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ORIGINAL PAPER

The Pro-inflammatory Cytokine TNF-α Regulates the Activity and Expression of the Serotonin Transporter (SERT) in Astrocytes

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Abstract Pro-inflammatory cytokines have been implicated in the precipitation of depression and related disorders, and the antidepressant sensitive serotonin transporter (SERT) may be a major target for immune regulation in these disorders. Here, we focus on astrocytes, a major class of immune competent cells in the brain, to examine the effects of pro-longed treatment with tumor necrosis factor-alpha (TNF-α) on SERT activity. We first established that high-affinity serotonin uptake into C6 glioma cells occurs through a SERT-dependent mechanism. Functional SERT expression is also confirmed for primary astrocytes. In both cell types, exposure to TNF-α resulted in a dose- and time-dependent increase in SERT-mediated 5-HT uptake, which was sustained for at least 48 h post-stimulation. Further analysis in primary astrocytes revealed that TNF-α enhanced the transport capacity (V_{max}) of SERT-specific 5-HT uptake, suggesting enhanced transporter expression, consistent with our observation of an increase in SERT mRNA levels. We confirmed that in both, primary astrocytes and C6 glioma cells, treatment with TNF-α activates the p38 mitogen-activated protein kinase (MAPK) signaling pathway. Pre-treatment with the p38 MAPK inhibitor SB203580 attenuated the TNF-α mediated stimulation of 5-HT transport in both, C6 glioma and primary astrocytes. In summary, we show that SERT gene expression and activity in astrocytes is subject to regulation by TNF-α, an effect that is at least in part dependent on p38 MAPK activation.

Keywords 5-HT · Serotonin transporter · TNF-α · Pro-inflammatory cytokines · Astrocytes · Depression

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Introduction

High-affinity re-uptake of the neurotransmitter serotonin (5-hydroxytryptamine, 5-HT) is mediated by the serotonin transporter (SERT), exerting precise spatial and temporal control over the physiological actions of this neurotransmitter. Consistent with the importance of the physiological processes governed by SERT, this protein is subjected to a network of regulatory influences. The regulation of SERT gene expression by numerous stimuli has been extensively studied in particular in a number of model cell lines of both central and peripheral origin, i.e. SERT gene expression appears to be subject to regulation by a variety of stimuli, including cAMP [1–5], BDNF [3], steroid hormones [6–8], and growth factors [9, 10]. In addition, correlations have been established between the expression and activity of SERT and a number of psychiatric disorders such as obsessive compulsive disorder (OCD), autism and major depressive disorder (MDD) [11].

Under physiological conditions there is intimate and reciprocal communication between the nervous, immune and endocrine systems and alterations in this equilibrium are known to be implicated in the onset of psychiatric illness, including major depression (for recent reviews see for example Ref. [12, 13]). Elevated levels of pro-inflammatory cytokines IL-1β, TNF-α and IL-6 has been reported in patients suffering from neuropsychiatric disorders [12, 14–16]. In addition, immune activation in human disease as

well as in animal models is often accompanied by a discrete set of psychiatric and behavioral alterations which bear many of the hallmarks of depressive illness [17, 18]. TNF-α is of particular interest as it is a pro-inflammatory cytokine that has been shown to be intimately associated with the development of MDD and is one of the few cytokines where decreases in its serum concentration has been shown to correlate with improvement of clinical symptoms of the disease [19].

As the serotonin transporter has been intimately linked to a variety of mood and behavioral disorders, not least as the primary target for the most widely prescribed antidepressant drugs, i.e. the selective serotonin reuptake inhibitors (SSRI's) such as fluoxetine, paroxetine and citalopram, SERT represents a potential target for cytokine-induced regulatory mechanisms contributing to the pathophysiological processes underlying depressive illnesses. Indeed, pro-inflammatory cytokines have been implicated in the regulation of serotonergic homeostasis in vivo through the modulation of central serotonin activity and metabolism [20, 21]. Studies assessing cytokine-mediated modulation of SERT expression and/or activity have predominately been conducted in SERT expressing cell lines of peripheral origin [4, 22–26]. Comparatively little is known about the mechanisms by which cytokines and other pro-inflammatory molecules may exert a functional influence on the transporter in cells of the central nervous system i.e. neurons or glia. Zhu et al. [27, 28] recently examined the acute regulation of SERT by the pro-inflammatory cytokines TNF-α and IL-1β in vitro and in vivo following peripheral immune challenge by lipopolysaccharide. However, given the chronic nature of depressive illness and related disorders as well as the fact that chronic inflammatory disorders are characterized by prolonged elevation of pro-inflammatory molecules, cytokine-dependent effects on mood and behavior potentially include gene expression alterations, in addition to their acute effects on SERT activity.

In the central nervous system, the serotonin transporter has traditionally been perceived to be expressed predominantly in neuronal cells. As a consequence, the potential physiological role of SERT expressed in non-neuronal cell populations has been largely overlooked. However, in recent years, several reports have been published providing compelling evidence for the expression of functionally active SERT in glial cells lines as well as in primary astrocytes [10, 29–31]. High-affinity uptake of 5-HT by astrocytes is sensitive to inhibition by antidepressants such as imipramine and SSRIs, with a high degree of correlation noted between the *Ki* values for inhibition of 5-HT uptake by antidepressants in astrocytes and neurons, thus suggesting that the pharmacologically indistinguishable, high-affinity 5-HT uptake is mediated by SERT in both, glial and neuronal cells [30, 32, 33]. Furthermore, in recent years glial-derived SERT has been successfully visualized in rat brain tissue [34, 35].

