Experimental Neurology 248 (2013) 262–274

Contents lists available at ScienceDirect

Experimental Neurology

journal homepage: www.elsevier.com/locate/yexnr

Oligodendrocyte differentiation and signaling after transferrin internalization: A mechanism of action

María Julia Pérez [a], Natalia Fernandez [b], Juana María Pasquini [a,*]

[a] Cátedra de Química Biológica Patológica, Departamento de Química Biológica, Facultad de Farmacia y Bioquímica (FFyB), Universidad de Buenos Aires (UBA), Instituto de Química y Fisicoquímica Biológica "Prof. Alejandro C. Paladini" (IQUIFIB), UBA-Consejo Nacional de Investigaciones Científicas y Técnicas (CONICET), Junin 965, CABA, Buenos Aires, Argentina

[b] Laboratorio de Farmacología de Receptores, Cátedra de Química Medicinal, Departamento de Farmacología, Facultad de Farmacia y Bioquímica (FFyB), Universidad de Buenos Aires (UBA), Junin 965, CABA, Buenos Aires, Argentina

ARTICLE INFO

Article history:
Received 10 January 2013
Revised 12 June 2013
Accepted 14 June 2013
Available online 21 June 2013

Keywords:
Fyn tyrosine kinase
PI3K/Akt pathway
MEK/ERK pathways
Oligodendrocyte differentiation and transferrin

ABSTRACT

Oligodendrocytes are the cells producing the myelin membrane around the axons in the central nervous system and, although apotransferrin (aTf) is required for oligodendrocyte differentiation, the underlying mechanisms are not fully understood.

Fyn tyrosine kinase, a member of the Src family of proteins, has been shown to play an important role in myelination by up-regulating the expression of myelin basic protein; however, a molecular link between aTf and Fyn kinase signaling pathway during oligodendrocytes differentiation has not been established yet. Our aim was to investigate whether Fyn kinase, MEK/ERK and PI3K/Akt signaling pathways are required for aTf-stimulation of oligodendrocyte differentiation and also to determine if the transferrin receptor is involved in these mechanisms.

Treatment of primary cultures of oligodendroglial precursor cells with aTf leads to Fyn kinase activation by a mechanism that involves transferrin receptor. In turn, Fyn kinase activation promotes MEK-mediated transient phosphorylation of ERK1/2. On the other hand, transferrin receptor internalization also produces rapid and sustained activation of Akt, which involves phosphatidylinositol 3-kinase (PI3K) activation. Finally, aTf incorporated through clathrin-mediated endocytosis increases myelin basic protein, F3-contactin and β-tubulin through Fyn/MEK/ERK pathways, as well as an activation of the PI3K/Akt pathway. Our results also demonstrate that the activation of the pathways necessary for oligodendroglial precursor cell maturation is dependent on AP2 recruitment onto the plasma membrane for clathrin-mediated endocytosis of transferrin receptor.

Introduction

Oligodendrocytes (OL) are responsible for myelination around axons in the central nervous system. However, the molecular mechanisms underlying oligodendrocyte progenitor cell (OPC) differentiation and myelinogenesis are complex and still remain under study.

Transferrin (Tf) is a glycoprotein synthesized in the liver and whose principal function is to act as an iron transport protein. Many investigators have demonstrated that Tf is also necessary for cellular growth (Kawabata et al., 2000; Suzuki et al., 2006) and has bacteriostatic properties (Artini et al., 2012; Wally and Buchanan, 2007).

Tf accumulation by OL is associated with myelin production (Espinosa de los Monteros et al., 1999). Moreover, Tf is synthesized by OL, which also express abundant Tf mRNA (Bartlett et al., 1991; Bloch et al., 1985; Rouault and Cooperman, 2006) and can secrete Tf when cultured (Espinosa de los Monteros et al., 1990). It has also been reported that apotransferrin (aTf) accelerates the myelination process *in vivo* (Escobar Cabrera et al, 1994, 1997; Marta et al., 2000; Saleh et al., 2003). aTf also prevents hypomyelination produced by iron deficiency in rats (Badaracco et al., 2008), reduces hypoxic/ischaemic white matter injury in rats (Guardia Clausi et al., 2012) and stimulates remyelination in cuprizone-induced demyelination, an animal model of multiple sclerosis (Adamo et al., 2006). Similarly,

Abbreviations: Ab-TfR, mouse antibody against Tf receptor (Ab-2) clone 42/6; aTf, apotransferrin; Cyt B, cytochalasin B; DIII, pEGFP-C2-Eps15 DIII plasmid, Eps15 dominant negative mutant; DIIIΔ2, pEGFP-C2-Eps15 DIIIΔ2 plasmid, Eps15 irrelevant mutant; EH29, pEGFP-C2-Eps15 EH29 plasmid, Eps15 dominant negative mutant; F3, F3-contactin; Fyn, Fyn tyrosine kinase; GAPDH, glyceraldehyde 3-phosphate dehydrogenase; LY, LY294002, PI3 kinase inhibitor; MBP, myelin basic protein; MDC, primary amine monodansylcadaverin; NG2, nerve/glia antigen 2; OL, oligodendrocytes; OPC, oligodendrocyte progenitor cell; PD, PD98059, MEK1/2 inhibitor; PDGFR-α, platelet-derived growth factor α; PI3K, phosphatidylinositol 3-kinase; PP2, Src family of tyrosine kinases inhibitor; Tf, transferrin; TfR, transferrin receptor; Tf-TR, Texas Red-labeled transferrin; U0126, MEK1/2 inhibitor; WM, wortmannin, PI3 kinase inhibitor; β-tub, β-tubulin.

* Corresponding author at: Cátedra de Química Biológica Patológica, Dpto. Química Biológica, FFyB, UBA, Junin 965, CABA, Buenos Aires, Argentina. Fax: +54 11 4964 8287/88.

E-mail addresses: mjuliaperez@gmail.com (M.J. Pérez), natyfernandez@hotmail.com (N. Fernandez), jpasquin@qb.ffyb.uba.ar (J.M. Pasquini).

0014-4886/$ – see front matter © 2013 Elsevier Inc. All rights reserved.
http://dx.doi.org/10.1016/j.expneurol.2013.06.014

aTf induces OPC differentiation *in vitro* (Garcia et al., 2004; Paez et al., 2002).

Fyn tyrosine kinase (Fyn) is a protein belonging to the Src-family of non-receptor tyrosine kinases, among which Fyn, Lyn and Src are expressed by OL (Colognato et al., 2004; Umemori et al., 1992). Fyn expression and kinase activity have been identified as mediators of different OL processes such as migration, differentiation, axonal contact and myelination start-up (Baer et al., 2009; Krämer et al., 1999; Osterhout et al., 1999; Umemori et al., 1994). In addition, previous reports have shown the relevance of MEK/ERK (Fyffe-Maricich et al., 2011) and PI3K/Akt signaling cascades in OPC differentiation (Bibollet-Bahena and Almazan, 2009; Cui et al., 2005; Flores et al., 2008).

The aim of our study was to investigate the molecular mechanisms and signaling pathways by which aTf promote OPC differentiation. We observed that (i) aTf treatment of OPC activates Fyn; (ii) aTf stimulates a transient phosphorylation of ERK downstream Fyn activation, as well as rapid and sustained Akt phosphorylation independent of Fyn activation; (iii) the activation of these signaling pathways depends on Tf receptor (TfR) internalization by clathrin-coated pits; (iv) aTf induction of the expression of proteins involved in the myelination process, such as myelin basic protein (MBP), β-tubulin and F3-contactin, is mediated by Fyn/MEK/ERK and PI3K/Akt pathways.

Materials and methods

Materials

Human apotransferrin (aTf), cytochalasin B (Cyt B), paraformaldehyde (PFA), serum albumin, poly-L-lysine, triiodothyronine (T3), Triton X-100, Hoechst (bis-Benzamide H 33258), DMSO and wortmannin were obtained from Sigma-Aldrich (St Louis, MO). DMEM/F12, Lipofectamine 2000, human transferrin conjugated to Texas Red (Tf-TR) and TMB Single Solution (3,3′,5,5′-TetraMethyl-Benzidine) were from Life Technologies (Argentina). Fetal calf serum was from Natocor (Argentina). Mowiol and PP2 (4-Amino-5-(4-chlorophenyl)-7-(t-butyl) pyrazolo [3,4-d]pyrimidine) were from Calbiochem (Nottingham, UK). PD 98059 (2′-amino-3′-methoxiflavone) and U0126 (1,4-diamino-2,3-dicyano-1,4-bis(2-aminophenyl-thio)butadiene) were from Promega (Madison, USA). LY 294002 [2-(4-Morpholinyl)-8-phenyl-4H-1-benzopyran-4-one] was purchased from Cell Signaling Technology (Danvers, MA). Immobilon-P^{sq} (PVDF transfer membrane) was from Millipore (Temecula, CA), while Hyperfim ECL and ECL Plus Western Blotting Detection Reagents were purchased from GE Healthcare (Buckinghamshire, UK). Human platelet-derived growth factor-AA (PDGF) and basic fibroblast growth factor (bFGF) were purchased from Peprotech (Mexico City, Mexico). Antibodies used were as follows, mouse anti-Active Src (dephosphorylated-Tyr 529) (Invitrogen); rabbit anti-total Fyn, rabbit anti-ERK1/2 (Santa Cruz Biotechnology); rabbit anti-phospho-ERK1/2 (p-Thr202/p-Tyr204), rabbit anti-phospho-Akt (p-Ser 473), rabbit anti-Akt (Cell Signaling Technology); mouse anti-β-tubulin (Chemicon International); goat anti-Tf (ICN Biomedicals); mouse anti-GAPDH (Abcam); rabbit anti-myelin basic protein (MBP) and mouse anti-O4 (generous gift from A. Campagnoni-UCLA); mouse anti-GFAP and anti-neurofilament NF200 (Sigma-Aldrich); rabbit anti-NG2 Chondroitinsulfate proteoglycan (Millipore, Temecula, CA), goat anti-PDGFR α (Neuromics); mouse anti-CD71 OX-26 transferrin receptor (BD Biosciences Pharmingen); rabbit anti-F3-contactin (generous gift from Dr.Watanabe) and mouse anti-Tf receptor (Ab-2) clone 42/6 (Calbiochem, Nottingham, UK). Horseradish peroxidase, Cy2, DyLyght 488, Cy3 and DyLight 549-conjugated secondary antibodies used for immunoblotting and immunocytochemistry were obtained from Jackson ImmunoResearch Laboratories (West Grove, PA). All other chemicals were analytical grade reagents.

Oligodendrocyte progenitor cell primary culture

Primary cultures of OPC from newborn Wistar rats of either sex were performed according to McCarthy and de Vellis (1980). After removing the meningeal membranes, newborn rat cerebral hemispheres were mechanically dissociated by gentle repetitive pipetting in a mixture of DMEM/F12 (1, 1 v/v) containing 5 g/ml streptomycin and 5 U/ml penicillin, supplemented with 10% fetal calf serum. The cell suspensions were seeded in poly-L-lysine-coated 75 cm^2 tissue culture flasks and incubated at 37 °C in 5% CO_2, with changes of medium every 4 days. After 14 days in culture, when cells reached confluence, the subpopulation of OPC was obtained by using a differential cell adhesion protocol. After first shake at 140 rpm/min during 1 h, the medium containing microglia was discarded. Then cultures were shaken overnight at 240 rpm/min. The cell suspension obtained was filtered through a 15 μm mesh filter and plated on bacterial grade Petri dishes for 1 h. Astrocytes and microglia were attached to the plastic surface while OPC remained in suspension. Then OPC were centrifuged at 1500 rpm during 10 min and the pellet was resuspended in glial defined medium (GDM) (Casaccia-Bonnefil et al., 1996), without the addition of aTf. OPC in suspension were seeded either on 12-mm poly-L-lysine-coated coverslips or 30-mm poly-L-lysine-coated Petri dishes with GDM in the presence of PDGF (10 ng/mL) and bFGF (10 ng/mL) during 24 h. Oligodendroglial cell cultures were evaluated quantitatively with anti-O4, anti-neurofilaments NF200 and anti-GFAP antibodies were 95% pure (Pasquini et al., 2003). For experiments involving intracellular signaling, OPC medium was changed to GDM without PDGF and bFGF during 4 h before aTf (100 μg/mL) treatment in order to initiate a mitogen starvation condition. Then, for experiments with kinase inhibitors, cells were pretreated with DMSO, 5 μM PP2 (Src family of tyrosine kinases inhibitor, Lck and Fyn) (Hanke et al., 1996), 10 μM U0126 and 2.5 μM PD 98059 (MEK1/2 inhibitors), 1 μM wortmannin and 50 μM LY 294002 (PI3 kinase inhibitors) for 30 min before the addition of aTf (100 μg/mL) during different times.

