The Proinflammatory Cytokines Interleukin-1beta and Tumor Necrosis Factor-Alpha Activate Serotonin Transporters

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Proinflammatory cytokines and serotonergic homeostasis have both been implicated in the pathophysiology of major psychiatric disorders. We have demonstrated that activation of p38 mitogen-activated protein kinase (MAPK) induces a catalytic activation of the serotonin transporter (SERT) arising from a reduction in the SERT K_m for 5-hydroxytryptamine (5-HT). As inflammatory cytokines can activate p38 MAPK, we hypothesized that they might also activate neuronal SERT. Indeed, Interleukin-1beta (IL-1β) and tumor necrosis factor alpha (TNF-α) stimulated serotonin uptake in both the rat embryonic raphe cell line, RN46A, and in mouse midbrain and striatal synaptosomes. In RN46A cells, IL-1β stimulated 5-HT uptake in a dose- and time-dependent manner, peaking in 20 min at 100 ng/ml. This was abolished by IL-1ra (20 ng/ml), an antagonist of the IL-1 receptor, and by SB203580 (5 µM), a p38 MAPK inhibitor. TNF-α also dose- and time-dependently stimulated 5-HT uptake that was only partially blocked by SB203580. Western blots showed that IL-1β and TNF-α activated p38 MAPK, in an SB203580-sensitive manner. IL-1β induced an SB203580-sensitive decrease in 5-HT K_m with no significant change in V_{max}. In contrast, TNF-α stimulation decreased 5-HT K_m and increased SERT V_{max}. SB203580 selectively blocked the TNF-α-induced change in SERT K_m. In mouse midbrain and striatal synaptosomes, maximal stimulatory effects on 5-HT uptake occurred at lower concentrations (IL-1β, 10 ng/ml; TNF-α, 20 ng/ml), and over shorter incubation times (10 min). As with RN46A cells, the effects of IL-1β and TNF-α were completely (IL-1β) or partially (TNF-α) blocked by SB203580. These results provide the first evidence that proinflammatory cytokines can acutely regulate neuronal SERT activity via p38 MAPK-linked pathways.
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INTRODUCTION

The role of the immune mediators in the pathogenesis of neuropsychiatric disorders has drawn significant interest over the last decade. Elevated proinflammatory cytokines levels have been found in a number of psychiatric conditions. High levels of tumor necrosis factor alpha (TNF-α) have been found in cerebral spinal fluid samples drawn from patients with obsessive-compulsive disorder (OCD), attention-deficit hyperactivity disorder, and schizophrenia (Mittleman *et al*, 1997). Likewise, serum levels of proinflammatory interleukins IL-1β, IL-6, and TNF-α are elevated in depressed patients (Maes, 1999; Tuglu *et al*, 2003). Conversely, therapeutic treatments with proinflammatory cytokines result in 'sickness behavior' that is similar in many respects to depression, with decreased appetite,

anorexia, weight loss, fatigue, loss of libido, sleep disturbances, and reduced social contact (Kent *et al*, 1996; Yirmiya *et al*, 2000; Dantzer, 2001), and depression is frequently observed in association with peripheral inflammatory cytokine activation in a number of medical conditions including viral infections, rheumatoid arthritis, cancer, and neurodegenerative diseases (Meijer *et al*, 1988; Hall and Smith, 1996; Yirmiya *et al*, 1999; Pollak *et al*, 2000). Additionally, in human volunteers, depression, anxiety, and memory impairment are associated with immune activation by the bacterial endotoxin, lipopolysaccharide, and are correlated with serum IL-1β and TNF-α levels induced by that treatment (Yirmiya *et al*, 2000).

Although the studies noted above suggest a correlation between immune activation and psychiatric symptoms, the underlying mechanisms for such a relationship have not been established. Peripheral cytokines have access to the CNS (Schiepers *et al*, 2005), and both IL-1β and TNF-α are expressed in the brain (Botchkina *et al*, 1997; Rivest *et al*, 2000; Sheng *et al*, 2001; Bette *et al*, 2003). Inflammatory cytokines are known to alter metabolism and release of central serotonin (5-hydroxytryptamine, 5-HT) in the CNS (Dunn, 1992; Palazzolo and Quadri, 1992; Cho *et al*, 1999). 5-HT is a neurotransmitter involved in the regulation of a

wide array of behaviors including those related to sleep, appetite, aggression, and mood (Nestler *et al*, 2001). Medications that modulate serotonergic signaling are used to treat many psychiatric disorders including eating disorders, depression, anxiety, OCD, impulse control disorders, and schizophrenia.

A primary target for serotonergic medications has been the 5-HT transporter (SERT, 5-HTT), a protein involved in termination of serotonergic signaling (Barker and Blakely, 1995). These medications block reuptake of serotonin into the serotonergic neuron, and thereby increase the available serotonin for postsynaptic interactions. Alterations in SERT expression and activity associated with variations in the human SERT gene have been associated with mental illness (Murphy *et al*, 2003). One genetic variant associated with elevated SERT activity has been associated with OCD as well as other psychiatric disturbances (Ozaki *et al*, 2003), whereas altered SERT expression associated with a different variant has been found to predispose individuals to depression and suicidal disorders (Caspi *et al*, 2003). Recently, we identified multiple SERT coding variants in autistic probands, and associated these variants with rigid-compulsive behavioral traits (Sutcliffe *et al*, 2005). As such, it is clear that modulation of SERT activity has profound implications in terms of emotional homeostasis.

Although the psychiatric effects of genetic disturbances in SERT activity have attracted significant attention, acute regulation of SERT activity by neurotransmitters and modulators has only been studied more recently using *in vitro* models that have revealed neurotransmitter receptor-linked intracellular cascades modulating 5-HT uptake. Protein kinase C (PKC) activation and protein phosphatase 2A inhibition trigger transporter phosphorylation, events that are correlated with a loss of SERT surface density (Qian *et al*, 1997; Ramamoorthy *et al*, 1998; Ramamoorthy and Blakely, 1999). Alpha-2 adrenergic receptor stimulation leads to a rapid downregulation of SERT activity in brain synaptosomes (Ansah *et al*, 2003), whereas histamine receptor stimulation induces protein kinase G (PKG)-linked increases in 5-HT transport in platelets. Miller and Hoffman (1994) demonstrated that adenosine receptor (AR) stimulation in rat basophilic leukemia cells (RBL-2H3) also leads to a PKG-linked increase in SERT activity. Using this RBL-2H3 cell model, as well as AR/SERT co-transfected CHO cells, we recently found that this PKG-linked increase in 5-HT uptake occurs via distinct SERT trafficking-dependent and trafficking-independent pathways (Zhu *et al*, 2004). Specifically, we found that PKG could activate p38 mitogen-activated protein kinase (MAPK) to catalytically activate SERT in a process independent of PKG-mediated trafficking of SERT to the plasma membrane (Blakely *et al*, 2005; Zhu *et al*, 2005). The catalytic activation of SERT was the result of a reduction in the SERT K_m for 5-HT, making the transporter more efficient at lower 5-HT concentrations. In RBL-2H3 and a serotonergic rat embryonic raphe cell line (RN46A; Eaton and Whittemore, 1996), we further demonstrated that activation of p38 MAPK by a variety of stimulus modalities (anisomycin, H_2O_2, UV radiation) enhanced 5-HT transport (Zhu *et al*, 2005). Saturation kinetic analyses revealed that p38 MAPK activation was associated with a selective reduction in 5-HT K_m accompanied by an increase in

5-HT potency in displacing surface SERT antagonist binding. Consistent with these changes, stimulation of p38 MAPK with anisomycin enhances SERT activity without altering surface SERT density.

