DEVELOPMENTAL DYNAMICS 243:676–689, 2014
DOI: 10.1002/DVDY.24114

RESEARCH ARTICLE

uPA-uPAR Molecular Complex is Involved in Cell Signaling During Neuronal Migration and Neuritogenesis

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Background: In the development of the central nervous system (CNS), neuronal migration and neuritogenesis are crucial processes for establishing functional neural circuits. This relies on the regulation exerted by several signaling molecules, which play important roles in axonal growth and guidance. The urokinase-type plasminogen activator (uPA)—in association with its receptor—triggers extracellular matrix proteolysis and other cellular processes through the activation of intracellular signaling pathways. Even though the uPA-uPAR complex is well characterized in nonneuronal systems, little is known about its signaling role during CNS development. Results: In response to uPA, neuronal migration and neuritogenesis are promoted in a dose-dependent manner. After stimulation, uPAR interacts with α_5- and β_1-integrin subunits, which may constitute an $\alpha\beta$-heterodimer that acts as a uPA-uPAR coreceptor favoring the activation of multiple kinases. This interaction may be responsible for the uPA-promoted phosphorylation of focal adhesion kinase (FAK) and its relocation toward growth cones, triggering cytoskeletal reorganization which, in turn, induces morphological changes related to neuronal migration and neuritogenesis. Conclusions: uPA has a key role during CNS development. In association with its receptor, it orchestrates both proteolytic and nonproteolytic events that govern the proper formation of neural networks. *Developmental Dynamics 243:676–689, 2014*.

Key words: uPA-uPAR complex; neuronal migration; neurite outgrowth; integrins; FAK activation

Submitted 26 July 2013; First Decision 23 January 2014; Accepted 24 January 2014; Published online 31 January 2014

Introduction

The development of the central nervous system (CNS) is characterized by a series of phenomena (i.e., cell proliferation, postmitotic neuronal migration, neuritogenesis, and synaptogenesis), that follows a precise spatiotemporal pattern. Among those events, neuronal migration is responsible for the establishment of the "inside-out" laminar organization typical of cortical structures (Rakic, 1990), which is essential for organ morphogenesis and formation of proper axonal networks and efficient synaptic connections.

Several signaling molecules are involved in regulating neuronal migration, either by inhibiting or stimulating cell motility and neurite outgrowth (Ward et al., 2003). One example is represented by the urokinase-type plasminogen activator (uPA) (Pittman et al., 1989; Siconolfi and Seed, 2001), which interacts with its receptor to form a macromolecular complex involved in a proteolytic cascade, that includes plasmin and matrix metalloproteases (Blasi, 1999; Mignatti and Rifkin, 2000; Blasi and Sidenius, 2010). Besides cellular migration, it has been shown in several nonneuronal systems that the uPA-uPAR complex induces cell adhesion, proliferation, and maintenance of differentiation programs (Farias-Eisner et al., 2000) through the activation of several signaling pathways both in vitro (Madsen et al., 2007) and in vivo (Waltz et al., 2000). Those pathways, in turn, promote cytoskeletal reorganization and modify cell adhesion (Webb et al., 2001; Kjøller, 2002).

Because uPAR is a GPI-anchored protein, the signaling events triggered by the formation of the uPA-uPAR complex should be necessarily mediated by the interaction of the receptor with transmembrane adaptor proteins, such as integrins, among others (Czekay et al., 2001; Engelholm et al., 2003; Degryse et al., 2005; Chaurasia et al., 2006). Within the CNS, integrins participate in multiple processes (Milner and Campbell, 2002). Several types of integrin subunits have been reported to act in cortical neurons during CNS development (Schmid et al., 2004). Moreover, it has been shown that β_1, β_2, and β_3 integrins can interact with uPAR (Tarui et al., 2003), leading to the activation of intracellular signaling pathways associated with cell migration (Yebra et al., 1996; Degryse et al., 2001). In this regard, it has been demonstrated that the interaction between uPA and uPAR induces the activation of specific signaling molecules,

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Grant sponsor: UBACyT 2008–2010; Grant number: M436; Grant sponsor: UBACyT 2010–2012; Grant number: 01/2078

Article is online at: http://onlinelibrary.wiley.com/doi/10.1002/dvdy.24114/abstract

such as the focal adhesion kinase (FAK) (Bernstein et al., 2004) and the extracellular signal-regulated kinase (ERK) (Mazzieri et al., 2006). In particular, Tang et al. (1998) have shown it is uPAR that mediates FAK phosphorylation in cultured endothelial cells. Furthermore, it has been described that FAK is abundantly expressed during CNS development, being enriched in neuronal growth cones (Burgaya et al., 1997; Contestabile et al., 2003).

In the present study, we investigated the nonproteolytic role of the uPA-uPAR complex in CNS development. We tested the hypothesis that the uPA-uPAR system acts as a macromolecular complex with both regulatory and effector functions in neuronal migration and neuritogenesis during CNS morphogenesis. For that purpose, the chick embryo retinotectal system was used as the experimental model, considered to be quite suitable for exploring the mechanisms involved in cortical lamination (Lavail and Cowan, 1971; Scicolone et al., 1995, Rapacioli et al., 2011, 2012). Initially, we performed migration and neurite outgrowth studies using optic tectum (OT) explants. We found that uPA stimulates neuronal migration and neuritogenesis, even in the presence of protease inhibitors. In addition, we tested the participation of the uPA-uPAR complex in the intracellular signaling pathways involved in the above-mentioned processes. We described an interaction between $\alpha_5\beta_1$ integrin and uPAR on postmitotic neurons, suggesting a potential role for integrins as coreceptors of the uPA-uPAR complex, which in turn triggers FAK activation. Together, these results demonstrate that uPA serves a critical role during CNS development by promoting neuronal migration and neuritogenesis in an unexpected nonproteolytic way.

Results

uPA Promotes Neuronal Migration and Neuritogenesis by a Combined Proteolytic and Nonproteolytic Mechanism

To study whether uPA plays a combined proteolytic and nonproteolytic role in CNS development, we initially examined the effect of uPA on neuronal migration. Embryonic day (E) 7 OT explants were cultured for 18 hr and later incubated with different concentrations of uPA (ranging from 0.4 to 15 ng/ml) for 4 hr. Postmitotic neurons appeared to migrate further in the treated explants as compared to controls (Fig. 1A–D). We observed a proportional increase in neuronal migration in accordance with uPA concentration; the maximum being reached at 10 ng/ml uPA (Fig. 1E). At this concentration, neuronal migration was approximately 39% greater than in control explants ($42 \pm 7\%$ vs. $3 \pm 0.8\%$, $P < 0.001$). A similar response was detected in explants incubated with 10 μM aprotinin before 3, 5, and 10 ng/ml uPA treatments (Fig. 1E). In this case, however, neuronal migration was only 30% greater in treated explants compared with controls ($30 \pm 2\%$ vs. $-0.4 \pm 0.6\%$, $P < 0.001$). Regardless of the concentration tested, when uPA was administered with its inhibitor, a reduction in the number of migrating neurons was noticed in comparison to those explants only exposed to uPA (Fig. 1E).

To further explore the role of the uPA-uPAR complex in CNS development, we then analyzed the influence of uPA on neuritogenesis. Once again, the uPA pulse led to a significant upregulation in the length of neurites, which was also directly proportional to uPA concentration (Fig. 1A–D,F). At 10 ng/ml uPA, a 31% increase was observed in neurites length compared with controls ($35 \pm 1.9\%$ vs. $4 \pm 0.8\%$, $P < 0.001$). In the presence of aprotinin, neuritogenesis was only 26% greater than in controls ($26 \pm 1\%$ vs. $0.6 \pm 0.3\%$; $P < 0.001$) (Fig. 1F).

In summary, our data show that uPA has a positive effect on neuronal migration and neuritogenesis, exerted by both proteolytic and nonproteolytic mechanisms. Nevertheless, it should be noted that the described effect was not evidenced at uPA concentrations greater than 10 ng/ml; in contrast, there was a reduction in both neuronal migration and neuritogenesis in those cases.

uPAR is Present in Postmitotic Neurons and Its Binding to uPA Induces Cytoskeletal Rearrangements

The next step was to confirm whether uPAR is located at neuronal cell bodies and neurite growth cones. Immunohistochemical assays for the detection of uPAR and actin filaments were performed on E7 OT explants previously exposed to 10 ng/ml uPA for 30 min. A confocal microscopy analysis of the explants revealed that uPAR was present in both neuronal cell bodies (Fig. 2A,C) and axonal growth cones (Fig. 2D,F). Actin filaments were observed forming a network within those regions (Fig. 2B,E).

