R., Jones, K. R., Feng, Y., Lu, B., and Xu, B. (2008) Distinct role of long 3′ UTR BDNF mRNA in spine morphology and synaptic plasticity in hippocampal neurons. *Cell* **134,** 175–187 CrossRef Medline
67. Jeanneret, V., Ospina, J. P., Diaz, A., Manrique, L. G., Merino, P., Gutierrez, L., Torre, E., Wu, F., Cheng, L., and Yepes, M. (2018) Tissue-type plasminogen activator protects the postsynaptic density in the ischemic brain. *J. Cereb. Blood Flow Metab.* **38,** 1896–1910 CrossRef Medline