As proinflammatory cytokines such as IL-1β and TNF-α were known to activate p38 MAPK in peripheral systems (Clerk *et al*, 1999; Saklatvala, 2004; Li *et al*, 2005; Pantouli *et al*, 2005), we hypothesized that these cytokines might acutely increase SERT activity through p38 MAPK activation, and might provide a critical link to physiologically significant systems known to regulate SERT *in vivo*. To this end, we examined the effects of IL-1β and TNF-α on serotonin uptake in the RN46A cell line. In addition, as both IL-1β and TNF-α are present in the brain, we examined the effects of these cytokines on serotonin transport in synaptosomal preparations from mouse midbrain and striatum. These regions allowed us to investigate alterations in SERT activity in the vicinity of the 5-HT-cell soma, and within an important distal terminal field, respectively. We provide evidence that IL-1β and TNF-α activate 5-HT transport in a p38 MAPK-sensitive manner, and do so via catalytic modulation (deceased K_m) of the SERT protein.

MATERIALS AND METHODS

Reagents

Interleukin-1beta (IL-1β), interleukin-1 receptor antagonist (IL-1ra), and tumor necrosis factor-alpha (TNF-α) were purchased from Santa Cruz Biotechnology Inc. (Santa Cruz, CA); anisomycin was ordered from Sigma Chemical (St Louis, MO). SB203580 was obtained from Alexis Biochemicals (San Diego, CA). Anti-total and -phospho-specific p38 MAPK polyclonal antibodies were purchased from Cell Signaling (La Jolla, CA). [^{3}H]5-HT (5-hydroxy[^{3}H]tryptamine trifluoroacetate, 107 Ci/mmol) was purchased from Amersham Biosciences Inc. (Piscataway, NJ); trypsin-EDTA, glutamine, and ampicillin/streptomycin were purchased from Invitrogen (Carlsbad, CA); modified Eagle's medium (MEM) and Dulbecco's MEM (DMEM) were derived from Invitrogen reagents and prepared in the Vanderbilt Media Core.

Cell Culture and Synaptosomal Preparation

RN46A cells (provided by Dr Whittemore, University of Miami School of Medicine, Miami, FL) were cultured at 37°C with DMEM:F12 (1:1 in volume) containing 250 mg/l G418, 10% FBS, 1% L-Gln, and 100 g/ml penicillin/streptomycin. For the preparation of synaptosomes, C57BL/6NHSD mice (Harlan Sprague Dawley Inc., Indianapolis, IN) were used as approved by the Institutional Animal Care Use Committee at Vanderbilt University. Synaptosomes were prepared as previously described (Ansah *et al*, 2003). Briefly, mouse midbrain and striatum were homogenized in 0.32 M glucose at 400 r.p.m. using a Teflon-glass tissue homogenizer (Wheaton Instruments, Millville, NJ). The homogenized tissue was centrifuged at 800*g* for 10 min at 4°C. The supernatant was transferred to new clean centrifuge tubes and centrifuged at 10 000*g* for 15 min at 4°C. The synaptosomal pellet was resuspended with Krebs–Ringer's HEPES (KRH) buffer containing 130 mM NaCl,

1.3 mM KCl, 2.2 mM CaCl$_2$, 1.2 mM MgSO$_4$, 1.2 mM KH$_2$PO$_4$, 1.8 g/l glucose, 10 mM HEPES, pH 7.4, 100 μM pargyline, and 100 μM ascorbic acid. The synaptosomal suspension was analyzed for protein content (Bio-Rad protein assay reagents) and used for both 5-HT transport assay and Western blots.

5-HT Transport Assays

[^{3}H]5-HT transport activity in RN46A cells was assayed as described previously (Zhu et al, 2005). Briefly, RN46A cells were plated at 200 000 cells/well 24 h before the 5-HT uptake assay. The medium was removed by aspiration and cells were washed once with KRH buffer. Cells were incubated in triplicate at 37°C in KRH buffer (0.2 ml/well) containing 100 μM pargyline and 100 μM L-ascorbic acid (Sigma), with or without modifiers. After 10 min incubation with [^{3}H]5-HT (100 nM) at 37°C, buffer was aspirated and the cells were washed three times with ice-cold KRH buffer. Cells were solubilized with 0.4 ml Microscint 20 (Packard Bioscience, Meriden, CT), and tritium-labeled monoamine accumulation was quantitated using a TopCount plate scintillation counter (Packard Bioscience, Meriden, CT). Specific 5-HT uptake was determined by subtracting the amount of [^{3}H]5-HT accumulated in the presence of 10 μM paroxetine. 5-HT saturation kinetics in RN46A cells were defined as described for standard transport assays, using varying concentrations of 0.5 Ci/mmol [^{3}H]5-HT produced using unlabeled 5-HT. Nonspecific uptake was defined with 10 μM paroxetine. Assays measuring transport of [^{3}H]5-HT in synaptosome were performed as described (Ansah et al, 2003). Generally, 30–50 μg synaptosomes per sample (a total volume of 200 μl) were preincubated at 37°C in a shaking water bath for 10 min. Vehicle or modifiers were then added for 10–60 min. After 5 min incubation with [^{3}H]5-HT (20 nM) at 37°C, uptake was terminated by filtration through GF/B Whatman paper. Filters were washed three times with ice-cold KRH buffer, immersed in scintillation liquid for 8 h, and counted by scintillation spectrometry. Counts obtained from the filtered samples were corrected for the nonspecific uptake obtained using parallel samples incubated with paroxetine (1 μM).