Because growth cones are active structures essential for mediating neurites elongation, we then wanted to examine whether uPA promotes changes in their cytoskeleton. Within a growth cone, three different regions can be distinguished: the peripheral (P) domain, consisting primarily of filopodia; the transitional (T) domain, a band situated at the interface between the P and the central (C) domains; and the C domain, which comprises thicker regions composed of microtubules, organelles and vesicles of varying sizes. To study those morphological changes, an immunofluorescence for β_{III}-tubulin and actin filaments was performed on E7 OT explants treated with 10 ng/ml uPA for 30 min or nonstimulated (control explants). In uPA-stimulated neurons, the expanded growth cones contained a bundle of stable microtubules at the C domain and a network of F-actin filaments within the lamellipodia (P domain) (Fig. 2J–L). In addition, an extended area of microtubules-actin association was located at the growth cones T region, suggesting a local stabilization/destabilization dynamic process of cytoskeletal reorganization during axonal growth. In control conditions, however, all these cytoskeletal structures appeared significantly less developed (Fig. 2G–I).

Finally, to further study growth cones behavior in a more quantitative way, another group of E7 OT explants exposed to 10 ng/ml uPA for 30 min was immunolabeled for synaptotagmin and the following parameters were analyzed: (i) growth cones area and (ii) number of filopodia along neurites. Growth cones area was significantly higher in uPA-treated neurons than in controls (40.96 ± 9.22 μm^2 vs. 24.89 ± 8.46 μm^2; $P < 0.001$) (Fig. 3A,C,E). Additionally, the number of filopodia (measured along entire neurites and normalized as number of filopodia/100 μm) was significantly higher in uPA stimulated neurons compared with controls ($18/100$ μm ± 1 vs. $7/100$ μm ± 2; $P < 0.001$) (Fig. 3B,D,F).

Together, these data allow us to ascertain a new role for uPA in neuronal migration and neurite outgrowth, involving the induction of cytoskeletal rearrangements within axon shafts and growth cones, which promote changes in their shape.

$\alpha_5\beta_1$ Integrin Interacts With uPAR in Postmitotic Neurons

To date, several integrins have been involved in uPA-uPAR-mediated signaling (Franco et al., 2006; Blasi and Sidenius,

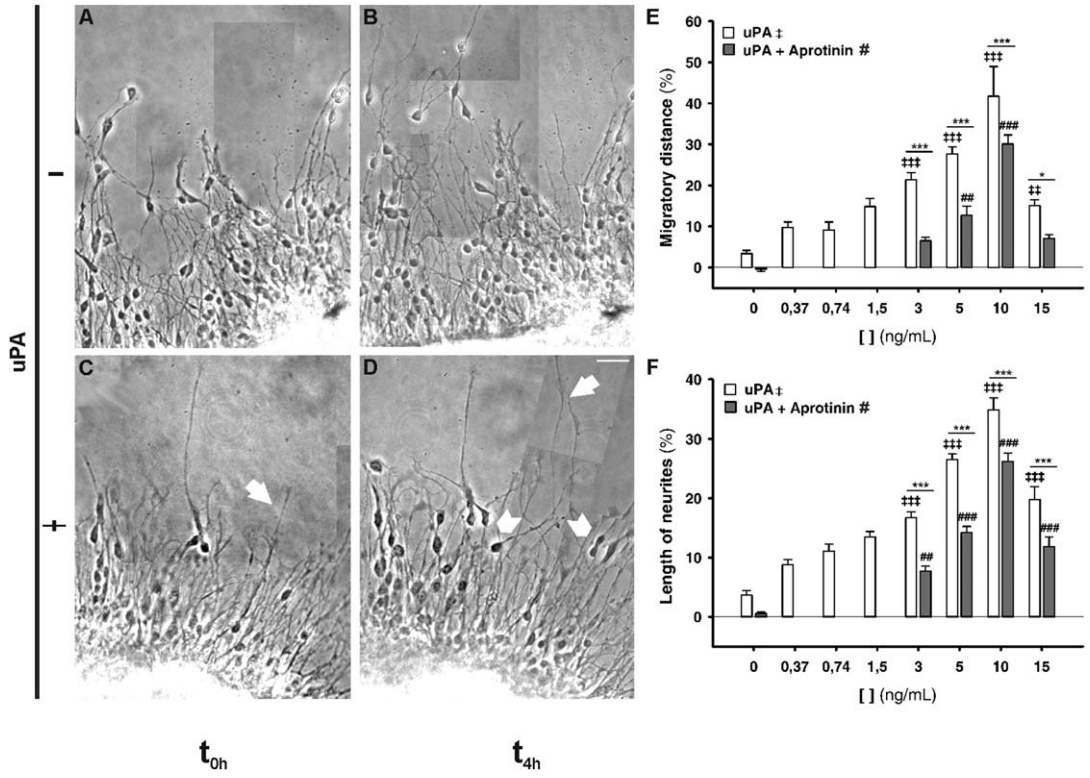

Fig. 1. uPA promotes neuronal migration and neuritogenesis. A–D: OT explants of E7 chick embryos showing uPA effects on neuronal migration and neurite outgrowth. The explants were incubated with uPA at different concentrations and analyzed before (t_{0h}) and 4 hr after uPA addition (t_{4h}). **A,B:** Control explants. **C,D:** uPA-stimulated explants. In response to uPA stimulation, neuronal somata moved away from the explant (as indicated by arrowheads), and neurites grew longer (as marked by arrows). **E,F:** Quantitative evaluation of neuronal migration and neurite outgrowth. Both neuronal migration and neurite outgrowth were enhanced concomitantly with uPA concentration, even in the presence of aprotinin. Data are presented as mean ± SEM ($n \geq 3$). Statistical analysis was performed using the one-way analysis of variance followed by the Tukey post hoc test. (The symbols illustrate statistical differences between treatments with and without aprotinin at a single uPA dose (*) or between controls and diverse uPA concentrations, in the absence (†) or presence (‡) of its inhibitor; e.g., $^{*}P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$). Scale bar = 25 μm.

2010); however, little is known about the specific interaction established between integrins and uPAR in migrating neurons. To study this interaction and the identity of the integrin subunits involved, double immunofluorescence staining for uPAR and α_5-, α_6-, or β_1-integrins were performed in explants nonstimulated and stimulated with a 2.5-min-long 10 ng/ml uPA pulse. In the absence of uPA, all the integrin subunits tested were found at neuronal cell bodies, but only α_5 and α_6 were also detected in neurites (Fig. 4A–C; upper row). Regarding the receptor, its expression was located primarily at the soma, and to a lesser extent at the initial part of some neurites. Following stimulation with uPA, a change in the expression pattern of both integrin subunits and uPAR was observed (Fig. 4A–C, lower row). α_5 and α_6 levels were significantly upregulated in neurites, while uPAR levels increased both in neurites and growth cones, as compared to untreated cells. Furthermore, β_1 expression was promoted in neurites by the uPA pulse. The quantitative analysis of the colocalization between uPAR and each of the integrin subunits tested was in good agreement with that observed by visual inspection. Exposure to uPA significantly increased the colocalization of uPAR with α_5 and β_1, but not with α_6 (Mander's colocalization coefficient (control vs. uPA): α_5: 0.6 ± 0.07 vs. 0.8 ± 0.04, $P = 0.032$; β_1: 0.7 ± 0.04 vs. 0.8 ± 0.05, $P = 0.011$; α_6: 0.6 ± 0.08 vs. 0.6 ± 0.2, $P = 0.01$) (Fig. 4D).

Considering the results derived from neuronal migration and neuritogenesis experiments, we then decided to study the interaction between integrins and uPAR in migrating neurons but after stimulation with a 15 ng/ml uPA pulse—a concentration at which both neuronal migration and neuritogenesis started to decrease. In control conditions, the pattern of expression was similar to the one described previously (Fig. 5A–C; upper row). However, following stimulation with uPA, the expression levels for uPAR and all the integrin subunits tested—but particularly for β_1—were considerably downregulated in neurites; while the corresponding levels at cell bodies remained unchanged (Fig. 5A–C, lower row).

