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Tumor Necrosis Factor-Like Weak Inducer of Apoptosis Induces Astrocyte Proliferation through the Activation of Transforming-Growth Factor-α/Epidermal Growth Factor Receptor Signaling Pathway

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ABSTRACT

Reactive astrogliosis is beneficial in many aspects; however, it is also detrimental in some pathological states such as the development of lethal brain tumors. It is therefore crucial to understand the mechanisms regulating astrocyte proliferation. Tumor necrosis factor-like weak inducer of apoptosis (TWEAK), a member of the tumor necrosis factor family, was shown to stimulate astrocyte proliferation in vitro. Herein, we further characterize the mitogenic potential of TWEAK on central nervous system cells. Among these cells, astrocytes express the highest level of TWEAK and Fn14 transcripts, suggesting that they are particularly sensitive to TWEAK stimulation. Using in vitro model systems, we found that TWEAK was as potent as epidermal growth factor (EGF) (a prototypical astrocyte mitogen) in mediating astrocyte proliferation. However, its mitogenic activity was delayed compared with that of EGF, suggesting distinct mechanisms of action. Using cell signaling pathway inhibitors, neutralizing antibodies, and protein assays, we further show that the mitogenic activity of TWEAK on primary astrocytes requires stimulation of the transforming growth factor-α (TGF-α) and of the epidermal growth factor receptor (EGFR) signaling pathway through extracellular signal-regulated kinase and p38 mitogen-activated protein kinase activation. In aggregates, our data demonstrate that TWEAK acts as a potent astrocyte mitogen through the induction of a TGF-α/EGFR signaling pathway. We anticipate that description of such a mechanism may allow novel approaches to human pathologies associated with astrocyte proliferation.

Introduction

Astrocytes, the most abundant cell type in the brain, play a crucial role in central nervous system (CNS) homeostasis (Rakic, 1991). In response to pathological insults, astrocytes can also participate in healing processes through the formation of neural/glial scars and the reconstitution of glial limitans (Pekny and Nilsson, 2005). However, it has also been suggested that reactive astrogliosis can be detrimental for nerve regeneration and survival in certain disease states. One of the most dramatic examples of astrocyte pathological involvement is the formation of the glioblastoma-type of brain tumors as a consequence of uncontrolled astrocyte proliferation (Furnari et al., 2007). Many factors can support

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ABBREVIATIONS: CNS, central nervous system; TWEAK, tumor necrosis factor-like weak inducer of apoptosis; TNF, tumor necrosis factor; Fn14, fibroblast growth factor-inducible 14; TNFR, tumor necrosis factor receptor; NF-κB, nuclear factor κB; MAPK, mitogen-activated protein kinase; TRAF, tumor necrosis factor receptor-associated factor; TGF-α, transforming growth factor-α; EGFR, epidermal growth factor receptor; DMEM, Dulbecco's modified Eagle's medium; FCS, fetal calf serum; MK-801, dizocilpine maleate; RT, reverse transcription; PCR, polymerase chain reaction; EGF, epidermal growth factor; bFGF, basic fibroblast growth factor; HB-EGF, human heparin-binding EGF-like growth factor; PD98059, 2′-amino-3′-methoxyflavone; ERK, extracellular signal-regulated kinase; SB203580, 4-(4-fluorophenyl)-2-(4-methylsulfinylphenyl)-5-(4-pyridyl)1*H*-imidazole; bis-tris, 2-[bis(2-hydroxyethyl)amino]-2-(hydroxymethyl)propane-1,3-diol; IκB, inhibitor of nuclear factor-κB; GFAP, glial fibrillary acidic protein.

astrocyte proliferation including growth factors and cytokines. TWEAK, a member of the tumor necrosis factor (TNF) family, has been shown to stimulate astrocyte proliferation in vitro (Desplat-Jégo et al., 2002), and its receptor, the fibroblast growth factor-inducible 14 (Fn14), has been reported to be up-regulated in glioma cell lines (Mariani et al., 2001) and in patients with glioblastoma multiforme (Tran et al., 2003).

TWEAK is synthesized as a type II transmembrane protein but often functions as a soluble cytokine because of its processing by furin-like proteases (Chicheportiche et al., 1997). The biological activities of TWEAK are mediated by its cognate receptor Fn14, a type I transmembrane protein (Wiley et al., 2001; Wiley and Winkles, 2003; Brown et al., 2006). Whereas Fn14 contains a short cytoplasmic tail lacking the classic TNFR death domain, it instead induces robust activation of both nuclear factor-κB (NF-κB) and mitogen-activated protein kinase (MAPK) transduction pathways by interacting with TNFR-associated factors (TRAFs) (Inoue et al., 2000; Brown et al., 2003; Han et al., 2003; Ando et al., 2006).

On the basis of both in vitro and in vivo animal studies, TWEAK can mediate multiple biological activities in the CNS including glial cell growth, stimulation of proinflammatory functions, demyelination, proliferation, and differentiation of adult neural progenitor cells, promotion of neurite outgrowth, survival, and migration, increased permeability of the blood-brain barrier, and, in some experimental conditions, induction of apoptosis (Saas et al., 2000; Desplat-Jégo et al., 2002; Tanabe et al., 2003; Tran et al., 2003, 2005; Potrovita et al., 2004; Polavarapu et al., 2005; Hamill et al., 2007; Iocca et al., 2008; Haile et al., 2010; Schölzke et al., 2011). In addition, TWEAK and Fn14 expression are strongly up-regulated in multiple CNS diseases such as multiple sclerosis (Desplat-Jégo et al., 2002, 2005, 2009; Ando et al., 2006; Locca et al., 2008; Serafini et al., 2008), stroke (Inta et al., 2008), and brain tumors (Tran et al., 2003, 2006), suggesting a pathological role of TWEAK signaling pathways in human diseases.

Whereas several groups have initiated investigations of the role of TWEAK/Fn14 in driving cell proliferation, the precise molecular mechanisms involved remain uncertain and are potentially context-dependent. In this study, we sought to explore in further detail the mechanism underlying the mitogenic potential of TWEAK on glial cells. Our results demonstrate that, in primary astrocyte cultures, TWEAK-induced cell proliferation requires the intermediate production of TGF-α and the activation of EGFR.

Materials and Methods

Astrocyte Cultures. Animals were treated in accordance with the *Guide for the Care and Use of Laboratory Animals* (Institute of Laboratory Animal Resources, 1996) and the guidelines of the local institutional animal care and use committee. Cultures were obtained from embryonic day 15.5 Wistar rat embryos (Janvier Breeding Center, Le Genest St. Isle, France). The ventral mesencephalon was dissected as described by Douhou et al. (2001). Astrocytes were obtained from mesencephalic cells in suspension, which were plated onto uncoated 25-cm^2 flask and maintained in 5 ml of DMEM/F-12 with 100 U/ml penicillin and streptomycin, 15 mM HEPES, and 10% fetal calf serum (FCS). After reaching confluence (approximately 7 days of culture), astrocyte cells were dissociated by trypsin treatment and plated onto polyethylenimine-precoated 24-well plates at 4×10^4 or 8×10^4 cells/well. To favor cell attachment, 10% FCS was also added during the 1st h. Next, astrocytes were washed in FCS-free DMEM and maintained in DMEM/F-12 supplemented with N2, 100 U/ml penicillin and streptomycin, and 15 mM HEPES. Astrocyte-enriched cultures were fed daily by replacing 300 μl of medium. Only one-passage cultures were used for all experiments.