SDS-PAGE and Western blot analysis

After treatment, cells were harvested in 150 μL of ice-cold lysis buffer which contained 20 mmol/L Tris-HCl (pH 8), 1% Nonidet P-40, 10% glycerol, 137 mmol/L NaCl, 1 mmol/L PMSF, 1 mmol/L aprotinin, 0.1 mmol/L sodium vanadate, and 20 mmol/L NaF. Protein content of cell lysates was determined with the BIO-RAD Protein Assay Kit and the samples were adjusted with loading buffer containing 2% sodium dodecyl sulfate (SDS), 5% glycerol, 5% b-mercaptoethanol and 0.01% bromophenol blue and boiled for 5 min. Aliquots containing 20 μg of protein were resolved by SDS polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to PVDF membranes. Membranes were blocked in 5% non-fat dried milk in 0.1% Tween 20 in TBS for 1 h at room temperature and incubated with an appropriate primary antibody overnight at 4 °C [mouse anti-Active Src (Tyr 529), rabbit anti-Fyn, rabbit anti-phospho-ERK1/2 (Thr202/Tyr204), rabbit anti-ERK1/2, rabbit anti-phospho-Akt (Ser 473), rabbit anti-Akt, goat anti-Tf, rabbit anti-MBP, rabbit anti-F3-contactin, mouse anti-β-tubulin, mouse anti-GAPDH]. After being washed, membranes were incubated in the corresponding horseradish peroxidase-conjugated secondary antibody. Bands were visualized by chemiluminescence with ECL Western Blotting Detection Kit on autoradiographic film. Films were scanned and quantified using Scion Image® software from National Institutes of Health (NIH). To normalize for sample loading and protein transfer, membrane-bound proteins were first exposed to anti-active or phospho-protein antibody, then stripped at 60 °C during 1 h, and finally incubated with anti-total antibody. The active or phospho-protein/total protein ratio was used to evaluate signaling activation.

Transferrin uptake assay

OPC were incubated with Tf–TR or human aTf (100 μg/mL) for different time periods (0–60 min) at 37 °C. After being washed, some cells were fixed and other cells were lysated to quantitate Tf incorporation by fluorescence microscopy or by Western blot (WB), respectively. Two controls of endocytosis were used: the assay was performed at 4 °C to calculate specific cell surface binding and actin polymerization was inhibited with 1 h treatment of 1 μM Cyt B before the addition of Tf-TR for 15 min.

Plasmids and transient transfections

The pEGFP-C2-Eps15 EH29, pEGFP-C2-Eps15 DIII, and pEGFP-C2-Eps15 DIIIΔ2 constructs were generous gifts from Dr. Benmerah (Universite' Paris 5, Institut Cochin, Departement de Maladies Infectieuses, Paris, France). For transient transfection, OPC were plated in GDM supplemented with PDGF and bFGF (10 ng/mL each) at 2.5×10^5 cells per well in six-well poly-L-lysine-coated culture dishes 1 d before transfection. The transfection protocol was optimized upon suppliers' (Invitrogen) recommendation as follows,

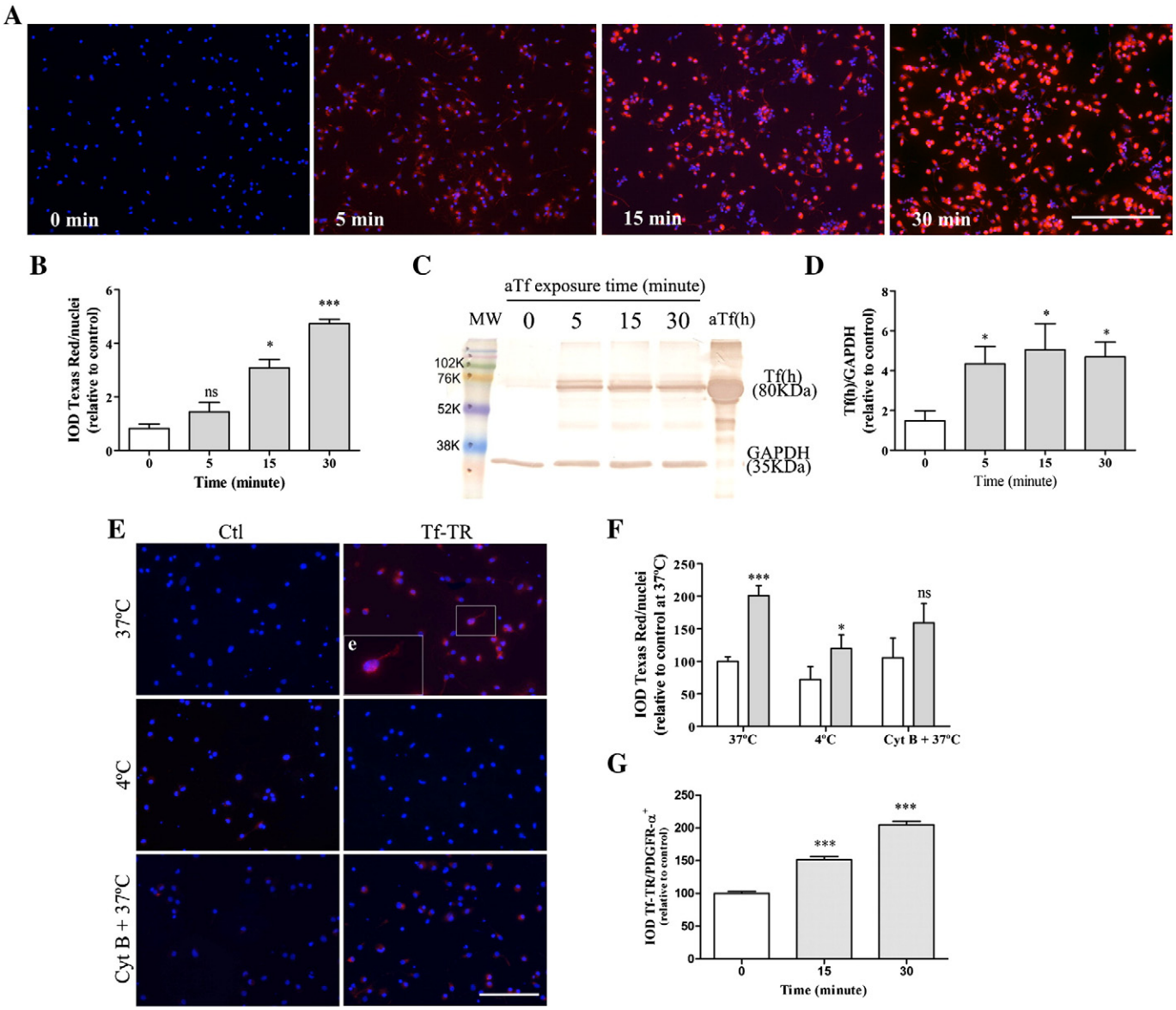


Fig. 1. OPC cultures incorporate Tf *in vitro*. A, Cultures were treated with Tf-TR during different time frames and Tf-TR uptake was evaluated by Texas Red fluorescence. B, Quantitation of Texas-Red fluorescence of images shown in A after normalizing the red IOD to the total nuclei in each image. C, WB analysis of total cell lysates after different exposure times to aTf using anti-human Tf antibodies and anti-GAPDH to detect immunopositive bands. D, Quantitative analysis of Tf uptake shown in C is represented as a Tf(h)/GAPDH ratio. E, Microscope imaging of Tf-TR incorporation at different temperatures (4 °C, 37 °C) or in the presence of an actin polymerization inhibitor (1 μM Cyt. B, 37 °C). Inset in the Tf-TR (37 °C) condition is shown in E at higher magnification. F, Quantitative analysis of images is shown in E as a red fluorescence IOD/nuclei ratio and compared to the Ctl of each condition. Differences among the different controls are not significant. G, Quantitation of red IOD of PDGFR-α^+ cells in each image at 15 and 30 min after Tf-TR exposure. Hoechst nuclear dye is shown in blue (A and E). Scale bar in A equals 200 μm for all images in the panel. Scale bar in E represents 100 μm for all images of the panel and 50 μm for the high magnification inset e. Bars in B, D and F represent the mean ± SEM belonging to three independent experiments. One-way ANOVA followed by Dunnett's Multiple Comparison Test were used in B, D and G; and followed by Neuman–Keuls Multiple Comparison Test were used in F to determine statistical significance; ***$p < 0.001$, *$p < 0.05$, ns non-significant, symbols above the bar indicate significance compared to corresponding control. aTf: human apotransferrin; Cyt. B: Cytochalasin B; GAPDH: Glyceraldehyde 3-phosphate dehydrogenase; IOD: integrated optical density; PDGFR-α: platelet-derived growth factor α; Tf-TR: Texas Red-labeled transferrin.

cells in 1.5 mL of culture medium received 2.7 μg of the specific Eps15 construct and 2.7 μg of mock in 300 μL of GDM with 12 μL of LipofectAmine 2000 per well. After 6 h of incubation, the medium was replaced. 48 h after transfection, cells were stimulated during 15 min with aTf (100 μg/mL) and total protein samples were obtained and analyzed by WB. The expression of the EGFP-Eps construct was confirmed by fluorescence microscopy.

Stimulation of transferrin receptor

TfR stimulation was performed by the crosslinking of TfR with mouse anti-Tf receptor (Ab-2) clone 42/6 (Ab-TfR) (Calbiochem, Nottingham, UK). Half an hour before aTf (100 μg/mL) treatment, OPC were incubated with 2 μg/mL of anti-Ab-TfR at 37 °C during 30 min. Non-treated OPC were used as controls. To stop stimulation, cells were cooled to 4 °C, washed with PBS and total protein content of cell lysates analyzed by WB.

TfR2 stimulation was evaluated with bovine Tf (100 μg/mL). OPC were treated with human aTf or bovine aTf during 15 min and total protein samples were analyzed by WB.

Pharmacological studies

OPC were pre-treated with 50 μM monodansylcadaverin (MDC) or DMSO during 10 min at 37 °C previous to the addition of Tf-TR for 15 min. MDC specifically inhibits the membrane-bound enzyme transglutaminase type I and interferes with clathrin-mediated vesicle formation. Cells were fixed to quantitate Tf incorporation by fluorescence microscopy. Total protein lysates were used to analyze signaling activation by WB.

Immunocytochemistry

Multiwells containing cultured OPC were kept for 1 d at 37 °C in GDM. Cells were fixed with 4% PFA in PBS for 30 min at room temperature. Fixed cells were permeabilized by incubation with 0.01% Triton X-100 in PBS for 15 min, blocked with 5% fetal calf serum in PBS for 2 h, and incubated overnight at 4 °C with the following primary antibodies, goat anti-Tf(h); rabbit anti-MBP; rabbit anti-NG2; goat anti-PDGFR α; and mouse anti-CD71. Then, coverslips were rinsed and incubated for 2 h at room temperature with the appropriate

