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Research article

Astroglial expression of the P-glycoprotein is controlled by intracellular CNTF

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Abstract

Background: The P-glycoprotein (P-gp), an ATP binding cassette transmembrane transporter, is expressed by astrocytes in the adult brain, and is positively modulated during astrogliosis. In a search for factors involved in this modulation, P-gp overexpression was studied in long-term *in vitro* astroglial cultures.

Results: Surprisingly, most factors that are known to induce astroglial activation in astroglial cultures failed to increase P-gp expression. The only effective proteins were IFNγ and those belonging to the IL-6 family of cytokines (IL-6, LIF, CT-1 and CNTF). As well as P-gp expression, the IL-6 type cytokines - but not IFNγ - stimulated the expression of endogenous CNTF in astrocytes. In order to see whether an increased intracellular level of CNTF was necessary for induction of P-gp overexpression by IL-6 type cytokines, by the same cytokines analysis was carried out on astrocytes obtained from CNTF knockout mice. In these conditions, IFNγ produced increased P-gp expression, but no overexpression of P-gp was observed with either IL-6, LIF or CT-1, pointing to a role of CNTF in the intracellular signalling pathway leading to P-gp overexpression. In agreement with this suggestion, application of exogenous CNTF -which is internalised with its receptor - produced an overexpression of P-gp in CNTF-deficient astrocytes.

Conclusions: These results reveal two different pathways regulating P-gp expression and activity in reactive astrocytes, one of which depends upon the intracellular concentration of CNTF. This regulation of P-gp may be one of the long searched for physiological roles of CNTF.

Background

Recent studies have reported expression of the transmembrane transporter P-glycoprotein (P-gp) in astrocytes, in addition to the previously determined endothelial locali-

sation. This second cellular location raises new questions as to the function of P-gp in the brain because, whereas endothelial P-gp clearly participates directly in the transport of substrates across the blood-brain barrier for a number of identified substrates [1–4], the physiological roles and substrates of its astroglial counterpart are unknown. One potential clue to these issues is the fact that P-gp expression is stimulated in astrocytes activated by various brain insults [5].

P-gp consists of a group of closely related, intrinsic membrane proteins encoded by the multidrug-resistance (mdr) genes [6]. It acts as an ATP-driven efflux pump [7] that accepts a wide range of structurally different substrates, among which are drugs, hormones, corticosteroids, cytokines or phospholipids [8–10]. The adult brain is a major site of expression of P-gp [2,11–15], and it had long been exclusively associated to the endothelial cells in capillary walls [2,4,16], until astrocytic endfeet were identified as another specific location *in vivo* [17,18], and *in vitro* [19].

In order to start deciphering the physiological role of P-gp in astrocytes, we have chosen to study its increase in expression in reactive astrocytes, by looking for the effects of a wide variety of molecules known to induce astroglial activation in *in vitro* culture conditions. This involved, in particular, several agents that had also been shown to stimulate P-gp expression in other cell types, such as Interferon-α and -γ (IFN-α, γ) [20–22], Tumor Necrosis Factor alpha (TNF-α) [21,23] and dibutyryl-cyclic-AMP [24], and also a number of other compounds classically used to induce astroglial differentiation *in vitro*. Surprisingly, only a few of these factors drove astroglial P-gp expression, indicating that P-gp overexpression is not just related to astroglial differentiation, but appears as a specific response to certain stimuli. This increase in P-gp expression thus seems to characterize a specific stage of differentiation, which, in one pathway at least, also involves an increased concentration of the ciliary neurotrophic factor (CNTF). The two proteins may be even more functionally linked to each other since some of the "astrogliotic" stimuli able to increase P-gp expression require CNTF as a key intracellular signal.

Results

Enriched astroglial cell cultures were readily obtained from cerebral hemispheres of Swiss mice and of CNTF knockout mice, and maintained for several weeks without signs of cell alteration.

Expression of P-gp in primary astrocyte culture

Astrocytes in culture expressed P-glycoprotein as early as 24 h after plating. The RT-PCR performed on a fragment of the P-gp transcript yielded a 167-bp product (Fig. 1a),