Microglial Cell Cultures. Highly pure cultures of amoeboid microglial cells were obtained as described previously by Théry et al. (1991). In brief, floating microglial cells were isolated from primary glial cell cultures prepared from the ventral mesencephalon of embryonic day 15.5 Wistar rats and grown in DMEM supplemented with 10% FCS. Harvested microglial cells were washed three times in FCS-free DMEM and plated in DMEM/F-12 supplemented with N2, 100 U/ml penicillin and streptomycin, and 15 mM HEPES. Microglial cells were maintained for 48 h after plating in at 8×10^4 cells/well in uncoated 96-well plates.

Neuron Cultures. Animals were treated in accordance with the *Guide for the Care and Use of Laboratory Animals* (Institute of Laboratory Animal Resources, 1996) and the guidelines of the local institutional animal care and use committee. Cultures were obtained from embryonic day 15.5 Wistar rat embryos (Janvier Breeding Center). The ventral mesencephalon was dissected as described by Douhou et al. (2001). After mechanical trituration, cells were seeded at 3.8×10^5 cells/well onto 24-well culture plates precoated with polyethylenimine (1 mg/ml; Sigma/RBI, Natick, MA). The cells were maintained in 500 μl of N5 medium (Kawamoto and Barrett, 1986) supplemented with 5 mM glucose, 5% horse serum, and 0.5% FCS, except for the first 2 days of culture during which the concentration of FCS was raised to 2.5% to favor cell attachment. To get neuron-enriched cultures, cells were treated with 3 μM 1-β-D-arabinofuranosylcytosine, and 1 μM dizocilpine maleate (MK-801) (Michel et al., 1997). Cultures were maintained for 6 days after plating and 300 μl of medium was daily replaced.

Total RNA Preparation. For extraction of total RNA, cell cultures were homogenized in TRIzol reagent (Invitrogen, Saint Aubin, France). The RNAs were isolated according to the manufacturer's instructions. RNA integrity was determined on agarose gel.

RT-PCR Analysis. Reverse transcription was performed according to the manufacturer's instructions (Invitrogen). In brief, total RNA was transcribed with ThermoScript RT (15 U), random hexamer DNA primers, and oligo(dT)$_{20}$. The following PCR conditions were used: 96°C for 30 s, 60°C for 45 s, and 72°C for 45 s. The primer sequences used in this study were 5′-CTCCGTTTGTGCCAGGTGTCT-3′ and 5′-GAGAGACAGGACTGGACCAAG-3′ for TWEAK, 5′-GACCTCGACAAGTGCATGGAC-3′ and 5′-TCACTGGATCAGTGCCACACC-3′ for Fn14, and 5′-GTGGGCCGCTCTAGGCACAA-3′and 5′-CTCTTTGATGTCACGCACGATTTC-3′ for β-actin.

Peptides and Pharmacological Agents. Fc-TWEAK (TWEAK), soluble TWEAK receptor (Fc-Fn14), and mutated Fc-TWEAK (Fc-TWEAK^{Y176A}) were kindly provided by Biogen Idec, Inc. (Cambridge, MA). All these peptides are fused to the IgG Fc domain to stabilize them. Recombinant rat EGF, human basic fibroblast growth factor (bFGF), and rat TNF-α were purchased from Biomedical Technologies (Stoughton, MA), Millipore (Billerica, MA), and PeproTech (Rocky Hill, NJ), respectively. Recombinant human TGF-α and human heparin-binding EGF-like growth factor (HB-EGF) were obtained from R&D Systems (Lille, France). Rabbit anti-rat EGF neutralizing antibody was obtained from Acris (Hiddenhausen, Germany) and was purified by this company to remove seric factors. Goat anti-human HB-EGF neutralizing antibody, known to inhibit rat HB-EGF (Zhang et al., 2004), was from R&D Systems. Mouse anti-rat TGF-α neutralizing antibody was from Merck/Calbiochem (Molsheim, France). 2′-Amino-3′-methoxyflavone (PD98059), a cell-permeable inhibitor of ERK and 4-(4-fluorophenyl)-2-(4-methylsulfinylphenyl)-5-(4-pyridyl)1*H*-imidazole (SB203580), an inhibitor of p38 MAPK, were purchased from Merck/Calbiochem and from Alexis (Villerbanne, France), respectively. Tyrphostin AG1478, which inhibits the kinase activity of EGFR, was obtained

from Sigma/RBI-Aldrich (Lyon, France). Treatments were added simultaneously with TWEAK and maintained during 72 h.

Western Blot Analysis. Cells were harvested from two wells at the indicated times and lysed in whole-cell lysis buffer (20 mM Tris/HCl, 150 mM NaCl, 2 mM EDTA, pH 8, 1% Triton, 10% glycerol, 1 mM sodium orthovanadate, 2 mM sodium pyrophosphate, and 50 mM sodium fluoride and protease inhibitors). For EGFR and TGF-α Western blotting, cells were lysed in the following whole-cell lysis buffer: 50 mM Tris, 150 mM NaCl, 0.5% NP40, 50 mM NaF, 1 mM Na_3VO_4, and protease inhibitors. Proteins (25 μg/lane) were separated by 8 and 10% SDS-polyacrylamide gel electrophoresis or by 12% bis-tris gel, blotted on polyvinylidene difluoride membranes, and incubated with mouse anti-phospho-ERK1/2 and rabbit anti-ERK1/2 (1:1000; Cell Signaling Technology, Danvers, MA), rabbit anti-phospho-p38 and mouse anti-p38 (1:1000; Cell Signaling Technology), mouse anti-phospho-IκB, rabbit anti-IκB (1:250; Cell Signaling Technology), mouse anti-TGF-α (Ab-3) (1:1000; Merck/Calbiochem), mouse anti-phospho EGFR (Tyr1068) and rabbit anti-EGFR (1:500; Cell Signaling Technology), or mouse anti-α-tubulin (1:1000; Sigma/RBI-Aldrich). Bound primary antibody was detected with the corresponding specific horseradish peroxidase-labeled secondary antibody (1/50,000; Jackson ImmunoResearch Laboratories Inc., West Grove, PA) and revealed by enhanced chemiluminescence (Pierce, Waltham, MA). Bands were quantified by using Scion Image software (available from the National Institutes of Health Web site).

[*methyl*-^{3}H]Thymidine Uptake. [*methyl*-^{3}H]Thymidine, a marker of DNA synthesis, was used to label proliferating cells. Astrocytes were maintained in regular serum-free medium. Cells were pulse-labeled for 18 h at 37°C with 1 μCi/ml [*methyl*-^{3}H]thymidine (GE Healthcare, Velizy-Villacoublay, France) in DMEM-F12 supplemented with N2. After washes, the cells were allowed to recover for 1 h in the same culture medium to remove unincorporated radioactivity and then were subjected to osmotic shock in water to release intracellular [*methyl*-^{3}H]thymidine. [*methyl*-^{3}H]Thymidine incorporation was measured using a beta scintillation counter (LS6500; Beckman Coulter, Villepinte, France).

For autoradiography, some cultures were fixed with 4% formaldehyde (15 min at 4°C) and washed twice with phosphate-buffered saline. Then, immunocytochemical analysis was performed according to the protocol described below, and [*methyl*-^{3}*H*]-thymidine incorporation was visualized using Hypercoat LM-1 photographic emulsion (Amersham Biosciences, Roissy, France) after 4 days of exposure at 4°C.