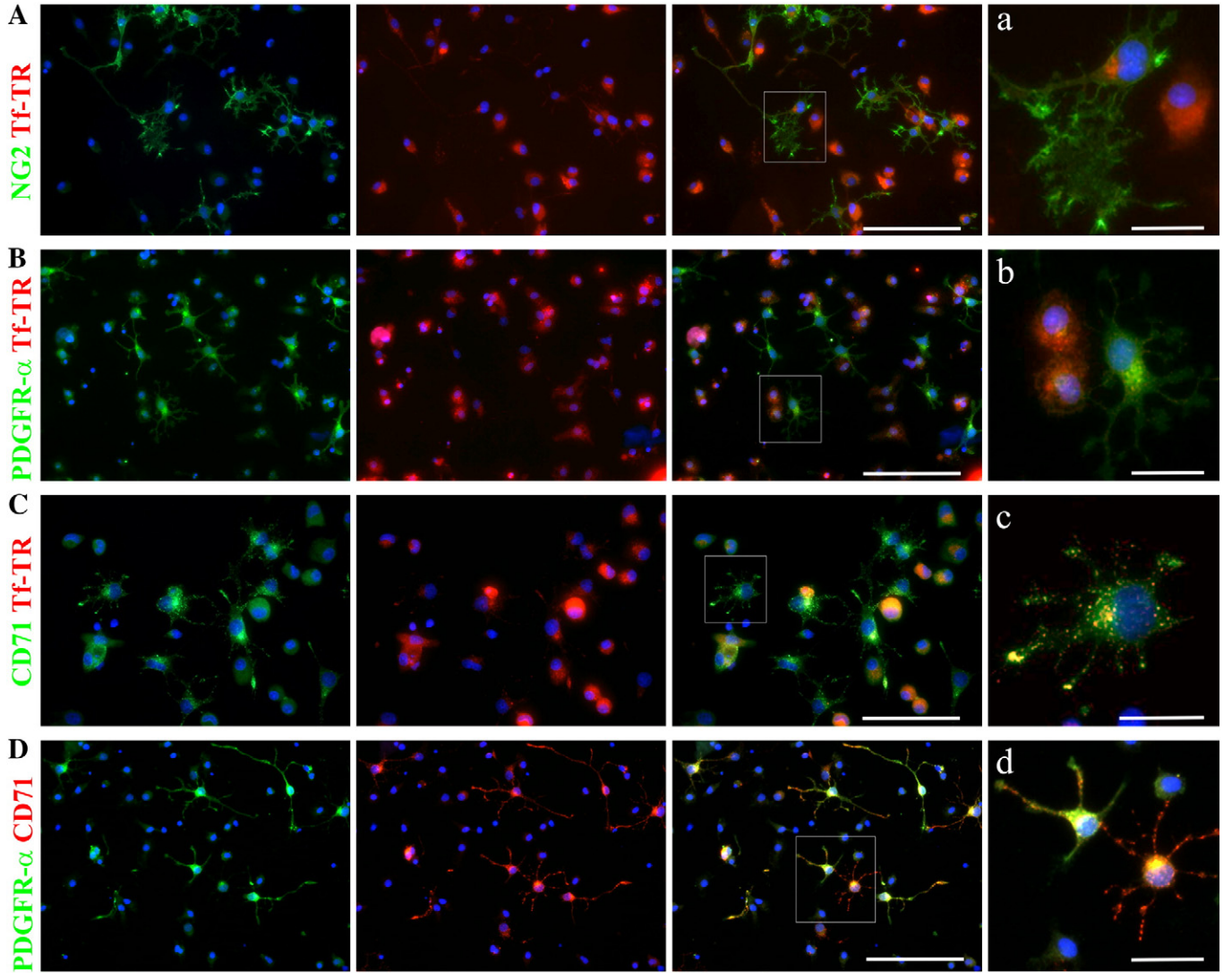


Fig. 2. OPC incorporate Tf-TR and express Tf receptor 1. A–C, Tf-TR (red) immunocytochemical analysis in cultured cells treated with Tf-TR (100 μg/ml) for 15 min. NG2 (A), PDGFR-α (B) and Tf receptor (CD71, C) are shown in green. D, PDGFR-α positive cells are shown in green and Tf receptor 1 immunodetection (CD71) is shown in red. Hoechst nuclear dye is shown in blue. The insets in A–D are shown at higher magnification on the right side of each panel (a–d). Scale bars equal 100 μm for all images in panels A, B and D. Scale bar in panel C equals 75 μm. Scale bars equal 25 μm for a, b and d images. Scale bar in c equals 20 μm. NG2: Nerve/glia antigen 2; Tf: Transferrin; Tf-TR: Texas Red-labeled transferrin; PDGFR-α: platelet-derived growth factor α.

fluorescent-conjugated secondary antibody. Finally, coverslips were mounted with Mowiol solution.

Microscopy image processing

Image acquisition was examined by epifluorescence microscopy (Olympus BX50) or confocal microscopy (Olympus FV1000). Epifluorescent images were analyzed with Image-Pro Plus Software (Media Cybernetics) and confocal images were analyzed using the Fluoview 2.1 software (Olympus).

Statistical analysis

Three independent experiments were performed for statistical analysis using GraphPad Prism 5.00 software. Data were represented as the mean ± SEM. Two-tailed Student's *t*-test and one-way ANOVA followed by Neuman–Keuls or Dunnett's Multiple Comparison Tests were used to determine statistical significance; ***$p < 0.001$, **$p < 0.01$, *$p < 0.05$, and ns non-significant.

Results

We previously analyzed the differentiation process of OPC culture in the presence of aTf, especially the increase in MBP expression (Paez et al., 2002) and the cytoskeletal reorganization (Perez et al., 2009). In order to describe the molecular mechanisms involved in these effects, we first focused on the analysis of Tf incorporation in the OPC culture.

aTf is internalized by oligodendrocyte cultures

Kinetic studies of (Tf-TR) uptake were done in cultured OPC. Analysis of Tf-TR incorporation showed a significant increase in the integrated optical density (IOD) values of Texas Red fluorescence as from 15 min of exposure and up to 30 min related to control (Figs. 1A and B). Similar results were obtained when aTf incorporation was measured by WB analysis. In this case, aTf incorporation was detected earlier (at 5 min) and remained with no significant differences up to 30 min of treatment (Figs. 1C and D). To evaluate whether aTf incorporation was due to endocytic mechanisms, we evaluated Tf-TR incorporation at 4 °C, to calculate specific cell surface binding, and in the presence of Cyt B, an actin polymerization inhibitor which acts as general endocytosis inhibitor. A significant decrease in Tf-TR incorporation was observed when cells were incubated at 4 °C or in the presence of Cyt B, which suggests the involvement of the endocytic machinery in aTf incorporation by OPC (Figs. 1E and F).

To confirm that Tf-TR-incorporating cells belong to the oligodendroglial lineage, we evaluated Tf-TR incorporation of PDGFR-α^+. The analysis at 15 min and 30 min after Tf-TR exposure showed a significant increase in IOD values with respect to control (Fig. 1G). Moreover, we showed the expression of OPC markers by immunocytochemistry. NG2$^+$ (Fig. 2A) and PDGFR-α^+ (Fig. 2B) cells showed Tf-TR incorporation with intense puncta in the cell soma, as magnified in Figs. 2A and B. Immunodetection for TfR (CD71) showed that the receptor has a subcellular distribution pattern that is coincident, in some points, with Tf-TR signal (Fig. 2C). Higher magnification images in Fig. 2C indicate that both TfR and Tf-TR have punctate patterns, which suggests that Tf internalization occurs through canonical-receptor-mediated endocytosis. Finally, immunodetection of PDGFR-α and CD71 in the same cells (Fig. 2D) confirmed that OPC express the TfR.

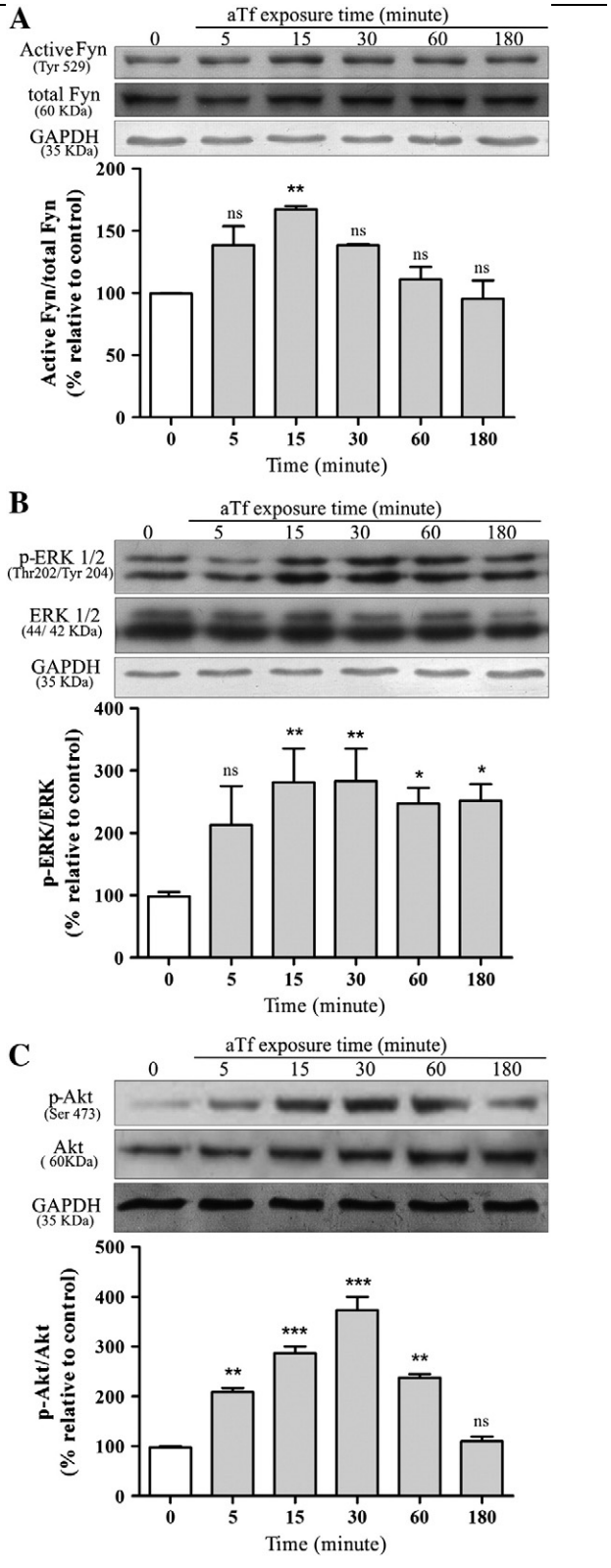


Fig. 3. aTf increases Fyn activation and phosphorylation of ERK and Akt in a time-dependent manner. Whole cell lysates were analyzed by WB at different time points after aTf addition to the culture medium. A, OPC cell lysate was immunoblotted by anti-Tyr 529 to indirectly measure the levels of active Fyn kinase. Fyn activation was analyzed as an active-Fyn/total Fyn ratio. B, ERK phosphorylation was evaluated as a p-ERK/ERK ratio. C, Akt phosphorylation was analyzed as a p-Akt/Akt ratio. The GAPDH immunopositive bands were used as sample loading. The bars in all graphs represent the mean ± SEM belonging to three independent experiments. One-way ANOVA followed by Dunnett's Multiple Comparison Test was used to determine statistical significance and the symbols above each bar represent the significance compared to the control condition (white bar); ***$p < 0.001$, **$p < 0.01$, *$p < 0.05$, ns non-significant. aTf: human apotransferrin; Fyn: Fyn tyrosine kinase; GAPDH: Glyceraldehyde 3-phosphate dehydrogenase.

aTf signaling involves Fyn/MEK/ERK and PI3K/Akt pathways through its receptor

To investigate the involvement of Fyn, MEK/ERK and PI3K/Akt signaling pathways in mediating aTf effect, we assessed the activation levels of Fyn, ERK 1/2 and Akt by WB with specific antibodies.

It has been reported that dephosphorylation of Tyr 529 and auto-phosphorylation of Tyr 418 of Fyn are the consecutive and crucial steps involved in Fyn activation (Cole et al., 2003). We used a monoclonal antibody against dephosphorylated Tyr 529 to indirectly measure the levels of active Fyn. Kinetic studies showed maximal stimulation of Fyn at 15 min of treatment with aTf (Fig. 3A). ERK1/2 phosphorylation was maximal at 15 min with a sustained pattern up to 180 min (Fig. 3B). Finally, Akt activation occurred faster (5 min), reaching its maximal levels at 30 min of treatment with aTf and decreasing afterwards. GAPDH expression remained with no significant differences up to 180 min of treatment. Moreover, when we assessed the activation levels of Fyn, ERK 1/2 and Akt related to GAPDH immunobands the activation pattern was similar than the analysis of phosphorylated protein/total protein ratio.

With the aim of evaluating whether the modulation of these signaling pathways depends on TfR, OPC were pre-incubated during 30 min with a specific antibody against TfR (Ab–TfR), which has been reported to block the binding of transferrin to its receptor (Lesley and Schulte, 1985; Trowbridge and Lopez, 1982).

In our hands, pre-treatment of OPC with the antibody before the addition of aTf showed no inhibition of aTf incorporation (Fig. 4A). By confocal microscopy, we observed that fluorescence of Tf-TR was incorporated into the cells in the presence of Ab-TfR (data not shown). Moreover, we did not find differences in the magnitude of Fyn activation by aTf, although we observed an increase in the basal levels of active Fyn after treatment with the antibody (Fig. 4B).

Consistently, neither Akt phosphorylation nor ERK1/2 phosphorylation were blocked by the monoclonal antibody. Again, pre-treatment with anti-TfR antibody led to an increase in the basal phosphorylated levels of ERK1/2 (Figs. 4C and D). These results agree with observations made by other authors, where anti-TfR antibody was shown to stimulate transferrin and iron uptake in rat reticulocytes by facilitating the formation of coated pits and, in consequence, by increasing the rate of turnover of TfR (McArdle and Morgan, 1984).

Kawabata et al. (2004) reported that bovine Tf could interact only with TfR2 but not with TfR1. Taking this advantage into consideration and in order to investigate whether TfR2 stimulation might lead to ERK activation in OPC cultures, we performed OPC treatment with bovine aTf in the same conditions as used with human aTf. Bovine aTf and human aTf produced similar effects on Fyn activation and ERK phosphorylation (Figs. 4E and F). On the other hand, bovine aTf was not able to stimulate Akt phosphorylation (Fig. 4G).

