

Interleukin-1 Receptor Activation by Systemic Lipopolysaccharide Induces Behavioral Despair Linked to MAPK Regulation of CNS Serotonin Transporters

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Serotonin (5-hydroxytryptamine, 5-HT) has long been implicated in regulation of mood. Medications that block the neuronal 5-HT transporter (SERT) are used as major pharmacological treatment for mood disorders. Conversely, stimuli that enhance SERT activity might be predicted to diminish synaptic 5-HT availability and increase the risk for 5-HT-related CNS disorders. We have shown that the inflammatory cytokines enhance brain SERT activity in cultured serotonergic cells and nerve terminal preparations *in vitro*. In this study, we establish that intraperitoneal injection of the cytokine-inducer lipopolysaccharide (LPS) stimulates brain SERT activity, acting at doses below those required to induce overt motor suppression. SERT stimulation by LPS is paralleled by increased immobility in both the tail suspension test (TST) and the forced swim test (FST); antidepressant-sensitive alterations are thought to model aspects of behavioral despair. Both the stimulation of SERT activity and induced immobility are absent when LPS is administered to interleukin-1 receptor (IL-1R)-deficient mice and in the presence of SB203580, an inhibitor of IL-1R-stimulated p38 MAPK. Moreover, the ability of LPS to enhance immobility in TST is lost in SERT knockout mice. These findings reveal an ability of peripheral inflammatory stimuli to enhance brain SERT activity through IL-1R and p38 MAPK pathways *in vivo* and identify a requirement for SERT expression in immune-system-modulated despair behaviors. Our studies identify IL-1R- and p38 MAPK-dependent regulation of SERT as one of the mechanisms by which environmentally driven immune system activation can trigger despair-like behavior in an animal model, encouraging future analysis of the pathway for risk factors in neuropsychiatric disorders.

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INTRODUCTION

A substantial amount of literature links alterations in the immune system with psychiatric illness (Dantzer *et al*, 2008; Anisman, 2009; Howren *et al*, 2009; Dowlati *et al*, 2010). Viral and bacterial infections that stimulate the production of proinflammatory cytokines can produce symptoms of depression (Dantzer *et al*, 2008). Proinflammatory cytokines such as interleukin-6 (IL-6) (Levine *et al*, 1999),

tumor necrosis factor- α (TNF- α) (Levine *et al*, 1999; Tuglu *et al*, 2003), and variably IL-1 β (Owen *et al*, 2001; Diniz *et al*, 2010) have been found to be elevated in the blood of depressed patients, and LPS-stimulated IL-1 production in macrophages derived from depressed patients is elevated compared with that observed in control subjects (Maes, 1999). Depression associated with peripheral inflammatory cytokine activation has also been described in rheumatoid arthritis, cancer, and neurodegenerative diseases (Meijer *et al*, 1988; Hall and Smith, 1996; Yirmiya *et al*, 1999; Pollak *et al*, 2000). In subjects without a psychiatric history, lipopolysaccharide (LPS), a known inducer of proinflammatory cytokines, induces a depressed mood associated with serum elevations of IL-1 β (van den Biggelaar *et al*, 2007). Moreover, levels of depression, anxiety, and memory impairment in human volunteers after LPS administration are positively correlated with serum IL-1 β , as well as with TNF- α levels (Yirmiya *et al*, 2000). In animal models, treatments with either proinflammatory cytokines or

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cytokine inducers can elicit 'sickness behavior' that, like depression, is associated with listlessness, loss of appetite, sleep disruption, and loss of interest in physical and social activities (Dantzer *et al*, 2008; Yirmiya *et al*, 2000).