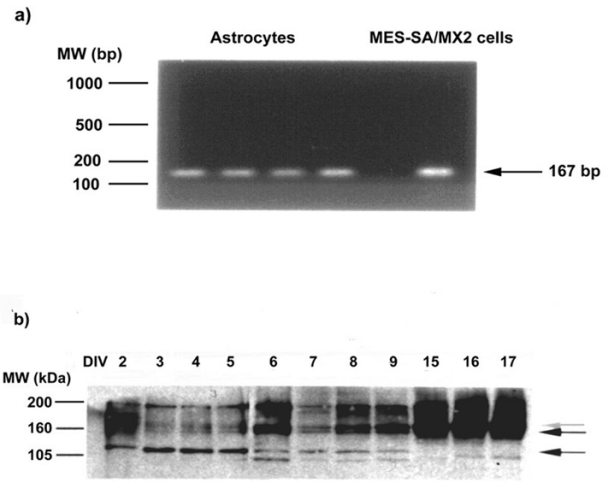

Figure 1
MDR1mRNA and P-gp expression in astroglial cultures. a/ RT-PCR showing MDR1mRNA. b/ Western blotting using rabbit anti-P-gp polyclonal antibody demonstrating the presence of P-gp as early as 2 div. Between 2 div and 5 div, a band is observed at about 120 kDa (lower black arrow); a doublet appears at 6 div at about 150–170 kDa (black arrows); the band corresponding to the mature form of P-gp, is expected at 170 kDa (grey arrow), and becomes increased after 15 div.

present in all astrocyte samples and in the MES-SA/MX2 cells used as a positive control. At the protein level, the results varied over time: until the 4th day in vitro (div), the antibody raised against P-gp detected a band at about 120 kDa; from the 6th div a doublet was detected at about 160–170 kDa; finally, at 15 div this doublet became predominant and its expression increased (Fig. 1b).

Effects of molecules promoting astrogliosis on P-gp expression

Among the agents known to activate astrocytes ("astrogliotic factors") that were tested for modulating P-gp expression, most failed to alter it (Table 1). This was in particular the case for dBcAMP, IL-1β, LPS, NGF, RA, rhIFNα, rhTGFα and TNFα. In contrast, all the cytokines that belong to the IL-6 family induced a 50% or more increase in expression of P-gp in long-term astrocyte culture. This effect was dose-dependent and was modulated by α receptor subunits, when relevant. For instance, rrCNTF was inefficient when used alone at 30 ng/ml in long-term cultures. However, an increase in the cellular content of P-gp was observed in a significant and reproducible manner when 100, and even further 250 ng/ml of rCNTF were used (Fig. 2a). As previously described for the effects of rCNTF on other astroglial markers of differentiation [25], addition

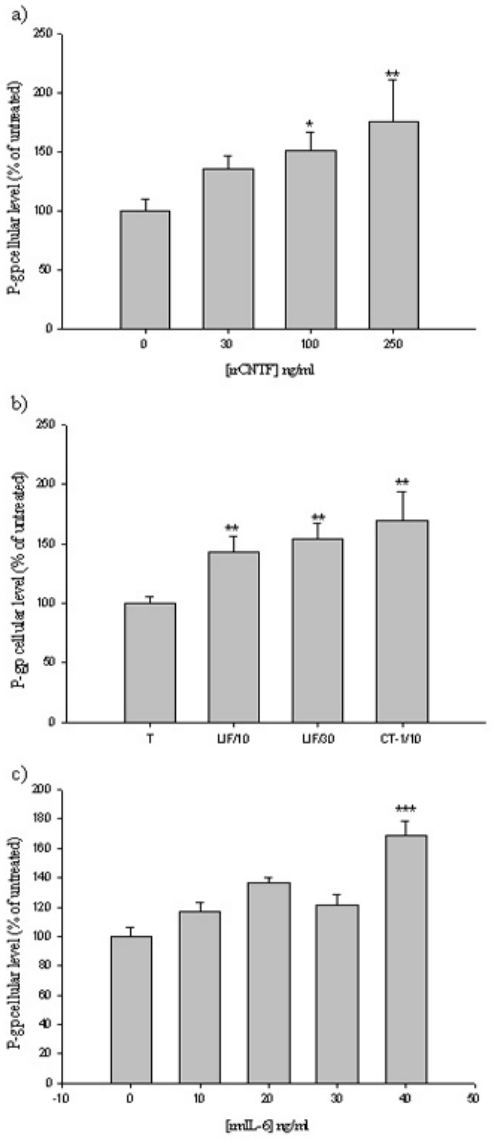

Figure 2
Effect of cytokines on P-gp cellular content in mature astrocytes. a/ Addition of 100 and 250 ng/ml rCNTF during 24 h induced a statistically significant increase as compared to untreated controls (+51.7% ± 15.6, factorial ANOVA significant at 95%, t-test $p < 0.01^{**}$ and +75.7 ± 30, factorial ANOVA significant at 95%, t-test $p < 0.05^{*}$, respectively). b/ Addition of rhLIF during 24 h induced a significant increase of P-gp expression both at 10 and 30 ng/ml (+42.7 % ± 14, factorial ANOVA significant at 95%, t-test $p < 0.05^{*}$ and +54.35 % ± 12, factorial ANOVA significant at 95%, t-test $p < 0.01^{**}$ respectively). The same pattern of regulation was observed with CT-1 at 10 ng/ml (+69.6 % ± 24, factorial ANOVA significant at 95%, t-test $p < 0.01^{**}$). c/ Addition of rmIL-6 elicited a significant increase of P-gp intracellular content at 40 ng/ml (+68.8 % ± 10, factorial ANOVA significant at 95%, t-test $p < 0.001^{***}$)