Confocal analysis of Tf-TR and TfR1 expression (Figs. 4H–J) showed some Tf-TR$^+$/TfR1$^+$ intracellular vesicles (arrowheads) indicating Tf internalization through the canonical pathway. However, we detected Tf-TR$^+$ vesicles lacking TfR1 signal located in OPC processes (Fig. 4H, arrows). It was reasonable to think that Tf, as well as Tf-TR, could be incorporated into cells through both receptors.

Fyn, ERK1/2 and Akt activation was then studied in the presence of specific inhibitors to further assess the role of these kinases and the sequence of activation events in the signaling pathways evoked by aTf stimulation. The inhibitor of the Src family of tyrosine kinases (PP2), PI3K inhibitors (LY294002 and wortmannin) or MEK inhibitors (PD98059 and U0126) were added to OPC cultures 30 min before aTf treatment and the phosphorylation levels of the kinases were examined by WB.

Pre-treatment of OPC with PP2 completely decreased Fyn activation by aTf (Fig. 5A), while MEK and PI3K inhibitors did not modify aTf-induced Fyn activation (Figs. 5B and C). Surprisingly, pre-treatment of cells with PD98059, led to an increase in basal phosphorylation of Fyn, which points at the existence of a feedback regulatory mechanism of Fyn mediated by ERK (Fig. 5B).

When ERK phosphorylation promoted by aTf was evaluated, we observed that PP2 inhibitor of Fyn tyrosine kinase, as well as PD98059 and U0126 MEK inhibitors, completely blocked ERK phosphorylation, which suggests that it depends on Fyn-MEK activity (Figs. 6A–C). Pre-treatment of cells with the PI3K inhibitors, LY294002 and wortmannin had no effect on aTf-stimulated phosphorylation of ERK1/2 (Figs. 6B and C). Finally, Akt activation was blocked by the PI3K inhibitors, which indicates that PI3K mediates Akt phosphorylation (Figs. 7B and C). PP2 inhibitor reduced basal level of Akt phosphorylation; however, had no effect on Akt phosphorylation by aTf stimulation. This result indicates that PI3K/Akt pathway is independent of Fyn activation (Fig. 7A).

Fyn activation by aTf requires clathrin-mediated endocytosis of TfR

To address the functional role of TfR internalization for intracellular signal distribution, we evaluated aTf-promoted Fyn activation in the presence of the MDC, which has been described as an endocytosis inhibitor (Ray and Samanta, 1996). We found that Tf-TR internalization was significantly inhibited in the presence of MDC (Figs. 8A and B). Moreover, Fyn activation by aTf was completely blocked in cells treated with this inhibitor (Fig. 8C). This result indicates that TfR endocytosis is necessary for Fyn stimulation and confirmed our previous observations suggesting that TfR internalization was involved in mediating aTf action on OPC.

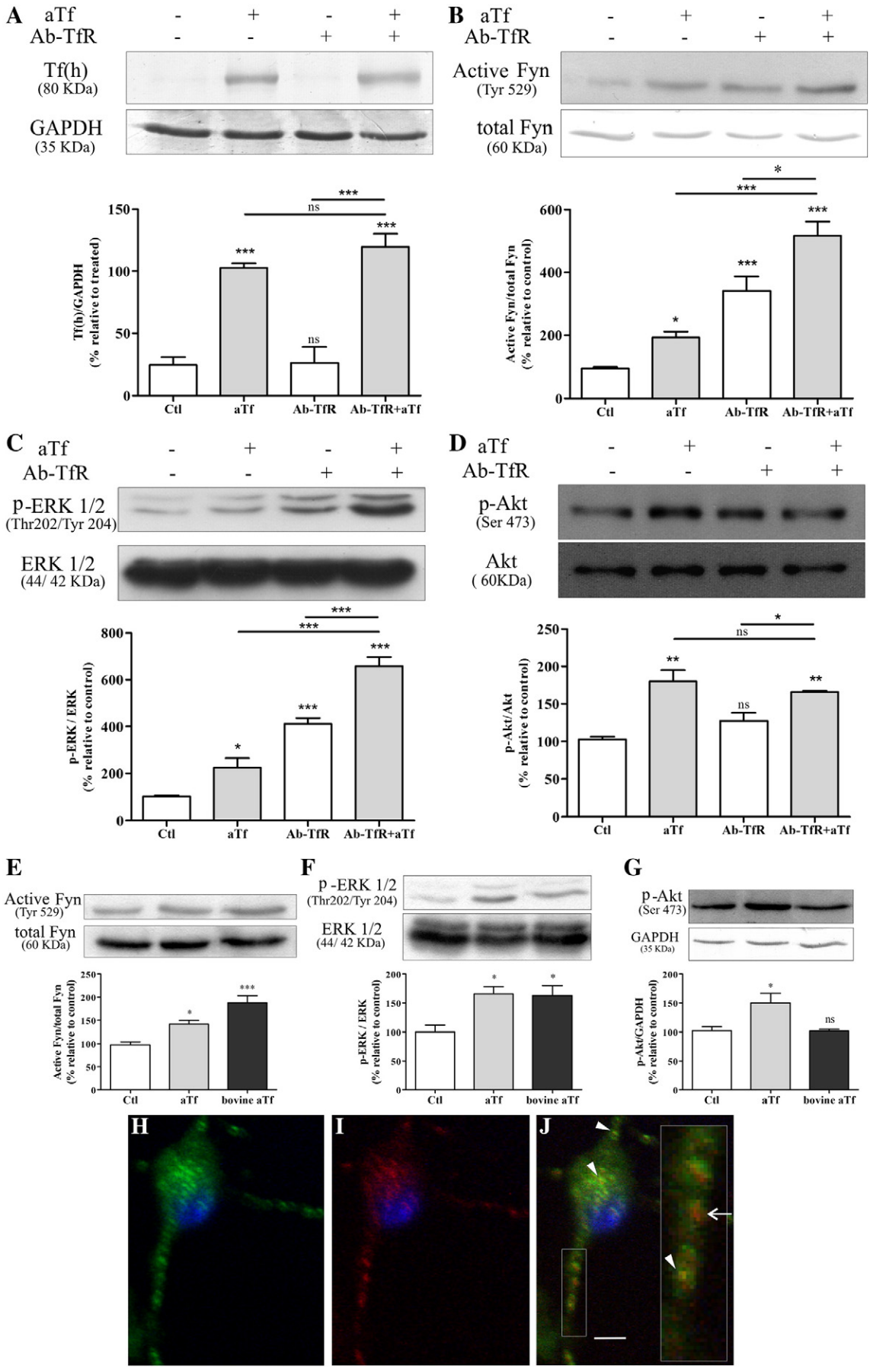
It has been previously reported that disruption of functional AP2 adaptor complex in HeLa cells eliminates endocytic clathrin-coated structures and blocks Tf uptake. To further investigate clathrin involvement in the activation of intracellular pathways by aTf, we used two dominant-negative mutants of Eps15 protein, one of the members of AP2 complex, which is specifically required for the correct assembly of functional clathrin-coated pits (Rappoport et al., 2004).

DIII and EH29, Eps15 dominant negative mutants, significantly reduced aTf-induced Fyn activation (Fig. 8D), which indicates that disruption of clathrin-coated-pit-mediated endocytosis (Benmerah et al., 1999) abolished aTf-stimulated Fyn activation. Moreover, the inhibition of the pathway was most noticeable at the level of ERK and Akt modulation, where the Eps15 dominant negative mutants significantly abolished ERK and Akt activation (Figs. 8E and F). Transfection with an irrelevant mutant (DIIIΔ2), used as a negative control, showed no effect on signaling activation by aTf. These results clearly showed that, when aTf was added to the culture medium, clathrin-mediated internalization was necessary for activation of Fyn, ERK and Akt signaling.

aTf induced OL maturation is mediated by Fyn/MEK/ERK and PI3K/Akt pathways

Since our results indicated that aTf treatment of OPC led to PI3K/Akt and Fyn/MEK/ERK pathway stimulation, we evaluated the involvement of these signaling cascades in OL maturation. Umemori et al. (1999) described that Fyn induces MBP gene transcription during myelination. In connection with this, we investigated whether the regulation of OPC maturation by aTf involves Fyn activity, by evaluating MBP, β-tubulin and F3-contactin levels after aTf treatment with or without PP2 inhibitor.

Immunocytochemical analysis showed that 4-day treatment of OPC with aTf resulted in an increase in MBP immunoreaction. This increase was abolished by the Fyn kinase inhibitor PP2 (Figs. 9A and B). Similarly, MBP analysis by WB showed that aTf induction of MBP was decreased by the presence of PP2 (Fig. 9C). Furthermore, the increase in β-tubulin levels after aTf treatment was blocked by PP2. Similarly, PP2 inhibitor also diminished the basal levels of this protein (Fig. 9D).


A
aTf - + - +
Ab-TfR - - + +
Tf(h)
(80 KDa)
GAPDH
(35 KDa)
Tf(h)/GAPDH
(% relative to treated)
Ctl
aTf
Ab-TfR
Ab-TfR+aTf
B
aTf - + - +
Ab-TfR - - + +
Active Fyn
(Tyr 529)
total Fyn
(60 KDa)
Active Fyn/total Fyn
(% relative to control)
C
aTf - + - +
Ab-TfR - - + +
p-ERK 1/2
(Thr202/Tyr 204)
ERK 1/2
(44/ 42 KDa)
p-ERK / ERK
(% relative to control)
D
aTf - + - +
Ab-TfR - - + +
p-Akt
(Ser 473)
Akt
(60KDa)
p-Akt/Akt
(% relative to control)
E
Active Fyn
(Tyr 529)
total Fyn
(60 KDa)
Active Fyn/total Fyn
(% relative to control)
Ctl
aTf
bovine aTf
F
p -ERK 1/2
(Thr202/Tyr 204)
ERK 1/2
(44/ 42 KDa)
p-ERK / ERK
(% relative to control)
G
p-Akt
(Ser 473)
GAPDH
(35 KDa)
p-Akt/GAPDH
(% relative to control)
H
I
J

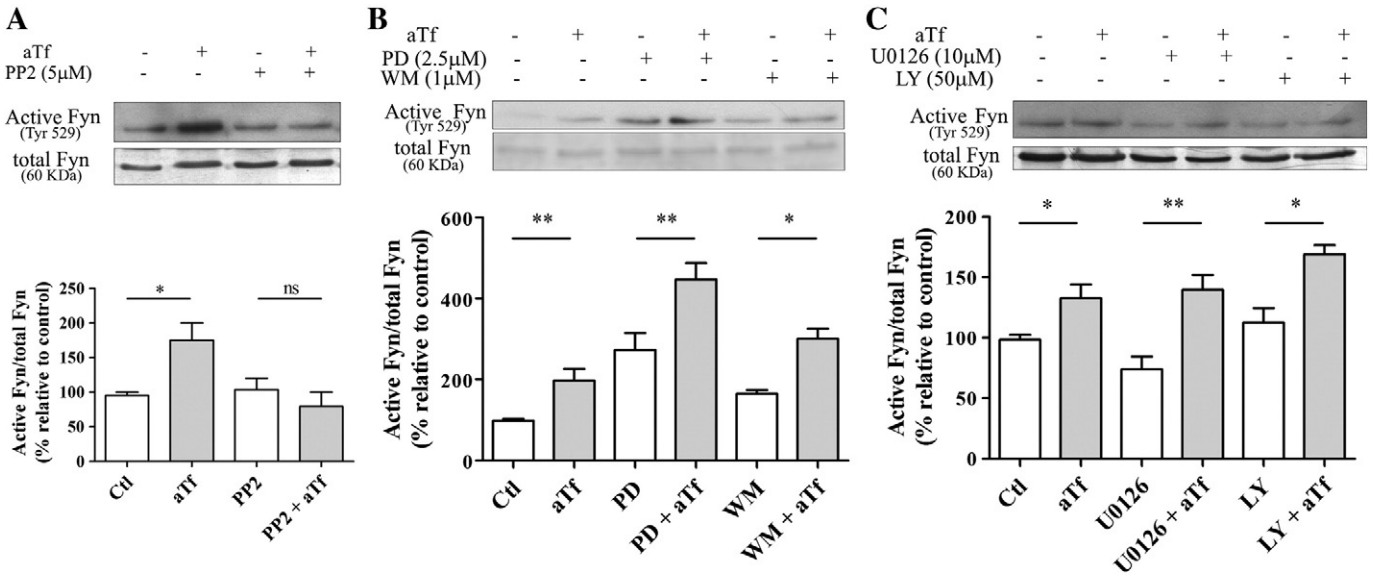


Fig. 5. Fyn is activated by aTf, regardless of downstream Erk and Akt inhibition. OPC were treated with PP2 (5 μM) in A, PD (2.5 μM) and WM (1 μM) in B, U0126 (10 μM) and LY (50 μM) in C for 30 min prior to the addition of aTf. Cells were then incubated for 15 min with aTf. Whole cell lysates were analyzed by WB with anti-Tyr 529 to indirectly measure the levels of active Fyn kinase and anti-total Fyn antibodies. The active-Fyn/total Fyn ratio was used to evaluate the Fyn pathway activation. The bars in all graphs represent the mean ± SEM of four independent experiments. Two-tailed Student's *t*-test was used to determine statistical significance; $^{**}p < 0.01$, $^{*}p < 0.05$, ns non-significant. aTf: human apotransferrin; Fyn: Fyn tyrosine kinase; PP2: Fyn kinase inhibitor; U0126 and PD (PD98059): MEK1/2 inhibitor; WM (wortmannin) and LY (LY294002): PI3 kinase inhibitor.

