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**CNTF protects oligodendrocytes from ammonia toxicity :
Intracellular signaling pathways involved**

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Abstract

In pediatric patients, hyperammonemia can provoke irreversible damages to developing CNS like cortical atrophy, ventricular enlargement, demyelination or gray and white matter hypodensities which are concordant with alterations of neurons and oligodendrocytes. Cerebral injury triggers endogenous protective mechanisms that can prevent or limit brain damage. Understanding these mechanisms may lead to new therapeutic strategies. We investigated whether ciliary neurotrophic factor (CNTF), a cytokine-like protein expressed by astrocytes and described as an injury-associated survival factor, was up-regulated by ammonia in developing reaggregated 3D brain cell cultures. We showed that CNTF is up-regulated by ammonia exposure, through mediation of p38 MAPK activation in astrocytes. We also observed that SAPK/JNK and Erk1/2 activations in oligodendrocytes and neurons, respectively, also play indirect roles in CNTF synthesis by astrocytes. Co-treatment with exogenous CNTF demonstrated strong protective effects on oligodendrocytes, but not on neurons, against ammonia toxicity. These protective effects involved JAK/STAT, SAPK/JNK and c-jun proteins.

Key words: hyperammonemia, CNTF, MAPK, oligodendrocytes, c-jun, neuroprotection.

Introduction

Hyperammonemia in neonates and infants is mainly due to defects of the urea cycle enzymes or other inborn errors of metabolism, and causes irreversible damages in the developing CNS such as cortical atrophy, ventricular enlargement, demyelination, and hypodensities of gray and white matter, which are compatible with alterations of neurons and oligodendrocytes. Irreversible lesions occur in prolonged hyperammonemia and/or high blood ammonia levels (for recent reviews, see Cagnon and Braissant, 2007, and Gropman et al., 2007). The recent use of alternative-pathway therapies detoxifying ammonia improved the survival of patients (Enns et al., 2007). However, the improved survival was correlated with a worsened neurological and cognitive outcome. Thus, the development of neuroprotective therapeutics is crucial for the improvement of the neurological outcome of neonates and infants experiencing hyperammonemia. Cerebral injury triggers endogenous protective mechanisms that can prevent or limit brain damage. Understanding these mechanisms may lead to new therapeutic strategies.

Among neurotrophic factors, ciliary neurotrophic factor (CNTF) is of particular interest regarding ammonia neurotoxicity. CNTF is a cytokine-like protein, specifically expressed in CNS by astrocytes and acting through a heteromeric receptor complex formed by CNTF receptor subunit (CNTFR α), glycoprotein 130 and leukemia inhibitory factor receptor (Sleeman et al., 2000). CNTF expression is strongly up-regulated in reactive astrocytes adjacent to lesions following several CNS injuries such as focal cerebral ischemia, entorhinal cortex lesion, kainic acid-induced excitotoxicity or intracerebral hemorrhage (Lee et al. 1997; Lin et al., 1998; Choi et al., 2004; Yokota et al., 2005). Numerous studies have demonstrated strong protective effects of CNTF on neurons and oligodendrocytes in various models of brain injury (Louis et al., 1993; Mitchell et al., 1998; Semkova et al., 1999; Mittoux et al., 2000;

Oshitari and chi-Usami, 2003; Ozog et al., 2007). Moreover, CNTF induces astrocyte swelling (Hudgins and Levison, 1998), which is often observed in the brain submitted to hyperammonemia (Cordoba and Blei, 1996; Norenberg et al., 2005). We hypothesized that ammonia could induce CNTF synthesis.

Many of the external signals encountered by cells are transduced to the nucleus by a highly conserved signaling mechanism, the mitogen-activated protein kinase (MAPK) cascades. MAPKs are serine/threonine kinases transducing extracellular signals to intracellular responses that balance survival, differentiation and death of the cell (Xia et al., 1995; Pearson et al., 2001; Weston and Davis, 2007). Three major conserved pathways of MAPKs have been described: the extracellular signal regulated kinases (Erk1/2 isoforms or p44/p42), the c-Jun NH₂-terminal kinases (JNK-1, -2, -3 isoforms; also named Stress-Activated Protein Kinase or SAPK) and the p38 kinases (p38 α , β , γ and δ isoforms). We hypothesized that MAPKs pathways could be involved in alterations of CNTF by ammonia.

In this study, reaggregated primary 3D cultures of developing brain cells, derived from fetal rat telencephalon and exposed to ammonia, were used as an experimental model for the developing brain exposed to hyperammonemia (Honegger and Monnet-Tschudi, 2001; Braissant et al., 2002; Braissant et al., 2008; Cagnon and Braissant, 2008). These cultures are a model of choice to study the ammonia-induced intracellular and extracellular crosstalks between glia and neurons which are intermingled in these aggregates, and are grown in absence of serum. We first studied how ammonia exposure alters the expression of CNTF and we investigated whether MAPKs were involved. Finally, we tested whether a treatment with exogenous CNTF could exert neuroprotective effects against ammonia toxicity, and analyzed the intracellular pathways involved.

Material and methods

Reagent and antibodies

DMEM powder without sodium pyruvate and sodium bicarbonate was purchased from Invitrogen (AG, Carlsbad, CA, USA). Gentamicin sulfate, insulin, linoleic acid, alpha-tocopherol, 3,3',5-triiodo-L-thyronine, apo-transferrin, choline chloride, hydrocortisone 21-hemisuccinate, alpha-lipoic acid, Basal Medium Eagle vitamin solution and ammonium-chloride (NH₄Cl) were purchased from Sigma (St Louis, MO, USA). Retinol, sodium bicarbonate, L-carnitine and vitamin B12 were purchased from Fluka Chemie AG (Buchs, Switzerland). Rabbit polyclonal anti-SAPK/JNK and phospho-SAPK/JNK (Thr183/Tyr185), anti-p38 and phospho-p38 MAPK (Thr180/Tyr182), anti-Erk1/2 and phospho-Erk1/2 (Thr202/Tyr204), anti-MAPKAPK2 and phospho-MAPKAPK2 (Thr334), anti-ATF2 (20F1) and phospho-ATF2 as well as anti-phospho-c-jun (Ser73) antibodies were purchased from Cell Signaling (Beverly, MA, USA). Mouse monoclonal anti-CNTF (clone 4-68), anti-microtubule-associated protein 2 (MAP2), anti-glia fibrillary acidic protein (GFAP) (clone GA5) and anti-galactocerebroside (GalC) antibodies were purchased from Chemicon (Temecula, CA, USA). Mouse monoclonal anti-histone H1 and anti-neurofilament 160 kD (NF-M) (clone NF09), rabbit polyclonal anti-total-c-jun as well as goat polyclonal anti-myelin basic protein (MBP) and anti-CNTFR α (C-20) antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). U0126 and AG490 were purchased from Calbiochem (San Diego, CA, USA), SP600125 from Alexis Corporation (Lausen, Switzerland) and SB203580 from Promega (Madison, Wisc., USA). Recombinant rat CNTF was purchased from R&D Systems Europe (Minneapolis, MN).