To further prove the evidence derived from immunohistochemistry, coimmunoprecipitation analyses were performed in entire OTs in control conditions or following incubation with 10 ng/ml uPA for 2.5 min. The immunoprecipitates (IPs) obtained using antibodies to uPAR, α_5-, α_6-, or β_1-integrin subunits were tested by Western blotting for all the integrin subunits studied (α_5-, α_6-, and β_1-subunits) or with uPAR polyclonal antibody (Fig. 6). After uPA treatment, an upregulation of uPAR, α_5 and β_1 levels was observed in the corresponding IPs. By contrast, the coimmunoprecipitation between α_6 and β_1 showed a discrete reduction in the levels of both integrins in response to uPA. These results were in agreement with what was observed by immunohistochemistry,

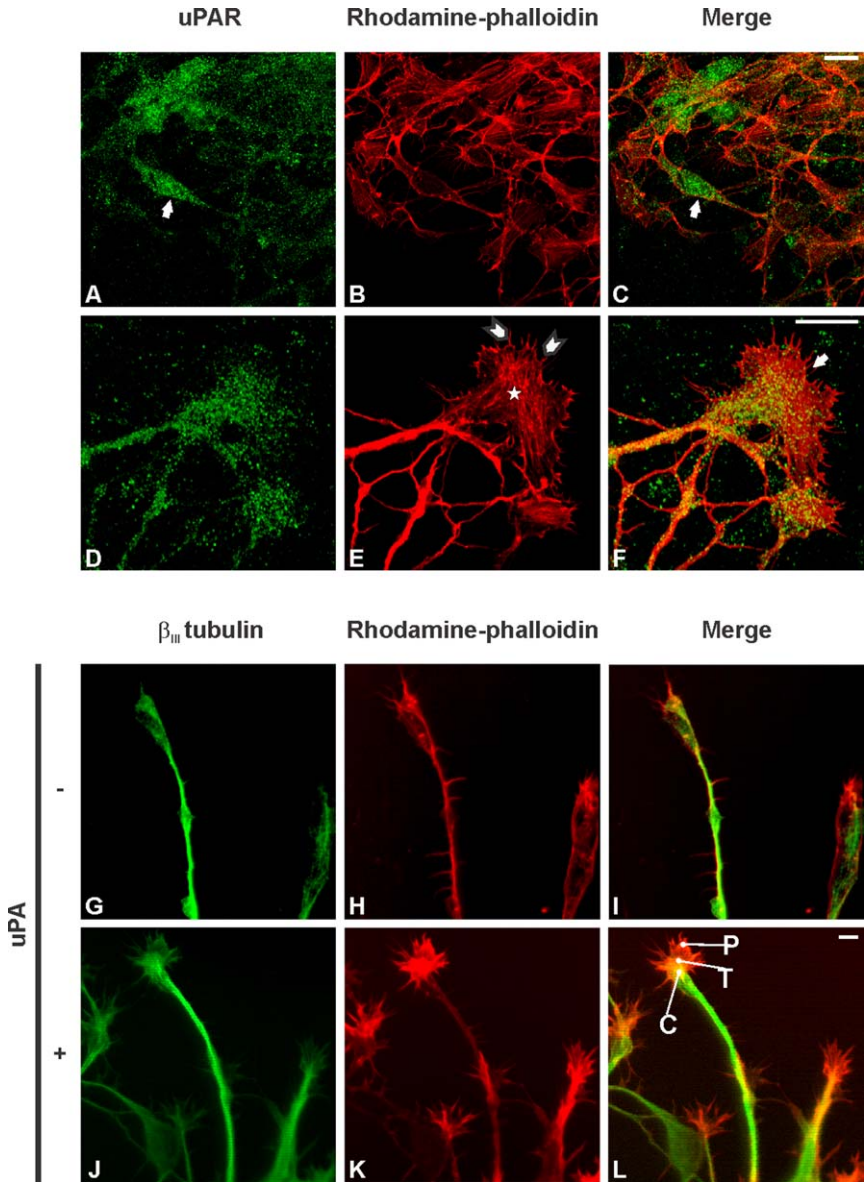

Fig. 2. uPAR is present in postmitotic neurons. **A–L**: Confocal micrographs of E7 chick embryos OT explants treated with 10 ng/ml uPA and immunostained with (A,D) anti-uPAR or (G,J) anti-β_{III}-tubulin (green), and (B,E,H,K) phalloidin-rhodamine (red) to mark actin filaments. C,F,I,L: Merges. Scale bar = 10.0 μm. As indicated by arrows, uPAR was located in both neuronal cell bodies (A,C) and axonal growth cones (D,F). Within the growth cone, two types of F-actin arrangements could be identified: an F-actin rib (star) and intrapodia (arrowheads) (E). G–L: In response to uPA treatment, the three characteristic domains of a growth cone (P, peripheral; T, transitional; C, central) could be clearly identified, being significantly more developed than in control conditions.

leading us to conclude that uPA induces colocalization among uPAR, α_5-, and β_1-integrin subunits in the developing OT.

uPA-uPAR Complex Promotes FAK Activation

It has been reported that both uPA and integrins promote FAK phosphorylation in several cellular models (Tang et al., 1998; Mitra and Schlaepfer, 2006). Besides, Tang et al. (2008) have shown, in human lung adenocarcinoma cell lines, that signaling through uPA and uPAR requires interactions with β_1-integrins. Given this evidence, we were interested in exploring whether a similar pathway may be involved in the uPA-uPAR-mediated signaling that results in stimulation of neuronal migration and neurite outgrowth in postmitotic neurons. To address this, we searched for changes in the level and intracellular distribution of phosphorylated FAK (pFAK) in response to uPA. Immunohistochemical assays showed that in untreated neurons pFAK was restricted to the soma (Fig. 7A), but after a 2.5 min uPA pulse it was also observed at neuronal processes (Fig. 7B). Doubling the incubation time produced an upregulation of pFAK within growth cones, which now reached the more distal end of lamellipodia (Fig. 7C). Similar results were observed when aprotinin was added previously to uPA stimulation

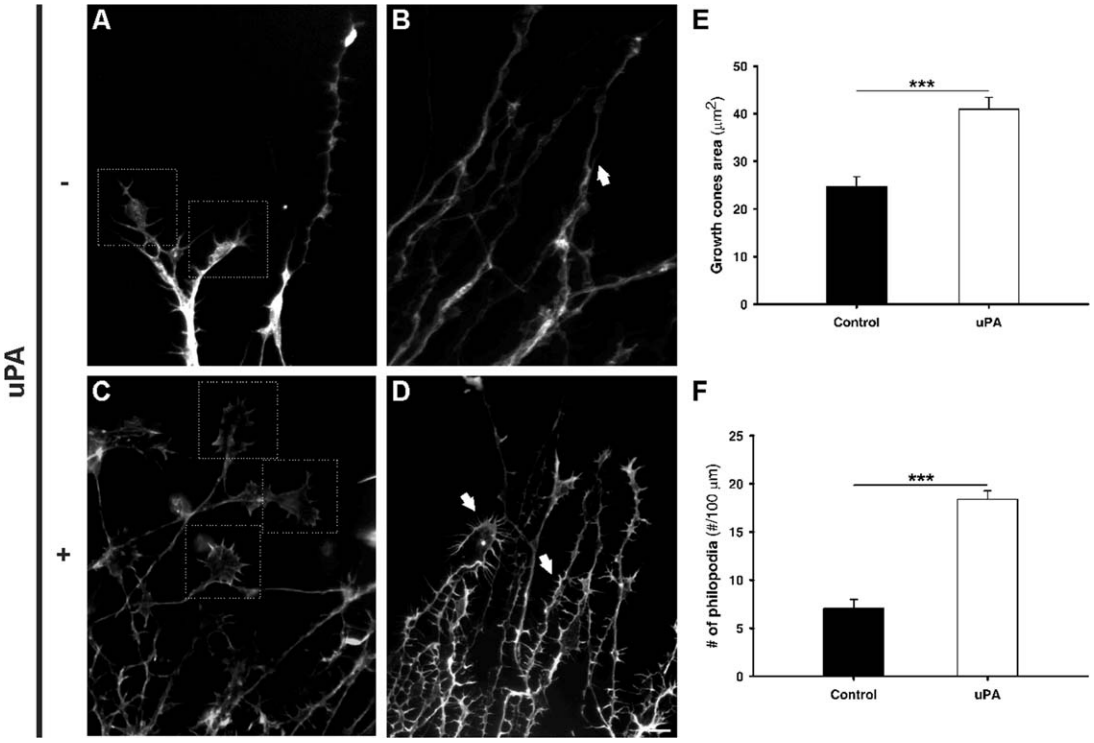

Fig. 3. uPA promotes morphological changes in growth cones. Representative images of E7 chick embryo OT explants showing uPA effects on growth cones area (**A,C**; boxes) and density of filopodia (**B,D**; arrows) (Scale bar = 20.0 μm). Explants were treated with 10 ng/ml uPA and photographed 30 min later. (**E,F**) Quantitative analysis of growth cones area and number of filopodia along neurites, respectively. Both parameters were significantly increased after uPA treatment (C,D) as compared to controls (A,B). Data are presented as mean ± SEM ($n \geq 3$). Statistical analysis was performed using the Student's *t*-test (***$P < 0.001$)

(Fig. 7D–F). Furthermore, it was noticed that both pFAK and actin filaments were placed quite close to each other at filopodia of uPA-stimulated neurites (Fig. 7G–I).