An expansive literature has proposed an important role of altered serotonergic neurotransmission in the origin, manifestations, and treatment of mood disorders, as well as other psychiatric illnesses (Lesch, 2001; Gaspar *et al*, 2003; Firk and Markus, 2007). The plasma membrane serotonin (5-hydroxytryptamine, 5-HT) transporter (SERT, *SLC6A4*) has received special attention as a genetic risk determinant in these disorders due to its central role in synaptic 5-HT inactivation (Caspi *et al*, 2003; Blakely *et al*, 2005; Vergne and Nemeroff, 2006). Genetic and functional variation in SERT is associated with psychiatric disorders, including alcoholism (Ait-Daoud *et al*, 2009), autism (Prasad *et al*, 2009), anxiety (Laucht *et al*, 2009), obsessive-compulsive disorder (OCD) (Ozaki *et al*, 2003), depression, and suicide (Segal *et al*, 2009). Inhibitors of SERT, the serotonin-selective reuptake inhibitors (SSRIs), represent a frontline pharmacological therapy for a number of these prevalent psychiatric conditions, including anxiety, depression, and OCD (Vaswani *et al*, 2003).

Ramamoorthy *et al* (1995) first provided a link between SERT and altered immune function, denoting the ability of prolonged IL-1 β treatment to elevate SERT mRNA and protein levels in cultured, placental-derived JAR cells. Mössner *et al* (1998, 2001) reported similar activities for TNF- α , IL-6 and IL-4. Using raphe neuron-derived RN46A cells and nerve terminal preparations, we established that both TNF- α and IL-1 β produce rapid catalytic activation of SERT, depending on p38 MAPK activation (Zhu *et al*, 2006). Samuvel *et al* (2005) also reported an important role for basal p38 MAPK activity in sustaining SERT surface expression. Together, these findings define the elements of a cytokine-modulated pathway for SERT activation having the potential to diminish extracellular synaptic 5-HT levels. To date, however, no reports describe the ability of systemic immune system activation to enhance brain SERT activity, nor do they tie such activation to alterations in behavior.

In this study, we examine the effect of systemic administration of the proinflammatory cytokine-inducer LPS on central SERT activity, monitored *ex vivo* in mouse brain synaptosomes and *in vivo* using chronoamperometry. Peripheral administration of LPS, an outer membrane component of Gram-negative bacteria, produces a rapid elevation of inflammatory cytokines, including IL-1 β , IL-6, and TNF- α (Loppnow *et al*, 1990; Dunn, 2006; Qin *et al*, 2008). These effects trigger a secondary wave of cytokine production in the CNS coincident with multiple neurophysiological and behavioral effects (Chen *et al*, 2005). As *in vitro* culture experiments and *ex vivo* synaptosomal studies reveal that SERT expression and/or activity can be modulated by inflammatory cytokines, we tested the critical question as to whether a peripheral inflammatory stimulus can modulate the brain SERT. We describe a time- and dose-dependent stimulation of SERT activity that is paralleled by behavioral changes in the tail suspension test (TST) and forced swim test (FST), frequently used to predict the efficacy of antidepressants. We also provide evidence that both the SERT activation and behavioral despair triggered by cytokine induction share the requirements

for IL-1 receptors (IL-1Rs), p38 MAPK activation, and intact SERT protein, as revealed using genetic and pharmacological approaches.

MATERIALS AND METHODS

Animals and Housing

Male C57BL/6 and CD1 mice (Harlan Sprague Dawley, Indianapolis, IN, 7–12 weeks), as well as IL-1R (Jackson Laboratories, Bar Harbor, ME) and SERT knockout mice (a gift by D Murphy, NIMH), both on a C57BL/6 background, were used in the experiments described. Animals were housed in AAALAC-approved facilities at either Vanderbilt University or at the University of Texas Health Science Center at San Antonio (UTHSCSA), with water and food provided *ad libitum*. All protocols used were approved by The Institutional Animal Care Use Committee at the Vanderbilt University or UTHSCSA.

Reagents

Lipopolysaccharide (LPS, *E. coli* serotype), interleukin-1 β (IL-1 β), paroxetine, fluoxetine hydrochloride, and SB202474 were purchased from Sigma Chemical (St Louis, MO). SB203580 was obtained from Calbiochem (La Jolla, CA). [3 H]5-HT (5-hydroxy[3 H]tryptamine trifluoroacetate, 107 Ci/mmol) and [3 H]NE (1-[7,8- 3 H]noradrenaline, 38 Ci/mmol) were purchased from Amersham Biosciences (Piscataway, NJ); [3 H]paroxetine, [3 H]DA (3,4-[7- 3 H]-dihydroxyphenylethylamine, 28 Ci/mmol) and [3 H]GABA (γ -[2,3- 3 H(N)]-aminobutyric acid, 35 Ci/mmol) were obtained from Perkin-Elmer (Boston, MA).