Reaggregated developing brain cell cultures

Rotation-mediated reaggregated brain cell cultures derived from fetal rat telencephalon were prepared as previously described (Honegger and Monnet-Tschudi, 2001; Braissant et al., 2002). In brief, the pooled forebrains of 15-day fetal rats (Sprague-Dawley, Harlan, Netherlands; animals handled according to the rules of the Swiss Academy for Medical Sciences) were dissociated mechanically, washed and resuspended in serum-free, chemically defined medium consisting of DMEM with high glucose (25 mM) supplemented with insulin (0.8 μ M), triiodothyronine (30 nM), hydrocortisone-21-phosphate (20 nM), transferrin (1 μ g/ml), biotin (4 μ M), vitamin B₁₂ (1 μ M), linoleate (10 μ M), lipoic acid (1 μ M), L-carnitine (10 μ M), choline chloride (2.5 g/l), sodium chloride (3.7 g/l) and trace vitamins and ions. Gentamicin sulfate (25 μ g/ml) was used as an antibiotic. The cultures were incubated at 3.6×10^7 cells per flask and maintained under constant gyratory agitation at 37°C, in an atmosphere of 10% CO₂ and 90% humidified air. Media were replenished from day-in-vitro (DIV) 5 every 3 days, by exchanging 5 ml of medium (of a total of 8 ml) per flask. On the day of harvest (DIV 13), aggregate pellets were washed three times with ice-cold PBS, embedded for histology in cryoform (O.C.T. compound tissue-tek, Digitana, Switzerland) or frozen in liquid nitrogen and kept at -80°C until analysis.

Treatments

Cultures were treated with NH₄Cl (5mM) (Braissant et al., 2002; Braissant et al., 2008) alone or in combination with 2 μ M U0126 (a selective inhibitor of Erk1/2), 2 μ M SP600125 (a selective inhibitor of SAPK/JNK), 2 μ M SB203580 (a selective inhibitor of p38 MAPK), 2 μ M AG490 (a selective inhibitor of JAK/STAT) and/or CNTF (100, 150 or 200 ng/ml) from DIV 5 to 13. NH₄Cl and CNTF were added after replenishing the culture medium at DIV 5, 8 and 11, whereas inhibitors were added every day from DIV 5 to 12. NH₄Cl stock solution was

prepared in milliQ water at 170 mM. The pH and osmolarity of each media with and without NH₄Cl and inhibitors was stable at value of 7.4 and 340 mOsmol, respectively. Inhibitors were diluted in dimethyl-sulfoxide (DMSO) and the highest DMSO concentration in the culture media was 0.06 %. CNTF was diluted in culture medium. The corresponding controls, only treated with vehicle, were assessed.

Western blot analysis

Cell cultures were homogenized in 10 mM Tris-HCl, pH 7.5, containing 6 M urea, 0.1% SDS, protease inhibitors (Complete; Roche, Switzerland) and phosphatase inhibitors (cocktail 2 aqueous solution, Sigma, St Louis, MO, USA). Homogenates were centrifuged at 16,000 x g for 10 min, and supernatants were recovered. Supernatant proteins were measured by the bicinchoninic acid assay (Pierce, Rockford, IL, USA) and diluted at a final concentration of 3 µg/µl in NuPage® LDS sample buffer and NuPage® reducing agent (Invitrogen, Carlsbad, CA, USA). Samples were heated at 70°C for 10 min and proteins were separated by SDS-PAGE (7 or 12% total acrylamide). After transfer of the proteins in a Transblot SD semi-dry transfer cell (Biorad) to polyvinylidene difluoride membranes (Immobilon; Millipore, Bedford, MA, USA), blots were probed with antibodies. All antibodies were diluted 1:1000, except anti-phospho-p38 MAPK antibody that was diluted 1:500. As CNTFR α expression was undetectable with “classical” immunoblot technique, a kit allowing signal amplification with avidin-biotin system was used following the manufacturer’s guidelines (Vectastain Elite ABC kit, Vector laboratories, Burlingame, CA, USA). Anti-CNTFR α antibody was diluted 1:5000. Western blots were revealed by chemiluminescence (ECL; Amersham Biosciences, Buckinghamshire, UK). Blots were stripped (Re-Blot Plus Mild antibody stripping solution; Chemicon, Temecula, CA, USA) and re probed with antibody against histone H1 to demonstrate equal loading of protein in each lane. The autoradiograms (Hyperfilm ECL,

Amersham Biosciences, UK) were scanned with an ImageScanner (Amersham Biosciences, UK) and processed by image analysis (ImageMaster 1D; Amersham Biosciences, UK). Data were acquired in arbitrary densitometric units and transformed to percentages of the densitometric levels obtained from scans of control samples visualized on the same blots.

Immunofluorescent staining

In-situ detection of MAPKs in cell cultures was performed with Tyramide Signal Amplification (TSA) kit (Molecular Probes, Eugene, OR, USA). 16 μm -thick aggregate cryosections were postfixed 1 h in 4% paraformaldehyde (PFA) in PBS at room temperature. Sections were washed in PBS and incubated in 1 % H_2O_2 (TraceSelect 30%, Fluka Chemie AG, Buchs, Switzerland) in PBS for 10 min to quench endogenous peroxidase activity. Nonspecific antibody binding sites were blocked for 1 h at room temperature with the blocking buffer of the kit. The primary antibody (1:100) diluted in blocking buffer was applied to sections overnight at 4° C. After washing, sections were incubated with a Horseradish Peroxidase-coupled anti-rabbit IgG secondary antibody (provided by the kit) for 1 h. Peroxidase staining was performed using Alexa Fluor® 555-labeled tyramide diluted at 1:200 in the amplification buffer (provided by the kit) and applied to sections for 5 min. For negative controls, primary antibody was omitted resulting in no staining. Sections were then incubated overnight at 4°C with the second primary antibody directed towards GFAP (1:200), MAP-2 (1:100) or MBP (1:100) to reveal astrocytes, neurons and oligodendrocytes, respectively. After washing, sections were incubated for 1 h with a secondary antibody directed towards mouse-IgG (for GFAP and MAP-2) or goat-IgG (for MBP) and labeled with Alexa Fluor® 350 (1/200) (Molecular Probes, Eugene, OR, USA). Immunohistochemistry against GalC was also performed to reveal oligodendrocytes, incubating sections overnight at 4°C with the anti-GalC primary antibody (1:100). After washing, sections were also incubated

for 1 h with a secondary antibody directed towards mouse-IgG and labeled with Alexa Fluor® 350 (1/200). Sections were mounted under FluorSave Reagent (Calbiochem, San Diego, CA, USA).

The sections were examined and photographed by the use of an Olympus BX50 fluorescence microscope equipped with a Olympus Color View 2 camera and appropriate filter combinations for fluorescence. The contrast of structures was slightly enhanced and images were superposed using image-processing software (Cell Imaging Software, Olympus). For the clarity of the merged panels of figures 2, 3 and 4, images were pseudocolored in red (activated MAPKs) and green (MAP-2, GFAP or MBP), colocalization appearing in yellow.

Statistics

All data points are expressed as mean \pm SEM. Experiments were independent in that separate cultures were not from the same primary preparation. Statistical difference was determined using Student's *t*-test or one-way ANOVA followed by Tukey's or Fisher's LSD *post hoc* test for multiple groups. Data were considered significantly different when $p < 0.05$.