To ascertain whether the rise observed in pFAK levels entailed an increase in FAK expression, different cellular fractions were obtained from uPA-stimulated E7 OTs and analyzed for the detection of both pFAK and total FAK (tFAK). In perfect agreement with our previous findings, expression of pFAK was considerably raised following uPA stimulation, not only in the whole cell lysate (Fig. 8A), but also in both cytosolic and crude membrane fractions, as compared to control conditions (Fig. 8C,D). This raise was parallel with an increase in the amount of phospho-tyrosine (pTyr) after uPA treatment, as observed by western blot analysis of a cell lysate immunoprecipitated for tFAK (Fig. 8B). In contrast, tFAK levels were not significantly altered by the uPA pulse in any of the cellular fractions tested.

Finally, we evaluated pFAK levels in explants stimulated with a 15 ng/ml uPA pulse for 2.5 min. Our results show that FAK phosphorylation was markedly reduced at both cell lysates and subcellular fractions, although this decrease was more evident at the CMF (Fig. 9).

Together, these data suggest that uPA promotes FAK phosphorylation, but does not induce its expression, in the developing OT.

uPAR is Expressed in Migratory Cell Compartments During OT Development

In previous reports, we showed that uPA activity is spatiotemporally correlated with massive neuronal migration, neurite outgrowth, and synapse formation and maturation in the developing OT (Pereyra-Alfonso et al., 1997). In this study, we evaluated whether uPAR expression presents a developmental pattern compatible with the previously mentioned cellular processes (Fig. 10). The figure shows sections of the OT wall at different embryonic ages (E) and OT developmental stages (DS).

Between DS1 (E2) and DS4 (E6), all neuroepithelial cells and postmitotic neurons display high uPAR cytoplasmic immunoreactivity. During DS1 and DS2, the premigratory zone (PMZ) is mainly occupied by neurons from the transitory cell compartment 1 (TCC1) whose basal processes originate uPAR positive ($^+$) tangential axons (Fig. 10B).

From DS3 onward, the PMZ is gradually occupied by the cohort of neurons that perform radial migration, TCC1 becomes occupied by uPAR$^+$ tangential neurons that invade the marginal zone, uPAR$^+$ neuroepithelial cells basal processes and uPAR$^+$ radial migrating neurons. When TCC2 emerges (DS4-DS5), it becomes occupied by high uPAR$^+$ migrating neurons and slight uPAR$^+$ round-shaped neurons.

Between DS4 and DS6 (E6–E10), neuroepithelial cells and migrating neurons display high uPAR immunoreactivity. Differentiating neurons also display uPAR immunoreactivity, but with some heterogeneity. The generation zone (GZ) and the PMZ still display high uPAR reactivity. Compartment "SGP" (stratum griseum periventriculare) neurons, that are generating a complex pattern of neurites, display intense uPAR immunoreactivity. The appearance of the C "SAC" (stratum albus central) allows the identification of radial uPAR$^+$ migrating neurons that display the periodic pattern of neuroepithelial cells basal processes (Fig. 10B).

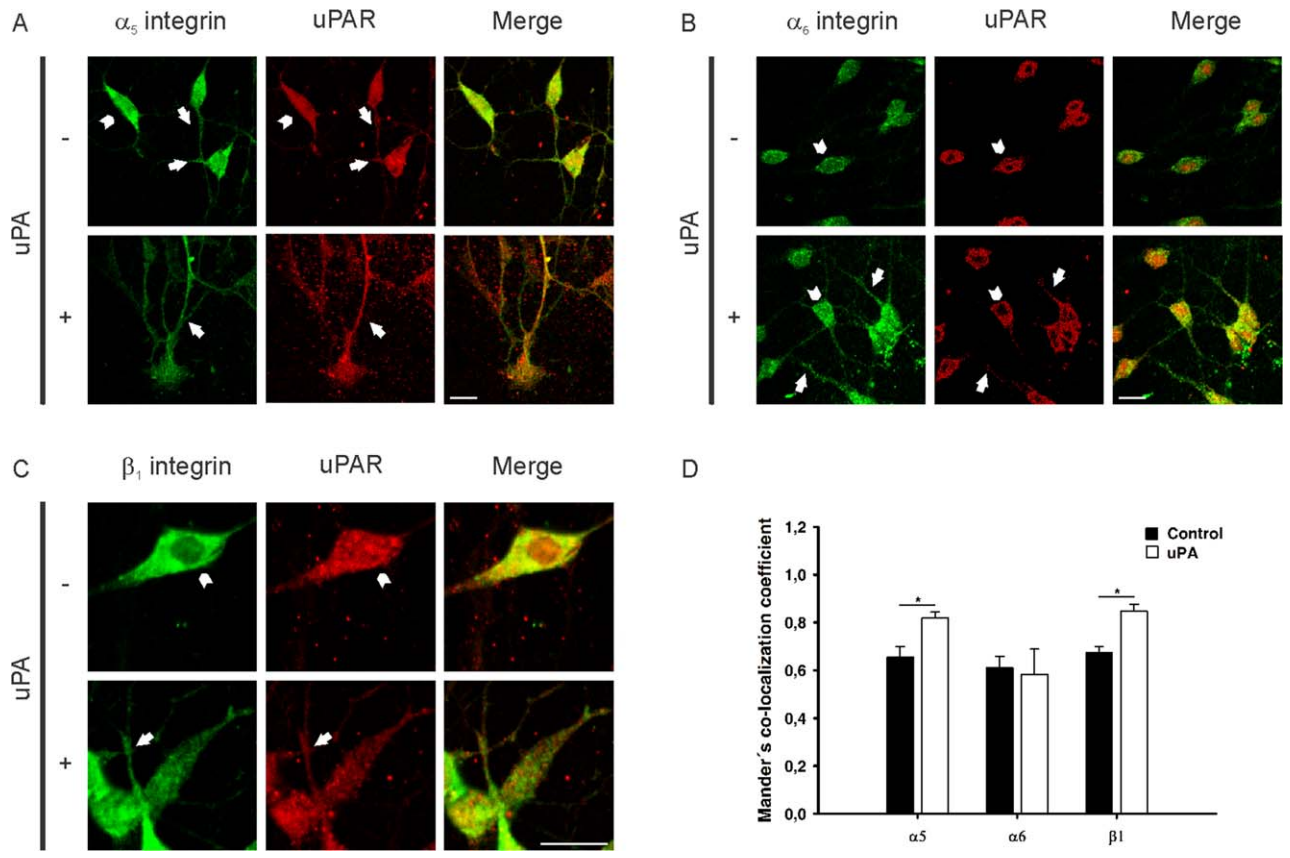

Fig. 4. uPA promotes the interaction between uPAR and both α_5- and β_1-integrins in postmitotic neurons. **A–C:** Confocal microphotographs of E7 chick embryo OT explants treated with 10 ng/ml uPA and immunostained with anti-uPAR and (A) anti-α_5-integrin, (B) anti-α_6-integrin, or (C) anti-β_1-integrin, respectively. uPA modifies the localization of both uPAR and the integrin subunits within migrating neurons, up-regulating their levels in neurites and growth cones (arrows; the arrowheads denote neuronal somas). **D:** Colocalization analysis for uPAR with each of the integrin subunits considered. A significant increase in colocalization of α5- and β1-integrin subunits was evidence following uPA treatment. Data are presented as mean ± SEM ($n \geq 3$). Statistical analysis was performed using the Student's *t*-test (*$P < 0.05$). Scale bar = 10 μm.

Differentiating C "SGC" (stratum griseum centrale) neurons display slight immunoreactivity. High uPAR$^+$ radial migrating neurons can also be observed in transit through superficial transient cell compartments intermingled with slight uPAR$^+$ differentiating neurons.

Between DS6 and DS7, PMZ thickness and radial migration decrease. The higher uPAR reactivity can be observed in scattered migrating neurons that can be observed through all transitory cell compartments and in the soma of big differentiating neurons corresponding to the C "SGC" and to the C "i", that are entering synaptogenesis.

These results indicate that uPAR expression is spatiotemporally related to neuronal migration during the lamination process and to events of cell differentiation such as neuritogenesis and synaptogenesis.

Discussion

Neuronal migration and neuritogenesis are essential for the architectural and functional development of the CNS. During this stage, migrating neurons undergo a series of modifications which involve (I) the interaction of extracellular signals with cell surface receptors and (II) the activation of downstream signaling pathways that ultimately lead to the regulation of cytoskeletal dynamics. Studies of the cellular and molecular mechanisms that control those morphological changes are vital to further understand neural development. In fact, defects in either of these processes have been linked with several human diseases (Valiente and Marín, 2010; Spreafico and Blümcke, 2010).

In accordance with the aforementioned, our aim was to investigate the nonproteolytic role of uPA and its influence on neuronal migration and neuritogenesis during development of cortical structures. In previous reports, we have analyzed the expression and the proteolytic activity of uPA in the developing OT (Pereyra-Alfonso et al., 1997), describing a bimodal pattern of enzymatic activity with two peaks that temporally match with events involving neuronal migration and neurite outgrowth (E6–E12) and subsequent synaptogenesis and plasticity (E18–E21). However, the relationship between the mentioned processes and the nonproteolytic role of the uPA-uPAR complex has not been explored until now.