Cell adhesion glycoprotein F3-contactin has been shown to trigger Fyn activation in OL (Colognato et al., 2004; Krämer et al., 1999) and it is implicated in axogenesis and myelination (Krämer-Albers and White, 2011; White et al., 2008). In order to evaluate if Fyn activation by aTf mediates changes in F3-contactin expression levels, we performed WB analysis of cellular lysates from aTf or control OPC cultures. We found augmented F3-contactin expression when aTf was added to the medium. Furthermore, this effect was inhibited in the presence of PP2 Src inhibitor (Fig. 9E).

When OPC were treated with 10 μM U0126 or 1 μM wortmannin, the increase in the MBP levels induced by aTf was significantly inhibited (Figs. 10A and B). Similar results were obtained when tubulin expression levels were evaluated by WB (Fig. 10C), indicating that both signaling pathways, Fyn/MEK/ERK and PI3K/Akt, need to be functional to achieve proper OPC maturation by aTf.

Discussion

The aim of this study was to better understand the molecular mechanism of OPC maturation by aTf. Our data show that the stimulatory effect of aTf on OPC maturation is regulated by Fyn/MEK/ERK signaling as well as by PI3K/Akt signaling. Moreover, TfR clathrin-AP2-dependent endocytosis is fundamental for signal transduction.

Fyn has multiple functions during OL differentiation and neuron-glial interaction during myelination (Krämer-Albers and White, 2011; Laursen et al., 2009), although there is no evidence of how Fyn stimulated MBP expression by aTf. In a previous paper studying the participation of Fyn in morphological differentiation, we described that aTf interacts with TfR in the soma and then probably activates signals that could induce a modification in the cytoskeletal arrangement (Ortiz et al., 2005). We also previously reported that aTf increased Fyn protein expression, induced Fyn kinase activation at 12 h and changed the Fyn protein localization. All these events are associated with morphological differentiation and cytoskeleton rearrangement that leads to a more mature OL in the presence of aTf (Perez et al., 2009). Fyn-Tau-microtubule interaction was an important step in OL growth, as clearly demonstrated by Klein et al. (2002). Our results indicated that besides the fact that aTf promotes morphological differentiation, MBP induction by aTf is impeded when Fyn is inhibited.

The regulatory mechanism of Fyn activity during OL maturation is associated with a change from PI3K to MAPK in neuregulin signaling, thereby switching the response from proliferation to differentiation (Colognato et al., 2004). Our results indicate that transient Fyn activation associated to the endocytic process occurs prior to MEK/ERK activation, while aTf signaling by PI3K/Akt pathway does not involve Fyn activity. Also, Fyn activation is needed for ERK phosphorylation in the aTf-triggered pathway of OPC maturation. In agreement with this, Fyffe-Maricich et al. (2011) demonstrated that ERK is important for the maturation of early OPC into mature OL *in vivo*, but not for survival or proliferation. Moreover, pharmacological inhibition of ERK activation in the presence of different extracellular signaling induces fewer mature OL (Baron et al., 2000; Chew et al., 2010; Du et al., 2006; Galvin et al., 2010; Xiao et al., 2012; Younes-Rapozo et al., 2009). In contrast, several studies have

Fig. 4. Transduction signaling pathways are activated after TfR stimulation. A–D, OPC were treated with Ab-TfR (2 μg/ml) for 30 min prior to the addition of aTf. Cells were then incubated for 15 min with aTf. Whole cell lysates were analyzed by WB with the corresponding antibodies. E–G, OPC were treated with human aTf or bovine aTf (100 μg/ml) during 15 min and analyzed by WB. A, Quantitative analysis of aTf uptake is represented as a Tf(h)/GAPDH ratio. B and E, Fyn activation is shown as an active-Fyn/total Fyn ratio. C and F, ERK phosphorylation is shown as a p-ERK/ERK ratio. D and G, Akt phosphorylation is represented as p-Akt/Akt and p-Akt/GAPDH ratios respectively. Bars in all graphs represent the mean ± SEM of three independent experiments. H–J, OPC were treated with Tf-TR (100 μg/ml) for 5 min (red, I). Confocal image shows Tf receptor 1 (CD71) in green (H). The inset in merge image of H shows a magnified OPC process. Tf-TR co-localization with TfR1 immunolabeling is indicated with arrowheads and $TR^{+}/TfR1^{-}$ are indicated with arrows. Hoechst nuclear dye is shown in blue. Scale bar in H equals 5 μm. One-way ANOVA followed by Neuman–Keuls Multiple Comparison Test was used in A–D and one-way ANOVA followed by Dunnett's Multiple Comparison Test were used in E–G to determine statistical significance; $^{***}p < 0.001$, $^{**}p < 0.01$, $^{*}p < 0.05$, ns non-significant. Ab-TfR: Antibody against Tf receptor; aTf: human apotransferrin; Fyn: Fyn tyrosine kinase; GAPDH: Glyceraldehyde 3-phosphate dehydrogenase.

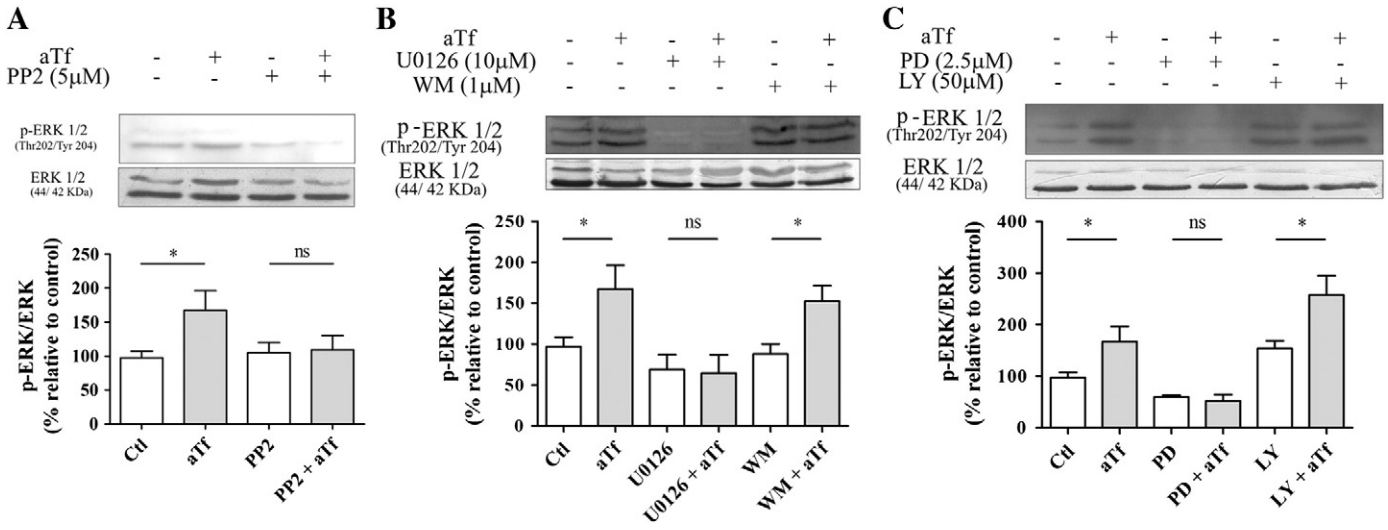


Fig. 6. aTf activates ERK through Fyn. OPC were treated with PP2 (5 μM) in A, U0126 (10 μM) and WM (1 μM) in B, PD (2.5 μM) and LY (50 μM) in C for 30 min prior to the addition of aTf. Cells were then incubated for 15 min with aTf. Whole cell lysates were analyzed by WB with anti-phospho ERK and anti-ERK1/2 antibodies. The p-ERK/ERK ratio was used to evaluate ERK pathway activation. Bars in all graphs represent the mean ± SEM of four independent experiments. Two-tailed Student's *t*-test was used to determine statistical significance; $^{*}p < 0.05$, ns non-significant. aTf: human apotransferrin; PP2: Fyn kinase inhibitor; U0126 and PD (PD98059): MEK1/2 inhibitor; WM (wortmannin) and LY (LY294002): PI3 kinase inhibitor.

suggested that MAPK signaling is important for OL proliferation (Cui and Almazan, 2007; Okada et al., 2010; Stariha and Kim, 2001).

There are two membrane receptors of Tf that have different functions and expression patterns. TfR1 is expressed in all cells and mediates the cellular uptake of transferrin-bound iron. TfR2 is only expressed in some cells and acts as an iron sensor to regulate hepcidin expression. In the central nervous system, TfR1 is expressed in normal brain and in brain tumors (Han et al., 2003; Recht et al., 1990) and TfR2 is predominantly expressed in hepatocytes, although Moos et al. (1998) report their expression in neurons. Hänninen et al. (2009) describe TfR2 expression in human brain and brain tumors and Calzolari et al. (2010) do so in astrocytomas. Furthermore, ERK signaling activation is associated with TfR2 in different cells regulating iron metabolism (Calzolari et al., 2006; Poli et al., 2010; Ramey et al., 2009). Our experiments showed that the signaling pathway of human aTf is different from that of bovine aTf, which suggests that PI3K/Akt is independent from Fyn/MEK/ERK pathway. Thus the induction of ERK phosphorylation triggered by aTf seems to be linked to TfR2. It was probably more interesting to observe that the MEK/ERK pathway was directly associated with MBP expression protein. The precise identification and the role of TfR in glial cells remain unexplored. However, the fact that aTf has maturational effects in a temporal window when TfR is present in OPC gives support to our hypothesis that differentiation is due to receptor internalization and that the activation of the signal transduction

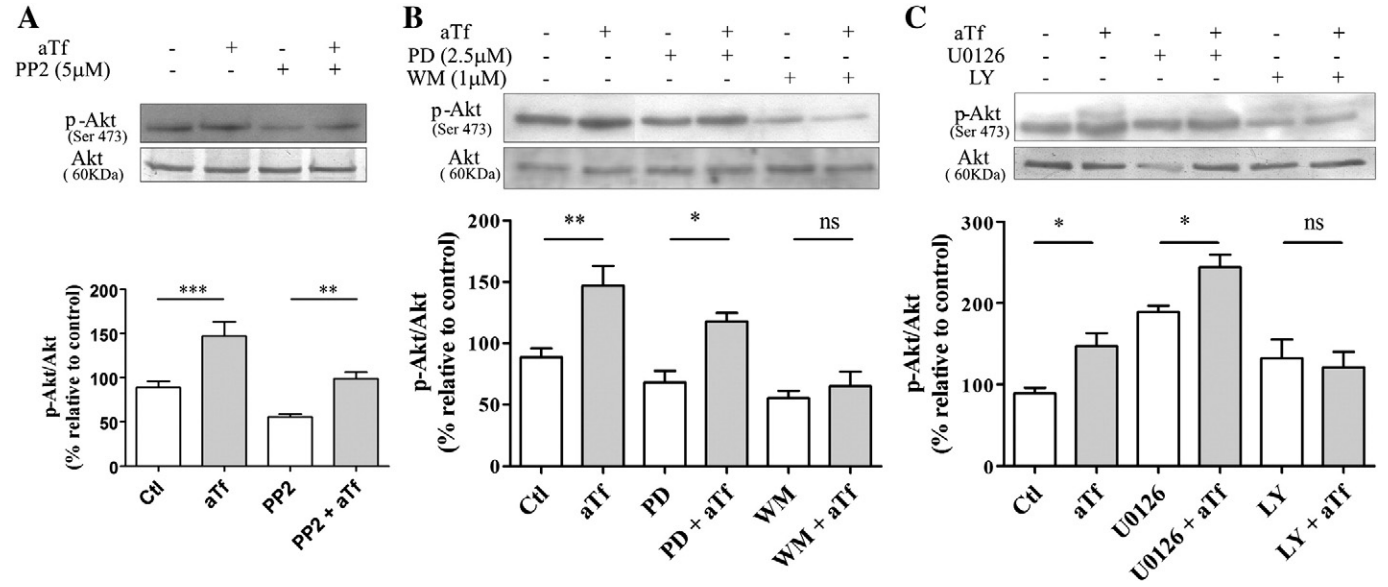


Fig. 7. PI3K/Akt pathway is independent of Fyn and ERK activation. OPC were treated with PP2 (5 μM) in A, PD (2.5 μM) and WM (1 μM) in B, U0126 (10 μM) and LY (50 μM) in C for 30 min prior to the addition of aTf. Cells were then incubated for 15 min with aTf. Whole cell lysates were analyzed by WB with anti-phospho Akt and anti-total Akt antibodies. p-Akt/Akt ratio was used to evaluate Akt pathway activation. Bars in all graphs represent the mean ± SEM of four independent experiments. Two-tailed Student's *t*-test was used to determine statistical significance; $^{***}p < 0.001$, $^{**}p < 0.01$, $^{*}p < 0.05$, ns non-significant. aTf: human apotransferrin; PP2: Fyn kinase inhibitor; U0126 and PD (PD98059): MEK1/2 inhibitor; WM (wortmannin) and LY (LY294002): PI3 kinase inhibitor.