Assays of p38 MAPK Activation

To monitor activation of p38 MAPK by IL-1β or TNF-α, RN46A cells were seeded in six-well plates (500 000 cells/well) and cultured for 24 h. Cells were washed once with KRH buffer followed by treatment with IL-1β or TNF-α in the presence or absence of SB203580 or relevant antagonist. Following the treatment, the KRH buffer containing modifiers was removed and cells were lysed with radio-immunoprecipitation assay (RIPA) buffer (10 mM Tris-HCl, pH 7.5, 150 mM NaCl, 1 mM EDTA, 0.1% SDS, 1% Triton X-100, 1% Na-deoxycholic acid) containing protease inhibitor cocktails (Sigma). Cell lysates were centrifuged at 10 000g for 20 min. For monitoring the activation of p38 MAPK in synaptosomes, an aliquot of synaptosomal suspension was centrifuged and lysed with RIPA buffer at different time points during incubation. Up to 50 μg protein from RN46A supernatant or 100 μg protein from the synaptosomal supernatant was mixed with Laemmli buffer (62.5 mM Tris pH 6.8, 20% glycerol, 2% SDS, 5% mercaptoethanol, and 0.01% bromophenol blue) and separated by SDS-PAGE, before transfer to PVDF membranes (Millipore, Bedford, MA) at 25 V for 30 min using Trans-Blot® SD Semi-Dry Electrophoretic Transfer Cell (Bio-Rad, Hercules, CA). Blotted membranes were subsequently blocked with 5% non-fat dry milk in PBS with 0.1% Triton X-100 or Odyssey Blocking Buffer (Li-Cor Biosciences, Lincoln, NE), and probed with anti-total or phospho-p38 MAPK polyclonal antibodies (1 : 1000; Cell Signaling) in 5% milk/PBS with 0.1% Triton X-100 or Odyssey Blocking Buffer, while gently shaking for 1 h at room temperature. Bound antibody was detected with HRP-conjugated goat anti-rabbit secondary antibody (for milk-blocked membrane, 1 : 10 000; Jackson ImmunoResearch, West Grove, PA), or fluorescence-labeled secondary antibody-Alexa Fluor 680 (for Odyssey-blocked membrane, avoiding exposure to light; 1 : 5000 in Odyssey Blocking Buffer; Molecular Probes Inc., Eugene, OR) for 1 h at room temperature. HRP signals were developed with ECL-plus solution (Amersham). To estimate the relative abundance of proteins, films were scanned on an AGFA Duoscan T1200, and the captured images were analyzed in Adobe Photoshop and quantified with NIH Image software, taking care through multiple exposures to work in the linear range of the film. The membrane processed with Odyssey reagents was scanned, and the captured image of the signal was processed and quantified with the Odyssey™ Infrared Imaging System (Li-Cor Biosciences, Lincoln, NE).

Statistical Analyses

All experiments were performed at least three times. Statistical analyses, comparing baseline and compound-modified uptake, or p38MAPK activation, were performed with GraphPad Prism (GraphPad, San Diego, CA) using one- and two-way analyses of variance (ANOVA) with subsequent planned comparisons (Dunnett, Bonferroni), as well as t-tests as noted in the legends.

RESULTS

Stimulation of 5-HT Uptake in RN46A Cells by IL-1β and TNF-α

IL-1β exerted a concentration- and time-dependent stimulation of 5-HT transport activity in cultured RN46A cells (Figure 1a and b). IL-1β stimulation of the 5-HT transport peaked at 100 ng/ml, ∼180% of control levels at 20 min, and declined to basal levels by 60 min. Using 20 min pretreatment, IL-1β stimulation peaked at 100 ng/ml and diminished over 200 ng/ml. Consistent with previous findings, the effects of IL-1β on 5-HT uptake were blocked by preincubation with the p38 MAPK inhibitor, SB203580 (Figure 3). Similarly, TNF-α also induced 5-HT uptake in a concentration- and time-dependent manner (Figure 2a and b). At 100 ng/ml, TNF-α stimulation of 5-HT transport was transient, peaking at 20 min and declining to basal levels by 60 min. At 20 min incubation, TNF-α stimulation peaked at 100 ng/ml, diminishing at 1 μg/ml. Unlike IL-1β, the effect of TNF-α was only partially blocked by SB203580, indicating a more complicated mechanism underlying TNF-α action (Figure 3).
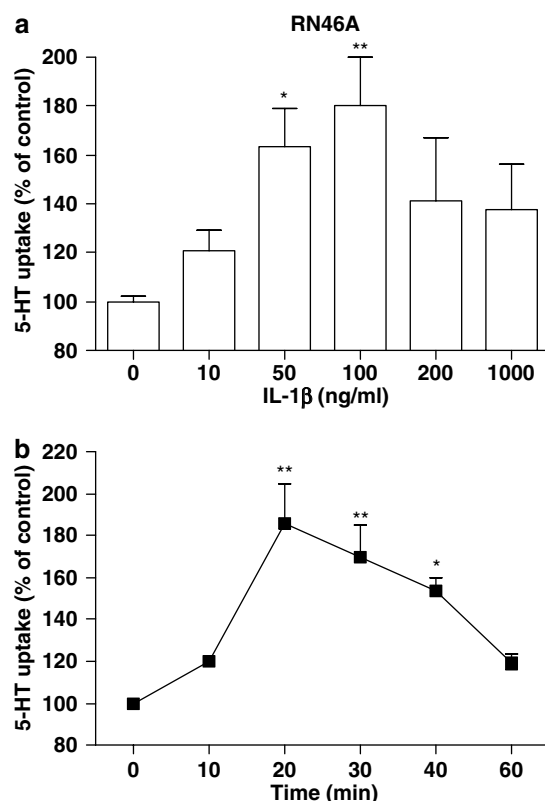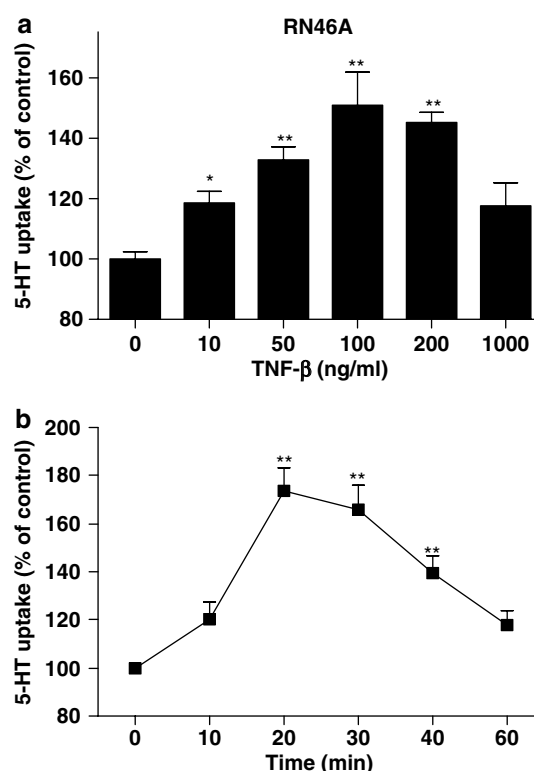
Figure 1 Effects of IL-1β on 5-HT uptake in RN46A cells. (a) Dose response: Cells were treated with varying concentrations of IL-1β for 20 min before transport assay. (b) Time course: Influence of pretreatment duration on IL-1β (100 ng/ml) stimulation of 5-HT uptake. Values in (a) and (b) are expressed as the mean of at least three experiments $\pm$ SEM. *$p < 0.05$, **$p < 0.01$ vs controls (one-way ANOVA, Dunnett).