The data presented in this study demonstrate that, besides its proteolytic role, uPA promotes neuronal migration and neuritogenesis through a nonproteolytic mechanism in a dose-dependent manner. These findings are consistent with previous evidence showing that uPA promotes cell migration in both MCF-7 breast cancer cells (Carriero et al., 1999) and HT 1080 fibrosarcoma cells (Nguyen et al., 1999) by triggering intracellular signaling cascades. This evidence, together with our findings, prompted us to hypothesize that the interaction between uPA and its receptor at the surface of migrating neurons would be

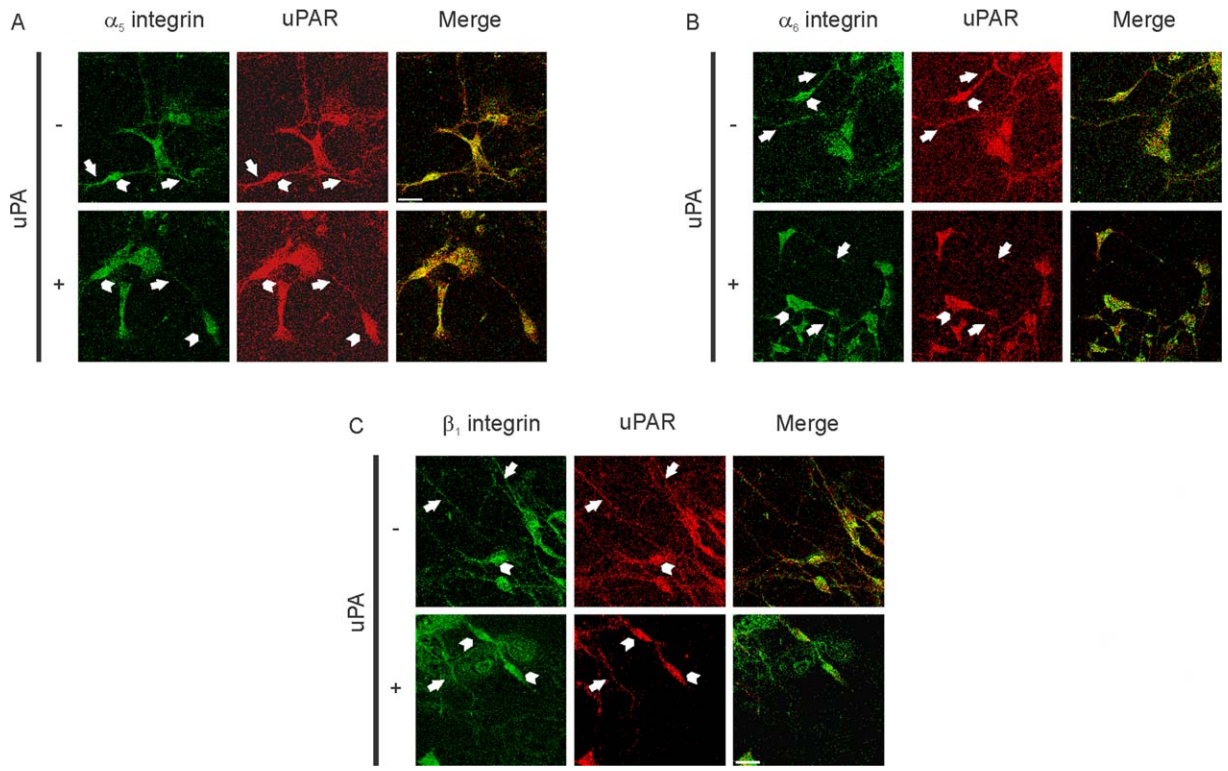

Fig. 5. uPA effect upon the interaction between uPAR and its coreceptors is concentration-dependent. **A–C:** Confocal microphotographs of E7 chick embryo OT explants treated with 15 ng/ml uPA and immunostained with anti-uPAR and (A) anti-α_5-integrin, (B) anti-α_6-integrin, or (C) anti-β_1-integrin, respectively. (Arrows denote neurites and the arrowheads signalize neuronal somas). Contrary to what was observed following 10 ng/ml uPA treatment, the expression levels for uPAR and all the integrins subunits tested were upregulated in neurites, remaining unchanged at cell bodies. Scale bar = 10 μm.

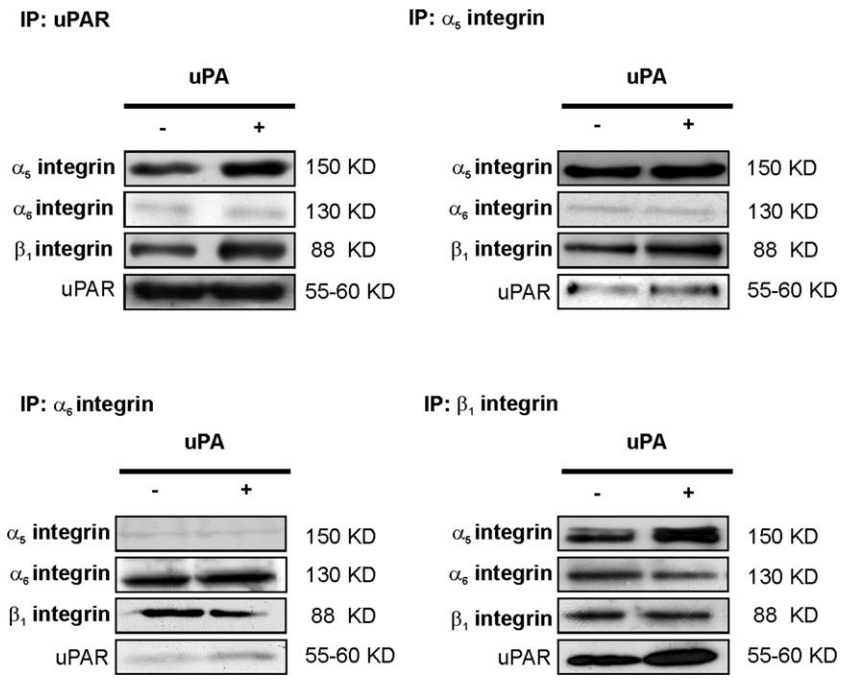

Fig. 6. uPA induces colocalization of uPAR with α_5- and β_1-integrins in the developing OT. Cell lysates of E7 chick embryo OT were stimulated with uPA and immunoprecipitated (IP) for uPAR, α_5-, α_6- or β_1-integrin subunit. Immunoblots for detection of all integrin subunits and uPAR are shown. The degree of uPAR and α5- and β1-integrin subunits coimmunoprecipitation was markedly increased in the developing OT after uPA treatment.

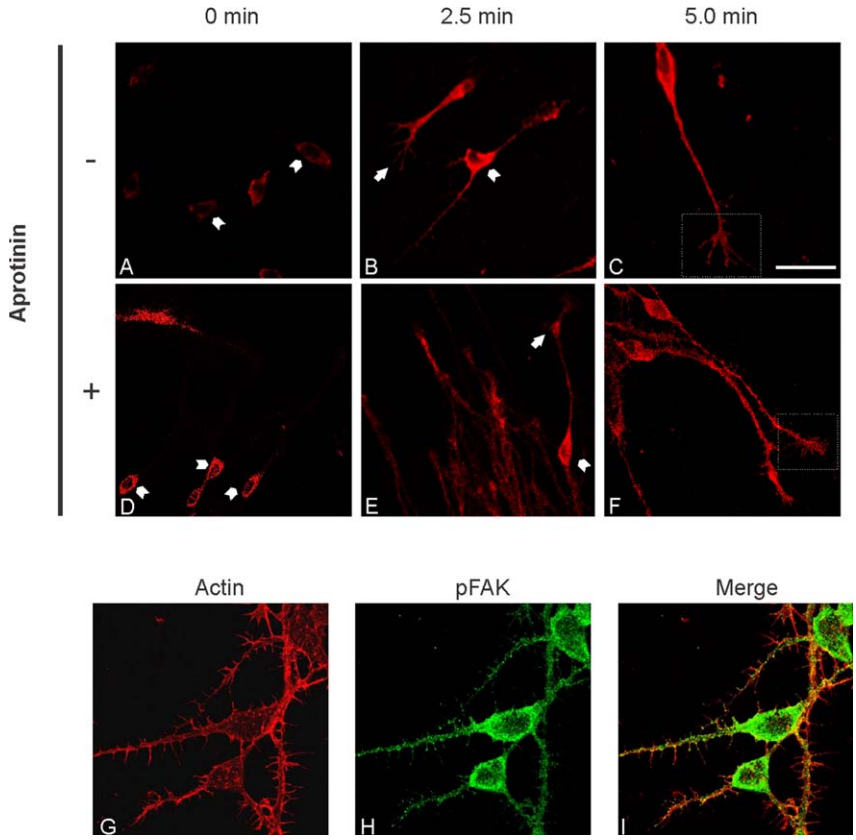

Fig. 7. uPA promotes pFAK expression within growth cones. **A–C:** Confocal microphotographs of E7 chick embryo OT explants treated with uPA at different times and immunolabeled for pFAK. In response to uPA stimulation, the intracellular levels of pFAK increased. At 0 min, pFAK was observed only in the soma (A; arrowheads). However, after 2.5-min incubation with uPA, it also appeared at neuronal processes at first (B; arrow), and later, within growth cones (C; box), reaching the more distal end of the lamellipodia. **D–F:** Comparable results were observed when 10 μM aprotinin was added to the explants before uPA stimulation. **G–I:** Immunohistochemical localization of actin and pFAK in somas and neurites of postmitotic neurons in response to uPA stimulation. Both molecules were located at growing neurites filopodia. Scale bar = 10 μm.