Results

Up-regulation of CNTF and its receptor CNTFR α following ammonia exposure in reaggregated developing brain cell cultures

Immunoblot analysis revealed that in untreated cultures, CNTF expression was barely detectable at DIV 8, while its level increased slightly at DIV 11 and 13, indicating the maturation of the cultures (Fig. 1A). In ammonia-exposed cultures, CNTF expression increased slightly at DIV 8, and markedly thereafter with a maximal tenfold increase at DIV 13, as compared to respective control (Fig. 1A and 1B). We also examined CNTFR α expression, which was slightly up-regulated by ammonia exposure at DIV 8 but returned to basal level at DIV 11 and 13 (Fig. 1A and C).

Regulation of MAPKs activation by ammonia exposure

In order to evaluate the possible involvement of MAPKs in the ammonia-induced expression of CNTF, we studied the activation levels of MAPKs at DIV 13 after 8 days of ammonia exposure, as well as their cell-type distribution in cultures.

Immunoblots revealed that ammonia did not affect significantly total and phosphorylated-Erk1/2 levels (Fig. 2A). Double labeling for MAP-2 protein (a neuronal marker) and phospho-Erk1/2 revealed that Erk1/2 was activated within nuclei of neurons both in untreated and ammonia-exposed cultures (Fig. 2B and C). No activated Erk1/2 could be detected in astrocytes and oligodendrocytes (data not shown).

Ammonia exposure significantly repressed SAPK/JNK phosphorylation, while total SAPK/JNK expression remained unchanged (Fig. 3A). Immunofluorescent labeling show that in control condition, phospho-SAPK/JNK expression was cytoplasmic and was more intense

at the periphery of the aggregates (Fig. 3B₁), where axonal outgrowth process takes place (Honegger and Monnet-Tschudi, 2001; Braissant et al., 2002). Ammonia exposure induced a drastic down-regulation of activated SAPK/JNK staining, with only few cells expressing phospho-SAPK/JNK at the periphery of the aggregates (Fig. 3C₁). Double immuno-labeling show that in control condition SAPK/JNK was activated in neurons (MAP-2 positive cells) (Fig. 3B₁₋₂₋₃₋₄), while in ammonia-exposed cultures, SAPK/JNK was activated in oligodendrocytes (MBP-positive cells) (Fig. 3C₁₋₂₋₃₋₄). In untreated cultures, no oligodendrocytes expressing phospho-SAPK/JNK could be observed (data not shown), suggesting that ammonia exposure induced SAPK/JNK activation in oligodendrocytes. Activated SAPK/JNK could not be detected in astrocytes (data not shown).

Ammonia exposure significantly increased the phosphorylation of p38 MAPK, while total p38 MAPK level remained unchanged (Fig. 4A). Immuno-labeling showed that phospho-p38 MAPK staining was very weak in control condition (Fig. 4B₁) while it was intense under ammonia exposure (Fig. 4C₁). Double immuno-labeling showed that ammonia exposure activated p38 MAPK in cellular bodies and fibers of astrocytes (GFAP-positive cells) (Fig. 4C₃₋₄), as well as in numerous cells that were negative for GFAP (Fig. 4C₁₋₂₋₃₋₄), MAP-2 and MBP (data not shown).

The activation of p38 MAPK by ammonia exposure prompted us to evaluate the activation state of two targets of p38 MAPK: MAPKAPK2 and ATF2. We showed that phospho-MAPKAPK2 level was increased while total MAPKAPK2 level was decreased (Fig. 4D). The ratio of phospho-MAPKAPK2 on total MAPKAPK2 revealed that MAPKAPK2 was significantly activated by ammonia exposure (Fig. 4E). In contrast, ATF2 was significantly deactivated by ammonia exposure (Fig. 4D and F).

The inhibition of SAPK/JNK and p38 MAPK repressed the ammonia-induced up-regulation of CNTF expression, while inhibition of Erk1/2 over-activated CNTF

In order to examine the role of MAPKs activation in ammonia-induced up-regulation of CNTF expression, cultures were co-treated with U0126, SP600125 and SB203580, which are inhibitors of Erk1/2, SAPK/JNK and p38 MAPK, respectively. Erk1/2 inhibition amplified the ammonia-induced up-regulation of CNTF expression while inhibition of SAPK/JNK and p38 MAPK significantly repressed CNTF up-regulation (Fig. 5). U0126 alone induced an increase of CNTF expression as compared to controls, while SP600125 and SB203580 alone had no significant effect on CNTF expression (Fig. 5). These results suggest that ammonia-induced CNTF up-regulation was mediated by SAPK/JNK and p38 MAPK activation, while Erk1/2 negatively regulated CNTF expression, independently of ammonia exposure. Because CNTF is specifically synthesized by astrocytes and because p38 MAPK is activated in these cells by ammonia, the inhibition of p38 MAPK in astrocytes most probably directly inhibits CNTF synthesis. In contrast, as SAPK/JNK and Erk1/2 are not activated in astrocytes, their inhibition probably affect CNTF synthesis indirectly via other brain cells.

Co-treatment with exogenous CNTF protected oligodendrocytes but not neurons

In reaction to brain injury, astrocytes become reactive and increase CNTF expression, which is known to have strong protective effects on neurons and oligodendrocytes. The ammonia-induced up-regulation of CNTF may be an endogenous protective mechanism against ammonia toxicity. To evaluate potential protective effects of exogenous CNTF in developing brain cells exposed to ammonia, cultures were co-treated with CNTF from DIV 5 to 13. CNTF at 150 ng/ml did not protect the expression of neuronal markers such as NF-M and MAP-2, but efficiently protected the expression of the oligodendroglial marker MBP (Fig. 6A and B). Other doses of CNTF were also tested (100 and 200 ng/ml), leading to the same result (data not shown). In particular, the higher dose of 200 ng/ml CNTF did not protect neurons

either. Immunohistochemistry against another oligodendroglial marker, GalC, demonstrated also that ammonia exposure not only inhibits MBP expression, but also leads to a decrease in the number of oligodendrocytes (Fig. 6C). CNTF also appeared to protect the number of oligodendrocytes differentiating in ammonia-exposed cultures (Fig. 6C). Because the expression of MBP closely parallels the course of oligodendrocytes differentiation (Staugaitis et al., 1996), our results suggest that CNTF co-treatment protected oligodendrocytes survival and differentiation against ammonia toxicity.

The protective effects of CNTF on oligodendrocytes were mediated by c-jun activation

CNTF was previously shown to up-regulate c-jun expression (Lu et al., 2003), a component of the transcription factor AP-1. Moreover, an AP-1-like site was observed within the promoter of MBP gene (Miskimins and Miskimins, 2001). Thus, we hypothesized that the protection of MBP expression by CNTF may be mediated by AP-1 signaling. We investigated the effect of CNTF treatment on c-jun expression and phosphorylation, in cultures exposed to ammonia. Immunoblot analysis revealed that ammonia inhibited the expression of both total and phosphorylated c-jun, while CNTF co-treatment protected both c-jun phosphorylation and expression (Fig. 7A, B and C).

To determine whether c-jun signaling was required for the protective effects of CNTF on MBP expression, we investigated whether the inhibition of c-jun synthesis and phosphorylation could abolish CNTF effects on MBP expression. We tested an inhibitor of SAPK/JNK (SP600125), which is an upstream regulator of both c-jun expression and phosphorylation (Pearson et al., 2001; Brantley-Finley et al., 2003). As several studies demonstrated that the JAK/STAT pathway mediates CNTF intracellular effects (Stahl and Yancopoulos, 1994; Narazaki et al., 1994; Alonzi et al., 2001), we also tested an inhibitor of JAK/STAT, named AG490. Inhibition of either the SAPK/JNK pathway with SP600125 or

JAK/STAT with AG490 abolished the protective effects of CNTF on MBP expression (Fig. 7 D and E). SP600125 and AG490 alone produced a small decrease of MBP levels. Moreover, both SP600125 and AG490 abolished the protective effects of CNTF on phosphorylated-c jun expression (Fig. 7 D and F).