Figure 2 Effects of TNF-α on 5-HT uptake in RN46A cells. (a) Dose response: Cells were treated with the indicated concentrations of TNF-α for 20 min before transport assay. (b) Time course: Influence of pretreatment duration on TNF-α (100 ng/ml) stimulation of 5-HT uptake. Values in (a) and (b) are expressed as the mean of at least three experiments $\pm$ SEM. *$p < 0.05$, **$p < 0.01$ vs controls (one-way ANOVA, Dunnett).
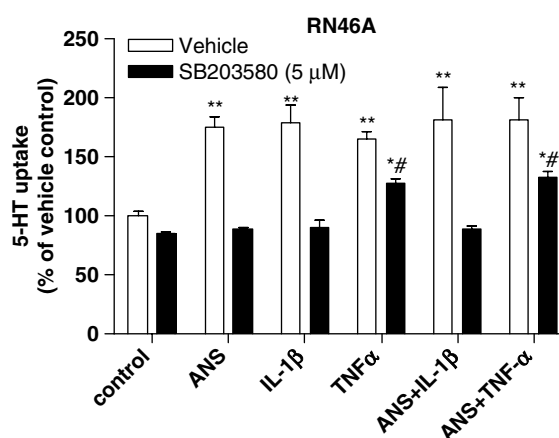
p38 MAPK must be dually phosphorylated to achieve catalytic activation (New and Han, 1998). To test whether IL-1β and TNF-α treatments of RN46A cells trigger p38 MAPK activation, we monitored the dual-phosphorylation status of the kinase with total and phospho-specific p38 MAPK antibodies using Western blotting techniques. As shown in Figure 4a and b, both IL-1β and TNF-α stimulated increases in phosphorylated p38 MAPK, effects that were blocked by pretreatment with SB203580. No changes were observed in total p38 MAPK levels.

IL-1β-Induced 5-HT Uptake is Abolished by IL-1 Receptor Antagonist in RN46A Cells

The initial events in the signaling cascades triggered by these cytokines is the binding and activation of specific receptors for TNF-α and IL-1 cytokines (Stauber et al, 1988; Hohmann et al, 1989; Brockhaus and Loetscher, 1990; Sims et al, 1988; O'Neill and Dinarello, 2000). Whereas the IL-1 receptor antagonist, IL-1ra, is commercially available, there is currently no such selective receptor antagonist available for TNF-α receptors. To assess whether IL-1β stimulates SERT activity via IL-1 receptors, IL-1ra was preincubated with RN46A cells followed by treatment with IL-1β. Anisomycin, a direct p38 MAPK stimulator and activator of SERT (Zhu et al, 2005), was used as a positive control


Figure 3 Effects of SB203580 on IL-1β- or TNF-α-stimulated 5-HT uptake. RN46A cells were incubated with the specific p38 MAPK inhibitor SB203580 (5 μM) or vehicle for 15 min, followed by treatment with anisomycin (1.0 μM), IL-1β (100 ng/ml), TNF-α (100 ng/ml), or a combination of anisomycin and IL-1β or TNF-α. Values are expressed as mean values ($n = 4$) $\pm$ SEM. *$p < 0.05$, **$p < 0.01$ vs vehicle control; #$p < 0.05$ vs TNF-$\alpha \pm$ anisomycin (two-way ANOVA). ANS: anisomycin; SB: SB203580.

that bypasses requirements for cell surface receptors. IL-1ra (10 ng/ml) blocked IL-1β-stimulated 5-HT transport, as did the p38 MAPK inhibitor SB203580 (Figure 5). In contrast,
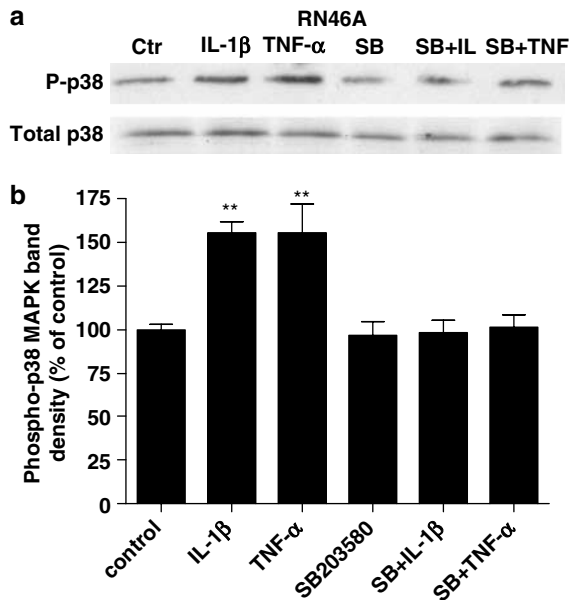
Figure 4 IL-1β or TNF-α triggers p38 MAPK phosphorylation in RN46A cells in an SB203580-sensitive manner. RN46A cells were seeded in a six-well plate and cultured overnight. The cells were then treated with vehicle (assay buffer) or SB203580 (5 μM) for 15 min followed by 20 min treatment with IL-1β or TNF-α (100 ng/ml for each). The cells were then processed for activated and total p38 MAPK immunoblots as described in Materials and methods. (a) Representative blots of phosphorylated (top) and total p3 (bottom). (b) Average values of activated p38 immunoblots from densitometry of experiments ($n = 3$). Values are expressed as the mean ± SEM. ** $p < 0.01$ vs vehicle (one-way ANOVA, Dunnett).
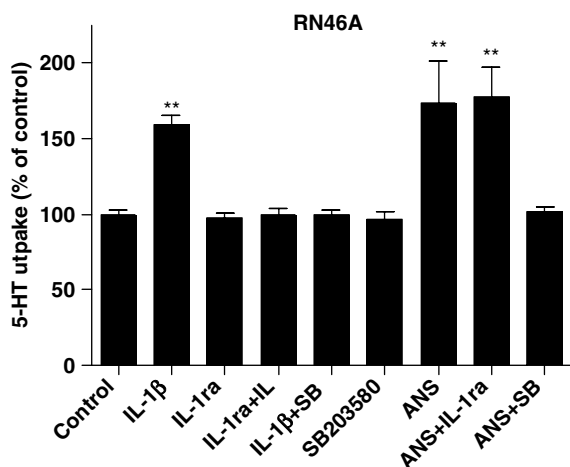
Figure 5 Effect of IL-1 receptor antagonist on IL-1β-induced 5-HT uptake. RN46A cells were treated for 10 min with the antagonist IL-1ra, SB203580, or vehicle, followed by IL-1β (20 min) or anisomycin (10 min) treatment. IL-1ra (10 ng/ml) completely blocked IL-1β- but not anisomycin-induced 5-HT uptake. SB203580 (5 μM) blocked the effect of both. Values are expressed as mean values ($n = 3$) ± SEM. ** $p < 0.01$ vs vehicle (one-way ANOVA, Dunnett). ANS: anisomycin; SB: SB203580.