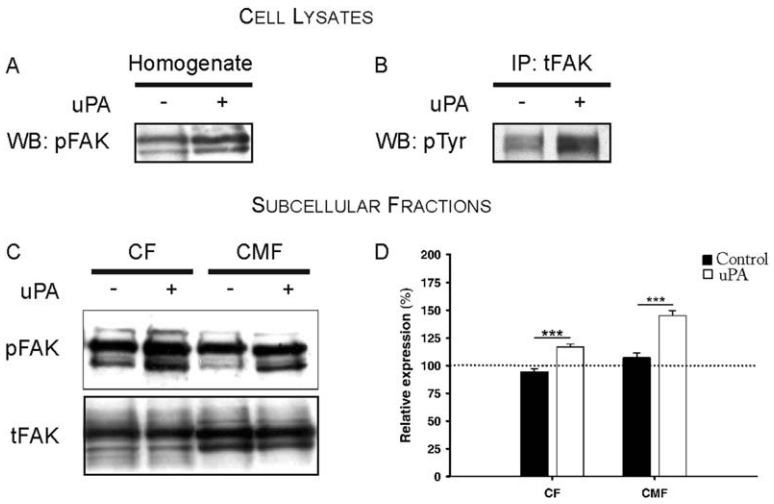

Fig. 8. uPA promotes FAK phosphorylation in the developing OT. E7 chick embryo OTs were incubated with uPA and homogenized. **A:** Immunoblotting for pFAK. **B:** Western blot for the detection of pTyr in a fraction immunoprecipitated for tFAK. **C:** Immunoblotting for pFAK and tFAK of both cytosolic (CF) and crude membrane (CMF) subcellular fractions. **D:** Quantitative evaluation of pFAK and tFAK expression. Differences in the levels of both molecules in experimental conditions were normalized to their corresponding levels in control conditions. The analysis reveals a significant increase in the levels of pFAK, but not of tFAK, in response to uPA stimulation. Data are presented as mean ± SEM ($n \geq 3$). Statistical analysis was performed using the Student's *t*-test (***$P \leq 0.001$)

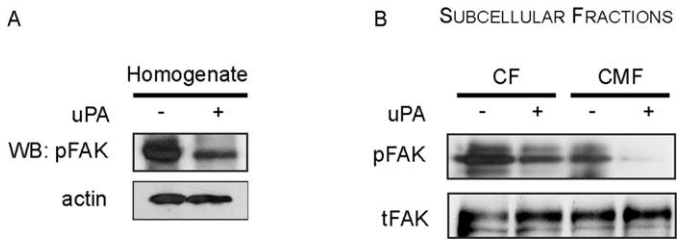

Fig. 9. uPA effect upon phosphorylation is concentration dependent. E7 chick embryo OT were incubated with uPA and homogenized. **A:** Immunoblotting of the homogenate for pFAK detection. **B:** Immunoblotting for pFAK and tFAK of both cytosolic (CF) and crude membrane (CMF) subcellular fractions. A decrease in the levels of pFAK was observed in both the homogenate and the CMF following stimulation with a 15 ng/ml uPA pulse.

responsible for the activation of intracellular signaling cascades which promote cytoskeletal reorganization, culminating in neuronal migration and neuritogenesis. The corresponding mechanism is not dependent on uPA proteolytic activity, because aprotinin was ineffective in blocking those processes.

Because uPAR is a GPI-anchored protein, it can be inferred that an intracellular coreceptor is required to develop uPA-dependent signaling. In this regard, there is substantial evidence suggesting that integrins function as coreceptors in pathways involving uPAR. Several studies show that, at the developing CNS, β_1 integrins are vital for mediating cell adhesion and migration of neuronal populations. Other evidence arises from a report indicating that neuronal migration in the developing OT is markedly reduced after retroviral infection with β_1- and α_6-integrin subunits antisense mRNAs (Zhang and Galileo, 1998). Additionally, Anton et al. (1999) have shown that $\alpha_3\beta_1$ integrin is a key component in cell adhesion during migration of cortical neurons along glial cells. Our data show that, following uPA stimulation, uPAR interacts with α_5- and β_1-integrin subunits, leading to the activation of protein kinases. In the absence of uPA, there is a weak interaction between uPAR and the corresponding integrin subunits, which becomes strengthened when uPA binds to its receptor, as evidenced using immunological methods. In addition, we suggest that the α_6-integrin subunit may not be participating as a coreceptor in the signaling pathway mediated by the uPA-uPAR complex; an idea supported by diverse evidence. Several studies have shown that, among the numerous integrins involved in the development of the NS, $\alpha_5\beta_1$—together with $\alpha_3\beta_1$—are those which have the major affinity for uPAR (Blasi and Carmeliet, 2002). In addition, it is well known that $\alpha_6\beta_1$ is involved in cortical lamination (Georges-Labousse et al., 1998), during which it functions as a laminin receptor. Thus, even though an increase in the expression of α_6-integrin subunit at neurites would be expected—because the interaction of $\alpha_6\beta_1$ with laminin is necessary for triggering neuronal migration and neurite outgrowth—it could be the case that the role of α_6 in those processes would be related to the activation of signaling pathways different from those stimulated by uPA. Consequently, an increase in the colocalization levels for uPAR and α_6 would not be detected. Moreover, it should also be considered that—at the developing chick embryo—α_6 expression is considerably lower in the OT compared with other tissues (Bronner-Fraser et al., 1992). In conclusion, it would be unlikely that a substantial increase in colocalization between this integrin subunit and uPAR could be noticed. According to these results, we suggest that $\alpha_5\beta_1$-integrin functions as a coreceptor for the uPA-uPAR complex, mediating the uPA nonproteolytic response at the developing OT. Nonetheless, we cannot exclude the possibility that other molecules, such as END0180 or LRP, could be acting as coreceptors too. In fact, these coreceptors could be responsible for what was observed at uPA concentrations above 10 ng/ml, which may be explained by a change in the affinity of uPAR for integrins, resulting in a novel interaction between uPAR and other coreceptors that may activate other signaling pathways.

Considering the fact that motile behaviors of growth cones at the ends of elongating axons require the dynamic reorganization of the cytoskeleton, and because it has been described that both uPAR and integrins interact with molecules commonly related to cytoskeletal structures, we proposed that the role of the formers in the OT development may involve the regulation of growth cones actin cytoskeleton (Berstein et al., 2004); actin filaments play a central role in neuronal migration and neurite outgrowth, and are primarily located at the leading edge of growth cones, being the main target for signaling molecules. Our findings suggest that the coupling between the uPA-uPAR complex and the $\alpha_5\beta_1$-integrin occurs within a very short time, and probably triggers downstream signaling pathways which include kinases activation and F-actin redistribution inside axonal growth cones.

Further evidence indicates that uPAR colocalizes with integrin-containing adhesion complexes and coimmunoprecipitates with signaling molecules, such as FAK and Src kinases (Wei et al., 2001). Accordingly, Tang et al. (2008) have shown—in lung cancer cells—that a particular region at one of uPAR domains (D3 domain) has a corresponding binding site for β_1-integrin, being the interaction between uPAR and β_1-integrin crucial for ERK activation. In our model, a comparable interaction proved to be essential for FAK activation, an event that occurs within the first 2 min that follow uPA stimulation, which is in accordance with existing evidence from different cell lines (Tang et al., 1998). Our results show that total FAK levels were similar in both control and experimental conditions. However, its subcellular distribution differed. This observation suggests that uPA stimulation promotes tFAK redistribution from the cytosolic fraction (CF) to the crude membrane fraction (CMF), which includes the growth cones. After uPA treatment, FAK phosporilation increased in experimental neuronal populations compared with control ones. In addition, the analysis of the subcellular distribution of pFAK showed significant differences between control and experimental conditions. In control neurons, most pFAK was present in the CF, but, after uPA stimulation, pFAK was found mostly in the CMF.