In this study, we provide evidence that glial cells may contribute to the effects of the pro-inflammatory cytokine on serotonin homeostasis. Following an initial thorough characterization of SERT-mediated high-affinity 5-HT uptake in C6 glioma cells and primary astrocytes, we show that pro-longed treatment with pro-inflammatory cytokine TNF-α substantially enhances SERT gene expression and activity in these cells.

Materials and Methods

Reagents

TNF-α was purchased from R&D systems, Europe (Oxon, UK). SB203580 was obtained from Calbiochem (Darmstadt, Germany). Anti-total and anti-phospho specific antibodies for signaling intermediates were purchased from Cell Signaling Technology (La Jolla, CA). [^{3}H]5-HT (5-hydroxytryptamine creatine sulphate, 28.1 Ci/mmol) was obtained from Perkin Elmer Life Sciences (Boston, MA). All chemicals unless otherwise stated were obtained from Sigma (Ayrshire, UK).

Cell Culture and Primary Astrocyte Preparation

C6 glioma cells were maintained at 37 °C in a humidified atmosphere of 5 % CO_2-95 % air in DMEM containing 10 % FBS, 2 mM L-glutamine, 100 units/ml penicillin and 100 μg/ml streptomycin. Primary astrocytes were prepared as follows. P0 Wistar rats were humanely killed by cervical dislocation, the cerebral cortices removed and separated from the meninges. The cortical tissue was dissociated by mechanical digestion and the cell suspension centrifuged at 1,000*g* for 5 min. Cell pellets were resuspended and cultured in pre-equilibrated flasks in DMEM containing 10 % FBS, 2 mM L-glutamine, 100 units/ml penicillin and 100 μg/ml streptomycin. Cells were cultured for 10 days at 37 °C in a humidified 5 % CO_2-95 % air atmosphere. Five to 7 days prior to experiments cells were trypsinised and re-plated onto 6 and 24 well dishes. The cellular composition of the astrocytic cultures was evaluated by immunofluoresence studies with the astrocytic marker glial fibrillary acidic protein (GFAP). Immunocytochemistry showed that approximately 95 % of the cells in these cultures stained positively for GFAP.

5-HT Transport Assay

[^{3}H]5-HT transport activity was assayed through minor modification of a technique described previously [36].

Briefly, C6 glioma cells were plated 48 h, while the primary astrocytes were seeded down 5–7 days prior to commencing the 5-HT transport assay. At the time of assay, the medium was removed by aspiration and cells washed with transport buffer (TB) containing 10 mM HEPES, pH 7.5, 150 mM NaCl, 2 mM KCl, 1 mM $CaCl_2$, 1 mM $MgCl_2$, 1.8 g/l glucose, 100 μM pargyline and 100 μM ascorbic acid. The TB was removed and cells were incubated in triplicate in TB at 37 °C for a further 10 min. In order to eliminate any non-specific, low affinity uptake of [^{3}H]5-HT by the norepinephrine transporter (NET), the NET-specific inhibitor nisoxetine (100 nM final concentration) was included in the TB. The 5-HT transport assay was initiated by the addition of [^{3}H]5-HT (100 nM) and terminated 10 min later by the aspiration of the reaction buffer followed by three rapid washes in ice-cold TB containing 1 μM paroxetine. Cells were then solubilized in 2 % SDS and [^{3}H]5-HT accumulation was evaluated by scintillation counting. Specific 5-HT uptake was calculated by determining the amount of [^{3}H]5-HT accumulated in the presence or absence of 1 μM escitalopram or paroxetine. Saturation kinetics analysis of 5-HT transport was carried out using increasing concentrations of 5-HT ranging from 0 to 4 μM. IC_{50} values for inhibition of 5-HT uptake by various uptake inhibitors were carried out by adding solutions of [^{3}H]5-HT (final concentration 50nM) containing a suitable concentrations range for each the respective uptake inhibitor. To evaluate the effect of p38 MAPK inhibition by SB203580 (SB) on SERT activity, primary astrocytes and C6 glioma cells were treated with the specific p38 MAPK inhibitor SB203580 (10 μM) or vehicle alone (DMSO, 1 % v/v final concentration) for 20 min followed by incubation for an additional 12 h in the presence or absence of TNF-α (100 ng/ml) or with TNF-α alone. Cells were then subjected to 5-HT transport assay under conditions described above. Data analysis was carried out using the SigmaPlot 8.0 software and kinetic parameters were determined by non-linear regression analysis.

Analysis of Signaling Kinase Activation

To monitor the activation of a number of previously characterized signaling pathways in response to TNF-α treatment, primary astrocytes and C6 glioma cells were cultured in 6 well plates and treated for the indicated times with TNF-α (100 ng/ml). Cells were lysed with RIPA buffer (PBS containing 0.1 % SDS, 1 % Triton X-100, 0.1 % NP-40, 1X protease inhibitors (Roche), 50 mM sodium orthovanadate, 50 mM sodium fluoride, 10 mM beta-glycerophosphate) followed by centrifugation at 13,000 *g* for 20 min at 4 °C, supernatants were mixed with 5 × Laemmli buffer (0.3 M Tris, pH 6.8, 10 % (w/v) SDS, 0.1 % (w/v) bromophenol blue, 50 % (v/v) glycerol and 100 mM DL-dithiothreitol) and analysed by Western blotting. Samples were subjected to SDS-PAGE followed by transfer onto nitrocellulose membrane (Whatman). Blotted membranes were subsequently blocked in 5 % (w/v) non-fat dry milk or 3 % (w/v) BSA in TBST (137 mM NaCl, 20 mM Tris pH 8.3, 0.5 % (v/v) Tween-20). Blots were then incubated with anti-total and anti-phospho specific antibodies (1:1,000, Cell Signaling Technology) in either 3 % (w/v) BSA or 5 % (w/v) non-fat dry milk overnight at 4 °C followed by incubation with either HRP-conjugated anti-rabbit or anti-mouse secondary antibodies (1:5,000, Cell Signaling Technology). Immunoreactive bands were visualized following incubation of immunoblots with ECL solution (Pierce).