whereas anisomycin-induced 5-HT uptake was abolished by SB203580, no attenuation was observed with IL-1ra (Figure 5), consistent with p38 MAPK being downstream of the IL-1 receptor.
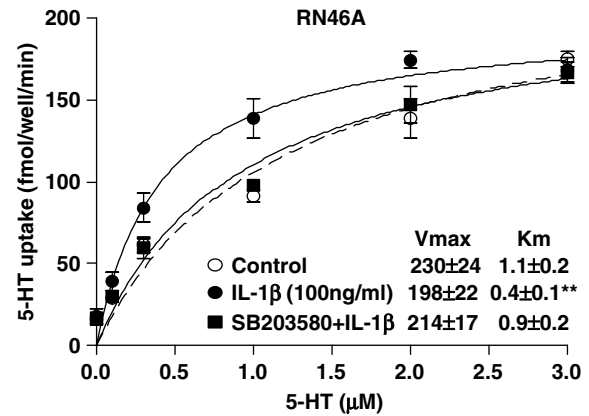
Figure 6 Saturation kinetic analyses of IL-1β stimulation. RN46A cells were treated with vehicle or SB203580 (5 μM) for 15 min followed by IL-1β (100 ng/ml) for 20 min before transport assays. Data were fit to a Michaelis–Menten equation (single binding site) to derive 5-HT K_m and V_{max} values (expressed as mean values ($n = 3$) ± SEM). ** $p < 0.01$ vs control (Student's t-test).

IL-1β Treatment of RN46A Cells Selectively Reduces the 5-HT-Transport K_m

Our recent study revealed that anisomycin-induced SERT stimulation does not arise from altered surface trafficking, but induces p38 MAPK-mediated catalytic activation of SERT (Zhu et al, 2005). Consistent with these findings, IL-1β treatments significantly reduced the SERT K_m for 5-HT in RN46A cells (1.1 ± 0.2 vs 0.4 ± 0.1 μM, $p < 0.01$), but effected no significant change in maximal transport capacity (V_{max}; Figure 6). Saturation kinetic analyses of 5-TH uptake revealed that incubation of RN46A cells with SB203580 before the treatment with IL-1β reversed the effects on 5-HT K_m.

TNF-α Stimulation of SERT Activity Involves Changes in Both V_{max} and K_m in 5-HT Transport

To evaluate the impact of TNF-α on the V_{max} and K_m of 5-HT transport, RN46A cells were treated as above with vehicle or TNF-α, with and without SB203580. Saturation kinetic analyses (Figure 7) showed that TNF-α treatment not only induced a reduction of K_m (0.7 ± 0.11 vs 1.0 ± 0.12 μM in control), but also an increase in V_{max} of 5-HT transport (394 ± 33 vs 294 ± 22 fmol/well/min in control). Notably, pretreatment with SB203580 blocked the shift of K_m (1.1 ± 0.21 μM), but did not affect significantly the increase of V_{max} (346 ± 16 fmol/well/min). These results suggest that TNF-α-induced 5-HT transport arises from a more complex form of stimulation than does IL-1β-stimulated transport, although here as well, p38 MAPK activation accounts for the changes in 5-HT K_m.

Stimulation of 5-HT Uptake in Mouse Synaptosomes by IL-1β and TNF-α

To assess the relevance of our findings with cultured raphe cells for native brain systems, synaptosomes were prepared from midbrain and striatum of C57BL/6 mice and treated with IL-1β or TNF-α. As seen in RN46A cells, the stimulation of 5-HT transport in synaptosomes induced by IL-1β or TNF-α was transient, peaking in this case at 10 min and diminishing 20 min after treatment in both

midbrain and striatum (data not shown). Using 10 min pretreatment, IL-1β dose-dependently stimulated an increase in 5-HT uptake in both midbrain (Figure 8a) and striatum (Figure 8c), peaking at 10 ng/ml. At 100 ng/ml, the IL-1β effect on SERT became inhibitory in both brain regions. This inhibition could represent a toxic effect; however, it was not investigated further. Treatment of synaptosomes with TNF-α for 10 min also induced an elevation in 5-HT transport in a concentration-dependent manner, with a maximal effect at 20 ng/ml. Stimulation declined to basal levels at concentrations over 50 ng/ml (Figure 8b and d).
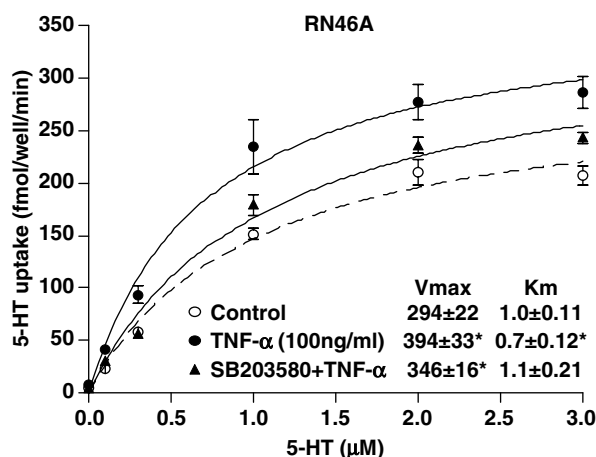

Figure 7 Saturation kinetic analyses of TNF-α stimulation. RN46A cells were treated with vehicle or SB203580 (5 μM) for 15 min followed by TNF-α (100 ng/ml) for 20 min before transport assays. Values are expressed as mean values ($n = 3$) ± SEM. *$p < 0.05$ vs control (Student's t-test).

p38 MAPK is Involved in IL-1β- and TNF-α-Stimulated 5-HT Transport in Mouse Synaptosomes

To assess p38 MAPK activation in synaptosomes, samples from mouse midbrain or striatum were preincubated with IL-1β or TNF-α in the presence/absence of p38 MAPK-specific inhibitor SB203580. As shown in Figure 9a, IL-1β stimulated an increase of 5-HT uptake in mouse midbrain. SB203580 pretreatment at this concentration (1 μM) did not affect basal uptake, but at 5 μM it reduced SERT activity. SB203580 blocked IL-1β-induced 5-HT transport at both concentrations. As seen in RN46A cells, TNF-α stimulation of 5-HT uptake in midbrain was only partially blocked by SB203580 at a concentration (1 μM) that did not affect basal 5-HT uptake (Figure 9b), whereas TNF-α stimulation of uptake was abolished by SB203580 at 5 μM, a concentration that reduced basal uptake. In striatum, the 5-HT transport induced by either IL-1β or TNF-α was blocked by SB203580 at 5 μM (Figure 9c). In addition, we observed that pretreatment of midbrain synaptosomes with IL-1β or TNF-α enhanced the phosphorylation of p38 MAPK, effects that were blocked by SB203580. Total p38 MAPK levels remained unchanged (Figure 10). We found similar effects of IL-1β or TNF-α on p38 MAPK in striatal synaptosomes (data not shown). These results support the idea that p38 MAPK is involved in IL-1β- or TNF-α-stimulated SERT activity in brain.