Immunocytochemistry. Cell phenotype was identified by immunodetection of specific cellular markers: GFAP for mature and vimentin for immature astrocytes. Cells were fixed with 4% formaldehyde (15 min at 4°C) and incubated overnight at 4°C with rabbit anti-GFAP (1:1000; Dako Corporation, Carpenteria, CA) and mouse anti-vimentin-Cy3 (1:400; Sigma/RBI-Aldrich). Anti-GFAP was detected by Alexa Fluor 568-conjugated anti-rabbit IgG (1:800 in phosphate-buffered saline plus 10% horse serum; Jackson ImmunoResearch Laboratories, Inc.) for 2 h at room temperature.

Statistical Analysis. All results are expressed as a percentage of untreated cultures ± S.E.M. from at least three independent experiments. Intergroup differences were compared by Student-Newman-Keuls test or, in the event of a failure in a normality test or equal variances test, by Dunn's method.

Results

TWEAK and Fn14 Expression in CNS-Derived Cells. To determine which cells of the CNS produce and/or respond to TWEAK, we first investigated by RT-PCR TWEAK and Fn14 mRNA expression in different cellular fractions prepared from the rat embryonic brain. The TWEAK expression level was low in both astrocyte and microglial cell cultures (Fig. 1) and totally absent in neuron cultures. The TWEAK mRNA level in astrocyte cultures was approximately 2 times higher than that in microglial cell cultures. In contrast, Fn14 mRNA was detected in all primary cell cultures (Fig. 1) with the highest level seen in astrocytes. Thus, the Fn14 mRNA level in microglial cell cultures was ~2 times less expressed than in astrocyte cultures and was barely detectable in neurons (~7.5 times less than in astrocyte cultures). These results suggest that astrocytes probably represent the main TWEAK-responsive cell population in the brain. We therefore focused our investigation on the molecular mechanisms underlying TWEAK-induced astrocyte proliferation.

TWEAK Is a Strong Astrocyte Mitogen as Potent as EGF. To determine the signaling pathway involved in TWEAK-induced astrocyte proliferation, we treated primary mesencephalon astrocytes with the soluble recombinant protein Fc-TWEAK. Primary astrocyte proliferation, measured by [^{3}H]thymidine incorporation, was promoted by TWEAK in a dose-dependant manner (Fig. 2a). To prove the specificity of TWEAK activity, astrocytes were also treated with the recombinant protein Fc-TWEAK carrying a single mutation, which abolishes its ability to bind Fn14 (Fc-TWEAK^{Y176A}) and with a neutralizing soluble TWEAK receptor Fc-Fn14. Astrocyte proliferation after treatment with the recombinant protein Fc-TWEAK carrying a mutation (50 ng/ml) or cotreatment with wild-type TWEAK and a neutralizing soluble TWEAK receptor Fc-Fn14 (10 μg/ml) was comparable to that of untreated cell cultures (Fig. 2, c and d). Of interest, astrocyte proliferation induced by TWEAK was similar to that induced by EGF, a well known growth factor with a strong proliferative effect on astrocytes in vitro (Leutz and Schachner, 1981) (Fig. 2, a, b, and d). Taken together, these findings confirm that TWEAK is a strong mitogenic factor for astrocytes.

TWEAK Induces ERK and p38 MAPK Pathway Signaling. The ability of the TWEAK/Fn14 pathway to activate MAPK through TRAF interaction is of particular relevance to the present study because of their critical involvement in regulating cell proliferation (Zhang and Liu, 2002). We tested whether these signaling pathways could mediate the mitogenic property of TWEAK in our culture system. We first showed by Western blotting that TWEAK (50 ng/ml) induced a time-dependent activation of both ERK and p38 MAPK (Fig. 3). However, several differences could be noticed. First, whereas both ERK and p38 MAPK were rapidly activated after the addition of TWEAK in the culture medium, only p38 MAPK activation was sustained up to 72 h. In contrast, ERK activation declined at 3 h but then became maximal at 72 h.

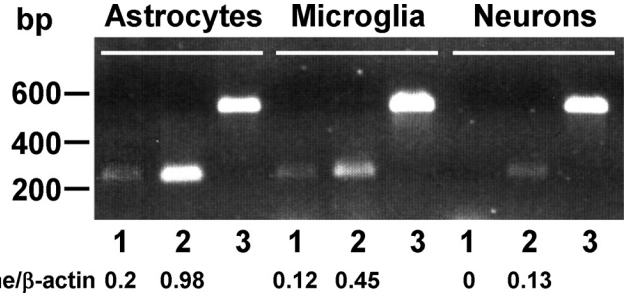

Fig. 1. Expression analysis of TWEAK (1), Fn14 (2), and β-actin (3) transcripts by RT-PCR in enriched astrocytic, microglial, and neuronal cultures. The expected sizes of the PCR products were 251, 258, and 539 base pairs (bp), respectively. These data are representative of three independent experiments.