RNA Isolation and Quantitative Real-Time PCR

Total RNA was isolated from cells using TRI Reagent® (Sigma) following manufacturer's instructions. RNA was quantitated using the Nanodrop ND-1000 spectrophotometer system (Labtech). Double stranded cDNA was synthesized from total RNA (2 μg) following DNAse I treatment using oligo dT primers and M-MLV Reverse Transcriptase (Promega). The expression of the SERT gene was examined using quantitative real-time reverse transcription PCR using gene specific primers (Forward 5′ GGCCTGGAAGGTGTGATCA 3′ and Reverse 5′GCGCT TGGCCCAGATGT 3′) and SYBR Green detection chemistry. The real-time PCR reaction was carried out in a Prism 7700HT Sequence Detection System (Applied Biosystems, Foster City, CA, USA). Each reaction was performed in duplicate and the fluorescent data was converted into cycle threshold (C_T) measurements and the $\Delta\Delta C_T$ method was used to calculate fold expression changes. A β-actin control primer and probe set (Applied Biosystems) was used for normalization purposes.

Statistical Analysis

Experimental data was analyzed by Student's *t* test for single comparisons. In each case, the differences between means were considered significant at *p* values of less than 0.05. For all transport experiments data represented are the mean percentage of control values ± SEM obtained from 3 independent experiments as indicated in the figure legends. Total uptake data was corrected for non-specific uptake and analyzed by fitting to a Michaelis–Menten kinetics using SigmaPlot version 8.0. Data represented in Figs. 4e, f were subjected to a one-way ANOVA using a Newman-keuls multiple comparison test.

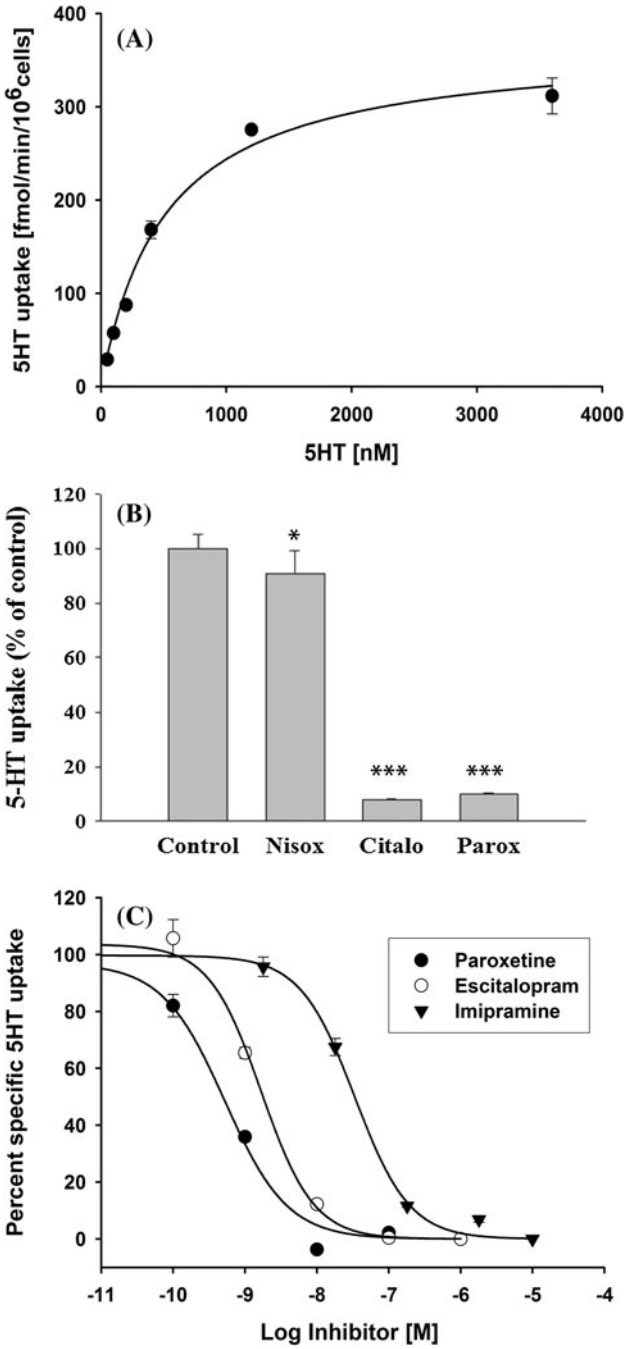

Fig. 1 C6 glioma cells exhibit high-affinity, SERT-mediated 5-HT uptake. **a** Saturation kinetics of 5-HT uptake into C6 cells was carried out as described in "Materials and Methods". Kinetic parameters were determined by non-linear regressing analysis using the Michaelis-Menton equation, values are: $V_{max} = 368 \pm 19$ fmol/min/10^6 cells, $K_m = 510 \pm 76$ nM. The *graph* shown is representative of three independent experiments, each giving similar results. **b** Pharmacological characterization of 5-HT uptake. 5-HT uptake was carried out in the absence and presence of monoamine transport inhibitors nisoxetine (Nisox, 100 nM, specific inhibitor of norepinephrine tranporter), paroxetine (Parox, 100 nM, SERT-selective) and citalopram (Citalo, 100 nM, highly SERT-specific). Values are expressed as the mean percentage of control ± SEM of triplicate values and are representative of 3–5 independent experiments. *Asterix* indicate a statistically significant change in 5-HT uptake as compared to control, $*p < 0.05$, $***p < 0.001$ (Student's *t* test). **c** IC_{50} values for inhibition of 5-HT uptake were determined as described in "Materials and Methods". Background was defined as uptake in presence of 1 μM paroxetine and subtracted from raw data. The *graph* shown is a representative of 2–3 independent experiments. Mean IC_{50} values obtained were paroxetine, 0.66 ± 0.14 nM; escitalopram, 1.2 ± 0.35 nM; imipramine, 35.4 ± 1.6 nM