IL-1β Stimulation of 5-HT Uptake is Blocked by IL-1ra in Mouse Synaptosomes

To evaluate whether the cytokine stimulation of SERT activity in brain is mediated by cytokine receptors, synaptosomes from both midbrain and striatum of mouse
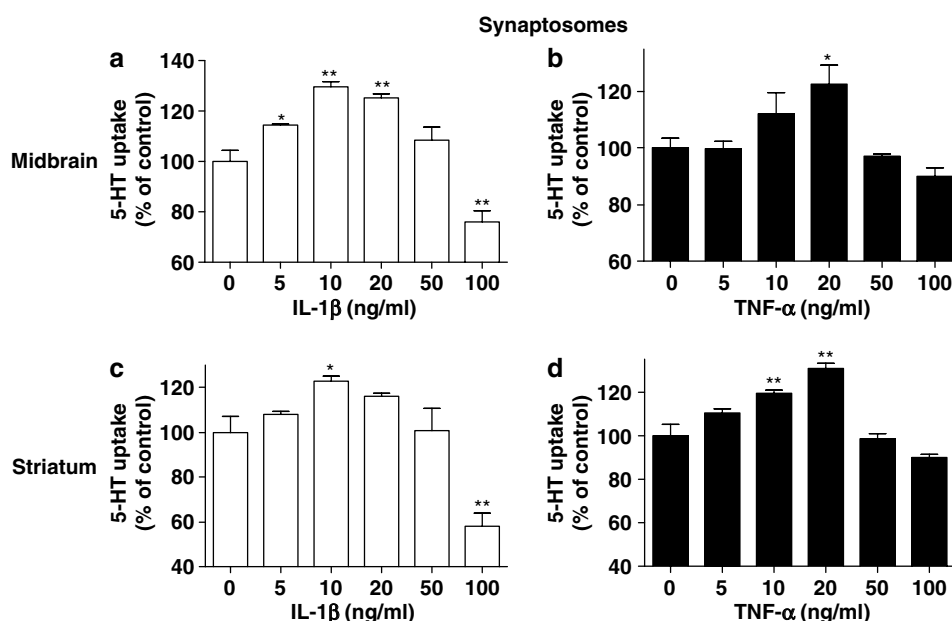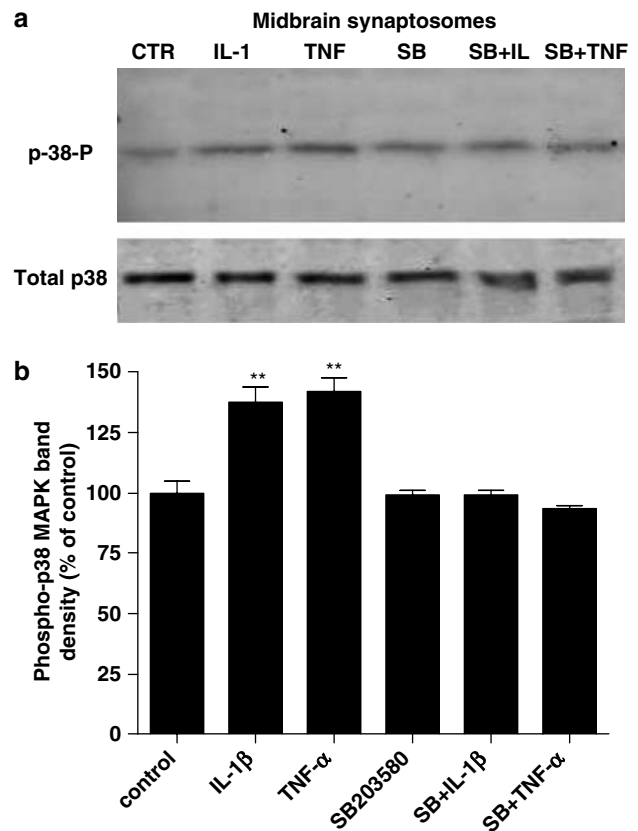

Figure 8 Effects of IL-1β and TNF-α on 5-HT uptake in mouse synaptosomes. Synaptosomes were preincubated at 37°C for 10 min before the cytokine treatment. IL-1β or TNF-α at varying concentrations was incubated with synaptosomes for 10 min before a 5 min uptake assay. (a) IL-1β effect on midbrain synaptosomes. (b) Influence of TNF-α on 5-HT uptake in midbrain synaptosomes. (c) Effect of IL-1β on SERT activity in striatal synaptosomes. (d) Influence of TNF-α on 5-HT uptake in striatal synaptosomes. Values are expressed as the mean of at least three experiments ± SEM. *$p < 0.05$, **$p < 0.01$ vs respective controls (one-way ANOVA, Dunnett).
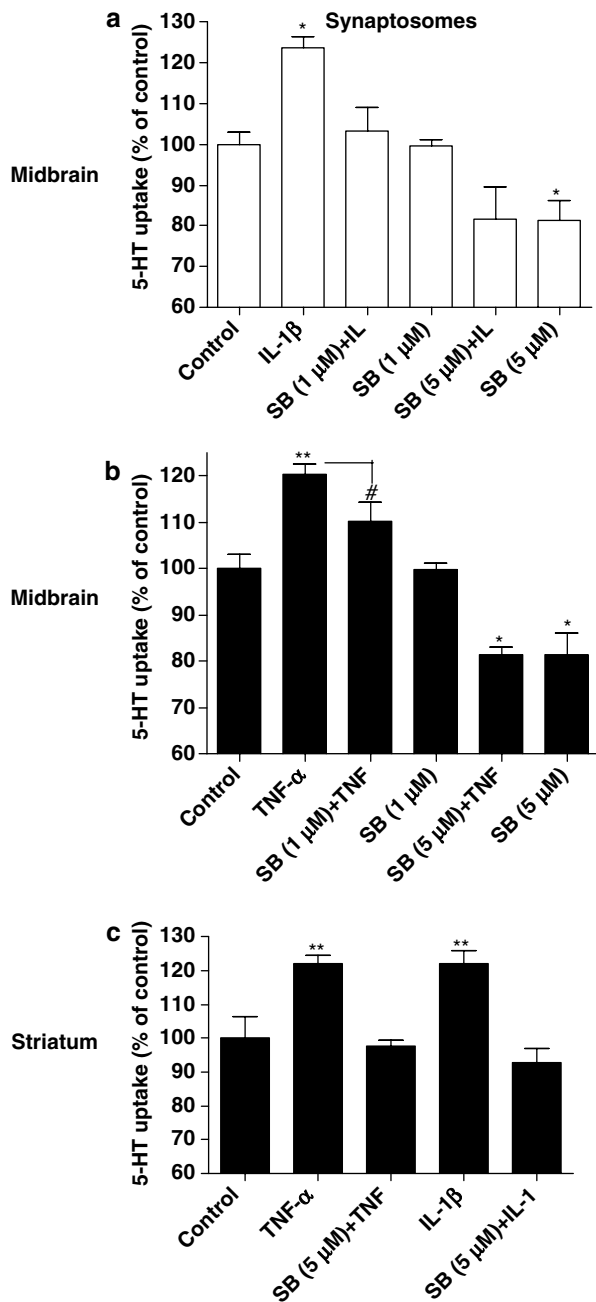
Figure 9 Effects of SB203580 on IL-1β- or TNF-α-stimulated 5-HT uptake in mouse synaptosomes. Synaptosomes were incubated with the specific p38 MAPK inhibitor SB203580 or vehicle for 10 min, followed by treatment with IL-1β (10 ng/ml) or TNF-α (20 ng/ml) for 10 min. (a) IL-1β stimulation of 5-HT uptake in midbrain synaptosomes was blocked by SB203580 at 1 and 5 μM. (b) Effect of SB203580 on TNF-α-induced 5-HT transport in midbrain synaptosomes. (c) SB203580 at 5 μM blocked the effect of both IL-1β and TNF-α in striatal synaptosomes. Values are expressed as mean values ($n = 4$) ± SEM. *$p < 0.05$, **$p < 0.01$ *vs* respective control; #$p < 0.05$ *vs* TNF-α (two-way ANOVA).