Discussion

Ammonia-induced up-regulation of CNTF expression is mediated by MAPKs

In CNS, CNTF is a factor synthesized exclusively by astrocytes in response to various brain injuries (Lee et al., 1997; Lin et al., 1998; Choi et al., 2004; Yokota et al., 2005). CNTF expression was up-regulated by ammonia exposure, an effect amplified with the duration of exposure. In contrast, CNTFR α was slightly up-regulated after 3 days of ammonia exposure but returned to basal level thereafter. Strong up-regulation of CNTF together with a shorter, transient up-regulation of its receptor CNTFR α have been observed in brain injuries such as kainic acid-induced excitotoxicity, entorhinal cortex lesion, transient global ischemia and intracerebral hemorrhage (Lee et al., 1997; Choi et al., 2004; Sarup et al., 2004; Yokota et al., 2005). So far, the mechanism leading to this differential regulation of CNTF and CNTFR α under these various injuries is unknown. The slight up-regulation of CNTF observed in control cultures is attributable to culture maturation and suggests a physiological role for CNTF. Indeed, CNTF was shown involved in the generation, survival, maturation and myelination rate of oligodendrocytes (Mayer et al., 1994; Barres et al., 1996; Stankoff et al., 2002).

Moreover, p38 MAPK was activated in astrocytes by ammonia exposure, as well as its cytoplasmic target, MAPKAPK2. P38 MAPK was also activated in cells negative for MAP-2, MBP and GFAP, which may be Alzheimer's type II astrocytes, which are induced by ammonia exposure and known to downregulate their GFAP expression (Norenberg et al., 1990). The inhibition of p38 MAPK repressed the ammonia-induced CNTF synthesis, suggesting that the activation of p38 MAPK in astrocytes mediates ammonia-induced CNTF synthesis. In contrast to MAPKAPK2, ATF2, a nuclear target of p38 MAPK, was not activated by ammonia exposure. As ATF2 is also target of SAPK/JNK, our data suggest that

ATF2 deactivation by ammonia may be due to the concomitant deactivation of SAPK/JNK. SAPK/JNK deactivation in ammonia-exposed cultures was observed in neurons, suggesting that ATF2 deactivation also occurs in neurons. The deactivation of SAPK/JNK pathway in neurons may play a role in the inhibition of neurite outgrowth observed previously under ammonia exposure (Braissant et al., 2002) as SAPK/JNK regulates neuronal differentiation (Heasley et al., 1996; Giasson et al., 1999; Xiao and Liu, 2003). Although ammonia exposure did not activate SAPK/JNK in astrocytes, the inhibition of SAPK/JNK pathway abolished the ammonia-induced CNTF expression. The induction of phosphorylated-SAPK/JNK in oligodendrocytes was observed under ammonia exposure, suggesting that the inhibition of SAPK/JNK in oligodendrocytes may indirectly prevent the up-regulation of CNTF expression in astrocytes through an alteration of the extracellular crosstalk between oligodendrocytes and astrocytes. In contrast, the inhibition of Erk1/2 increased CNTF expression both in control and ammonia-exposed cultures suggesting that the Erk1/2 pathway negatively regulates CNTF synthesis. Because phosphorylated-Erk1/2 was detected only in neurons, we can hypothesize that the effect of Erk1/2 inhibition on CNTF expression in astrocytes is also indirect and mediated by neurons.

Our results are in contrast with data showing that ammonia induces a down-regulation of CNTF in primary cultures of astrocytes (Bodega et al., 2006; Bodega et al., 2007a). However, these latter experiments were conducted in primary astrocytes, while our model contains intermingled brain cells. We can thus hypothesize that astrocytes deprived of natural communications with other brain cells may react differently to ammonia exposure from astrocytes *in vivo*. Astrocytes might up-regulate CNTF synthesis in reaction to ammonia injury on neurons or oligodendrocytes, which might release a specific factor of injury, as it was suggested for epidermal growth factor (EGF; Kamiguchi et al., 1995; Zeleniaia et al., 2000).

Treatment with CNTF prevents ammonia-induced inhibition of MBP expression and c-jun expression and phosphorylation

Ammonia exposure repressed NF-M, MAP-2 and MBP expression, confirming our previous results (Braissant et al., 1999; Braissant et al., 2002; Braissant et al., 2008) (Cagnon and Braissant, 2008). Because MBP expression reflects the maturation of oligodendrocytes and their capacity of myelination (Staugaitis et al., 1996), the toxic effect of ammonia on MBP expression is in accordance with clinical data showing hypomyelination in patients with neonatal onset of hyperammonemia (Takanashi et al., 2003; Majoie et al., 2004).

Exogenous treatment with CNTF protected both the number of oligodendrocytes developing in the culture, and their MBP expression, against ammonia toxicity. These results are in line with data showing that CNTF promotes survival and maturation of oligodendrocytes (Mayer et al., 1994; Barres et al., 1996; Stankoff et al., 2002), increases myelin synthesis (Stankoff et al., 2002) and protects oligodendrocytes against Tumor Necrosis Factor-induced death (Louis et al., 1993). CNTF might directly act on oligodendrocytes as CNTFR α is expressed in oligodendrocytes and this expression can be up-regulated following injury (Miotke et al., 2007). However, because our cultures contain intermingled brain cell types, the protective effect of CNTF on MBP expression may be indirect, through astrocyte activation, which could release a trophic factor for oligodendrocytes as recently demonstrated (Albrecht et al., 2007), one likely candidate being platelet-derived growth factor (PDGF; Richardson et al., 1988; Hu et al., 2008). Despite its protective effects on oligodendrocytes against ammonia toxicity, CNTF was unable to protect neurons. In a model of glutamate-mediated excitotoxicity on neurons, CNTF alone had no protective effects while the CNTF complexed with soluble CNTFR α protected both neuron survival and neurite outgrowth (Ozog et al., 2007). However, more work is requested to determine whether soluble CNTFR α -CNTF

complex can protect neurons in our model. Indeed, ammonia-exposed neurons may be completely unresponsive to CNTF as ammonia is neurotoxic through many other gene-independent mechanisms (see Cagnon and Braissant, 2007, for a review).

We then investigated the signaling pathway mediating the protective effects of CNTF on MBP expression. It was previously observed that the AP-1 signaling pathway mediates CNTF-dependent transcription (Symes et al., 1997; Lu et al., 2003) and that the MBP gene promoter contains an AP-1-like site (Miskimins and Miskimins, 2001). Thus, we hypothesized that the protection of MBP expression by CNTF could be mediated by AP-1 signaling. AP-1 is composed of Fos and Jun family proteins. Homo- and heterodimers of Jun proteins can bind DNA directly, whereas Fos members require interaction with any of the Jun proteins to act as transcriptional activators (Angel and Karin, 1991). Phosphorylation of c-Jun potentiates its ability to activate transcription either as a homodimer or a heterodimer with c-Fos (Karin, 1995). Thus, we investigated the level of c-jun expression and phosphorylation in cultures exposed to ammonia alone or in combination with CNTF. Ammonia-exposure inhibited expression and phosphorylation of c-jun. CNTF protected both total and phosphorylated c-jun protein expression in cultures exposed to ammonia, suggesting that c-jun synthesis and activation may mediate the effects of CNTF on MBP expression. This parallel increase in the expression of total and phosphorylated c-jun was expected as activated c-jun can induce the expression of its own gene, together with increased stability of the phosphorylated protein (Angel et al., 1988; Kolomeichuk et al., 2008).