of 200 ng/ml of c-myc-sCNTFRα induced a potentiation of the effects, and an increase of P-gp cellular content was then observed with the lowest concentration of rCNTF studied (10 ng/ml), and maintained for all other concentrations used (data not shown). Addition of rhLIF and rhCT-1, in the culture medium provoked an increase of about 1.5 to 2-fold of P-gp cellular content (Fig. 2b). Addition of rmIL-6 at concentrations below 40 ng/ml in the culture medium was inefficient, but a significant increase of P-gp intracellular content was observed when this concentration was used (Fig. 2c). Addition of rhsIL-6Rα to the culture medium induced a major potentiation of the effects, since, in these conditions, all concentrations used triggered a significant increase in P-gp content (Fig. 3).

Selective blockade of P-gp transporter activity was studied using two different specific blockers, S9788 and verapamyl. When used at concentrations of 5 μM and 10 pM, a complete blockade of the effects of all IL-6 family cytokines was observed. Even the presence of the specific α receptor subunit for IL-6 and CNTF failed to reverse the neutralising effects of S 9788 (Fig. 5) and verapamyl (data not shown).

Besides the IL-6 family of cytokines, the only compound to trigger P-gp overexpression in astrocytes was IFNγ (Fig. 6a). This IFNγ effect was very likely direct since hLIF05, which inhibits the LIF receptor pathway, could not reverse it.

Regulation of P-gp expression in CNTF-/- astrocytes

The progressive maturation of P-gp during development and the regulation of its expression by members of the IL-6 family of cytokines are two features that P-gp shares with CNTF in astrocytes (see discussion section). Moreover, like P-gp, CNTF regulation is very specific, since, among different molecules that normally trigger astrogliosis, only those belonging to the IL-6 family provoked an increase of CNTF (Fig. 7). We, therefore, explored the possibility of a functional link between the two molecules by analysing whether the presence of endogenous CNTF in astrocytes was required for the observed modulation of P-gp expression by IL-6 type cytokines and IFN-γ. This was tested by analysing the effects of the cytokines in astrocytes taken from CNTF knockout mice. In these cultures, the modulation of P-gp by LIF, CT-1 or IL-6 (even in presence of its specific α receptor subunit) was completely lost (Fig. 4). In contrast, addition of rrCNTF (250 ng/ml) produced a significant increase of P-gp intracellular level. In the CNTF -/- astrocytes, addition of IFN-γ to the medium triggered an increase of P-gp cellular level comparable to that observed in wild-type astrocytes, underlining its direct effect on P-gp modulation (Fig. 6b).

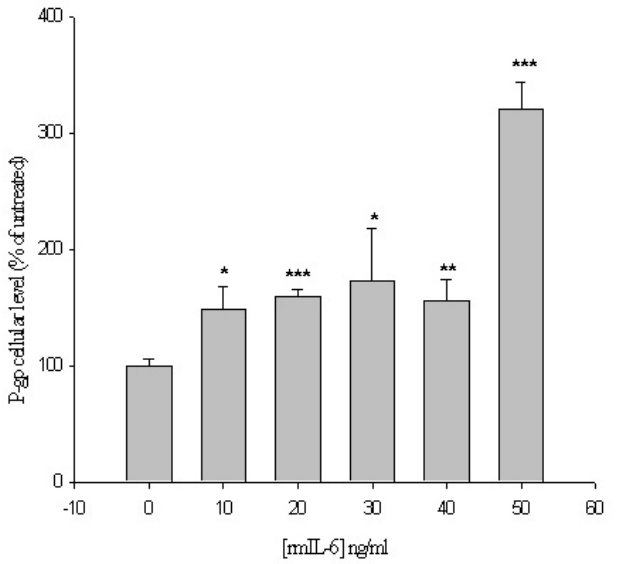

Figure 3
Potentiation of IL-6 effects on P-gp expression by adding its specific α receptor subunits. Addition of soluble IL-6R induced a major potentiation of rmIL-6 whatever the concentration of the factor used.