Figure 10 IL-1β and TNF-α activate p38 MAPK in mouse synaptosomes. Midbrain synaptosomes were prepared as described in Materials and methods. Synaptosomes were treated with vehicle (assay buffer) or SB203580 (5 μM) for 10 min followed by 10 min treatment with IL-1β or TNF-α (10 and 20 ng/ml, respectively). The synaptosomes were then processed for activated and total p38 MAPK immunoblots. (a) Representative blots of dual-phosphorylated (top) and total p38 MAPK (bottom). (b) Average values of activated p38 MAPK immunoblots from densitometry experiments ($n = 3$). Values are expressed as the mean ± SEM. **$p < 0.01$ *vs* vehicle (one-way ANOVA, Dunnett).

were treated with IL-1β and/or IL-1ra, the antagonist at IL-1 receptors. Consistent with the results from RN46A cells, IL-1β pretreatment of synaptosomes from both midbrain and striatum increased the 5-HT uptake, effects that were abolished by IL-1ra at a concentration that did not affect the basal SERT activity. In contrast, anisomycin stimulation of

5-HT uptake was not affected by IL-1ra (Figure 11). These data further indicate that IL-1 receptors are critical in mediating IL-1β stimulation of p38 MAPK and SERT activity.

DISCUSSION

The influence of cytokines on CNS processing and their potential role in psychiatric disorders has drawn increased scientific attention over the years. In the current study, we found that the proinflammatory cytokines, IL-1β and TNF-α, acutely stimulate SERT activity in both the raphe cultured cell line and in mouse synaptosomal preparations. This finding supports our hypothesis that proinflammatory cytokines might acutely alter SERT activity in CNS serotonergic neurons, providing a mechanism by which cytokines can modulate serotonergic signaling, and influence emotional cognitive processing. The similarity of the cytokine effects on SERT in midbrain and striatal synaptosomes suggests a consistent effect of these cytokines on SERT over the entirety of the serotonergic neuron, acting to
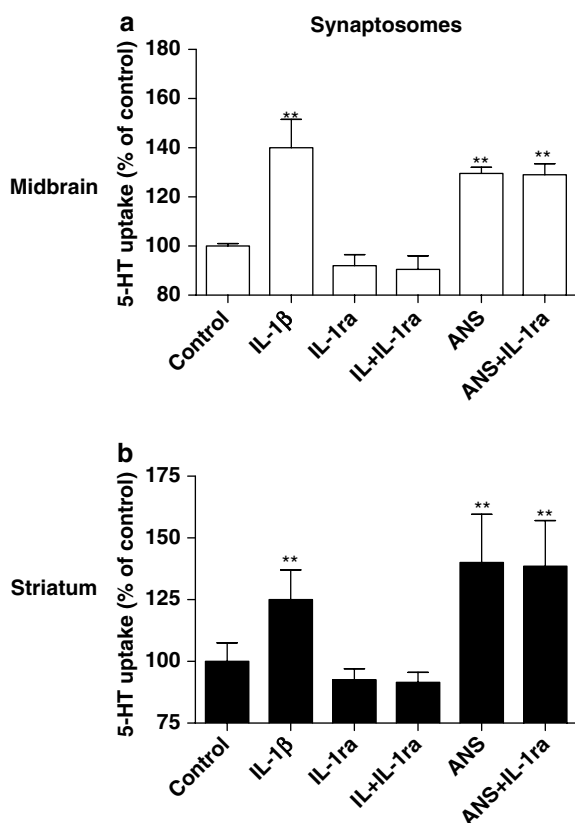
Figure 11 Effect of IL-1ra on IL-1β-induced 5-HT uptake in mouse synaptosomes. Synaptosomes from either midbrain or striatum were treated for 10 min with the IL-1 receptor antagonist IL-1ra or vehicle, followed by IL-1β (10 min) or anisomycin (10 min) treatment. IL-1ra (10 ng/ml) completely blocked IL-1β- but not anisomycin-induced 5-HT uptake in both (a) midbrain and (b) striatum. Values are expressed as mean values ($n = 3$) ± SEM. **$p < 0.01$ vs control (one-way ANOVA, Dunnett). ANS: anisomycin.

reduce extracellular 5-HT concentrations in both the peri-somatodendritic and distal terminal fields. In the case of IL-1β, we confirmed that this stimulation was mediated through an IL-1 receptor present in both the RN46A raphe cell line and on terminals in midbrain and striatal synaptosomes. IL-1β and TNF-α do not exhibit these effects on 5-HT uptake in human platelets (data not shown), which have high levels of SERT, suggesting that the presence of the transporter alone is not sufficient for cytokine inducibility. However, the underlying mechanism proposed here, p38 MAPK activation, does augment platelet SERT activity (Zhu et al, 2005), suggesting that other peripheral cytokines having the capacity to stimulate p38 MAPK might target these formed blood elements.

SB203580 completely abolished IL-1β stimulation of SERT activity in both preparations, but only partially blocked TNF-α-induced 5-HT transport, indicating that while both cytokines activate p38 MAPK, TNF-α has an additional mode of enhancing transport. As confirmation of p38 MAPK involvement, we demonstrated that stimulation of 5-HT transport by IL-1β and TNF-α was paralleled by the activation of p38 MAPK, with increased levels of dual-phosphorylated p38 MAPK—an effect that was blocked by SB203580 in both cases.