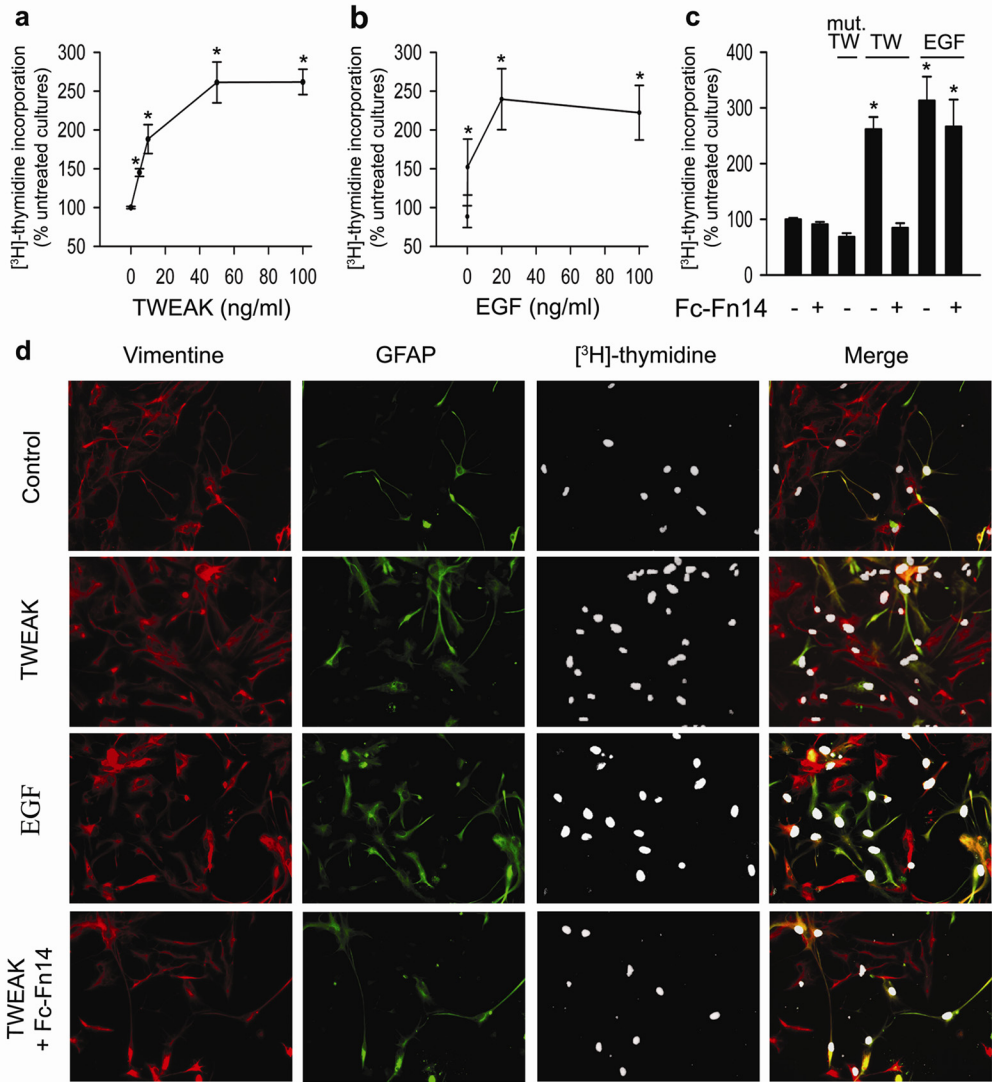

Fig. 2. Mitogenic properties of TWEAK and EGF on cultured astrocytes. a and b, proliferation assay using [*methyl*-^{3}H]thymidine incorporation in TWEAK- or EGF-treated astrocyte cultures. c, in sister cultures, astrocytes were treated either with a recombinant Fc-TWEAK^{Y176A} protein carrying a mutation in the receptor-binding domain of the ligand (mut. TW) or with soluble Fn14 receptor (Fc-Fn14) to neutralize the biological activity of TWEAK. Results are expressed as a percentage of untreated cultures ± S.E.M. *, $p < 0.05$ compared with untreated cultures (Student-Newman-Keuls or Dunn's test). d, phenotypic characterization of [*methyl*-^{3}H]thymidine-positive nuclei (white) was performed by immunofluorescent detection of GFAP (green) and vimentin (red). Values are representative of at least three independent experiments performed in triplicate.

Second, compared with EGF treatment (20 ng/ml) used as a positive control, TWEAK-mediated ERK and p38 MAPK activation appeared much milder. The slight activation of ERK and p38 MAPK in untreated astrocyte cultures may be related to the basal cell proliferation observed in this condition (Fig. 2d). In addition, we could never observe IκB phosphorylation and degradation in TWEAK-stimulated astrocytes, suggesting that the NF-κB signaling pathway was not activated under our experimental conditions (Fig. 3c). As expected, TNF-α (50 ng/ml), used as a positive control, induced IκB phosphorylation and degradation in these cells. Overall, the increase in ERK and p38 MAPK phosphorylation after TWEAK treatment suggests a role of these signaling pathways in its mitogenic properties. To test this hypothesis, we first blocked the ERK transduction pathway using the specific chemical inhibitor PD98059. PD98059 (20 μM) completely prevented ERK phosphorylation (Fig. 4a), which was associated with a complete blockade of astrocyte proliferation induced by both TWEAK and EGF (Fig. 4b). Similar results were obtained with an inhibitor of the p38 MAPK pathway, SB203580 (20 μM) (Fig. 5, a and b). Finally, it is also worth noting that PD98059 and SB203580 partially inhibited basal astrocyte proliferation in untreated cultures. Taken together, these data suggest that both ERK1/2 and p38 MAPK signaling pathways are critical in mediating the mitogenic function of TWEAK on astrocytes because they cannot compensate for each other.

Delayed Proliferative Response Induced by TWEAK. Although TWEAK-induced ERK and p38 MAPK activation was much weaker than that triggered by EGF (Fig. 3), TWEAK was as potent as EGF in inducing astrocyte proliferation by 72 h (Fig. 2). This result suggests that the molecular mechanisms involved in TWEAK-induced astrocyte proliferation may differ from those induced by EGF. In line with this, we made the striking observation that the kinetic features of astrocyte proliferation were different between TWEAK and EGF. Whereas astrocyte proliferation was statistically increased by 24 h after EGF treatment, 72 h was required for TWEAK to induce the same level of proliferation (Fig. 6). Therefore, despite TWEAK and EGF being equally potent to induce astrocyte proliferation at 72 h, the effect of TWEAK was much delayed compared with that of EGF. This finding suggests that TWEAK-induced astrocyte proliferation requires the stimulation of additional factors. Of importance, TWEAK did not potentiate EGF mitogenic activity (data not shown), suggesting that TWEAK-induced astrocyte proliferation may involve the activation of the EGFR pathway.

TWEAK-Induced Astrocyte Proliferation Is Abolished by EGFR Inhibition. Because of the delayed proliferative response upon TWEAK treatment, we next tested the possibility that TWEAK-induced astrocyte proliferation may require intermediate factors. We hypothesized that EGFR signaling could be involved in the TWEAK response. To assess the involvement of EGFR activation in TWEAK-induced astrocyte proliferation, we blocked EGFR activity in TWEAK-treated cell cultures using the highly potent and specific EGFR inhibitor, tyrphostin AG1478. At a concentration of 3 μM, tyrphostin AG1478 specifically inhibits the EGF receptor tyrosine kinase (Santiskulvong et al., 2001). As expected, tyrphostin AG1478 treatment completely prevented EGF-induced astrocyte proliferation (Fig. 7a). Remarkably, this EGFR inhibitor almost totally prevented cell proliferation in TWEAK-treated astrocyte cultures (Fig. 7a). Because Fn14 receptor does not display tyrosine kinase activity in its cytoplasmic tail, this suggests that the binding of TWEAK to Fn14 triggers a cascade of molecular events that leads to EGFR activation and subsequent cell proliferation. To further test the specificity of tyrphostin AG1478 on EGFR, we tested its inhibitory properties on bFGF, another growth factor known to induce astrocyte proliferation (Kniss and Burry, 1988; Morrison et al., 1994) through the binding and activation of tyrosine kinase-type receptors. As expected, tyrphostin AG1478 did not inhibit astrocyte proliferation in-