Results

High-Affinity 5-HT Uptake in C6 Glioma Cells is Mediated by SERT

SERT-specific 5-HT uptake has been detected in primary astrocytes and various glial cells lines [10, 29, 30], but to our knowledge has not been reported for C6 glioma cells. Thus, we utilized saturation kinetics and various monoamine transporter inhibitors to characterize the high-affinity 5-HT uptake components in these cells. Serotonin uptake was carried out as described in Materials and Methods; preliminary experiments showed that 5-HT uptake in these cells was linear for up to 20 min (data not shown). Uptake specificity was determined in the presence of the highly specific SERT inhibitor escitalopram. The kinetic parameters obtained were: $V_{max} = 368 \pm 19$ fmol/min/10^6 cells, $K_m = 510 \pm 76$ nM (Fig. 1a), indicating high-affinity serotonin uptake in these cells. Furthermore, using 100 nM 5-HT pre-treatment with the NET inhibitor nisoxetine resulted in only a marginal reduction of 5-HT uptake to 91 ± 8 % of control, suggesting that at low serotonin concentration non-specific uptake of 5-HT by NET accounted for no more than approximately 10 % of total uptake in C6 glioma cells. In contrast, citalopram and paroxetine, highly selective SERT inhibitors almost completely abolished 5-HT uptake in these cells at an inhibitor concentration (100 nM), which effectively blocks SERT, but does not significantly affect any other monoamine uptake systems (Fig. 1b). Using 50nM [^{3}H]5-HT, we obtained average IC_{50} values for three well characterized SERT inhibitors: paroxetine, 0.66 ± 0.14 nM; escitalopram, 1.2 ± 0.35 nM; imipramine, 35.4 ± 1.6 nM. These values as well as the order of potency of these inhibitors, i.e. paroxetine > escitalopram > imipramine, is highly consistent with previously reported findings for SERT-mediated 5-HT uptake [37, 38]. Thus, we conclude that high-affinity 5-HT uptake into C6 glioma cells is largely SERT-dependent. Although, NET-mediated uptake of 5-HT accounts for only a minor portion of the 5-HT uptake C6 glioma cells, NET-mediated 5-HT uptake was inhibited in all subsequent 5-HT transport assays by adding nisoxetine to the uptake buffer at 100 nM, a concentration that does not significantly affect SERT-specific 5-HT uptake.

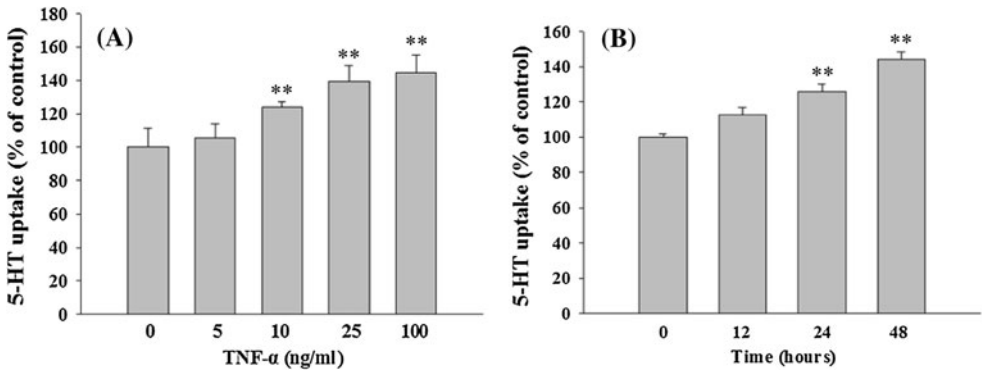

Fig. 2 TNF-α treatment results in a time and dose-dependent increase in SERT activity in C6 glioma cells. **a** Dose dependence: C6 glioma cells were treated with the indicated concentrations of TNF-α for 24 h prior to 5-HT transport assay. **b** Time course: C6 glioma cells were treated with TNF-α (100 ng/ml) for the times indicated and alteration in SERT activity was monitored by 5-HT transport assay. Values in **a** and **b** are expressed as the mean percentage of control ± SEM of triplicate values from three independent experiments. *Asterix* indicate a statistically significant change in 5-HT uptake as compared to control, $^{**}p < 0.01$ (Student's *t* test)

TNF-α Stimulates SERT Activity in C6 Glioma Cells

Having established SERT-specific 5-HT uptake in C6 cells, we used this cell culture model to investigate the regulatory effects of pro-inflammatory cytokines in isolated glial cells, focusing in this study on TNF-α. As shown in Fig. 2a, b, TNF-α treatment results in a dose- and time-dependent increase in SERT-mediated 5-HT uptake in C6 glioma cells. TNF-α mediated stimulation of 5-HT transport in C6 glioma reached statistical significance upon treatment with 10 ng/ml of TNF-α, however, this increased further upon treatment with up to 100 ng/ml TNF-α (144.5 ± 10.1 % of control, Fig. 2a). Using 100 ng/ml TNF-α stimulation of 5-HT transport was found to reach statistical significance at 24 h post-stimulation and was sustained thereafter with a further increase in SERT-mediated 5-HT uptake at 48 h post-stimulation (144.3 ± 4.8 % compared to control, Fig. 2b).