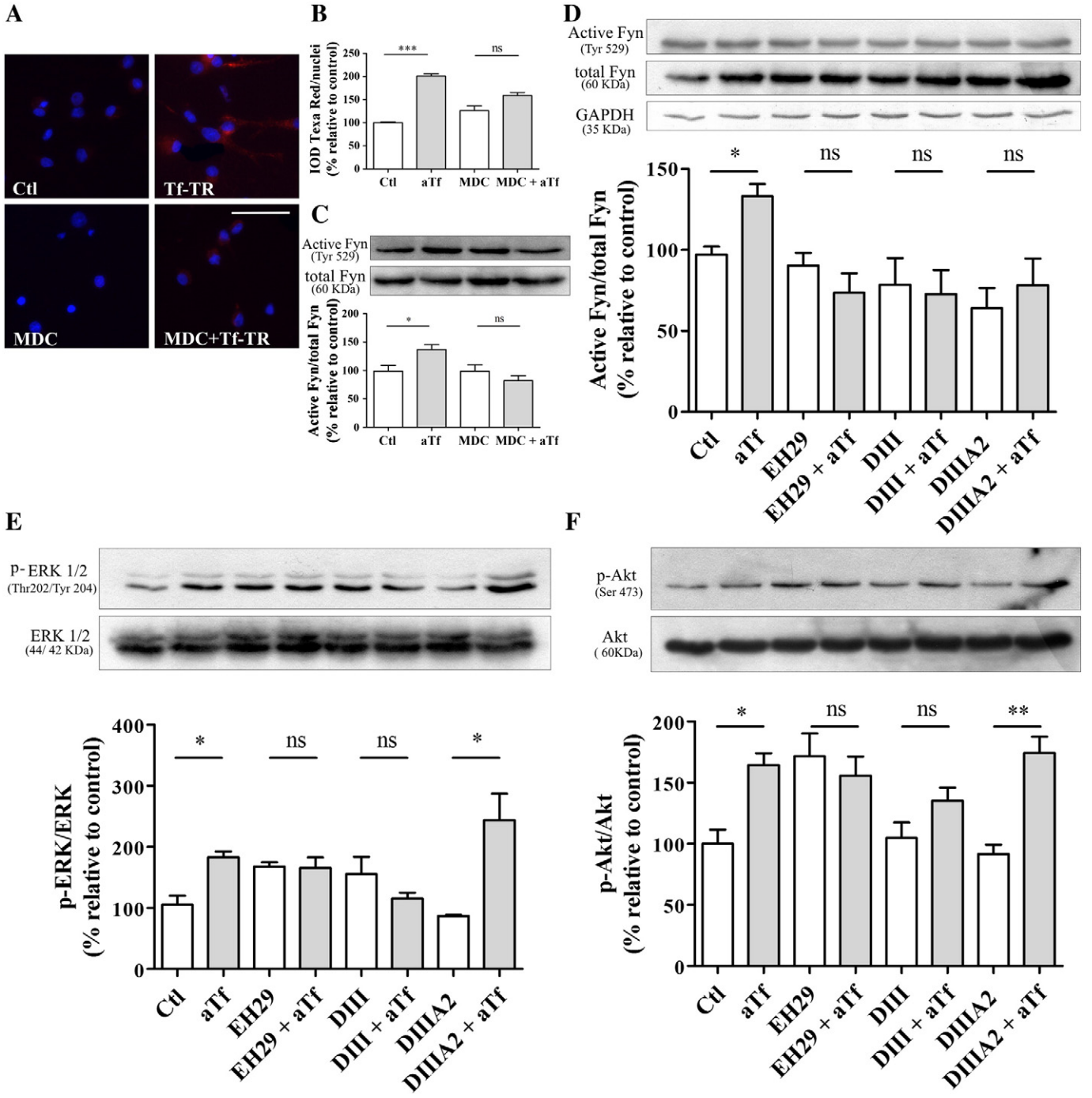


Fig. 8. Signaling activation by aTf requires clathrin-mediated endocytosis of TfR. A, Cultures were treated with MDC (50 μM) during 10 min previous to the addition of Tf-TR. Cells were then incubated for 15 min with Tf-TR. Scale bar equals 50 μm for all panels. Tf-TR fluorescence is shown in red and Hoechst nuclear dye is shown in blue. B, The red fluorescence shown in A is quantitated and shown as a red fluorescence/nuclei ratio. C, OPC were treated with MDC (50 μM) for 10 min prior to the addition of aTf. Cells were then incubated for 15 min with aTf. Whole cell lysates were analyzed by WB. The active-Fyn/total Fyn ratio was used to evaluate Fyn activation. D-F, OPC were transfected with Mock (Ctl and aTf), EH29, DIII, and DIIIΔ2 plasmid as described in the Materials and methods section, followed by treatment with aTf for 15 min. Whole cell lysates were analyzed by WB. D, Fyn activation was analyzed as an active-Fyn/total Fyn ratio. The GAPDH immunopositive bands were used as sample loading. E, ERK phosphorylation was evaluated as a p-ERK/ERK ratio. F, Akt phosphorylation was analyzed as a p-Akt/Akt ratio. The bars in all graphs represent the mean ± SEM of three independent experiments. Two-tailed Student's *t*-test was used to determine statistical significance; $^{***}p < 0.001$, $^{**}p < 0.01$, $^{*}p < 0.05$, ns non-significant. aTf: human apotransferrin; Fyn: Fyn tyrosine kinase; GAPDH: Glyceraldehyde 3-phosphate dehydrogenase; IOD: integrated optical density; MDC: primary amine monodansylcadaverin; Tf-TR: Texas Red-labeled transferrin; EH29: Eps15 dominant negative mutant -pEGFP-C2-Eps15 EH29 plasmid-; DIII: Eps15 dominant negative mutant -pEGFP-C2-Eps15 DIII plasmid-; DIIIΔ2: Eps15 irrelevant mutant-pEGFP-C2-Eps15 DIIIΔ2 plasmid-.

cascade is triggered by this phenomenon. Although mature OL do not express TfR, Todorich et al. (2008) suggested that Tim-2, an alternative iron delivery mechanism, exists in these cells.

Depending on the stage of OL development, a growth factor can mediate cell proliferation, survival or progression of differentiation through the PI3K pathway (Baron et al., 2000; Cui and Almazan, 2007; Flores et al., 2008; Guardiola-Diaz et al., 2012; Xu et al., 2012). It is possible to speculate that enhancement of OPC maturation by aTf is due to Akt phosphorylation. The PI3K/Akt and the MAPK signaling pathways have been implicated in different processes such as

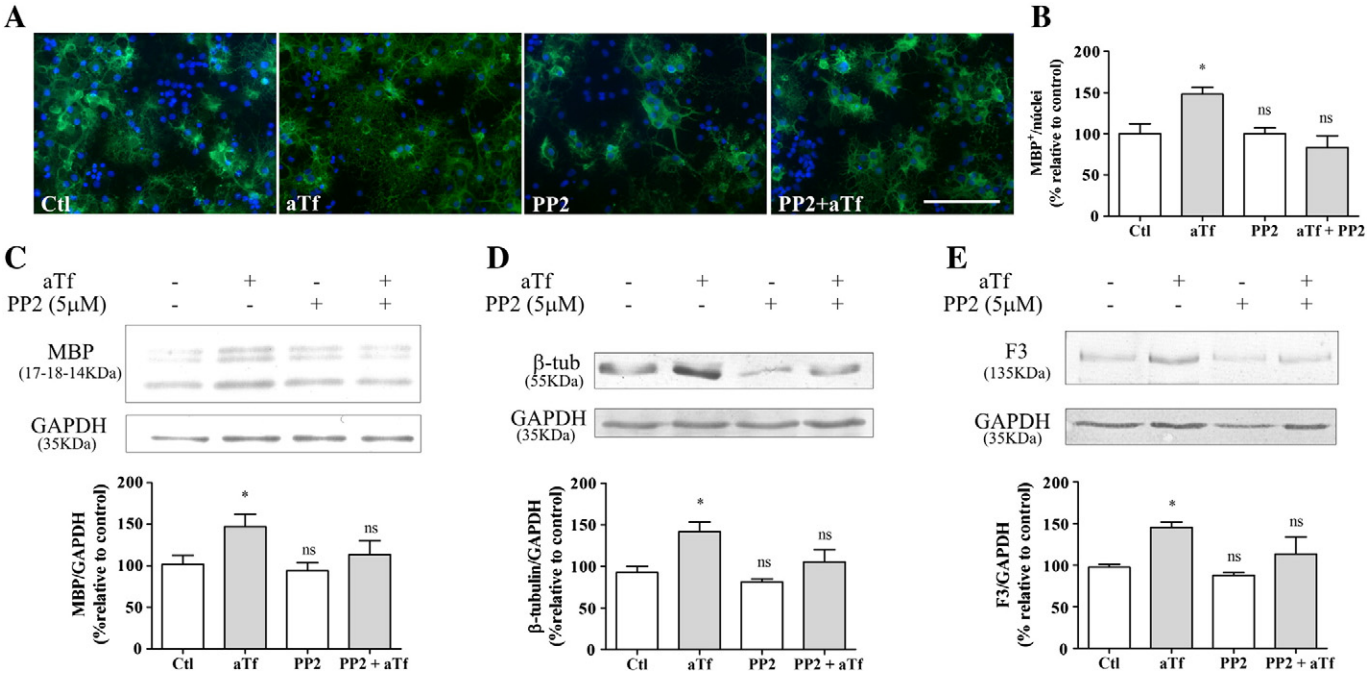


Fig. 9. aTf induces OPC maturation through Fyn activation. OPC were treated with or without PP2 (5 μM) for 30 min, followed by aTf addition for four days. A, MBP is shown in green and Hoechst nuclear dye is shown in blue. Scale bar equals 100 μm for all panels. B, MBP cells were quantitated and normalized to the total nuclei in each image. C–E, Whole cell lysates were analyzed by WB four days after aTf treatment. The IOD of MBP (C), β-tubulin (D), F3-contactin (E) immunopositive bands was quantitated and expressed as a normalized value compared to GAPDH. Bars in graphs represent the mean ± SEM belonging to five (A, B and C) or three (D and E) independent experiments. One-way ANOVA followed by Neuman–Keuls Multiple Comparison Test was used to determine statistical significance for C–E; *$p < 0.05$, ns non-significant, symbols above bars indicate significance compared to the control condition (Ctl). aTf: human apotransferrin; β-tub: β-tubulin; F3: F3-contactin; GAPDH: Glyceraldehyde 3-phosphate dehydrogenase; IOD: integrated optical density; PP2: Fyn kinase inhibitor; MBP: myelin basic protein.

trophic support from astrocytes to OPC against injury (Arai and Lo, 2010), PDGF-AA induction of OPC differentiation from embryonic neural stem cells (Hu et al., 2012) and the neurotrophin-3 stimulatory effect on protein translation during OL maturation (Coelho et al., 2009). Our data suggest that both signaling pathways are implicated in OPC maturation by aTf, since the stimulation of MBP, β-tubulin and F3-contactin expression is decreased in the presence of PI3K/Akt and MAPK inhibitors.