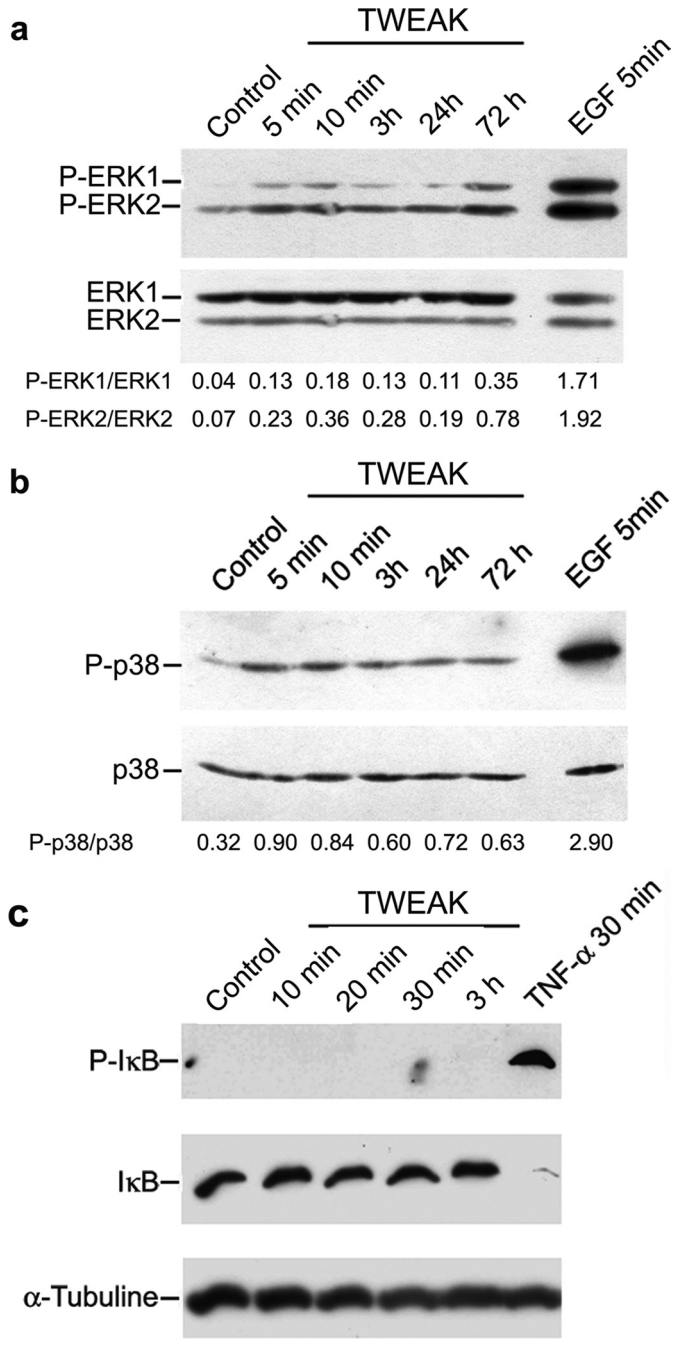

Fig. 3. Time course analysis of ERK1/2, p38 MAPK, and NF-κB signaling pathway activation in TWEAK-treated astrocytes. Phosphorylation of ERK1/2 (a) and p38 MAPK (b) was analyzed by Western blotting using phospho (P)-specific primary antibodies. Protein loading was controlled by reprobing the blots with an anti-ERK1/2 or anti-p38 antibody. EGF-treated astrocytes were used as a positive control for ERK1/2 and p38 MAPK pathway activation. Levels of phospho- and total MAPK expression were quantified by optical density measurement of the bands. The ratio between the phosphorylated form and the total form is given under the blot. c, IκB phosphorylation and degradation, as assayed by Western blotting, in TWEAK-stimulated astrocytes, was taken as an index of NF-κB activation. TNF-α-treated cultures (50 ng) were used as a positive control for NF-κB activation. Protein loading was controlled by striping and reprobing the blots with an anti-α-tubulin antibody. These data are representative of three independent experiments.

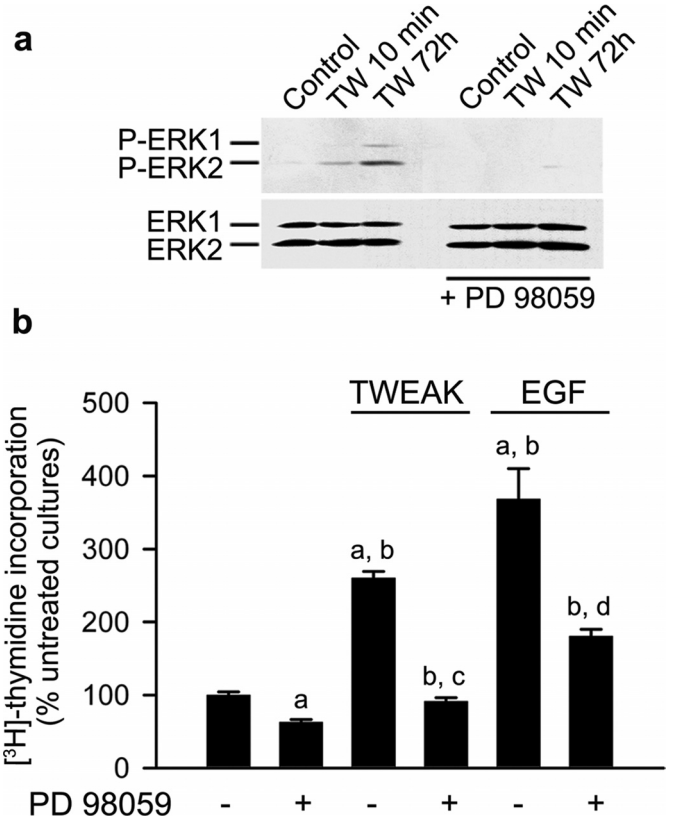

Fig. 4. Role of the ERK1/2 transduction pathway in astrocyte proliferation induced by TWEAK. a, Western blotting analysis of ERK1/2 phosphorylation in TWEAK (TW)-treated astrocytes with or without addition of the ERK1/2 chemical inhibitor PD98059. b, proliferation assay in TWEAK- or EGF-treated astrocytes with or without the addition of the ERK1/2 chemical inhibitor PD98059. Values are representative of at least three independent experiments performed in triplicate, and results are expressed as a percentage of untreated cultures ± S.E.M. a, $p < 0.05$ compared with untreated cultures; b, $p < 0.05$ compared with PD98059 alone-treated cultures; c, $p < 0.05$ compared with TWEAK-treated alone cultures; d, $p < 0.05$ compared with EGF-treated alone cultures (Student-Newman-Keuls or Dunn's test). P, phospho.

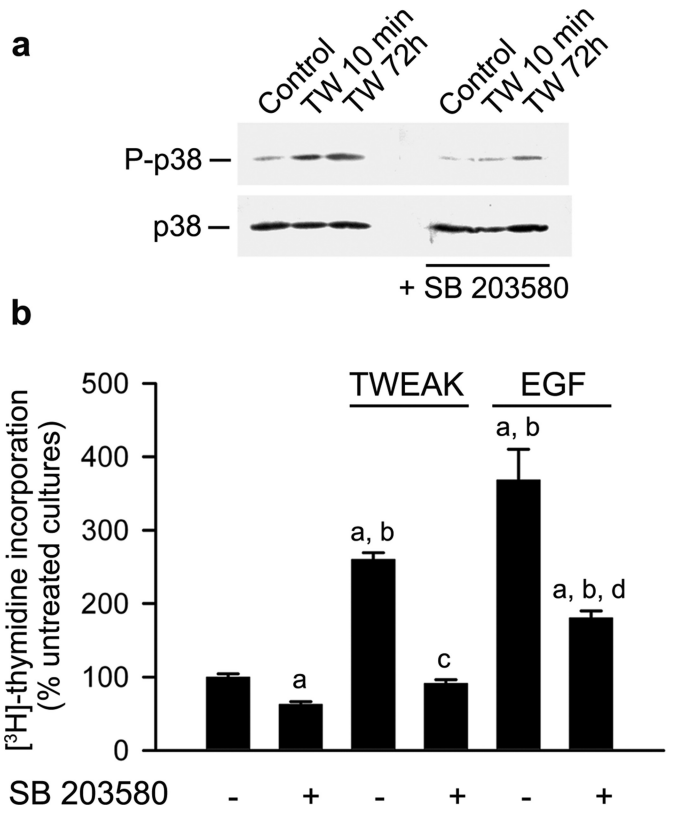

Fig. 5. Role of the p38 MAPK transduction pathway in astrocyte proliferation induced by TWEAK. a, Western blotting analysis of p38 MAPK phosphorylation in TWEAK (TW)-treated astrocytes with or without addition of the p38 MAPK chemical inhibitor SB203580. b, proliferation assay in TWEAK- or EGF-treated astrocytes with or without addition of the p38 MAPK chemical inhibitor SB203580. Values are representative of at least three independent experiments performed in triplicate, and results are expressed as a percentage of untreated cultures ± S.E.M. a, $p < 0.05$ compared with untreated cultures; b, $p < 0.05$ compared with SB203580-treated alone cultures; c, $p < 0.05$ compared with TWEAK alone-treated cultures; d, $p < 0.05$ compared with EGF alone-treated cultures (Student-Newman-Keuls or Dunn's test). P, phospho.

duced by bFGF (20 ng/ml) (Fig. 7a). Taken together, these data suggest that TWEAK-induced astrocyte proliferation requires signaling through the EGF receptor. Although we cannot fully rule out the possibility that TWEAK can bind to EGFR, the most likely scenario would be that TWEAK promotes astrocyte proliferation by inducing the stimulation of EGFR ligands.