To determine whether c-jun signaling was required for the protective effects of CNTF on MBP expression, we investigated whether the inhibition of c-jun synthesis and phosphorylation could abolish CNTF effects on MBP expression. We tested an inhibitor of SAPK/JNK (SP600125), which is an upstream regulator of both c-jun expression and phosphorylation (Pearson et al., 2001; Brantley-Finley et al., 2003). As several studies

demonstrated that the JAK/STAT pathway mediates CNTF intracellular effects (Stahl and Yancopoulos, 1994; Narazaki et al., 1994; Alonzi et al., 2001), we also tested an inhibitor of JAK/STAT, named AG490. Inhibition of either the SAPK/JNK pathway with SP600125 or JAK/STAT with AG490 abolished the protective effects of CNTF on MBP expression under ammonia exposure. Similarly, both SP600125 and AG490 abolished the protective effects of CNTF on phosphorylated-c jun expression

Our data suggest that JAK/STAT, SAPK/JNK and phosphorylated-c jun mediate the protective effects of CNTF on MBP. It was shown that SAPK/JNK up-regulates c-jun (Pearson et al., 2001; Brantley-Finley et al., 2003) and that JAK/STAT can up-regulate SAPK/JNK (Okugawa, et al., 2003). Altogether, these results suggest that the protective effects of CNTF on the maturation of oligodendrocytes exposed to ammonia were mediated by the activation of c-jun, through the successive activation of JAK/STAT and SAPK/JNK.

Conclusion

Our work showed that CNTF expression was induced by ammonia toxicity through the activation of p38 MAPK in astrocytes. Intracellular signaling occurring in other brain cells could have indirect inhibiting or activating effects on synthesis of CNTF in astrocytes. Our observations stress the importance of investigating the toxic effects of ammonia in a model containing all brain cell types. While we have recently shown that creatine, as well as cdk5 inhibition, exerts protective effects on neurons exposed to ammonia (Braissant et al., 2002; Braissant et al., 2008; Cagnon and Braissant, 2008), this new study demonstrates that a treatment with CNTF protects oligodendrocytes against ammonia toxicity, and that JAK/STAT, SAPK/JNK and c-jun are involved in this process. *In vivo* experiments are clearly needed to assess whether CNTF may have therapeutic implications for the treatment of demyelination observed in the brain of young patients experiencing hyperammonemia.

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Figure legends

Figure 1. Time course of CNTF and CNTFR α expressions in cultures exposed to ammonia. **A:** Immunoblot analysis of CNTF and CNTFR α expressions. Histone H1 was used as loading control. **B** and **C:** Data quantification of CNTF (**B**) and CNTFR α (**C**) expressions after 3 (DIV 5-8), 6 (DIV 5-11) and 8 (DIV 5-13) days of ammonia exposure. The values are expressed as percentage of respective controls and represent the mean \pm SEM from at least 5 replicates taken from 3 independent cultures. *t*-test: ** $p < 0.01$ *** $p < 0.001$ as compared to corresponding controls.

Figure 2. Effect of ammonia exposure on Erk1/2 activation. **A:** Immunoblots and data quantification of total and phosphorylated levels of Erk1/2 in cultures exposed for 8 days (DIV 5-13) to ammonia. Histone H1 was used as loading control. The values are expressed as the ratio of phosphorylated Erk1/2 on total Erk1/2 and represent the mean \pm SEM from 10 replicates taken from 3 independent cultures. **B** and **C:** Double immunofluorescent staining of phospho-Erk1/2 (red) and MAP-2 (green) in control (**B**) and ammonia-exposed (**C**) cultures, showing that phospho-Erk1/2 is expressed in nuclei of neurons. Scale bar = 50 μ m.

Figure 3. Effect of ammonia exposure on SAPK/JNK activation. **A:** Immunoblots and data quantification of total and phosphorylated levels of SAPK/JNK in cultures exposed for 8 days (DIV 5-13) to ammonia. Histone H1 was used as loading control. The values are expressed as the ratio of phosphorylated SAPK/JNK on total SAPK/JNK and represent the mean \pm SEM from 10 replicates taken from 3 independent cultures. *t*-test: *** $p < 0.001$ as compared to control. **B:** Double immunofluorescent staining of phospho-SAPK/JNK (red) and MAP-2 (green) showed that SAPK/JNK is activated in cytoplasm of neurons in control cultures. **C:** Double immunofluorescent staining of phospho-SAPK/JNK (red) and MBP (green) showed

that SAPK/JNK activation is induced in oligodendrocytes by ammonia exposure. Scale bar = 50 μm .

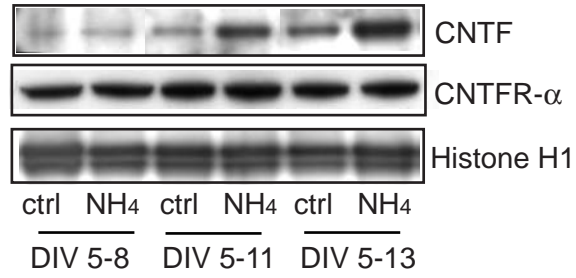
Figure 4. Effects of ammonia exposure on p38 MAPK activation. **A:** Immunoblots and data quantification of total and phosphorylated levels of p38 MAPK in cultures exposed for 8 days (DIV 5-13) to ammonia. Histone H1 was used as loading control. The values are expressed as the ratio of phosphorylated p38 MAPK on total p38 MAPK and represent the mean \pm SEM from 9 replicates taken from 3 independent cultures. *t*-test: *** $p < 0.001$ as compared to control. **B** and **C:** Double immunofluorescent staining of phospho-p38 MAPK (red) and GFAP (green) in control (B) and ammonia-exposed (C) cultures, showing that p38 MAPK is activated in cellular bodies and fibers of astrocytes by ammonia exposure. Scale bar = 50 μm . **D:** Immunoblots of phosphorylated and total MAPKAPK2 and ATF2. Histone H1 was used as loading control. **E** and **F:** Data quantification of immunoblots presented in D. The values are expressed as the ratio of phosphorylated-MAPKAPK2 on total MAPKAPK2 and of phosphorylated-ATF2 on total ATF2 and represent the mean \pm SEM from 13 replicates and 4 replicates, respectively, taken from 3 independent cultures. *t*-test: ** $p < 0.01$ and *** $p < 0.001$ as compared to controls.

Figure 5. Effects of MAPKs inhibitors on CNTF synthesis under ammonia exposure. Immunoblot analysis and corresponding data quantification of CNTF expression in cultures exposed or not to ammonia for 8 days (DIV 5-13) and treated or not with MAPKs inhibitors. Histone H1 was used as loading control. The values are expressed as percentage of control and represent the mean \pm SEM from 6 replicates taken from 2 independent cultures. * $p < 0.05$, ** $p < 0.01$ as compared to control, and ^o $p < 0.05$, ^{ooo} $p < 0.001$ as compared to ammonia (one-way ANOVA followed by Fisher's LSD *post-hoc* test).