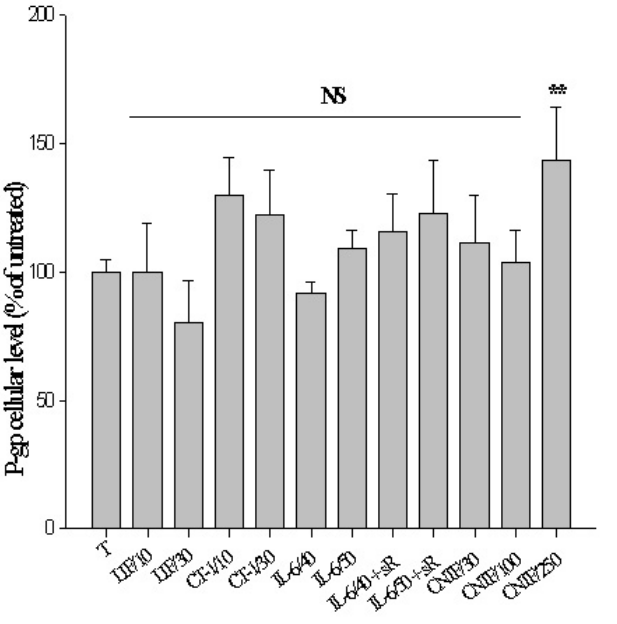

Figure 4
Effect of cytokines of the IL-6 family on P-gp intracellular content in CNTF-/- astrocytes. Only rrCNTF (250 ng/ml) provoked a significant increase in P-gp intracellular content (+43.8 % ± 20, factorial ANOVA significant at 95%, t-test $p < 0.01^{**}$).

Discussion

The main result of this study is the demonstration of a close interaction between expression of two intrinsic astroglial proteins, the IL-6 cytokine CNTF and the ATP-binding cassette transmembrane transporter P-gp. Besides a parallel development of expression, the concentration of the two proteins appears, in particular, to be similarly increased following stimulation by IL-6-type cytokines whereas a number of other agents eliciting activation of astrocytes are ineffective. Moreover, astroglial expression of CNTF appeared to be necessary for the effects of IL-6-type cytokines on P-gp expression. Reciprocally, blockade of P-gp activity eliminated the effects of cytokines, even CNTF, on its own expression, suggesting a functional link between the two systems.

P-gp and CNTF may be involved together in a specific type of astrocytic activation

The recent discovery of P-gp expression on astrocytic endfeet [17,18] and its induction after a stress [5] has raised the issue of a role of this protein in astrocytes. To address this issue, we have undertaken, here, an analysis of the expression of P-gp over time during development and of its stimulation in mature cells. The first feature in this study is the striking similarity between the results obtained for astroglial P-gp and those previously gathered on the astroglial cytokine CNTF. Indeed, P-gp and CNTF seem to share parallel modulations of expression both during development and under specific stimulations. First, like CNTF [26], P-gp is not detectable in the early stages of postnatal development in the central nervous system. Both proteins are detected from P7, and their concentration levels gradually increase, reaching a plateau at P28 [15,26]. Results obtained in *in vitro* astroglial cultures are also indicative of a parallel maturation of the two proteins. P-gp, both at mRNA and protein levels, was expressed in our study as soon as after 24 hours in culture, but Western blot studies revealed that the protein detected in those early cultures did not have the 170 kDa mature form [15], that became predominant only after 14 days, the point at which astrocytes exhibit a mature phenotype (see discussion in 25). A very similar time course of expression was observed previously for CNTF [25]. Before 7 days in vitro, immature astrocytes did not express detectable levels of CNTF, whereas at 14 div, when astrocytes form a confluent monolayer and express high levels of GFAP, they exhibit a significant intracellular content of CNTF.

In vitro, astrocytes activation experiments further indicate a parallel regulation of the two proteins. Even though *in vivo* models of astrogliosis have revealed a concomitant overexpression of both proteins [5,27], most factors that readily induce astroglial activation *in vitro* fail to increase their expression [28,29] and the present study). Indeed,

Table 1: Modulation of P-gp expression by agents that induce astroglial reactivity.

Treatment	*Concentration*	*Effect*
dBcAMP	0,5 mM	- 3.1 ± 8
IL-1β	50 ng/ml	+10.5 ± 11
Lesion	/	- 12.1 ± 8
LPS	1 μg/ml	+ 2.3 ± 8
NGF	10 ng/ml	- 21.7 ± 22
RA	10^{-7} M	- 28.6 ± 20.2
rhIFN-β	600 U/ml	-20 ± 11
rhIFNγ	**300 U/ml**	**+50 ± 30**
rhTGFα	50 ng/ml	-9 ± 5
TNFα	100 ng/ml	+ 7.6 ± 9.1
rhCT-1	**10 ng/ml**	**+ 69.6 ± 24****
rhLIF	**30 ng/ml**	**+ 54.3 ± 12****
rmIL-6	**40 ng/ml**	**+68.8 ± 10*****
RmIL-6	**50 ng/ml**	**+220.28 ± 22*****
+		
rhsIL-6R	**500 ng/ml**	
rrCNTF	**250 ng/ml**	**+ 75.7 ± 30***

*: ANOVA significant at 95%, t-test p < 0.05; **: ANOVA significant at 95%, t-test p < 0.01; ***: ANOVA significant at 95%, t-test p < 0.001.