TWEAK-Induced Astrocyte Proliferation Requires TGF-α/EGFR Signaling. Several EGFR ligands have been identified including EGF, HB-EGF, and TGF-α (Kornblum et al., 1999; Sharif et al., 2006). Using specific neutralizing antibodies, we next attempted to identify which of these ligands may be involved in TWEAK-mediated astrocyte proliferation. Despite its potent inhibitory effect on EGF-induced astrocyte proliferation (Fig. 7b), anti-EGF neutralizing antibody (3.8 μg/ml) had no effect on TWEAK-induced astrocyte proliferation. Likewise, anti-HB-EGF neutralizing antibody (4 μg/ml) only exhibited a slight effect on TWEAK-induced astrocyte proliferation (Fig. 7c). In stark contrast, the addition of anti-TGF-α neutralizing antibody (4 μg/ml) totally abrogated the ability of TWEAK to promote astrocyte proliferation (Fig. 7d). This raises the possibility that astrocytes are prone to produce TGF-α upon TWEAK treatment. In support of this view, we could show a significant increase in mature TGF-α levels in TWEAK-treated astrocyte cultures at 12 and 24 h after treatment (Fig. 8a). It is interesting to note that TGF-α levels were similar (~140% of untreated cultures) at 24 and 48 h after TWEAK exposure, suggesting that the production of mature TGF-α reaches a threshold at 24 h (data not shown). Taken together, these data strongly indicate that the production of mature TGF-α represents the predominant TWEAK-induced growth factor that is responsible for astrocyte proliferation through EGFR activation. Further evidence for this statement is the observation that astrocyte exposure to TWEAK resulted in EGFR activation as shown by the increased phosphorylation status of this receptor 24 and 48 h after cytokine treatment (Fig. 8b). In line with the delayed proliferative response induced by TWEAK compared with that obtained with EGF, TWEAK-induced EGFR phosphorylation was much milder at 24 than at 48 h. As expected, a 5-min treatment of primary astrocytes with recombinant EGF, used as a positive control, highly increased EGFR phosphorylation. Of importance, TWEAK-induced EGFR phosphorylation was completely abolished by anti-TGF-α neutralizing antibodies, suggesting that TWEAK-stimulated EGFR activation strictly requires intermediate TGF-α production (Fig. 8b).

ERK1/2 and p38 MAPK Pathways Act Upstream of TGFα/EGFR Signaling. Activation of ERK1/2 and p38 MAPK in astrocytes within minutes after TWEAK exposure suggests that these cell transduction pathways may assume upstream regulation of the TGFα/EGFR signaling. To test this hypothesis, TGFα expression was assessed in astrocyte cultures exposed to TWEAK and cotreated with either PD98059 or SB203580 inhibitors. Figure 8a shows that inhibition of either ERK1/2 by PD98059 or p38 MAPK by SB203580 completely prevented the production of mature TGF-α in cultured astrocytes treated by TWEAK. This result suggests that both ERK1/2 and p38 MAPK activation are

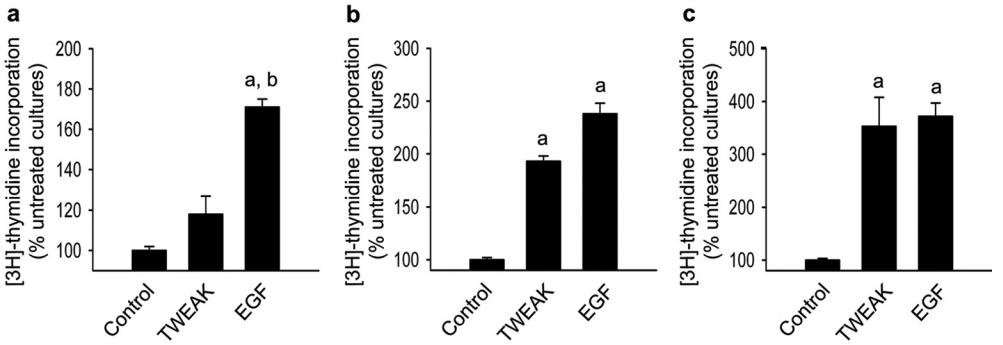

Fig. 6. Delayed proliferative response induced by TWEAK. TWEAK- and EGF-induced astrocyte proliferation was compared at 24 h (a), 48 h (b) and 72 h (c) after stimulation. Values are representative of at least three independent experiments performed in triplicate, and results are expressed as a percentage of untreated cultures ± S.E.M. a, $p < 0.05$ compared with untreated cultures; b, $p < 0.05$ compared with TWEAK-treated cultures.