TNF-α Stimulates SERT Gene Expression and Activity in Primary Astrocytes

In order to rule out that the TNF-α mediated stimulation of SERT activity was confined to C6 glioma cells and thus, potentially an artifact of the immortalization of this cell line, we investigated whether this effect could also be observed in primary astrocytes. In primary astrocytes, nisoxetine-sensitive NET mediated non-specific uptake of 5-HT accounted for no more than 20 % of total uptake of 5-HT (82 ± 2 % of control). SSRI antidepressants paroxetine and citalopram reduced 5-HT uptake to 34 ± 1.8 and 42 ± 2.6 % of control, respectively (Fig. 3a). Thus, under the conditions used here approximately 60 % of total 5-HT uptake into primary astrocytes is SERT-mediated. The remaining approximately 20 % 5-HT uptake may be mediated by low-affinity uptake systems, such as the extraneuronal monoamine transporter, also known as the organic cation transporter 3, which was found to be expressed in human astrocytes [39].

As in C6 glioma cells, treatment with TNF-α enhances SERT-specific 5-HT uptake in these cells in a concentration and time-dependent manner. A significant increase of 5-HT uptake was observed at 10 ng/ml TNF-α, saturation was reached at approximately 25 ng/ml, with only a small additional increase seen at 100 ng/ml, resulting in a maximal enhancement of SERT-mediated 5-HT uptake to 178.2 ± 18.0 % of control values (Fig. 3b). Furthermore, TNF-α treatment (100 ng/ml) resulted in a significant stimulation of SERT activity in these cells at 24 h, while the most pronounced increase was observed at 48 h (258.0 ± 19.1 % compared to control, Fig. 3c). We noted that even at 12 h TNF-α induced an increase in SERT activity, although this was not found to be statistically significant, most likely due to a high degree of variability of basal SERT activity in control cells. However, as shown below (Fig. 4), strict control of experimental conditions, such as the number of days in vitro (DIV) and the use of the same batch of serum in the cell culture medium, results in a reduced variability in basal SERT activity levels and consequently a clear, statistically significant difference between control and TNF-α treated cells at 12 h. Similar effects of TNF-α were observed in both, C6 cells and primary astrocytes, however, it is notable that primary astrocytes are more sensitive to TNFα, i.e. the magnitude of the increase in 5HT uptake activity was substantially greater when compared to C6 glioma cells (258 vs. 144 % of control, 100 ng/ml TNF-α, 48 h).

Kinetic analysis of SERT activity in primary astrocytes revealed that TNF-α treatment resulted in a significant increase in the maximal transport capacity V_{max} (83.3 ± 4.0 vs. 153.4 ± 7.8 fmol/min/well) without significant alteration of the K_m value (203.0 ± 37.6 vs. 149.5 ± 29.3 nM) (Fig. 3d).