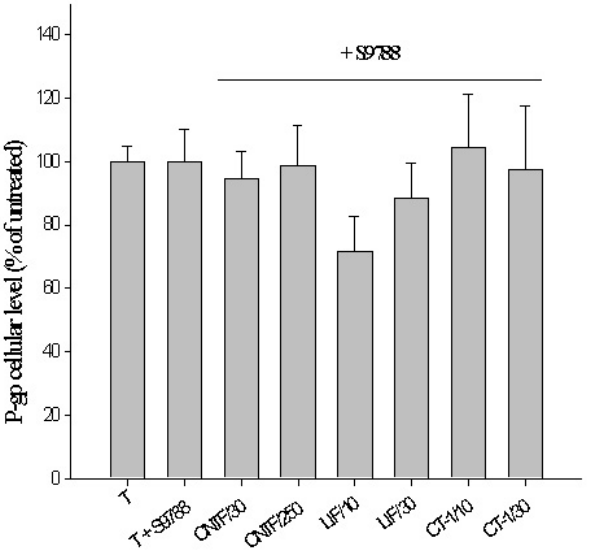

Figure 5
Effect of P-gp blockers. Addition of the P-gp blocker S9788 in the culture medium blocked completely the effect of rrCNTF at 250 ng/ml (-1.5 vs. +75.7 %), rhLIF at 30 ng/ml (-11.5 vs. + 54.3%) and rhCT-1 at 30 ng/ml (-2.5 vs. +26.1%) on P-gp expression in astrocytes.

for both proteins, only IFNγ (in the study by Carroll and colleagues, 31, for CNTF) and the IL-6 family cytokines could trigger a quantifiable increase in expression in astrocytes (this study).

All these results suggest that the overexpression of CNTF and P-gp participate in the definition of a particular stage of astroglial differentiation. So-called astrogliosis has long been considered as a discrete "activation" stage of astrocytes, on the basis of its characteristic morphological features. However, more recent studies exploring the expression of specific molecules have questioned this view, drawing attention to the fact that reactive gliosis varies qualitatively and quantitatively depending on both the nature of the injury and the microenvironment of the injury site (see references and discussion 30 and 31). The nature of the injury and the microenvironment necessary for the differentiation stage of astrocytes that involves CNTF and P-gp overexpression is, at this point, a matter of speculation. It is interesting to note, nevertheless, that, in agreement with the *in vitro* data, intra-cerebral administration of adenovirus recombinant for CNTF produced an increase in the astroglial content of endogenous CNTF without triggering massive "astrogliotic" morphological changes [32].

Intracellular concentration of CNTF as a modulator of P-gp expression in astrocytes

Out of the present study, the relationship between CNTF and P-gp in astrocytes appears as a functional link rather than as a mere co-regulation. Indeed, one main result obtained in this study was the apparent requirement of an intracellular level of CNTF for the expression of P-gp to be modulated by IL-6-type cytokines, since this modulation was not seen in cells that did not express the CNTF gene. The only exogenous cytokine that remained effective in CNTF-/- cells was CNTF itself. This may be explained by the fact that exogenous CNTF, when bound to its receptor, is internalised into the cells [33]. As discussed by these authors, endocytosed CNTF may be active inside the cell, as has been demonstrated for neurotrophins/Trks complexes (see 34 for a review). Through this mechanism, therefore, restoration of an intracellular content of CNTF may become sufficient to trigger a increased P-gp expression.

How an increase in the cellular content of CNTF increases the expression of P-gp was not directly addressed in this study. Nevertheless, a number of elements help to suggest a working hypothesis. A direct transcriptional role of CNTF on the P-gp gene promoter is unlikely for three reasons. First, it has been well demonstrated that the intracellular signalling systems triggered by CNTF are essentially similar to those triggered by LIF [35,36]. Why would LIF not be effective in CNTF-/- astrocytes when CNTF is,