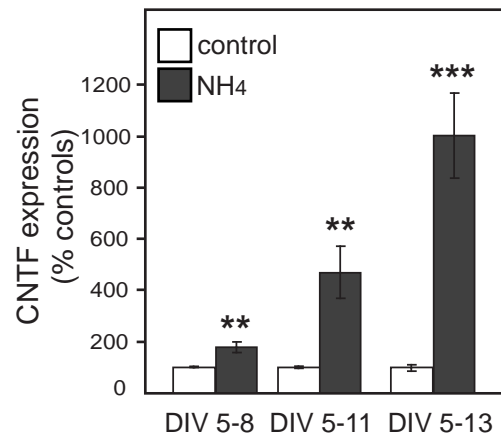
Figure 6. Effect of exogenous CNTF treatment on neurons and oligodendrocytes under ammonia exposure. **A:** Immunoblot analysis of the neuronal (NF-M and MAP-2) and oligodendroglial (MBP) markers in cultures exposed to ammonia and co-treated or not with CNTF. Histone H1 was used as loading control. **B:** Data quantification of MBP expression. The values are expressed as percentage of control and represent the mean \pm SEM from 6 replicates taken from 2 independent cultures. *** $p < 0.001$ as compared to control and ^{ooo} $p < 0.001$ as compared to ammonia (one-way ANOVA followed by Tukey *post-hoc* test). **C:** Immunohistochemical analysis of GalC, an oligodendroglial marker, in cultures exposed to ammonia and co-treated or not with CNTF. Bar: 50 μ m.

Figure 7. Mediation of the oligodendroglial protective effects of CNTF by c-jun. **A:** Immunoblot analysis of total and phosphorylated c-jun expressions in cultures exposed to ammonia and co-treated or not with CNTF. Histone H1 was used as loading control. **B** and **C:** Data quantification of total (B) and phosphorylated (C) c-jun expression. The values are expressed as percentage of control and represent the mean \pm SEM from 6 replicates taken from 2 independent cultures. * $p < 0.05$ and *** $p < 0.001$ as compared to control and ^{ooo} $p < 0.001$ as compared to ammonia (one-way ANOVA followed by Tukey's *post-hoc* test). **D:** Immunoblot analysis of MBP and phosphorylated c-jun expression in cultures exposed to ammonia, CNTF and/or SP600125 and AG490. Histone H1 was used as loading control. **E** and **F:** Data quantification of MBP and phosphorylated c-jun expression in cultures exposed to ammonia, CNTF and/or SP600125 and AG490. The values are expressed as percentage of control and represent the mean \pm SEM from 6 replicates. *t*-test: * $p < 0.05$ and *** $p < 0.001$ as compared to ammonia.