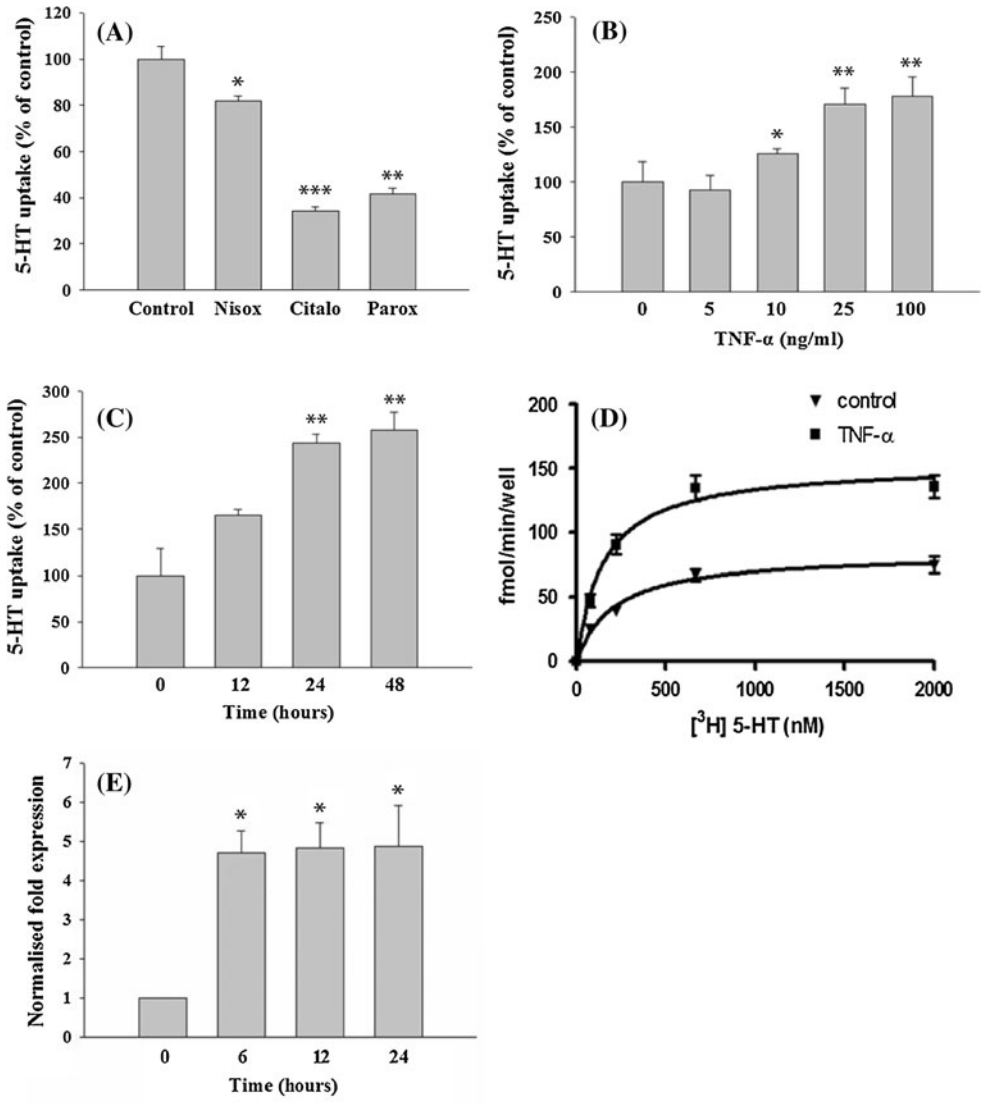

Fig. 3 TNF-α treatment results in a time and dose-dependent stimulation of SERT gene expression and activity in primary astrocytes. **a** Pharmacological characterization of 5-HT uptake in primary astrocytes. 5-HT uptake was carried out in the absence and presence of monoamine transport inhibitors nisoxetine (Nisox, 100 nM), paroxetine (Parox, 100 nM) and citalopram (Citalo, 100 nM). Values are expressed as the mean percentage of control ± SEM of triplicate values and are representative of three independent experiments. *Asterix* indicate a statistically significant change in 5-HT uptake as compared to control, $*p < 0.05$, $**p < 0.01$, $***p < 0.001$ (Student's *t* test). **b** Dose dependence and **c** time course of TNF-α treatment: Primary astrocytes were treated with the indicated concentrations of TNF-α for 24 h (**b**) or with 100 ng/ml TNF-α for the indicated times (**c**) prior to 5-HT transport assay. Values in **b** and **c** are expressed as the mean percentage of control ± SEM of triplicate values from three independent experiments. *Asterix* indicate a statistically significant change in 5-HT uptake as compared to control, $*p < 0.05$, $**p < 0.01$ (Student's *t* test). **d** Analysis of transport kinetics upon TNF-α mediated stimulation of SERT activity in primary astrocytes. Cells were treated with TNF-α (100 ng/ml) for 24 h prior to transport assay carried out with increasing concentrations of 5-HT as indicated. The graph shown is representative of three independent experiments; values were determined in triplicate and plotted as mean ± SEM. **e** Quantitative analysis of alterations in SERT gene expression following treatment of primary astrocytes with TNF-α. Cells were treated with TNF-α (100 ng/ml) for the indicated times and SERT mRNA levels were monitored by quantitative real-time PCR. Values are expressed as the mean fold induction ± SEM from three independent experiments, $*p < 0.05$ (Student's *t* test)

Since the predominant effect on the V_{max} value for 5-HT uptake could be the result of enhances SERT expression, we monitored SERT gene expression in these cells by quantitative real-time PCR upon treatment with TNF-α (100 ng/ml). As seen in Fig. 3e, TNF-α treatment resulted in a significant, approximately 4.5 fold increase in SERT mRNA as early as 6 h post stimulation, with a sustained increase in SERT gene expression observed at 12 and 24 h. Thus, the TNF-α mediated stimulation of SERT activity in primary astrocytes is likely due to an increase in SERT gene expression.