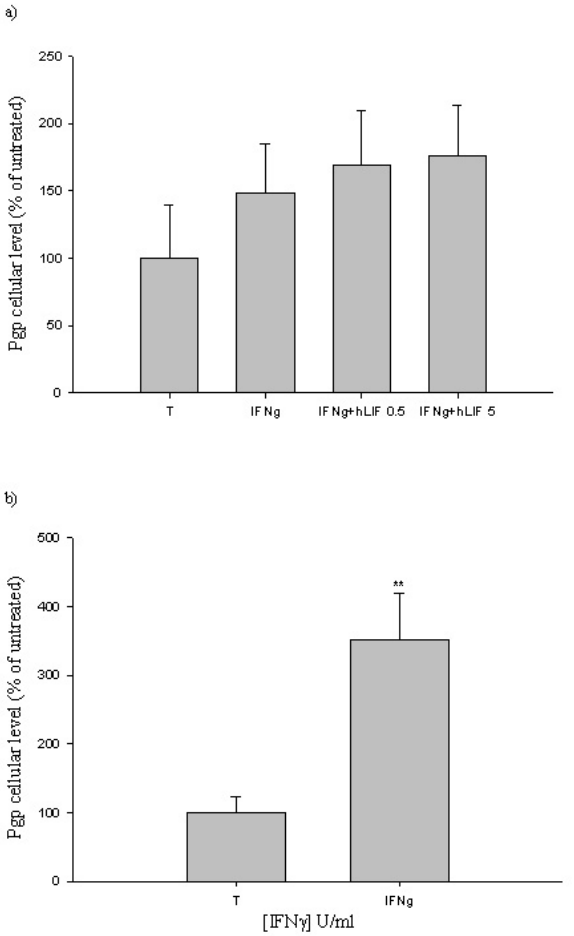

Figure 6
Evidence of another P-gp-CNTF-independent-regulation pathway. a) Addition of IFNγ at 300 U/ml in the culture medium induces an increase of the P-gp cellular level (+48.42% ± 30). This increase remains unchanged by the addition of the hLIF05, partial LIF receptor inhibitor at two concentrations (+69% ± 35 at 0.5 μg/ml and +76% ± 30 at 5 μg/ml). b) The same effect is observed in CNTF-deficient astrocyte culture. Addition of IFNγ at the same concentration induces a major significant increase of P-gp cellular level (+252% ± 68, factorial ANOVA significant at 95%, t-test $p < 0.01^{**}$).

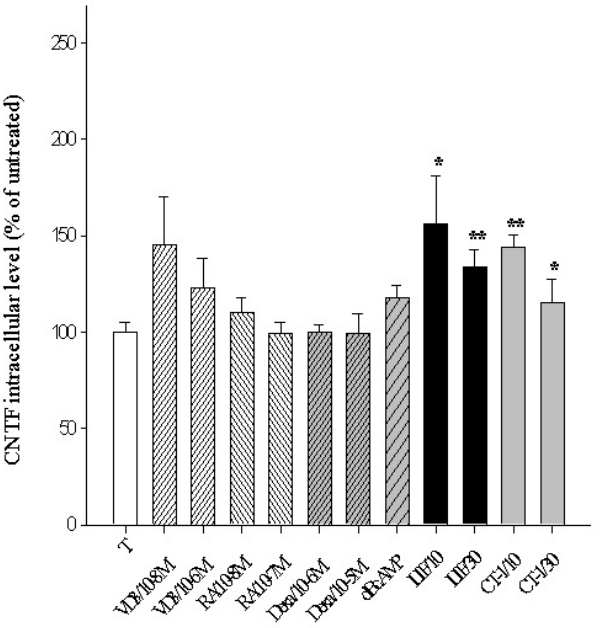

Figure 7
Effect of cytokines on CNTF-intracellular content astrocytes. Among all the molecules tested, only LIF and CT-1 induced a CNTF-modulation in long term astrocyte culture. Addition of rhLIF during 24 h elicited a significant increase of P-gp expression both at 10 and 30 ng/ml (+56.12 % ± 25, factorial ANOVA significant at 95%, t-test $p < 0.05^{*}$ and +33.8 % ± 8, factorial ANOVA significant at 95%, t-test $p < 0.01^{**}$ respectively). The same pattern of regulation was observed with CT-1 (+44.5 % ± 12, factorial ANOVA significant at 95%, t-test $p < 0.01^{**}$ and +15.1 % ± 5, factorial ANOVA significant at 95%, t-test $p < 0.05^{*}$).

would be difficult to explain in case of a direct transcriptional effect. Second, our results demonstrate that the control of P-gp expression requires a modulation of the intracellular content of the cytokine. Third, the stimulation of P-gp expression by cytokines was blocked with antagonists of the activity of the transporter. This indicates that the regulation of P-gp depends upon its own activity and suggests the existence in astrocytes of a positive feedback of the molecule upon its own expression. Such a suggestion is concordant with the well demonstrated positive feedback of P-gp activity upon its expression in various tumor cells, in the presence of non-organic substrates like cytotoxic drugs [37,38].

In a mechanistic model based upon this hypothesis, endogenous astroglial CNTF would most likely not appear as a usual cytokine activating signalling pathways after binding to a transmembrane receptor system, but as a "sensor", itself modulated by extrinsic cues. The changes induced in the intracellular concentration of this "sensor" might affect the activity of the P-gp transmembrane transporter and, eventually, lead to a modulation of its gene expression (Fig. 8). Such a scheme suggests a physiological role for CNTF, a cytokine which, because of the absence of a signal peptide and the lack of a quantifiable release in the culture medium of cells that synthesise it [32,39,40], was not thought to play a physiological role in the absence of a lesion [41]. Whether P-gp is the only target of the effects of CNTF, or just the first one identified is obviously an open question at this point.