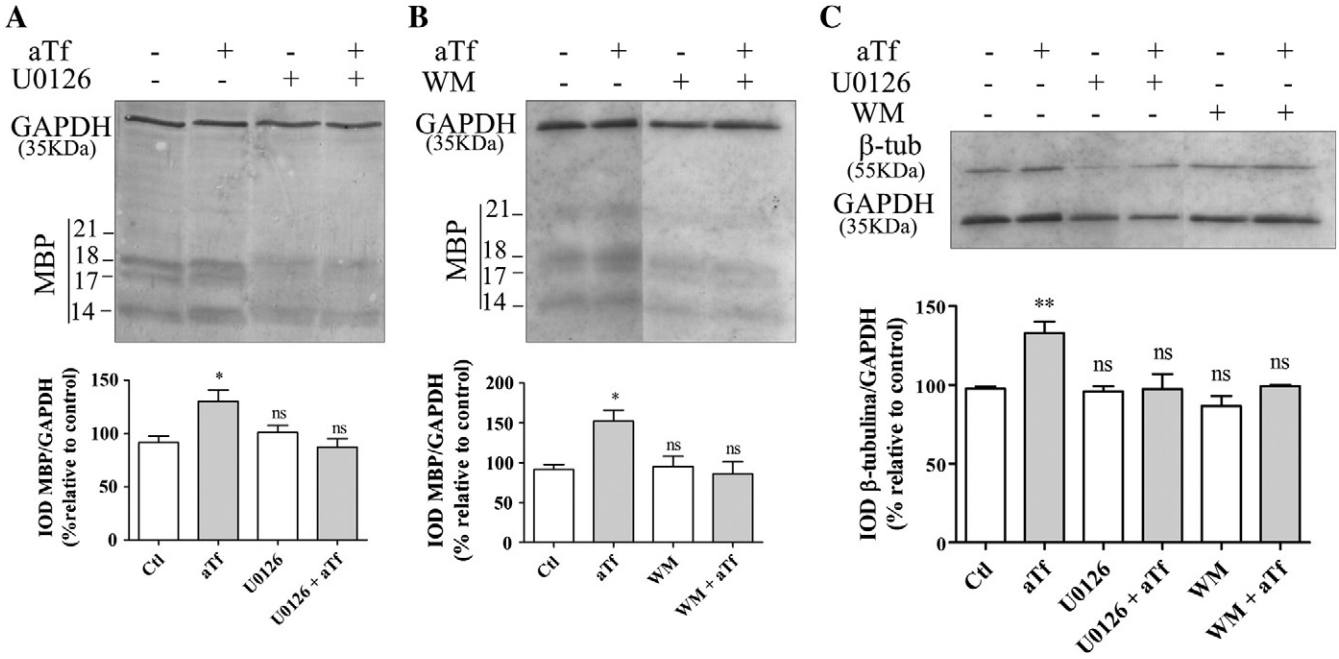


Fig. 10. Maturation effect of aTf is mediated by Fyn/MEK/ERK and PI3K/Akt pathways. OPC were treated with or without U0126 (10 μM) or WM (1 μM) for 30 min, followed by aTf treatment for four days. Whole cell lysates were analyzed by WB. The IOD of MBP (A and B) and β-tubulin (C) immunopositive bands was quantitated and expressed as a normalized value compared to GAPDH. Ratios are expressed as the mean ± SEM of three independent experiments for all graphs. One-way ANOVA followed by Neuman–Keuls Multiple Comparison Test was used to determine statistical significance; **$p < 0.01$, *$p < 0.05$, ns non-significant, symbols above bars indicate significance compared to the control condition (Ctl). aTf: human apotransferrin; β-tub: β-tubulin; GAPDH: Glyceraldehyde 3-phosphate dehydrogenase; IOD: integrated optical density; MBP: myelin basic protein; U0126: MEK1/2 inhibitor; WM: PI3 kinase inhibitor (wortmannin).

Clathrin-dependent endocytosis inhibitor MDC did not only inhibit TfR internalization but also blocked aTf-stimulated signaling. This result allows us to better understand the functional role of TfR endocytosis in intracellular signal transduction. The role for clathrin coated vesicles in TfR recycling, as well as the involvement of AP2 complex in the uptake of proteins with YXXϕ sorting signal, such as the TfR (Nesterov et al., 1999; Ohno et al, 1995) were also previously demonstrated. However, there is also evidence that alternative mechanisms mediated by caveolae could also lead to TfR internalization, which suggests the existence of mechanisms that may compensate for defects in the clathrin-dependent uptake route (Puri, 2009). On the other hand, AP2 depletion was found to cause inhibition of Tf uptake even though AP2 knock-down did not diminish clathrin-coated vesicle formation; this observation rules out the possibility of an AP2-independent clathrin-dependent internalization of TfR (Motley et al., 2003).

In the present work we observed that lack of Fyn/MEK/ERK and PI3K/Akt activation in EH29 and DIII (dominant negative of Eps15)-transfected cells indicated that TfR signaling occurs in the earlier steps of receptor endocytosis and that it depends on the proper formation of the AP2-Eps15 complex. Cao et al. (2010) demonstrated that activated Src kinase regulated TfR endocytosis in epithelial cells. In this context, if there is an alternative mechanism for TfR internalization that could take place, it seems to be insufficient to evoke Fyn activation. These results demonstrated that the activation of the pathways necessary for OPC maturation is dependent on AP2 recruitment onto the plasma membrane for clathrin-mediated endocytosis of TfR.

Together, the data presented here demonstrate that endocytosis of TfR accelerates OL differentiation. In addition, we provide evidence indicating that this effect is exerted through Fyn/MEK/ERK and PI3K/Akt signaling pathways. In agreement with this, insulin-like growth factor-1 also stimulates protein synthesis through PI3K/mTOR/Akt and MEK/ERK pathways in OL (Bibollet-Bahena and Almazan, 2009). The molecular mechanisms and signaling pathways by which aTf promotes OPC differentiation provide a key point to develop new therapies to counteract the loss of OPC maturation in demyelinating disorders.

Acknowledgments

Funding sources: This work was supported by grants from the Agencia Nacional de Promoción Científica y Tecnológica (BID-PICT 0791) and the University of Buenos Aires (UBA 20020100100395).

The authors are grateful to Dr. Paula Franco and Lucas Silvestroff for insightful comments and helpful discussions on this manuscript and to Dr. Benmerah for the kind gift of expression plasmids. We wish to thank Dr. Ferrari Alejandro for the excellent artwork.

References

Adamo, A.M., Paez, P.M., Escobar Cabrera, O.E., Wolfson, M., Franco, P.G., Pasquini, J.M., Soto, E.F., 2006. Remyelination after cuprizone-induced demyelination in the rat is stimulated by apotransferrin. Exp. Neurol. 198, 519–529.

Arai, K., Lo, E.H., 2010. Astrocytes protect oligodendrocyte precursor cells *via* MEK/ERK and PI3K/Akt signaling. J. Neurosci. Res. 88, 758–763.

Artini, M., Scoarughi, G.L., Cellini, A., Papa, R., Barbato, G., Selan, L., 2012. Holo and apo-transferrins interfere with adherence to abiotic surfaces and with adhesion/invasion to HeLa cells in Staphylococcus spp Biometals 25, 413–421.

Badaracco, M.E., Ortiz, E.H., Soto, E.F., Connor, J., Pasquini, J.M., 2008. Effect of transferrin on hypomyelination induced by iron deficiency. J. Neurosci. Res. 86, 2663–2673.

Baer, A.S., Syed, Y.A., Kang, S.U., Mitteregger, D., Vig, R., ffrench-Constant, C., Franklin, R.J.M., Altmann, F., Lubec, G., Kotter, M.R., 2009. Myelin-mediated inhibition of oligodendrocyte precursor differentiation can be overcome by pharmacological modulation of Fyn-RhoA and protein kinase C signaling. Brain 132, 465–481.

Baron, W., Metz, B., Bansal, R., Hoekstra, D., de Vries, H., 2000. PDGF and FGF-2 signaling in oligodendrocyte progenitor cells, regulation of proliferation and differentiation by multiple intracellular signaling pathways. Mol. Cell. Neurosci. 15, 314–329.

Bartlett, W.P., Li, X.S., Connor, J.R., 1991. Expression of transferrin mRNA in the CNS of normal and jumpy mice. J. Neurochem. 57, 318–322.

Benmerah, A., Bayrou, M., Cerf-Bensussan, N., Dautry-Varsat, A., 1999. Inhibition of clathrin-coated pit assembly by an Eps15 mutant. J. Cell Sci. 112, 1303–1311.

Bibollet-Bahena, O., Almazan, G., 2009. IGF-1-stimulated protein synthesis in oligodendrocyte progenitors requires PI3K/mTOR/Akt and MEK/ERK pathways. J. Neurochem. 109, 1440–1451.

Bloch, B., Popovici, T., Levin, M.J., Tuil, D., Kahn, A., 1985. Transferrin gene expression visualized in oligodendrocytes of the rat brain by using *in situ* hybridization and immunohistochemistry. Proc. Natl. Acad. Sci. U. S. A. 82, 6706–6710.

Calzolari, A., Raggi, C., Deaglio, S., Sposi, N.M., Stafsnes, M., Fecchi, K., Parolini, I., Malavasi, F., Peschle, C., Sargiacomo, M., Testa, U., 2006. TfR2 localizes in lipid raft domains and is released in exosomes to activate signal transduction along the MAPK pathway. J. Cell Sci. 119, 4486–4498.

Calzolari, A., Larocca, L.M., Deaglio, S., Finisguerra, V., Boe, A., Raggi, C., Ricci-Vitani, L., Pierconti, F., Malavasi, F., De Maria, R., Testa, U., Pallini, R., 2010. Transferrin receptor 2 is frequently and highly expressed in glioblastomas. Transl. Oncol. 3, 123–134.

Cao, H., Chen, J., Krueger, E.W., McNiven, M.A., 2010. SRC-mediated phosphorylation of dynamin and cortactin regulates the "constitutive" endocytosis of transferrin. Mol. Cell. Biol. 30, 781–792.

Casaccia-Bonnefil, P., Aibel, L., Chao, M.V., 1996. Central glial and neuronal populations display differential sensitivity to ceramide-dependent cell death. J. Neurosci. Res. 43, 382–389.

Chew, L.J., Coley, W., Cheng, Y., Gallo, V., 2010. Mechanisms of regulation of oligodendrocyte development by p38 mitogen-activated protein kinase. J. Neurosci. 30, 11011–11027.

Coelho, R.P., Yuelling, L.M., Fuss, B., Sato-Bigbee, C., 2009. Neurotrophin-3 targets the translational initiation machinery in oligodendrocytes. Glia 57, 1754–1764.

Cole, P.A., Shen, K., Qiao, Y., Wang, D., 2003. Protein tyrosine kinases Src and Csk, a tail's tale. Curr. Opin. Chem. Biol. 7, 580–585.

Colognato, H., Ramachandrappa, S., Olsen, I.M., ffrench-Constant, C., 2004. Integrins direct Src family kinases to regulate distinct phases of oligodendrocyte development. J. Cell Biol. 167, 365–375.

Cui, Q.L., Almazan, G., 2007. IGF-I induced oligodendrocyte progenitor proliferation requires PI3K/Akt, MEK/ERK, and Src-like tyrosine kinases. J. Neurochem. 100, 1480–1493.

Cui, Q.L., Zheng, W.H., Quirion, R., Almazan, G., 2005. Inhibition of Src-like kinases reveals Akt-dependent and -independent pathways in insulin-like growth factor I-mediated oligodendrocyte progenitor survival. J. Biol. Chem. 280, 8918–8928.

Du, Y., Lercher, L.D., Zhou, R., Dreyfus, C.F., 2006. Mitogen-activated protein kinase pathway mediates effects of brain-derived neurotrophic factor on differentiation of basal forebrain oligodendrocytes. J. Neurosci. Res. 84, 1692–1702.

Escobar Cabrera, O.E., Bongarzone, E.R., Soto, E.F., Pasquini, J.M., 1994. Single intracerebral injection of apotransferrin in young rats induces increased myelination. Dev. Neurosci. 16, 248–254.

Escobar Cabrera, O.E., Zakin, M.M., Soto, E.F., Pasquini, J.M., 1997. Single intracranial injection of apotransferrin in young rats increases the expression of specific myelin protein mRNA. J. Neurosci. Res. 47, 603–608.

Espinosa de los Monteros, A., Kumar, S., Scully, S., Cole, R., de Vellis, J., 1990. Transferrin gene expression and secretion by rat brain cells *in vitro*. J. Neurosci. Res. 25, 576–580.

Espinosa de los Monteros, A., Kumar, S., Zhao, P., Huang, C.J., Nazarian, R., Pan, T., Scully, S., Chang, R., de Vellis, J., 1999. Transferrin is an essential factor for myelination. Neurochem. Res. 24, 235–248.

Flores, A.I., Narayanan, S.P., Morse, E.N., Shick, H.E., Yin, X., Kidd, G., Avila, R.L., Kirschner, D.A., Macklin, W.B., 2008. Constitutively active Akt induces enhanced myelination in the CNS. J. Neurosci. 28, 7174–7183.