Synaptosomal Transport and Binding Assays

Mice were injected intraperitoneally (i.p.) with saline (vehicle), or with LPS, followed by preparation of crude brain synaptosomes (P2 fraction, hereafter termed synaptosomes) and assay of [3 H]5-HT, [3 H]NE, [3 H]DA, or [3 H]GABA transport as described previously (Zhu *et al*, 2006). In some experiments, SB203580 was injected i.p. 30 min before LPS i.p. injection to test the ability of systemic, pharmacological antagonism of p38 MAPK to suppress synaptosomal and behavioral effects. In some assays, LPS or SB203580 was applied *in vitro* to synaptosomes 10–15 min before transport assays to evaluate the potential for direct effects on the synaptosomal transport. Mice were killed by rapid decapitation at different time points after LPS treatment. Brain regions (midbrain, hippocampus, striatum, and frontal cortex) were homogenized in 0.32 M glucose using a Teflon-glass tissue homogenizer (400 r.p.m.) (Wheaton Instruments, Millville, NJ), followed by centrifugation at 800 g for 10 min at 4°C. Supernatants containing synaptosomes were transferred to 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 μ M pargyline, and 100 μ M ascorbic acid. Synaptosomal suspensions were analyzed for protein content using the Bio-Rad

protein assay (Bio-Rad). Synaptosomes (20–30 µg protein per sample, total volume 200 µl) were preincubated (10 min) at 37°C in a shaking water bath. [³H]5-HT (20 nM) was then added and incubated at 37°C for 5 min. Paroxetine (10 µM) was included in parallel assays to define non-specific 5-HT uptake. In some experiments, synaptosomes from LPS-treated animals were preincubated with SB203580 (2 µM), or with vehicle for 15 min on ice before the 10-min incubation at 37°C and subsequent 5-HT transport assay. In some assays, [³H]DA (50 nM), [³H]NE (50 nM), or [³H]GABA (50 nM) transport was also assessed (5 min at 37°C). Specific uptake was determined by subtracting the amount of [³H]DA, [³H]NE, or [³H]GABA accumulated by incubation on ice from the uptake detected at 37°C. Transport assays were terminated by filtration through GF/B Whatman paper using a Brandel Cell Harvester (Brandel, Gaithersburg, MD).

We used [³H]paroxetine binding assays to assess the total and surface density of SERT protein in synaptosomes as previously described (Zhu *et al*, 2005). For these binding assays, all procedures subsequent to resuspension of synaptosomes were performed on ice. Samples (20–30 µg protein per sample, total volume 200 µl) were preincubated on ice for 15 min with 1 × PBS/CM for total binding, 1 × PBS/CM containing 100 µM 5-HT for displacement of surface-bound [³H]paroxetine, or 1 × PBS/CM containing 100 µM fluoxetine hydrochloride to define the total specific binding. [³H]paroxetine (final concentration = 5 nM) was added and binding assays were terminated after 45 min by filtration. Filters were washed three times with ice-cold 1 × PBS buffer, immersed in scintillation liquid for 8 h, and counted by a scintillation spectrometer (Beckman, Fullerton, CA). All tests were performed in duplicate with three or more assays used to collect the mean values. Statistical tests were performed with Prism software (Graphpad).

***In Vivo* Chronoamperometry**

Recordings of exogenously applied 5-HT clearance were obtained from the CA3 region of the dorsal hippocampus (AP, −1.94 from bregma; ML, +2.0 from midline; and DV, −2.0 from dura) as previously described (Daws and Toney, 2007). Under the conditions used, basal 5-HT clearance is mediated primarily by SERT (Daws and Toney, 2007). Once reproducible 5-HT signals were obtained, saline or LPS was injected i.p. and 5 min later 5-HT was again ejected intrahippocampally and at 10-min intervals thereafter, until the 5-HT signal parameters returned to the levels obtained before drug injection. Recording sites were verified post-mortem by histological analysis of hippocampal sections. Details of the methods can be found in the Supplementary Methods.