As predicted from studies with the p38 MAPK activator anisomycin (Zhu et al, 2005), both IL-1β and TNF-α increased the apparent affinity of 5-HT for SERT. TNF-α additionally increased the V_{max} of this interaction. Kinetic assays showed that SB203580 blocked the changes in K_m induced by IL-1β and TNF-α, but, at concentrations that do not impact basal SERT activity, it did not impact the TNF-α-induced increase in V_{max}, consistent with an independent mode of action for this latter cytokine. Regardless, these data confirm that p38 MAPK participates in the acute regulation of SERT activity by IL-1β and TNF-α, with IL-1β stimulating SERT intrinsic activity and TNF-α enhancing both intrinsic activity and the surface density of the transporter. Alterations in SERT trafficking through PKC- or PKG-related mechanisms (Zhu et al, 2004; Qian et al, 1997) could potentially play a role in the TNF-α-induced increase in SERT density. Further studies are needed to define the p38 MAPK-independent mechanism by which TNF-α augments 5-HT uptake.

The parameters for activation of SERT did differ in the two preparations. Both IL-1β and TNF-α produced greater activation of SERT in RN46A cells (70–80%) as compared to the synaptosomal preparations (20–40%); however, higher concentrations and longer incubation times were required for optimal stimulation in the RN46A cells. There are a number of possible explanations for these differences. First, the process of preparing the synaptosomes, itself, elevates basal p38 MAPK activity (data not shown). This may have reduced the percentage increase inducible by cytokines in this preparation. RN46A cells are immature immortalized cells derived from rat raphe neurons, whereas our synaptosomes were derived from adult mouse brain. Additionally, as the RN46A cells do not form neurites under the conditions of these experiments, our findings with this preparation may reflect processes representative of cytokine interactions at somatodendritic regions of the serotonergic neuron. In contrast, the serotonergic response in synaptosomes might better represent cytokine interactions at serotonergic terminals. Finally, it should be noted that our synaptosomal preparation included non-serotonergic terminals. We cannot rule out the possibility that IL-1β and TNF-α acted at non-serotonergic terminals to induce changes in 5-HT uptake via a trans-synaptic process having different parameters of activation.

Previous studies using JAR choriocarcinoma cells have shown that longer-term exposure to either IL-1β (Ramamoorthy et al, 1995) or TNF-α (Mössner et al, 1998) will increase 5-HT uptake via an increase in transporter density. These effects, however, required prolonged exposure—16 h in the case of IL-1β and 48 h in the case of TNF-α. For IL-1β, the increase was attributed to an increase in production and/or stability of SERT mRNA (Ramamoorthy et al, 1995). Exposing this cell preparation to shorter durations (1–4 h for IL-1β; 24 h for TNF-α) produced no significant change in 5-HT uptake. By contrast, the cytokine-induced increase in uptake in our studies involved transcription-independent, short-term modulation of serotonin uptake via catalytic activation of the transporter, occurring within 10–20 min of exposure, and resolving within 20–60 min.

The short duration of cytokine effects in these experiments, relative to the more prolonged elevated circulating

cytokine levels associated with human illness, raises a question as to the functional significance of the acute effects demonstrated here. Although it is conceivable that the time-limited nature of SERT activation in these experiments was a product of our methodology (ie these cytokines might induce more prolonged activation of SERT *in vivo*), it is tempting to consider that the brevity of this effect reflects the capability of time-limited cytokine exposure to induce short-term reductions in 5-HT availability. Both TNF-α and IL-1β mRNA and protein are known to be present in specific CNS neuronal populations (Breder *et al*, 1988, 1993; Ignatowski and Spengler, 1994; Ignatowski *et al*, 1997), and receptors for these cytokines have differential brain regional distributions (Farrar *et al*, 1987; Takao *et al*, 1990; Ban *et al*, 1991; Kinouchi *et al*, 1991; Cunningham *et al*, 1992). Brief local exposure to these cytokines results in both documented neuronal and behavioral effects. IL-1β receptors are found in the medial hypothalamus, where they are colocalized with 5-HT-2C receptors (Hassanain *et al*, 2005). Microinjection of low concentrations of IL-1β into this region facilitates short-term defensive rage expression in the cat, an effect blocked by a 5-HT-2 antagonist (Hassanain *et al*, 2005). Brief *in vivo* exposure of orbitoprefrontal neurons to IL-1β is associated with either hyperpolarizing or depolarizing potentials in different neuronal populations (Lukáts *et al*, 2005). In guinea-pig brain slice preparations, IL-1β induces a rapid reduction in the firing rates of neurons in the serotonergic dorsal raphe nucleus (DRN; Manfridi *et al*, 2003), an area replete with IL receptors (Cunningham *et al*, 1992). The reduced firing rate is associated with a hyperpolarization of the neurons that is rapidly reversed with washout of IL-1β. Consistent with this effect, when microinjected into the DRN *in vivo*, IL-1β induces sleep without rapid eye movements (Manfridi *et al*, 2003), early in the sleep cycle.

Similarly, short-term exposure of hippocampal neurons to TNF-α (15–30 min) has been found to induce endocytosis of GABA-A receptors and increase trafficking of excitatory AMPA receptors to the plasma membrane (Stellwagen *et al*, 2005). TNF-α will also inhibit norepinephrine (NE) release in a manner dependent on alpha-2 adrenergic receptor activation (Ignatowski *et al*, 1997). In the aggregate, these findings suggest that short-term exposure to IL-1β or TNF-α induces rapid neuronal plasticity, independent of the effects associated with long-term exposure that might be seen in the setting of chronically elevated peripheral cytokines. These findings are consistent with the possibility that these cytokines, released locally, may act in the CNS as short-term neuromodulators that influence local synaptic 5-HT availability.

Irrespective of whether acute changes in SERT activity occur at the initial stages of prolonged cytokine elevations or are a result of shorter, acute fluctuations in CNS cytokine levels, our study provides first evidence that proinflammatory cytokines can alter SERT function acutely in preparations of serotonergic neuronal tissue, and further implicates a p38 MAPK-linked pathway in SERT regulation. Additionally, our synaptosomal findings suggest that functional p38 MAPK in distal terminals has the capability to regulate local 5-HT concentrations, independent of cellular processing occurring in the neuronal soma. Such a functional localization suggests a potential role for p38 MAPK in real-time modulation of neuronal activity at serotonergic terminals.

Recently, we established that multiple human SERT coding variants are refractory to p38 MAPK stimulation, effects also observed in primary lymphocytes derived from autistic probands (Prasad *et al*, 2005). Our studies suggest that CNS sites of SERT expression in these subjects would also lack flexibility for cytokine-triggered changes in 5-HT uptake. The detailed molecular mechanism of cytokine-induced SERT activity requires more study. Potential mechanisms may involve p38 MAPK-dependent alterations in phosphorylation states of SERT and associated proteins. Additional *in vivo* studies should be undertaken and may provide new perspectives on the relevance of cytokines for emotional cognitive processing, as well as the potential for the development of novel compounds that might target these signaling pathways as treatments for psychiatric disorders.

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