Similarly, immunohistochemical studies showed a redistribution of the phosphorylated enzyme to neuronal processes and growth cones after uPA stimulation. In agreement with these last results and the fact that one of the most important roles of FAK

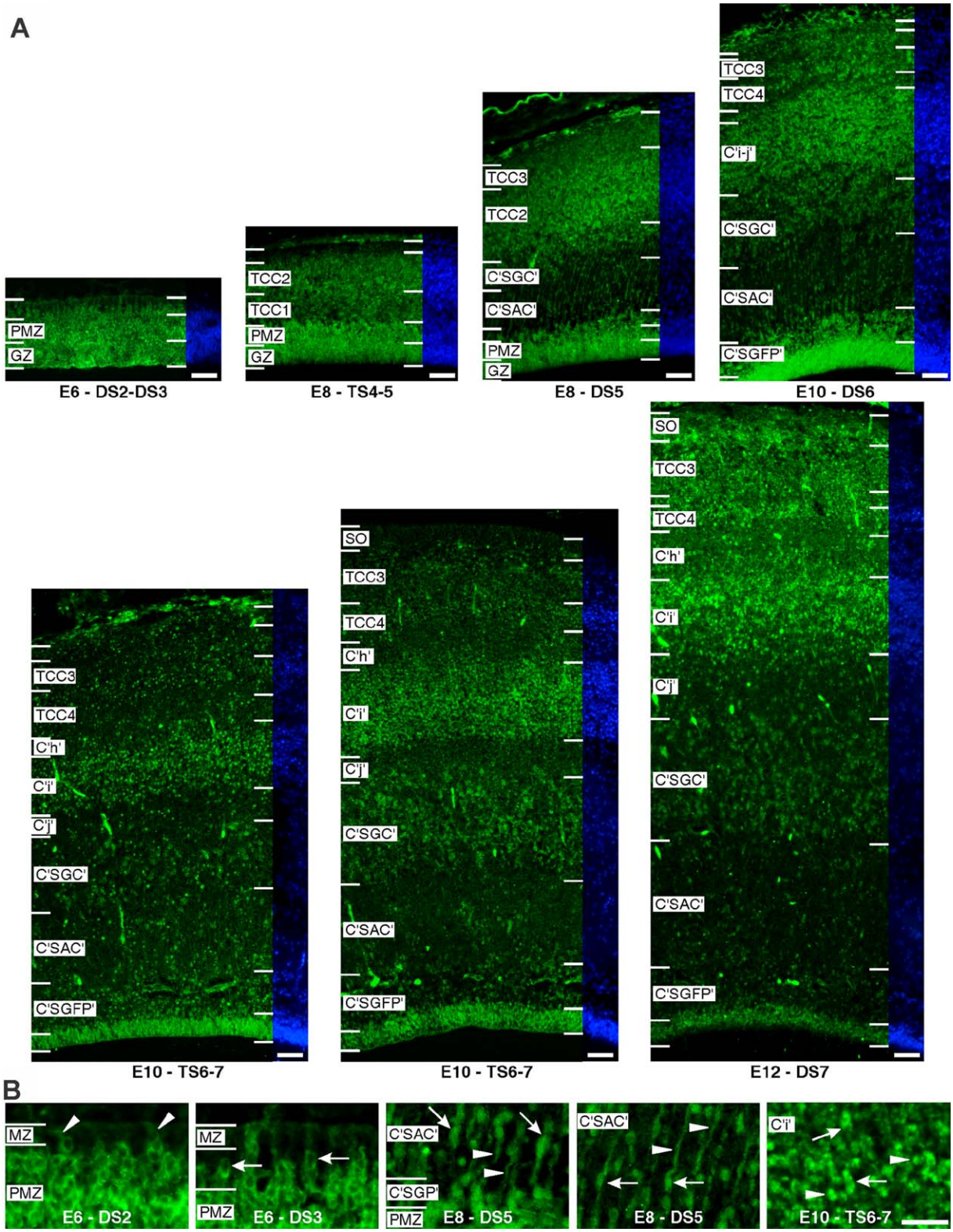

Fig. 10. uPAR expression in the developing optic tectum. **A:** Slices of OTs at different developmental stages immunostained with anti-uPAR. **B:** Detail of uPAR expression in different compartments during OT development. Arrowheads indicated neurites, and arrows, the soma; both uPAR positive. uPAR was preferably expressed in transitory cell compartments (TCC), which are composed of migrating neurons that will generate the future definitive laminar tectal structure. (References: Ventricular zone (VZ); subventricular zone (SVZ); premigratory zone (PMZ); the transitory cell compartment 1 (TCC1), TCC2, TCC3, TCC4; C "stratum griseum periventriculare" (C "SGP"); C "stratum album centrale" (C "SAC"); C "stratum griseum centrale" (C "SGC"); C "j"; C "i"; C "h"; layers a, b, c, d, e, f, g, h, i, and j of the "stratum griseum et fibrosum superficiale" (SGFS); "stratum opticum" (SO) (for nomenclature see Scicolone et al., 1995; Rapacioli et al., 2011)).

involves the rearrangement of the actin cytoskeleton, the analysis of growth cones revealed enlarged structures with a more developed actin network. Thus, we propose the existence of a close relationship between uPA, FAK activation and F-actin rearrangement. When higher doses of uPA were used, FAK phosphorilation was markedly reduced. According to what was stated previously, the interaction of uPAR with coreceptors different from integrins could lead to the activation of other signaling pathways which may not involved FAK activation. However, conclusive evidence is still lacking. Using different methodological approaches, we have demonstrated that a relatively short-term stimulation is sufficient to observe uPA effects. And given that this response is dose-dependent, those effects may depend on uPA availability. Hence, we suggest that uPA may act as a regulatory molecule, tuning neuronal migration and neurite outgrowth during CNS development.

The data presented in this study demonstrate that uPA nonproteolytic effect upon cellular migration and neurite outgrowth at the developing OT involves an interaction between the uPA-uPAR complex and $\alpha_5\beta_1$-integrin, which in turn could trigger cytoplasmic kinases cascades that ultimately catalyze cytoskeletal reorganization.

In summary, both proteolytic and nonproteolytic roles of uPA are mediated by uPAR, which proved to be essential for orchestrating the activation of intracellular signaling pathways and pericellular proteolysis, necessary for cellular migration. Even though this hypothesis will require further examination, our results are a great step forward for the better understanding of the complex interactions established between molecules that are implicated in controlling several aspects of cell behavior.

Experimental Procedures

Animals

Pathogen-free fertilized White Leghorn chicken eggs were obtained from commercial breeders (Rosenbusch Institute, Buenos Aires, Argentina) and maintained at 39°C in a humidified incubator. Embryos were staged according to Hamburger and Hamilton (HH) (Hamburger and Hamilton, 1992) and the corresponding embryonic age (in embryonic days, E) was indicated.

Explants Cultures

Explants cultures were prepared from OT cephalic portions of 6.5- to 7-day-old (E6.5–E7; HH29–HH30) chick embryos. Explants were plated on coverslips coated with 200 μg/ml poly-L-lysine (Invitrogen) and 20 μg/ml laminin (Invitrogen) in F12/DMEM (Invitrogen) supplemented with 0.4% methylcellulose (Sigma-Aldrich Co.), 2.5 mM glutamine (Invitrogen) and 1% N2 (Invitrogen), for 18 hr with 5% CO_2 at 37°C.

Neuronal Migration and Neuritogenesis Analysis

To study neuronal migration and neuritogenesis, explants were incubated with different uPA concentrations (0.4–20 ng/ml; EMD Chemicals Inc.) for 4 hr. A fraction of the explants stimulated with 3, 5, 10, and 15 ng/ml uPA was also treated with 10 μM aprotinin (Sigma-Aldrich Co.) before uPA addition. Aprotinin works as a potent serine protease inhibitor, completely blocking uPA proteolytic activity at that concentration (Stonelake et al., 1997; Delannoy-Courdent et al., 1998).

Explants were photographed before (t_0: 0 hr) and after uPA treatment (t_4: 4 hr) using a Nikon Eclipse TS100 inverted microscope coupled to a Nikon Coolpix 4500 digital camera. The corresponding micrographs were edited with Adobe Photoshop 7.0 (Adobe Systems). Image Pro Plus 6.0 (Media Cybernetics Inc.) was used for taking semi-automatic quantitative measurements of image data.

Neuronal migration pattern.

To analyze neuronal migration, the average distance between the edge of the explant and the 50-more distant somas was determined at t_0 and t_4 over the same section of a given explant. The distance covered by migrating neurons in experimental conditions was expressed as a ratio (t_4/t_0) and normalized in relation to the one corresponding to control conditions.

Neurite growth pattern.