A



B



C

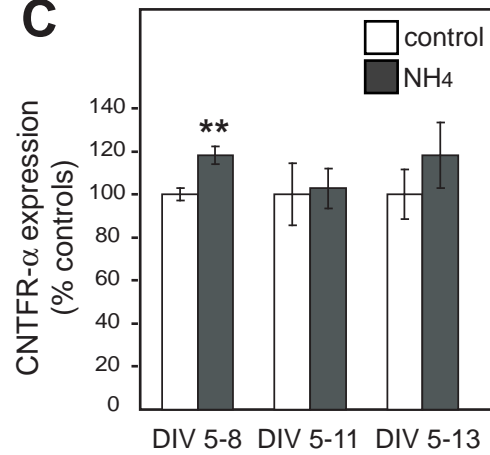


Figure 1

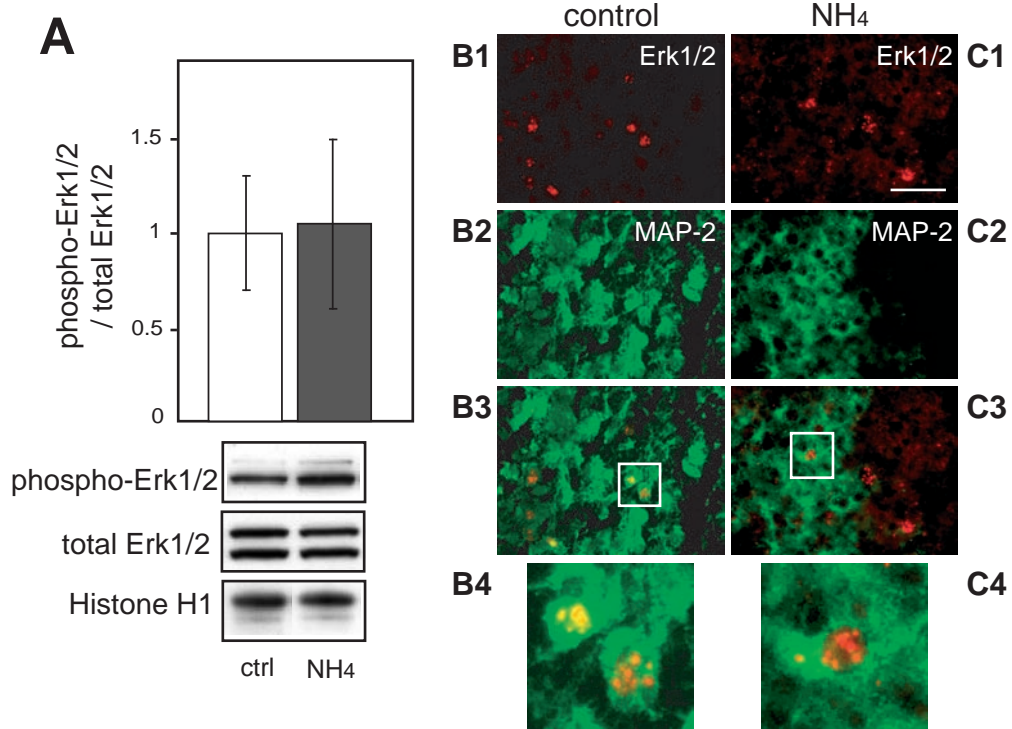


Figure 2

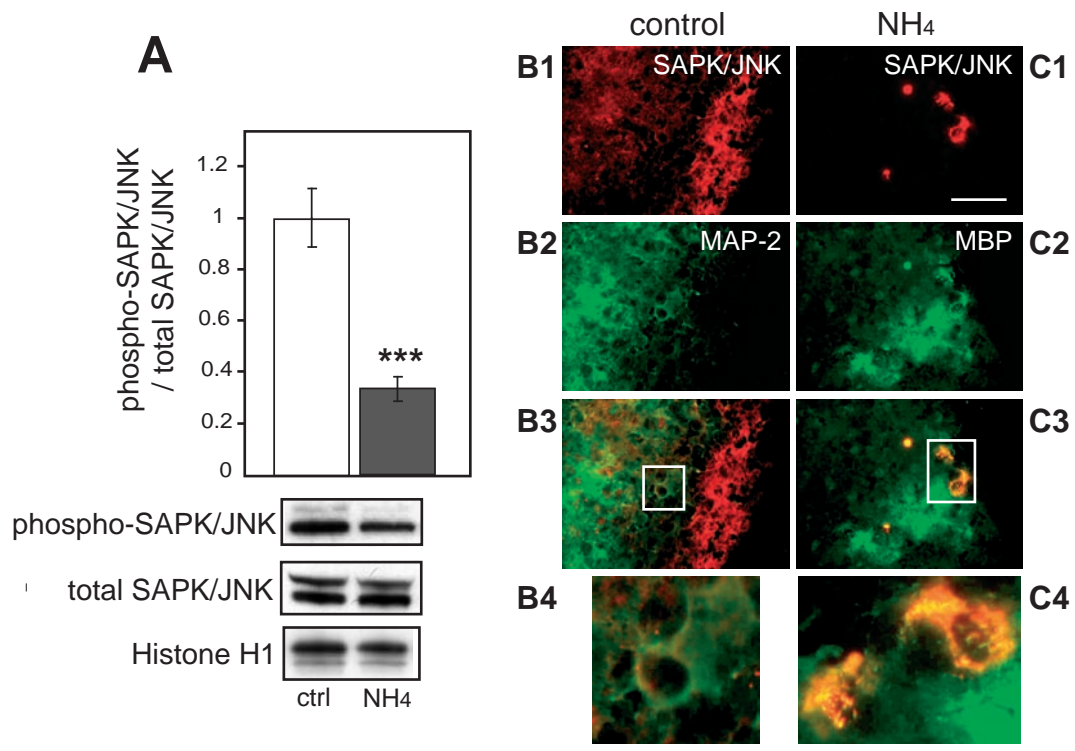


Figure 3