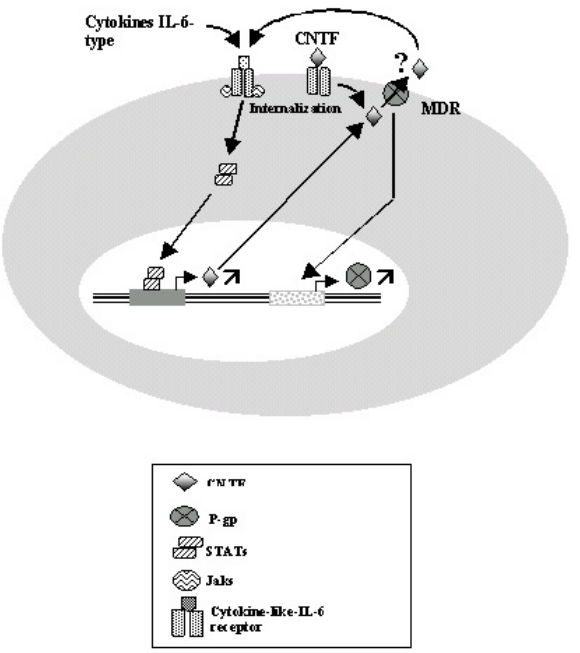

Figure 8
Tentative mechanistic model of P-gp regulation by CNTF based upon the results of the present study.

One directly related issue is the role of P-gp in astrocytes. Taking into account the functions of P-gp in other cells, in the protection against potentially harmful chemicals and metabolites, it is plausible that the transporter plays an important role in the response against cell stress [42], as suggested by its increase in reactive astrocytes. In addition, one may consider another role that has been demonstrated for P-gp and other transporters of the mdr family, namely their ability to transport various organic molecules through cell membranes, including various cytokines. Indeed, P-gp has been shown to transport interleukin-2 (IL-2) and interleukin-4 (IL-4) through cell membranes [9,21,43]. Whether, among other substrates, P-gp can similarly transport CNTF out of astrocytes is a tempting hypothesis that will require further studies.

Conclusions

In this study, we have demonstrated a close interaction between two proteins, CNTF and P-gp. These results suggest another role for the CNTF in which it could not be necessary for it to be secreted. Indeed, we have shown that an intrinsic astrocyte P-gp-regulation pathway, in which CNTF has a predominant role, can trigger biochemical changes in astrocytes. This pathway is directly related to the modification of the CNTF-intracellular concentration. Whether P-gp is the only target of CNTF, or just the first one identified and whether, among other substrates, P-gp can similarly transport CNTF out of astrocytes remains to be seen.

Methods

Astroglial cultures were prepared from cerebral hemispheres of neonatal Swiss mice (Iffa Credo, France) or CNTF knockout mice (BRL, Switzerland). Cultures were grown at confluence, for 14 days, thus defining "mature" cultures as previously described [25].

Recombinant rat CNTF (rrCNTF, Boehringer Mannheim, Germany), recombinant human Leukemia Inhibitory Factor and human Cardiotrophin-1 (rhLIF and rhCT-1, Dr Gascan, Angers), Tumor Necrosis Factor alpha (TNFα), Nerve Growth Factor (NGF, Promega, France), Retinoic Acid (RA, Sigma Aldrich, France), dibutyryl cyclic AMP (dBcAMP, Sigma Aldrich), bacterial lipopolysaccharides (LPS, Sigma Aldrich), Transforming Growth Factor alpha (rhTGFα, Promega, France), recombinant human Interleukin-1β (rhIL-1β, Sigma), Interferon-β (IFN-β, Promega, France), recombinant human Interferon-γ (rhIFN-γ, R&D systems, United Kingdom), recombinant mouse Interleukin-6 (rmIL-6, R&D systems, United Kingdom), recombinant human soluble Il-6 receptor (R&D systems) were used in these experiments.