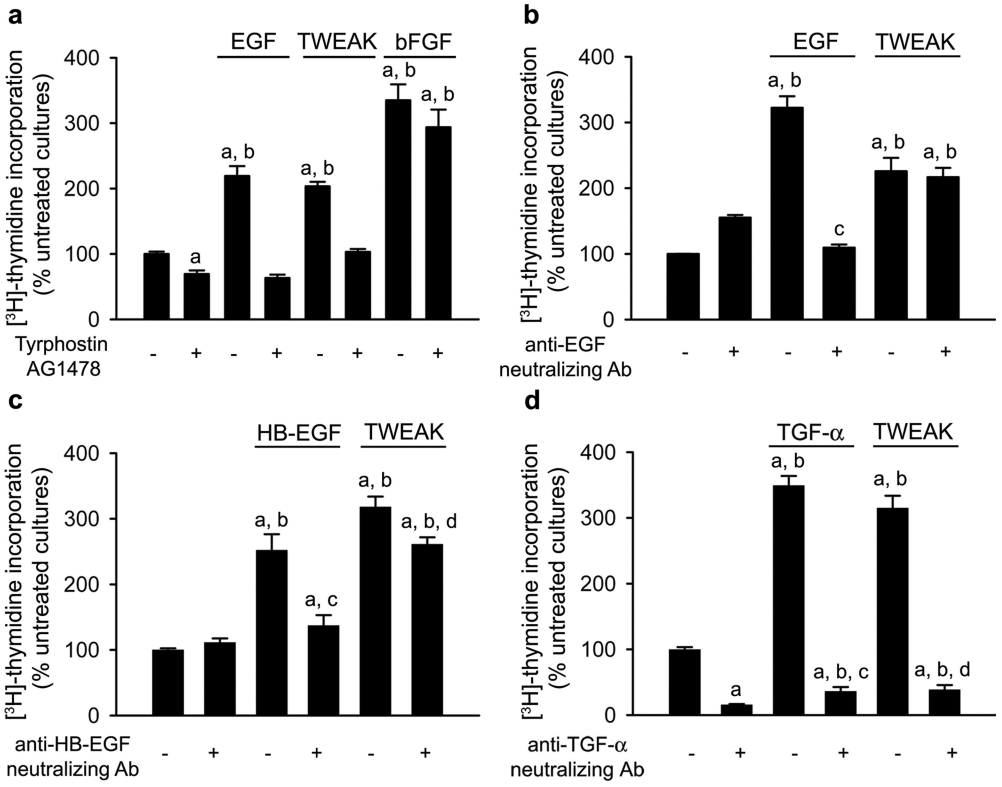

Fig. 7. Role of EGFR and EGFR ligands in TWEAK-induced astrocyte proliferation. Proliferation assay in TWEAK-stimulated astrocytic cultures treated with the chemical inhibitor tyrphostin AG1478 (a) or with EGFR ligand neutralizing antibodies: anti-EGF antibody (b), anti-HB-EGF antibody (c), and anti-TGF-α (d). In a, EGF-treated cultures were used as a positive control and bFGF-treated cultures as a negative control for tyrphostin AG1478 efficiency. In b, c, and d, EGF-, HB-EGF-, and TGF-α-treated cultures were used as a positive control for inhibition efficiency of neutralizing antibodies, respectively. Values are representative of at least three independent experiments performed in triplicate, and results are expressed as a percentage of untreated cultures ± S.E.M. a, $p < 0.05$ compared with untreated cultures; b, $p < 0.05$ compared with tyrphostin AG1478 or neutralizing antibody alone-treated cultures; c, $p < 0.05$ compared with TWEAK alone-treated cultures; d, $p < 0.05$ compared with growth factor alone-treated cultures. (Student-Newman-Keuls or Dunn's test).

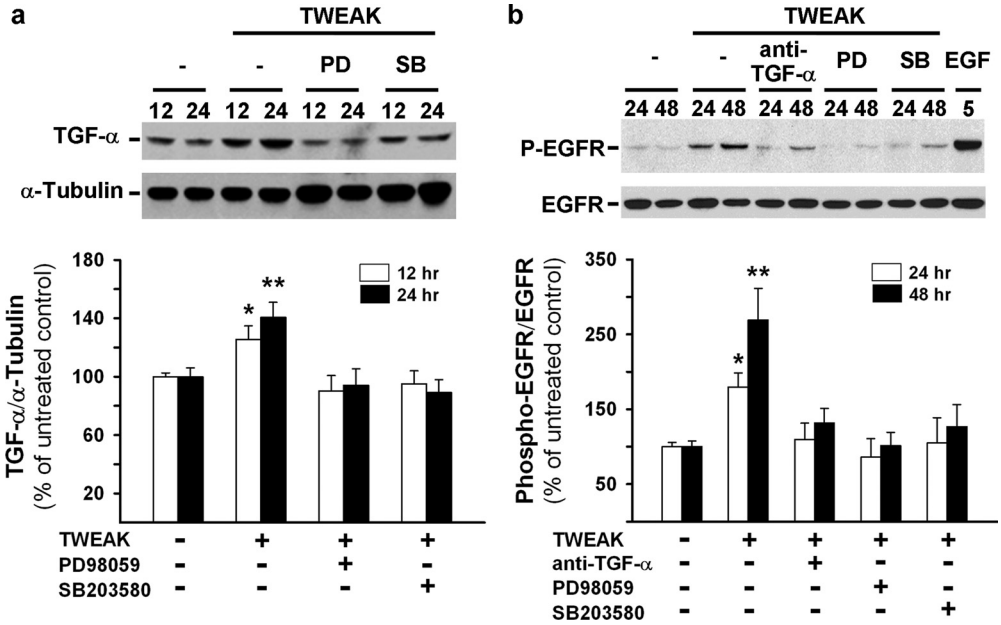

Fig. 8. ERK1/2 and p38 regulate the production of mature TGF-α and EGFR phosphorylation in TWEAK-stimulated astrocytes. a, Western blotting analysis of mature TGF-α in TWEAK-treated astrocytes with or without addition of either SB203580 (SB) or PD98059 (PD) inhibitors at 12 and 24 h after treatment. Protein loading was controlled by stripping and reprobing the blots with an anti-α-tubulin antibody. The ratios between mature TGF-α and α-tubulin levels are shown in the graph under the blot. Values are representative of three independent experiments performed in triplicate, and results are expressed as a percentage of untreated cultures ± S.E.M. *, $p < 0.05$; **, $p < 0.001$ compared with untreated cultures and cultures treated with PD98059 or SB203580 (Student-Newman-Keuls test). b, Western blotting analysis of EGFR phosphorylation in TWEAK-treated astrocytes with or without addition of anti-TGF-α neutralizing antibodies, PD98059 or SB203580 inhibitor 24 and 48 h after treatment. Protein loading was controlled by reprobing the blots with an anti-total EGFR antibody. EGF-treated astrocytes for 5 min were used as a positive control for EGFR phosphorylation. Levels of phospho (P)-EGFR and total-EGFR were quantified by optical density measurement of the bands. The ratios between phosphorylated and total EGFR are shown in the graph under the blot. Values are representative of three independent experiments performed in triplicate, and results are expressed as a percentage of untreated cultures ± S.E.M. *, $p < 0.05$; **, $p < 0.001$ compared with untreated cultures and cultures treated with PD98059, SB203580, or anti-TGFα antibody (Student-Newman-Keuls test).

required to induce the production of mature TGF-α. Therefore, EGFR phosphorylation was also inhibited upon treatment with ERK1/2 or p38 MAPK inhibitor (Fig. 8b) most likely because of the absence of mature TGF-α production. Taken together, these data suggest that both ERK1/2 and p38 MAPK activation are required to induce the production of mature TGF-α and consequently EGFR phosphorylation.

Discussion

TWEAK was reported previously to promote glial cell proliferation in vitro (Desplat-Jégo et al., 2002). In this report, we have further characterized the mitogenic properties of TWEAK on astrocytes. We demonstrate herein that the intermediate TGF-α/EGFR signaling pathway was essential for TWEAK-induced astrocyte proliferation, which is mediated by the activation of ERK and p38 MAPK transduction pathways (Fig. 9).

TWEAK is expressed in a variety of normal tissues including the brain (Chicheportiche et al., 1997). In cultured CNS cells, we found that TWEAK was moderately expressed in astroglial and microglial cells and it was absent in neurons. The low level of TWEAK transcript detected in our study contrasts with a previous report showing higher expression in postnatal cultured astrocytes and microglial cells (Desplat-Jégo et al., 2002). The TWEAK expression level may thus vary both during brain development and in different brain areas. In our hands, using a high-sensitivity detection method, we were unable to find any TWEAK transcript in neuron cultures, suggesting that neurons do not represent a primary source of TWEAK. This view is further supported by the finding that under pathological circumstances, astrocytes and microglia/macrophages were found to represent the main source of TWEAK in the CNS (Serafini et al., 2008). However, unlike the ligand, TWEAK receptor Fn14 was found to be expressed in both glial cells and neurons with the highest level in astrocytes, suggesting that astroglial cells might be particularly sensitive to TWEAK-mediated biological effects with respect to other CNS cell populations. Astrocytes, the most represented type of glial cells in the brain, participate in a great variety of developmental and physiological functions. Unlike postmitotic neurons, astrocytes can proliferate in response to brain injury, thereby participating in the healing process. However, anarchic astrocyte proliferation can also give rise to some of the most aggressive brain tumors in humans (Furnari et al., 2007). In agreement with a previous report (Desplat-Jégo et al., 2002), we here demonstrate that TWEAK can markedly induce astrocyte proliferation in vitro. Of importance, our study reveals that TWEAK is as effective as EGF in stimulating astrocyte proliferation, therefore emphasizing its potency.