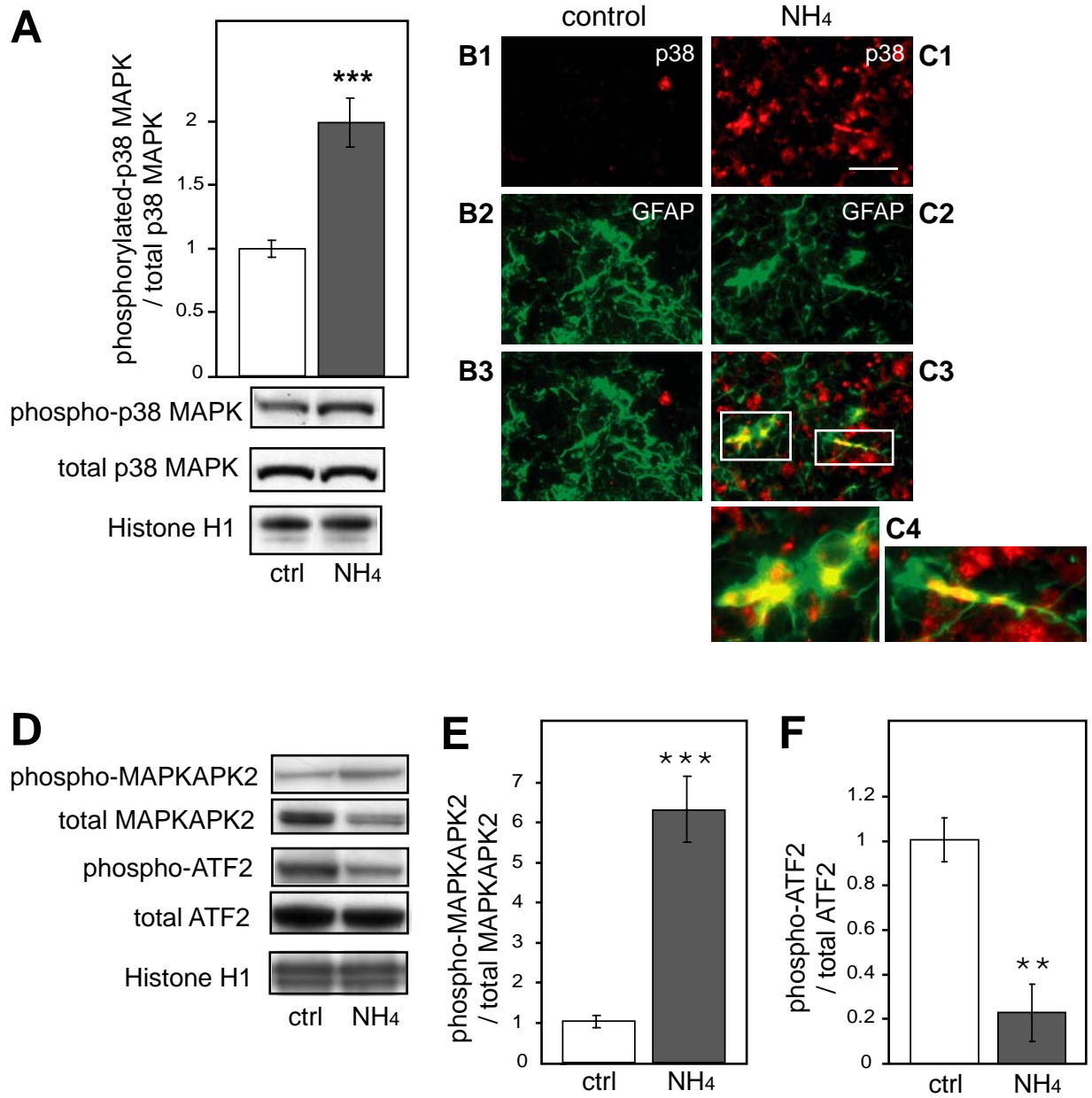


Figure 4

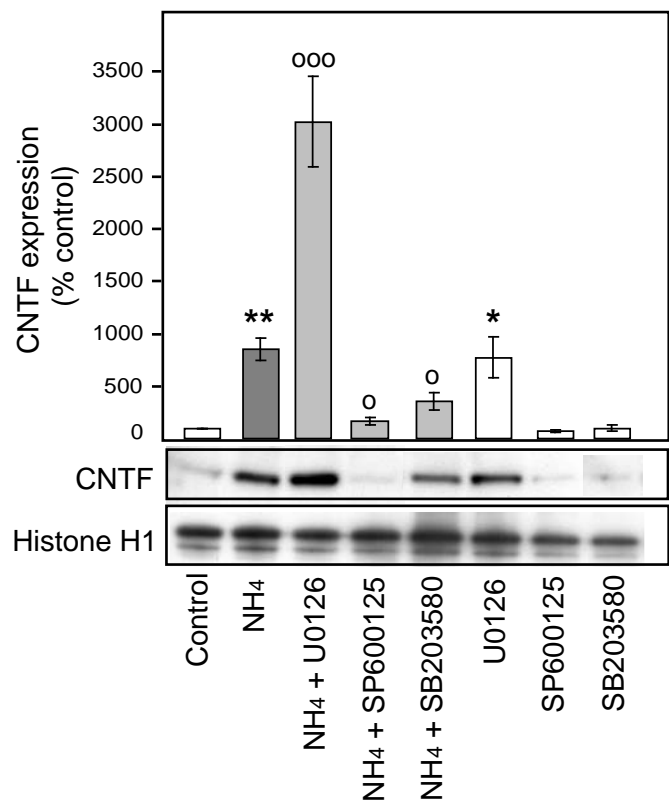


Figure 5

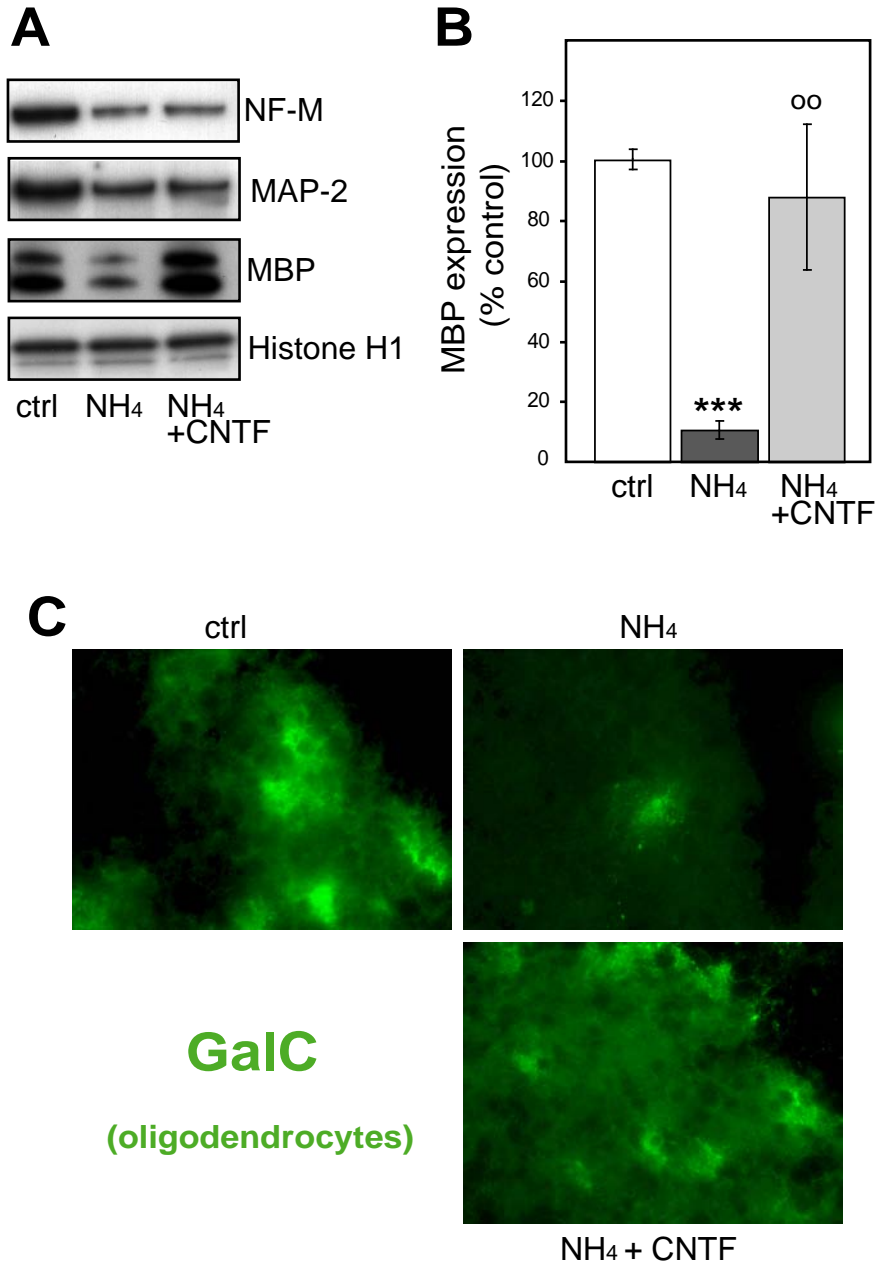


Figure 6

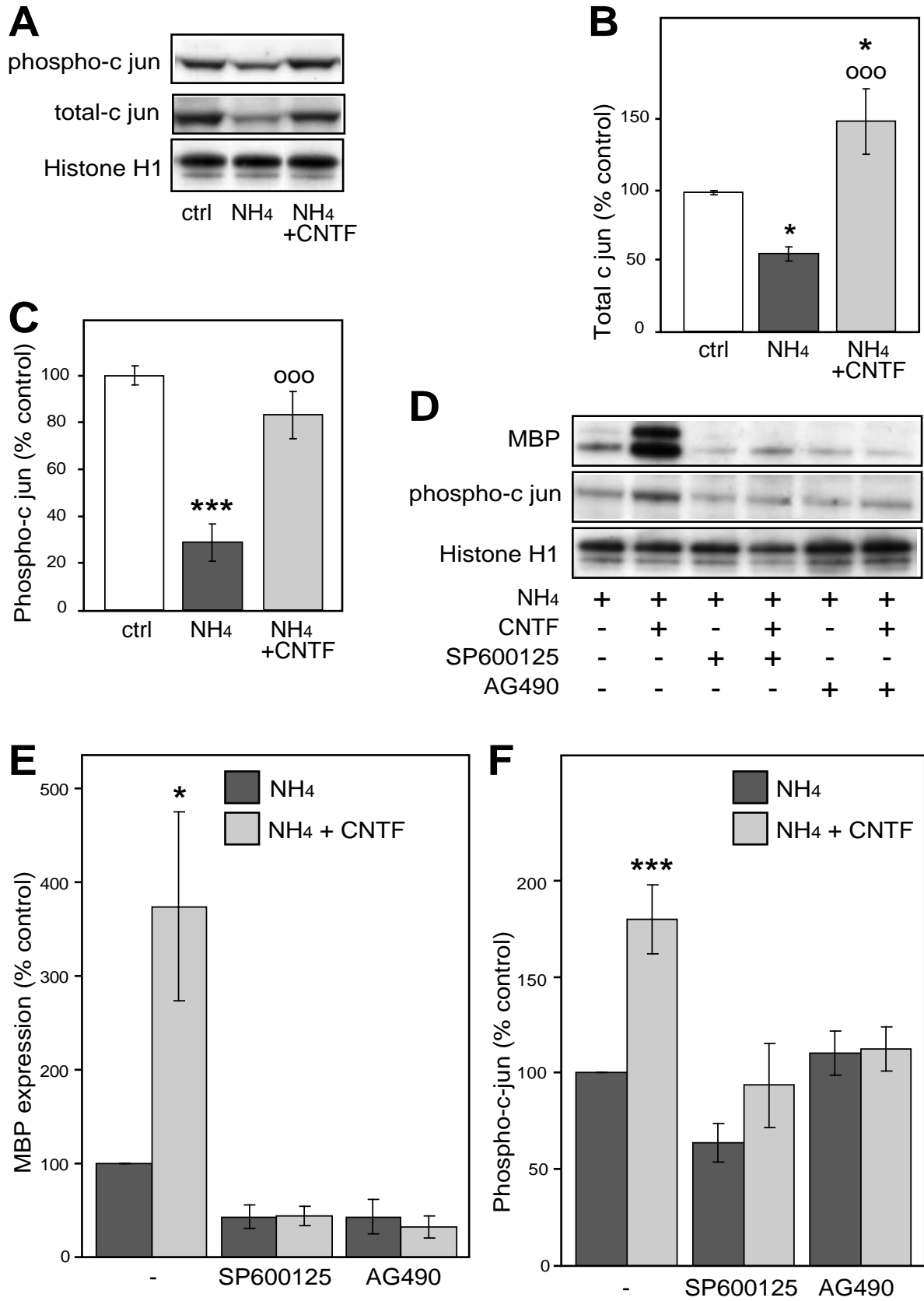


Figure 7