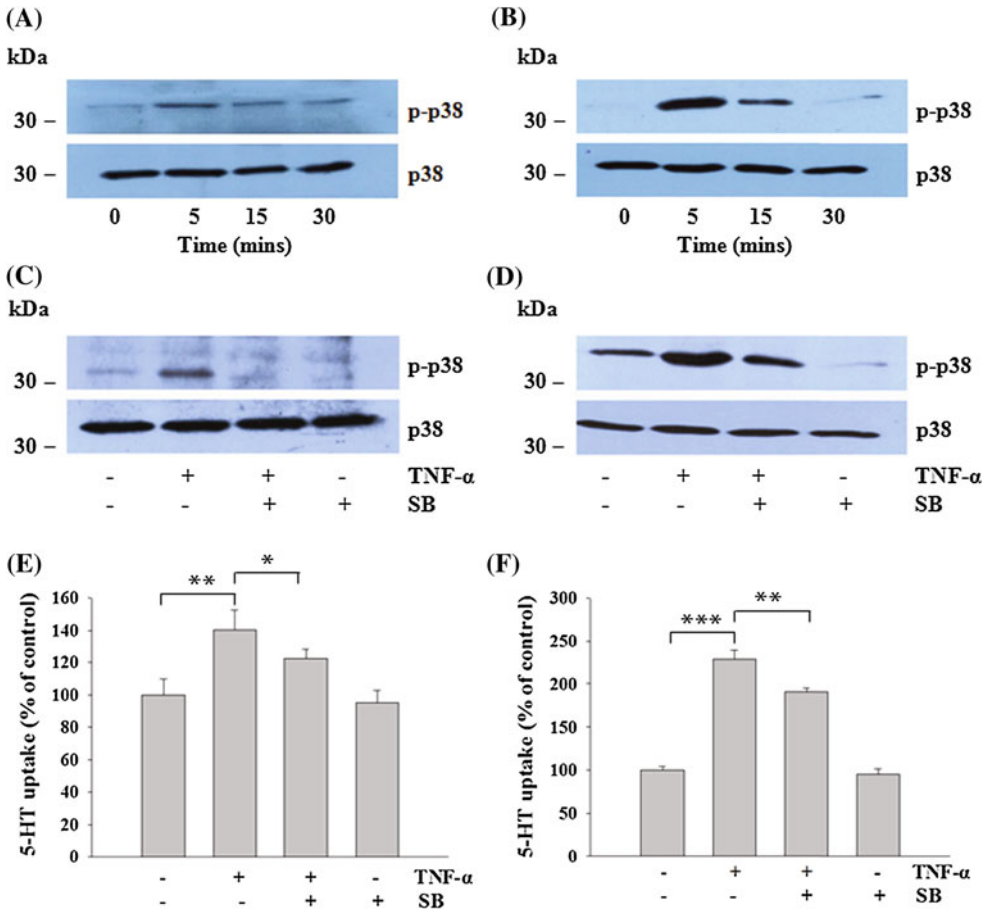

Fig. 4 Effect of the inhibition of p38 MAPK by SB203580 on the TNF-α mediated stimulation of SERT activity in primary astrocytes and C6 glioma cells. **a**, **b** TNF-α treatment activates the p38 MAPK pathway in C6 glioma cells (**a**) and primary astrocytes (**b**). Cells were treated for the indicated times with TNF-α (100 ng/ml), cell lysates were prepared and subjected to Western blotting, n = 2–3. **c, d** TNF-α triggers p38 MAPK phosphorylation in C6 glioma (**c**) and primary astrocytes (**d**) in an SB203580 (SB) sensitive manner. Cells were pre-treated with SB203580 (10 μM) or vehicle (DMSO) for 20 min prior to treatment with TNF-α (100 ng/ml) for 5 min. The ability of SB203580 to inhibit the activation of p38 MAPK was evaluated by Western blot analysis of cell lysates using a phospho-specific p38 antibody, n = 2. **e**, **f** Effect of p38 MAPK inhibition on the TNF-α-mediated stimulation of SERT activity in C6 glioma cells (**e**) and primary astrocytes (**f**). Cells were pre-treated with SB203580 (10 μM) for 20 min followed by stimulation with TNF-α (100 ng/ml) for 12 h. Alteration in SERT activity was evaluated by 5-HT transport assay. Values in **e** and **f** are expressed as the mean percentage of control ± SEM of triplicate values from three independent experiments. Changes in 5-HT uptake were compared to the corresponding control value, i.e. TNF-α versus control, TNF-α versus TNF-α + SB; $***p < 0.001$, $**p < 0.01$ or $*p < 0.05$ (one-way ANOVA using the Newman-Keuls multiple comparison test)

p38 MAPK Activation is Involved in the TNF-α Mediated Stimulation of SERT Activity in C6 Glioma Cells and Primary Astrocytes

Upon engagement of its cell surface receptors, TNF-α is known to stimulate a number of signaling cascades [40]. We focused our attention on MAP kinase pathways. As seen in Fig. 4a, b, p38 MAPK was transiently activated in response to TNF-α treatment in both, C6 glioma cells and primary astrocytes, as evaluated by an increase in the phospho-p38 MAPK signal. In both cell types, phospho-p38 immunoreactivity peaked at 5 min post-stimulation and decreased thereafter, consistent with previous observations in these cells types [40, 41].

We next sought to link the transient activation of p38 MAPK to the subsequential TNF-α mediated stimulation of SERT activity. Firstly, as seen in Fig. 4c, d, the activation of p38 MAPK in response to TNF-α treatment is effectively blocked by pre-treatment with SB203580 in C6 glioma cells (Fig. 4c) and primary astrocytes (Fig. 4d) respectively, as evaluated by Western blotting using total and phospho-specific p38 MAPK antibodies. C6 glioma cells and primary astrocytes were then stimulated with TNF-α in the presence or absence of SB203580 and subsequent alteration in SERT activity was monitored by 5-HT transport assay 12 h later. We chose the 12 h time point for this series of experiments, as incubation times with TNF-α in the presence of the SB203580 vehicle DMSO of 24 h or

more resulted in a reduction in cell viability (data not shown). Exposure to SB203580 alone did not affect basal 5-HT uptake in C6 glioma cells (95.4 ± 7.2 % of control), but upon pre-treatment with SB203580 the TNF-α mediated increase in 5-HT uptake was reduced (122.5 ± 5.6 % of control), compared to TNF-α treatment alone (140.1 ± 12.2 % of control, Fig. 4e). Similarly, in primary astrocytes SB203580 alone did not significantly affect basal 5-HT uptake (95.4 ± 7.2 % of control), but attenuated the TNF-α mediated stimulation of SERT activity (191.0 ± 4.1 % of control), compared to TNF-α treatment alone (228.5 ± 11.0 % of control, Fig. 4f). Thus, in both, C6 glioma cells and primary astrocytes, the TNF-α mediated stimulation of 5-HT uptake was partially blocked by inhibition of p38 MAPK. These results implicate the p38 MAPK pathway in the TNF-α mediated stimulation of SERT activity in astrocytes. However, our observation that TNF-α-induced stimulation of 5-HT uptake is only partially inhibited by the p38 inhibitor indicates that (an) additional signaling pathway(s) may work in conjunction with or by a mechanism distinct from p38 MAPK.

Discussion

In this study, we uncovered a functional link between the pro-inflammatory cytokine TNF-α and the regulation of serotonin transporter activity in glial cells. Although research so-far has primarily focused on neuronal SERT, it has been recognized that SERT is also expressed in glial cells, as outlined in detail in the Introduction. Thus, it seems plausible that glial SERT contributes significantly to the maintenance of serotonergic homeostasis and neurotransmission. Indeed, with their sensitivity to inflammatory signals and their close proximity to the synapse, astrocytes are ideally equipped to elicit such influences.

In this study, we utilized two cell culture models to isolate the glia-specific effect of pro-inflammatory cytokines, specifically TNF-α, on SERT expression. For one of our model systems, namely for primary astrocytes, evidence for functional SERT expression already exists, and we confirmed here results from earlier studies [30, 31, 33]. Although, the murine SERT promoter was found to be active in C6 glioma cells [42], to our knowledge, SERT-specific uptake had not previously been shown for our second model. However, kinetic and pharmacological parameters determined for high-affinity 5-HT uptake into C6 cells in this study, are highly consistent with values obtained for SERT-mediated serotonin uptake in other cells and tissues [37, 38].