Fn14 receptor binds TRAFs in its cytoplasmic tail (Han et al., 2003; Wiley and Winkles, 2003; Brown et al., 2006), which in turn can mediate activation of the NF-κB and MAPK signaling pathways. Unlike other studies in which various cell populations have been investigated (Marsters et al., 1998; Brown et al., 2003, 2010; Dai et al., 2009; Kumar et al., 2009; Sanz et al., 2010; Moreno et al., 2011), TWEAK stimulation of primary astrocytes was not associated with NF-κB but rather with ERK and p38 MAPK transduction pathway activation. This observation emphasizes the differences that exist between cell types in transducing signals from common receptors, thereby mediating distinct biological functions. In line with this finding, it has now been established that the biological activity of TWEAK can be independent of NF-κB and requires the activation of alternative cell signaling pathways such as ERK or p38 MAPK signaling (Ando et al., 2006; Sanz et al., 2009; Vendrell et al., 2010). We further demonstrated that ERK and p38 MAPK pathways are indispensable for TWEAK-dependent mitogenic function, a result compatible with the well known central role of these MAPKs in cell proliferation (Zhang and Liu, 2002). The rapid induction of these pathways after TWEAK exposure (as early as 5 min) indicates that they could be directly activated through TWEAK-mediated Fn14 activation. However, whereas the p38 MAPK activation level remains unchanged over time, ERK phosphorylation increases later on. Although we do not know exactly what further stimulates the ERK pathway at this point, it has been previously reported that the p38 MAPK pathway modulates ERK activity, raising the possibility of positive feedback between these two transduction pathways (Sanz-Moreno et al., 2003; Thorsen et al., 2003), which would explain why they could not compensate for each other. Hence, several lines of evidence from our findings suggest that TWEAK-induced astrocyte proliferation is not a direct mechanism but requires secondary extracellular stimuli. Indeed, both the inhibition of EGFR activation by the chemical inhibitor tyrphostin AG1478 and the extracellular sequestration of mature TGF-α with the anti-TGF-α neutralizing antibodies completely blocked TWEAK mitogenic activity. In contrast, neutralization of either EGF or bFGF (data not shown) in TWEAK-stimulated cultures did not affect astrocyte proliferation. Thus, TGF-α/EGFR signaling is required for TWEAK-induced astrocyte proliferation.

Of importance, such a mechanism is consistent with the delayed mitogenic response induced by TWEAK compared with that of EGF. Therefore, the production of mature TGF-α and EGFR phosphorylation increases in a time-dependent manner during TWEAK-induced astrocyte proliferation. The elevated activation of the ERK pathway 72 h after TWEAK stimulation could be explained, at least in part, by EGFR activity, which would be maintained and amplified by a positive feedback loop through ERK-dependent TGF-α induction. However, this is unlikely because increased EGFR phosphorylation at 24 h was not accompanied by a rise in ERK1/2 activation at this time point. Whether other pathways are

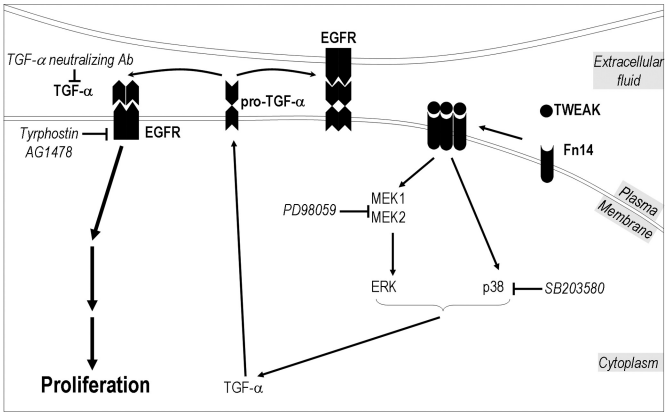

Fig. 9. Schematic diagram of the cell proliferation signaling pathway induced by TWEAK in primary astrocytes. MEK, mitogen-activated protein kinase kinase.

involved in EGFR-mediated astrocyte proliferation will need further clarification.

It is worth noting that TWEAK-induced cell proliferation may not always require secondary growth factors. Thus, Lynch et al. (1999) suggested that TWEAK could directly induce endothelial cell proliferation without intermediate factors. However, the authors investigated the role of vascular endothelial growth factor in this process but not that of other growth factors such as bFGF, which is also known to strongly stimulate endothelial cell proliferation (Presta et al., 1992; Gualandris et al., 1996). The TWEAK/Fn14 pathway is also associated with an increase in ovarian cancer cell metastasis through vascular endothelial growth factor expression (Dai et al., 2009). The TWEAK/Fn14 pathway may thus activate different growth factors in a cell type- and a cellular context-dependent manner. The link between TWEAK-induced Fn14 activation and TGFα/EGFR signaling is not fully known. However, we herein provide evidence that TWEAK-induced early activation of ERK and p38 MAPK pathways is probably involved in this process because pharmacological inhibition of these signaling pathways prevented the production of mature TGF-α and the phosphorylation of EGFR after TWEAK exposure. The increased level of mature TGF-α observed after TWEAK stimulation also suggests that TWEAK may stimulate the processing of TGF-α. Therefore, either transmembrane or soluble TGF-α could bind to EGFR and sustain the MAPK signaling pathway.

In summary, we propose that the mitogenic property of TWEAK on astrocytes is indirect and requires the activation of an intermediate growth factor. In our astrocyte culture model, TGF-α serves as a strong mitogenic factor for TWEAK-induced glial cell proliferation (Fig. 9). We anticipate that such a mechanism could be involved in a number of different pathological situations including glioblastoma multiforme. In this most common and aggressive primary malignant brain tumor with a poor survival rates, Fn14 mRNA and protein levels were found not only to be markedly increased but also to be directly implicated in the malignant behavior of this tumor (Tran et al., 2003, 2006). Although it is widely accepted that EGFR ligands/EGFR function as an important autocrine loop in supporting proliferation of high-grade gliomas, clinical trials with anti-EGFR agents in malignant gliomas have shown limited therapeutic efficiency (Voelzke et al., 2008). Because EGFR signaling in glioblastoma development has been widely proven (for review, see Taylor et al., 2012), our data provide a rationale for further studying TWEAK/Fn14 signaling as a potential target for glioma treatment.

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Authorship Contributions

Participated in research design: Rousselet and Hunot.

Conducted experiments: Rousselet, Traver, Monnet, Perrin, Mandjee, and Hild.

Contributed new reagents or analytic tools: Zheng.

Performed data analysis: Rousselet and Hunot.

Wrote or contributed to the writing of the manuscript: Rousselet, Hirsch, Zheng, and Hunot.

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