Fyffe-Maricich, S.L., Karlo, J.C., Landreth, G.E., Miller, R.H., 2011. The ERK2 mitogen-activated protein kinase regulates the timing of oligodendrocyte differentiation. J. Neurosci. 31, 843–850.

Galvin, J., Eyermann, C., Colognato, H., 2010. Dystroglycan modulates the ability of insulin-like growth factor-1 to promote oligodendrocyte differentiation. J. Neurosci. Res. 88, 3295–3307.

Garcia, C., Paez, P.M., Davio, C., Soto, E.F., Pasquini, J.M., 2004. Apotransferrin induces cAMP/CREB pathway and cell cycle exit in immature oligodendroglial cells. J. Neurosci. Res. 78, 338–346.

Guardia Clausi, M., Paez, P.M., Campagnoni, A.T., Pasquini, L.A., Pasquini, J.M., 2012. Intranasal administration of aTf protects and repairs the neonatal white matter after a cerebral hypoxic-ischemic event. Glia 60, 1540–1554.

Guardiola-Diaz, H.M., Ishii, A., Bansal, R., 2012. Erk1/2 MAPK and mTOR signaling sequentially regulates progression through distinct stages ofoligodendrocyte differentiation. Glia 3, 476–486.

Han, J., Day, J.R., Connor, J.R., Beard, J.L., 2003. Gene expression of transferrin and transferrin receptor in brains of control *vs.* iron-deficient rats. Nutr. Neurosci. 6, 1–10.

Hanke, J.H., Gardner, J.P., Dow, R.L., Changelian, P.S., Brissette, W.H., Weringer, E.J., Pollok, B.A., Connelly, P.A., 1996. Discovery of a novel, potent, and Src family-selective tyrosine kinase inhibitor. Study of Lck- and FynT-dependent T cell activation. J. Biol. Chem. 271, 695–701.

Hänninen, M.M., Haapasalo, J., Haapasalo, H., Fleming, R.E., Britton, R.S., Bacon, B.R., Parkkila, S., 2009. Expression of iron-related genes in human brain and brain tumors. BMC Neurosci. 10, 36.

Hu, J.G., Wang, Y.X., Wang, H.J., Bao, M.S., Wang, Z.H., Ge, X., Wang, F.C., Zhou, J.S., Lü, H.Z., 2012. PDGF-AA mediates B104 CM-induced oligodendrocyte precursor cell differentiation of embryonic neural stem cells through Erk, PI3K, and p38 signaling. J. Mol. Neurosci. 46, 644–653.

Kawabata, H., Germain, R.S., Vuong, P.T., Nakamaki, T., Said, J.W., Koeffler, H.P., 2000. Transferrin receptor 2-alpha supports cell growth both in iron-chelated cultured cells and *in vivo*. J. Biol. Chem. 275, 16618–16625.

Kawabata, H., Tong, X., Kawanami, T., Wano, Y., Hirose, Y., Sugai, S., Koeffler, H.P., 2004. Analyses for binding of the transferrin family of proteins to the transferrin receptor 2. Br. J. Haematol. 127, 464–473.
Klein, C., Kramer, E.M., Cardine, A.M., Schraven, B., Brandt, R., Trotter, J., 2002. Process outgrowth of oligodendrocytes is promoted by interaction of Fyn kinase with the cytoskeletal protein tau. J. Neurosci. 22, 698–707.
Krämer, E.M., Klein, C., Koch, T., Boytinck, M., Trotter, J., 1999. Compartmentation of Fyn kinase with glycosylphosphatidylinositol-anchored molecules in oligodendrocytes facilitates kinase activation during myelination. J. Biol. Chem. 274, 29042–29049.
Krämer-Albers, E.M., White, R., 2011. From axon-glial signalling to myelination, the integrating role of oligodendroglial Fyn kinase. Cell. Mol. Life Sci. 68, 2003–2012.
Laursen, L.S., Chan, C.W., ffrench-Constant, C., 2009. An integrin-contactin complex regulates CNS myelination by differential Fyn phosphorylation. J. Neurosci. 29, 9174–9185.
Lesley, J.F., Schulte, R.J., 1985. Inhibition of cell growth by monoclonal anti-transferrin receptor antibodies. Mol. Cell. Biol. 5, 1814–1821.
Marta, C.B., Escobar Cabrera, O.E., Garcia, C.I., Villar, M.J., Pasquini, J.M., Soto, E.F., 2000. Oligodendroglial cell differentiation in rat brain is accelerated by the intracranial injection of apotransferrin. Cell. Mol. Biol. 46, 529–539.
McArdle, H.J., Morgan, E.H., 1984. The effect of monoclonal antibodies to the human transferrin receptor on transferrin and iron uptake by rat and rabbit reticulocytes. J. Biol. Chem. 259, 1398–1400.
McCarthy, K.D., de Vellis, J., 1980. Preparation of separate astroglial and oligodendroglial cell cultures from rat cerebral tissue. J. Cell Biol. 85, 890–902.
Moos, T., Oates, P.S., Morgan, E.H., 1998. Expression of the neuronal transferrin receptor is age dependent and susceptible to iron deficiency. J. Comp. Neurol. 398, 420–430.
Motley, A., Bright, N.A., Seaman, M.N., Robinson, M.S., 2003. Clathrin-mediated endocytosis in AP-2-depleted cells. J. Cell Biol. 162, 909–918.
Nesterov, A., Carter, R.E., Sorkina, T., Gill, G.N., Sorkin, A., 1999. Inhibition of the receptor-binding function of clathrin adaptor protein AP-2 by dominant-negative mutant mu2 subunit and its effects on endocytosis. EMBO J. 18, 2489–2499.
Ohno, H., Stewart, J., Fournier, M.C., Bosshart, H., Rhee, I., Miyatake, S., Saito, T., Gallusser, A., Kirchhausen, T., Bonifacino, J.S., 1995. Interaction of tyrosine-based sorting signals with clathrin-associated proteins. Science 269, 1872–1875.
Okada, M., Makino, A., Nakajima, M., Okuyama, S., Furukawa, S., Furukawa, Y., 2010. Estrogen stimulates proliferation and differentiation of neural stem/progenitor cells through different signal transduction pathways. Int. J. Mol. Sci. 11, 4114–4123.
Ortiz, E.H., Pasquini, L.A., Soto, E.F., Pasquini, J.M., 2005. Apotransferrin and the cytoskeleton of oligodendroglial cells. J. Neurosci. Res. 82, 822–830.
Osterhout, D.J., Wolven, A., Wolf, R.M., Resh, M.D., Chao, M.V., 1999. Morphological differentiation of oligodendrocytes requires activation of Fyn tyrosine kinase. J. Cell Biol. 145, 1209–1218.
Paez, P.M., Marta, C.B., Moreno, M.B., Soto, E.F., Pasquini, J.M., 2002. Apotransferrin decreases migration and enhances differentiation of oligodendroglial progenitor cells in an *in vitro* system. Dev. Neurosci. 24, 47–58.
Pasquini, L.A., Paez, P.M., Moreno, M.A., Pasquini, J.M., Soto, E.F., 2003. Inhibition of the proteasome by lactacystin enhances oligodendroglial cell differentiation. J. Neurosci. 23, 4635–4644.
Perez, M.J., Ortiz, E.H., Roffé, M., Soto, E.F., Pasquini, J.M., 2009. Fyn kinase is involved in oligodendroglial cell differentiation induced by apotransferrin. J. Neurosci. Res. 15, 3378–3389.
Poli, M., Luscieti, S., Gandini, V., Maccarinelli, F., Finazzi, D., Silvestri, L., Roetto, A., Arosio, P., 2010. Transferrin receptor 2 and HFE regulate furin expression *via* mitogen-activated protein kinase/extracellular signal-regulated kinase (MAPK/Erk) signaling. Implications for transferrin-dependent hepcidin regulation. Haematologica 95, 1832–1840.
Puri, C., 2009. Loss of myosin VI no insert isoform (NoI) induces a defect in clathrin-mediated endocytosis and leads to caveolar endocytosis of transferrin receptor. J. Biol. Chem. 284, 34998–35014.
Ramey, G., Deschemin, J.C., Vaulont, S., 2009. Crosstalk between the mitogen activated protein kinase and bone morphogenetic protein/he mojuvelin pathways is required for the induction of hepcidin by holotransferrin in primary mouse hepatocytes. Haematologica 94, 765–772.
Rappoport, J.Z., Simon, S.M., Benmerah, A., 2004. Understanding living clathrin-coated pits. Traffic 5, 327–337.
Ray, E., Samanta, A.K., 1996. Dansyl cadaverine regulates ligand induced endocytosis of interleukin-8 receptor in human polymorphonuclear neutrophils. FEBS Lett. 378, 235–239.
Recht, L.D., Griffin, T.W., Raso, V., Salimi, A.R., 1990. Potent cytotoxicity of an antihuman transferrin receptor-ricin A-chain immunotoxin on human glioma cells *in vitro*. Cancer Res. 50, 6696–6700.
Rouault, T.A., Cooperman, S., 2006. Brain iron metabolism. Semin. Pediatr. Neurol. 13, 142–148.
Saleh, M.C., Espinosa de los Monteros, A., de Arriba Zerpa, G.A., Fontaine, I., Piaud, O., Djordjijevic, D., Baroukh, N., Garcia Otin, A.L., Ortiz, E., Lewis, S., Fiette, L., Santambrogio, P., Belzung, C., Connor, J.R., de Vellis, J., Pasquini, J.M., Zakin, M.M., Baron, B., Guillou, F., 2003. Myelination and motor coordination are increased in transferrin transgenic mice. J. Neurosci. Res. 72, 587–594.
Stariha, R.L., Kim, S.U., 2001. Protein kinase C and mitogen-activated protein kinase signalling in oligodendrocytes. Microsc. Res. Tech. 6, 680–688.
Suzuki, A., Raya, A., Kawakami, Y., Morita, M., Matsui, T., Nakashima, K., Gage, F.H., Rodríguez-Esteban, C., Izpisúa Belmonte, J.C., 2006. Maintenance of embryonic stem cell pluripotency by Nanog-mediated reversal of mesoderm specification. Nat. Clin. Pract. Cardiovasc. Med. (Suppl. 1), S114–S122.
Todorich, B., Zhang, X., Slagle-Webb, B., Seaman, W.E., Connor, J.R., 2008. Tim-2 is the receptor for H-ferritin on oligodendrocytes. J. Neurochem. 107, 1495–1505.
Trowbridge, I.S., Lopez, F., 1982. Monoclonal antibody to transferrin receptor blocks transferrin binding and inhibits human tumor cell growth *in vitro*. Proc. Natl. Acad. Sci. U. S. A. 79, 1175–1179.
Umemori, H., Wanaka, A., Kato, H., Takeuchi, M., Tohyama, M., Yamamoto, T., 1992. Specific expressions of Fyn and Lyn, lymphocyte antigen receptor-associated tyrosine kinases, in the central nervous system. Brain Res. Mol. Brain Res. 16, 303–310.
Umemori, H., Sato, S., Yagi, T., Aizawa, S., Yamamoto, T., 1994. Initial events of myelination involve Fyn tyrosine kinase signalling. Nature 367, 572–576.
Umemori, H., Kadowaki, Y., Hirosawa, K., Yoshida, Y., Hironaka, K., Okano, H., Yamamoto, T., 1999. Stimulation of myelin basic protein gene transcription by Fyn tyrosine kinase for myelination. J. Neurosci. 19, 1393–1397.
Wally, J., Buchanan, S.K., 2007. A structural comparison of human serum transferrin and human lactoferrin. Biometals 20, 249–262.
White, R., Gonsior, C., Krämer-Albers, E.M., Stöhr, N., Hüttelmaier, S., Trotter, J., 2008. Activation of oligodendroglial Fyn kinase enhances translation of mRNAs transported in hnRNP A2-dependent RNA granules. J. Cell Biol. 181, 579–586.
Xiao, L., Guo, D., Hu, C., Shen, W., Shan, L., Li, C., Liu, X., Yang, W., Zhang, W., He, C., 2012. Diosgenin promotes oligodendrocyte progenitor cell differentiation through estrogen receptor-mediated ERK1/2 activation to accelerate remyelination. Glia 60, 1037–1052.
Xu, C., Lv, L., Zheng, G., Li, B., Gao, L., Sun, Y., 2012. Neuregulin1β1 protects oligodendrocyte progenitor cells from oxygen glucose deprivation injury induced apoptosis *via* ErbB4-dependent activation of PI3-kinase/Akt. Brain Res. 1467, 104–112.
Younes-Rapozo, V., Felgueiras, L.O., Viana, N.L., Fierro, I.M., Barja-Fidalgo, C., Manhães, A.C., Barradas, P.C., 2009. A role for the MAPK/ERK pathway in oligodendroglial differentiation *in vitro*, stage specific effects on cell branching. Int. J. Dev. Neurosci. 27, 757–768.