Treatment of C6 glioma cells and primary astrocytes with TNF-α induced a significant and sustained increase in SERT-mediated 5-HT uptake in both cell types. As we show in primary astrocytes, the increase in SERT activity was due to an increase in the transport capacity (V_{max}) without a significant alteration of the apparent affinity of SERT for its substrate 5-HT (K_m). Together with our observation of increased SERT mRNA levels this suggests that the increase in the V_{max} of SERT-mediated 5-HT uptake is due to enhanced expression of the SERT gene.

The upregulation of SERT gene expression in response to TNF-α is in accordance with previous reports examining the consequences of long term exposure of SERT expressing human JAR choriocarcinoma cells to pro-inflammatory cytokines. In JAR cells, exposure to IL-1β for up to 16 h has been shown to mediate changes in SERT activity, namely a dose-dependent increase in SERT mRNA, an increase in SERT cell surface density and consequently an imipramine sensitive increase in [^{3}H]5-HT uptake [24]. Studies conducted in the same cell line following treatment for up to 72 h with the pro-inflammatory cytokines TNF-α and IL-6 revealed that TNF-α but not IL-6 treatment resulted in an increase in the V_{max} for 5-HT transport in these cells [25]. Others have found a down-regulation of SERT by TNF-α in enterocyte-like Caco-2 cells [43], suggesting cell-type specific effects of TNF-α.

We also observed that in primary astrocytes, TNF-α treatment induced a far greater increase in SERT-mediated 5-HT uptake (approximately 250 % of control) than in C6 glioma cells (approximately 145 % of control). There are a number of possible explanations for this discrepancy. C6 glioma cells are astrocytoma in nature, and the immortalization and maintenance of this cell line in culture may result in the modulation of normal signaling pathways, thus possibly blunting the stimulatory effect of TNF-α on SERT gene expression. C6 glioma cells may express a different profile of TNF-α receptors than primary astrocytes. For example, it has been shown that C6 glioma cells in the unstimulated state preferentially express TNF-R1, however, upon stimulation with TNF-α there is upregulation of the expression of TNF-R2. The balance between the differential expression of these receptors may result in altered responses to TNF-α [44]. Nonetheless, very similar time courses and concentration profiles for SERT activation were observed in both C6 glioma cells and primary astrocytes in this study.

As expected, upon treatment with TNF-α, the p38 MAPK signaling pathway becomes activated in these cells. This pathway has previously been implicated in the acute regulation of SERT activity in vitro [27, 45, 46] and in vivo [28, 47, 48]. Our data presented here suggest that p38 MAPK is also involved in the chronic regulation of SERT activity upon pro-longed cytokine exposure. The inhibition of p38 MAPK activation resulted in a partial attenuation of the TNF-α induced stimulation of SERT-mediated 5-HT uptake in astrocytes. Thus, it seems that p38 MAPK

activation is at least in part responsible for the TNF-α induced increase in SERT activity in astrocytes. These results also indicate that one or more additional mechanisms exist by which TNF-α enhances the activity of SERT.

There are a number of reasons to consider a potential role for NF-κB-dependent pathways in the regulation of SERT activity. Firstly, the SERT gene promoter is known to contain a consensus NF-κB binding site, suggesting that this transcription factor may be involved in the regulation of SERT gene expression in response to inflammatory stimulation [49]. Also, this transcription factor has previously been implicated in the precipitation of the clinical manifestation of sickness behavior as blockade of NF-κB activation following peripheral administration of IL-1β has been shown to attenuate the depressive-like behavioral effects of this peripherally administered cytokine in experimental animals [50]. Finally, the stimulation of both, primary astrocytes and C6 glioma cells, with TNF-α results in the activation of the NF-κB signaling pathway [40, 51, 52]. Preliminary experiments in our laboratory confirmed the activation of NF-κB upon TNFα treatment in C6 cells and primary astrocytes (data not shown). Furthermore, we sought to investigate the potential contribution of the NF-κB signaling pathway to the TNF-α mediated stimulation of SERT activity in primary astrocytes through the use of a number of inhibitors of this pathway. However, we were unable to clearly link NF-κB activation and SERT gene expression and activity in these cells. At this point we do not, however, exclude the possibility that the NF-κB signaling pathway may contribute to the increase in SERT gene expression and activity in response to TNF-α treatment. A complex relationship relevant to SERT regulation may exist in astrocytes between the NF-κB and other pathways activated by TNF-α, consequently, the role of NF-κB in the regulation of SERT gene expression remains to be elucidated.

The magnitude of the TNF-α induced increase in SERT mRNA levels and 5-HT uptake activity, observed in particular in primary astrocytes, indicates that these cells are very sensitive to TNF-α mediated alteration in SERT gene expression. This may in fact represent a molecular mechanism by which SERT expression is switched on under pathophysiological conditions in glial cells, which normally express very little SERT in vivo [34]. We postulate that under conditions of chronic inflammation, by enhancing SERT mediated 5-HT uptake, the pro-longed elevation of circulating pro-inflammatory cytokines such as TNF-α could significantly impact on available extracellular 5-HT. Since astrocytes are known to rapidly degrade 5-HT following uptake [53], enhanced astrocytic uptake would significantly affect the turnover rate of this neurotransmitter, resulting in a decrease in the total brain 5-HT content. In summary, our findings support the hypothesis that TNF-α may significantly influence central serotonergic homeostasis through the modulation of glial localized SERT activity, providing a possible link to molecular pathways underlying depressive symptoms associated with chronic inflammatory diseases.

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Conflict of interest The authors declare that they have no conflict of interest.

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