To analyze neurite outgrowth, the average length of the 50-longest neurites was measured at t_0 and t_4 over the same section of a given explant. The distance traversed by neurites in experimental conditions was expressed as a ratio (t_4/t_0) and normalized in relation to the corresponding control ratio.

Morphological changes in axonal growth cones.

For the analysis of morphological changes in neurites, both growth cones area and number of filopodia were measured in control and 10 ng/ml uPA-stimulated explants after 30 min of treatment. Then, the cultures were fixed with 4% paraformaldehyde and 4% sacarose (Sigma-Aldrich Co.) in 0.1 M phosphate buffered saline (PBS) for 30 min at room temperature (RT), and analyzed.

Immunohistochemistry

Cultured explants were treated with 10 or 15 ng/ml uPA as appropriate; in particular, for studying its effect over time, 2.5- and 5-min-long pulses were applied. Following stimulation, explants were fixed with 4% paraformaldehyde and 4% sucrose in 0.1 M PBS. After extensive washes with PBS, the tissue was soaked in blocking solution (4% normal goat serum [NGS] in 0.1 M PBS), and subsequently incubated with the following primary antibodies ON at 4°C: rabbit anti-uPAR (2 μg/ml; Santa Cruz Biotechnology Inc.; the specificity of the antibody for the chicken uPAR was confirmed by WB, being comparable with that corresponding to the mouse protein [data not shown]) and mouse anti-α_5-, α_6-, or β_1-integrin subunits (3 μg/ml; Developmental Studies Hybridoma Bank; these antibodies were developed by Horwitz AF specifically for chicken integrins detection [α_5: Muschler and Horwitz, 1991; Lakonishok et al., 1992; α_6: Bronner-Fraser et al., 1992; Zagris et al., 2009; β_1: Neff et al., 1982; Nagy et al., 2009]) for colocalization analysis; rabbit anti-total FAK (tFAK) and rabbit anti-phosphorylated FAK (pFAK) antibodies (1 μg/ml; Santa Cruz Biotechnology Inc. and Calbiochem Co., respectively) for FAK detection; Phalloidin-TRITC (1:1,500; Sigma-Aldrich Co.) and mouse anti-β_{III}-tubulin antibody (1:500; Abcam) for detection of cytoskeletal components; and mouse anti-

synaptotagmin I (0.5 μg/ml; Chemicon International) for measuring growth cones area and number of filopodia. Then, explants were incubated with the corresponding secondary antibodies: Alexa Fluor 594 goat anti-rabbit IgG or Alexa Fluor 488 $F(ab')_2$ fragment of goat anti-mouse IgG (2 μg/ml; Invitrogen Co.). Images were obtained using an Olympus FV300 laser confocal microscope and processed with Image Pro Plus 6.0 software. For colocalization analysis, Mander's overlap coefficient was used.

uPAR Expression Pattern in the Developing Optic Tectum

To describe the developmental pattern of uPAR expression, OTs from E6–E12 chick embryos were dissected in ice-cold 0.1 M PBS (pH 7.4) and immediately fixed by immersion in 4% paraformaldehyde for 4 hr at RT. After fixation, the specimens were cryoprotected with 20% sucrose in PBS and subsequently frozen in a 1:1 (v/v) mixture of 20% sucrose solution and tissue freezing medium (Tissue-Tek OCT Compound, Sakura Finetek). Cryosections (12 μm thick) were obtained (Leica cryostate CM 1900), collected on gelatinized slides and stored at −20°C until use.

Before the immunolabeling, sections were thawed and rinsed in PBS. Blocking of nonspecific binding and permeabilization were simultaneously performed by preincubating the sections with 5% NGS in PBS containing 0.3% Triton X-100 for 1 hr at RT in a humidification chamber. Afterward, sections were incubated ON with a rabbit anti-uPAR antibody (2 μg/ml; Santa Cruz Biotechnology Inc.) in blocking solution at 4°C in a humidification chamber, and subsequently rinsed in PBS and incubated with Alexa Fluor 488 goat anti-rabbit IgG (H1L) (1:1,000; A-11008, Molecular Probes) for 2 hr at RT in a dark humidification chamber. Sections were washed again with PBS and the slides finally mounted with polyvinyl alcohol mounting medium with DABCO antifading (10981, Fluka).

Nomenclature.

To characterize the differential expression of uPAR throughout embryonic development, we used a nomenclature that describes tectal lamination as a dynamic process of transient cell compartments (TCCs) segregation and establishes tectal developmental stages (DS) according to that process (Scicolone et al., 1995; Rapacioli et al., 2011). This categorization is based on histogenetic changes and expression of several proteins which regulate cell behavior in the developing OT. The designation of the embryonic layers as "transient cell compartments" emphasizes the fact that those are not precursors of particular adult layers, but transient aggregates of neurons which later segregate into several different definitive layers. In this nomenclature, the different TCCs that successively appear during development are designated with a number that refers to their chronological order of appearance. The embryonic layers are designated with a more specific name only when they can be topographically identified as precursors of particular definitive layers.

Immunoprecipitation of uPAR and Integrin Subunits

OT explants were, first, incubated with 10 ng/ml uPA for 2.5 min at 37°C. The incubation was halted by rapid aspiration of the culture media and the corresponding tissue was homogenized in cold lysis buffer (50 mM Tris-HCl, 150 mM NaCl, 0.5% Triton X-100, 1 mM PMSF, 1 mg/ml Na_3VO_4 and a protease inhibitors cocktail [1:100; catalogue #P2714, Sigma-Aldrich Co]). Protein concentration was assayed by using a BCA Protein Kit (Pierce Biotechnology). Equal amounts of cell lysates ([Proteins] = 2 mg/ml) were precleared with A/G sepharose (protein A sepharose for polyclonal antibodies and protein G sepharose for monoclonal antibodies) and centrifuged for 1 min at 14,000 rpm. The corresponding supernatants were incubated with 8 μg rabbit anti-uPAR antibody, 16 μg mouse anti-α_5-, α_6-, or β_1-integrin antibodies or 8 μg rabbit anti-tFAK antibody ON at 4°C, and then precipitated with protein A/G sepharose. The immunoprecipitates (IPs) were obtained by centrifugation for 10 min at 14,000 rpm, electrophoresed on 10% polyacrylamide gels, and then transferred to PVDF membranes (GE Healthcare Life Sciences). The immunoblots were probed with anti-α_5-, α_6-, or β_1-integrin antibodies, or with anti-uPAR antibody. Afterward, blots were incubated with a horseradish peroxidase conjugated-secondary antibody (1:1,000; Santa Cruz Biotechnology Inc.) and developed with Enhanced Chemiluminescence (ECL) Western Blotting Substrate (Pierce Biotechnology). Quantitative analysis was performed with Gel Pro Analyzer Software (Media Cybernetics).

Detection of FAK Activation

To assay FAK activation, differential centrifugation of whole cell lysates was performed. After an initial centrifugation at 800 g for 10 min, the resultant pellet—corresponding to the nuclear fraction (NF)—was isolated,. The supernatant was then subjected to a second centrifugation at 11,500 g for 30 min. The soluble fraction—designated as cytosolic fraction (CF)—contained cytosolic proteins, while the pellet—the crude membrane fraction (CMF)—included neurite endings.

Equal amounts of protein from cell lysates, CF and CMF samples, belonging to control and uPA-stimulated OTs, were electrophoresed on 10% polyacrylamide gels and transferred to PVDF membranes. The corresponding blots were incubated ON at 4°C with the following primary antibodies as appropriate: rabbit anti-tFAK (0.5 μg/ml), rabbit anti-pFAK (0.5 μg/ml) or mouse anti-pTyr (1 μg/ml; clone 4G10, Upstate); and then for 2 hr at RT with the corresponding secondary antibodies. Enhanced Chemiluminescence (ECL) Western Blotting Substrate (Pierce Biotechnology) was used for developing the blots.

Quantitative analysis was performed using Gel Pro Analyzer Software. For quantification of tFAK and pFAK, the sum of the Integrated Optic Density (IOD) belonging to both subcellular fractions (CF+CMF) from control and stimulated OTs was considered as 100% (IOD_t, total IOD). The percentage corresponding to each subcellular fraction was then calculated as the ratio between the IOD for each fraction (IOD_f) and IOD_t. Finally, a comparison between control and experimental conditions was made considering the subcellular distribution of tFAK or pFAK (%IOD corresponding to each fraction), respectively.

Statistical Analysis

All quantitative data are presented as mean ± SEM. Statistical analysis was performed using the statistical software GraphPad Prism 5.0 (GraphPad Software). For neuronal migration and neuritogenesis patterns evaluation, one-way analysis of variance

followed by a Tukey post hoc test was run. For morphological changes analysis and protein levels quantification the Student's *t*-test was performed. Statistical significance was set at $P < 0.05$.

Acknowledgments

We thank Florencia Mallou and Joaquín Díaz Carrizo for their unceasing collaboration and technical support.

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