Culture conditions

Astroglial cultures were prepared as previously described [44]. After 14 days in culture, the astrocytes had formed a confluent monolayer. Serum containing medium (Minimal Essential Medium containing 2 mM Glutamin, Essential Amino acids, 0.03% glucose, Penicillin-Streptomycin, foetal calf serum -FCS- 10%) was removed and serum free medium added for 24 h. To investigate the effects of various factors on P-gp expression, the following compounds were added for 24, 48 or 72 h: rrCNTF (30, 100, 250 ng/ml); rhLIF (10, 30 ng/ml); rhCT-1 (10 ng/ml), TNFα (10, 100 ng/ml), NGF (10 ng/ml), retinoic acid (10^{-7} M, 10^{-8} M), dBcAMP (0.5 mM), IFNβ (600 U/ml), LPS (1 μg/ml), TGFα (50 ng/ml), rhIL-1β (10, 50 ng/ml), rmIL-6 (10, 20, 30, 40, 50 ng/ml) with or without rhsIL-6R (100, 200, 300, 400, 500 ng/ml), rhIFN-γ (300 U/ml). In the experiments with soluble CNTFRα, 200 ng/ml of myc-sCNTFRα (kindly provided by Ralph Laufer, IRBM, Italy) were added to the medium without FCS after 14 days *in vitro*. Thirty minutes later, rrCNTF was added at 0, 10 (4.4×10^{-10} M), 30, 50 (2.2×10^{-9} M), 100 or 250 ng/ml (10^{-8} M).

Verapamil (5 μM, Sigma) and S9788 (10 pM, Servier, France) were used as blockers of P-gp. They were added in the culture medium 12 h before rrCNTF, rhCT-1 and rhLIF, used at the concentrations indicated above.

Since our preliminary results suggested that ligands of the LIF-receptor (LIFR) may modulate P-gp, a specific inhibi-

tor of this receptor (hLIF05) [45] was added to the culture medium in some experiments (0.5 and 5 μg/ml), 30 min before addition of potential stimulating agents. MES-SA/MX2 cells (ATCC, Biovalley, France) were used as a control. This cell line is a mitoxanthrone-resistant derivative of the human uterine sarcoma cell line MES-SA, that displays features of overexpression of the two classical multidrug resistance P-gps. The cells were cultured in a medium containing 1:1 mixture of Waymouth's MB 752/1 medium and McCoy's 5 a medium, 90%, foetal bovine serum, 10%.

In all cases, the medium was removed at the end of the experiments and each dish was rinsed three times with HBSS (Hank's Balanced Salt Solution, Seromed, Germany). The cells were collected by scraping into 62.5 mM Tris HCl (pH 6.8), 2 mM EDTA, 2 mM phenylmethylsulfonyl fluoride, 0.5 % Triton X-100 and 2.3 % sodium dodecyl sulfate.

Biochemical analysis

Total protein content was determined by the BCA protein assay kit (Pierce, Illinois, USA) with bovine serum albumin as a standard. The proteins were analysed by Western blotting. Briefly, samples were boiled for 5 min after addition of 10 % glycerol, 5 % mercaptoethanol (or 5 % Dithiotreitol for the MAPK-P) and 5 % bromophenol blue, then lysates were electrophoresed on 7.5% SDS polyacrylamid gels. Gels were blotted on nitrocellulose, blocked for one hour in 5 % non fat dry milk in TBS-T (20 mM Tris, pH 7.5/ 500 mM NaCl/0.1% Tween 20) and then probed overnight at 4°C, first with a polyclonal anti-P-glycoprotein antibody (mdr, Ab-1, Immunotech, France, 1/200), then with a monoclonal anti-α tubulin antibody (Sigma-Aldrich, France, 1/5000). After washing with TBS-T, membranes were incubated with a horseradish peroxidase-conjugated donkey anti-rabbit secondary antibody followed by the enhanced chemiluminescent reaction (Amersham, Sweden), according to the manufacturer's instructions. The levels of P-gp were measured by densitometry and normalised to the total protein loaded in each lane.

To limit variations in their processing extracts from the control and all experimental conditions were treated in parallel, on a single sheet, for each specific experiment. In addition, to evaluate the variability between specific experiments, control extracts were subsequently reloaded together on a single nitrocellulose membrane and processed together. Statistical analysis used one-factor ANOVA and unpaired t-test.

PCR experiment

For RT-PCR, RNA isolation was performed with the Trizol method (Life technologies, Cergy-Pontoise, France). PCR was carried out with cDNA derived from 2 μg of RNA, 2.5 unit of AmpliTaq Polymerase and reaction kits (Superscript preamplification systems, Gibco BRL, France) in a final volume of 50 μl. Each cycle of PCR included 30 sec of denaturation at 94°C, 1 min of primer annealing at 55°C, and 2 min of extension/synthesis at 72°C and one cycle of 72°C for 10 min. MDR1-specific sequences were amplified by using the sense-strand primer CCCATCATTGCAATAGCAGG (residues 2596–2615) and the antisense-strand primer GTTCAAACTTCTGCTCCTGA (residues 2733–2752) [46], which yield a 167-bp product. Each primer was added at 10 μM per reaction.

Authors' contributions

CM participated in the conception of this work, carried out the molecular and biological experiments and drafted the manuscript. CF carried out the study with the interferon gamma. AMF, VD supplied the probes and carried out the PCR. CG participated in the IL6 cytokines study. AV carried out hLIF preparation. HG participated to the design of the study. MP conceived the study, participated to its co-ordination and writing.

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