Behavioral Analysis

Animals were housed under conditions of a 12-h light–dark cycle (lights switched on from 0800 to 2000 hours), constant temperature (23–25°C), and with food and water *ad libitum*. All behavioral tests in the chambers for open-field and tail suspension testing were conducted under an illumination of 230–250 Lux starting at 0900 hours.

Open-Field Test

Open-field testing was conducted in eight identical commercially available activity monitors measuring 27 × 27 cm (MED Associates, Georgia, VT) at the Laboratory for Neurobehavior, Center for Molecular Neuroscience. Each apparatus contained 16 photocells in each horizontal direction and 16 photocells elevated by 4.0 cm to measure the rearing. Beam breaks were recorded and analyzed automatically by a Windows-based microcomputer using MED Associates software. At varying times after drug injection, mice were placed individually in the activity monitors and were allowed to explore freely. Total activity was calculated by summing the horizontal and vertical movements. An ambulatory episode was defined by the software as a bout of activity in which the mouse left a 2 × 2 beam area, broke three or more beams, and then rested for 2 s. Active episodes were calculated by adding ambulatory episodes to vertical beam breaks. Repetitive beam breaks were the breaks that occurred without leaving a 2 × 2 beam area. The periphery of the activity monitor was defined as the area within four beams of the wall.

Tail Suspension Test

At varying times of post i.p. injection of saline or LPS, mice were suspended from the edge of a shelf, 80 cm above the floor, by their tails. Tails were secured to the shelf by an adhesive tape placed approximately 1 cm away from the tip of the tail. Each trial was conducted for a period of 6 min, and the duration of immobility during this period was recorded. Immobility was measured using the program PORSOLT (Infallible Software, Rockville, MD). Mice were considered immobile only when they hung passively and were completely motionless.

Forced Swim Test

Mice were placed in transparent cylindrical 3-l glass beakers (height 25 cm, internal diameter 12 cm) containing water (24–25°C) to a level of 15 cm. The water was changed after each mouse was tested. The procedure consisted of a pretest exposure followed by the experimental test 24 h later. During the pretest, animals were acclimated to the test facility for not less than 1 h. They were subsequently placed into the test cylinders for 6 min. After this initial exposure, the animals were dried for 15 min and then returned to their home cages. The next day, the animals were transferred into the experimental room and allowed to acclimate for at least 1 h. Animals were administered the test compounds (i.p. saline or LPS 60 min before testing) and placed in the test cylinders. The behavior of animals was measured during a 6-min period by an observer blind to the treatment conditions. A mouse was considered immobile when floating motionless or making only sufficient movement to keep its head above water. The time spent immobile during the 6-min test period was measured in seconds.

Body Temperature Assessment

Mice were injected i.p. with saline or LPS and body temperature was monitored using BAT-12 Microprobe

Thermometer (Physitemp Model Bat-12, Clifton, NJ) at constant room temperature (23.2–23.8°C). The temperature was recorded up to 2 h after injection.

Statistical Analyses

All experiments were performed at least three times. Statistical analyses, comparing baseline and drug-induced changes in uptake/clearance or animal behavior, 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

Synaptosomal 5-HT transport is Elevated Following Systemic Injection of LPS

C57BL/6 mice were injected with LPS (0.2 mg/kg i.p.) and midbrain synaptosomes were assayed for SERT activity at different time points over a 120-min interval post injection. As shown in Figure 1a, LPS induced a time-dependent stimulation of SERT activity, with a significant and stable response of ~40% over that of the saline control achieved at 60 min ($p < 0.05$, one-way ANOVA). Dose-response analysis (0.02–5.0 mg/kg) of LPS modulation of 5-HT transport, performed at 60 min post injection, demonstrated

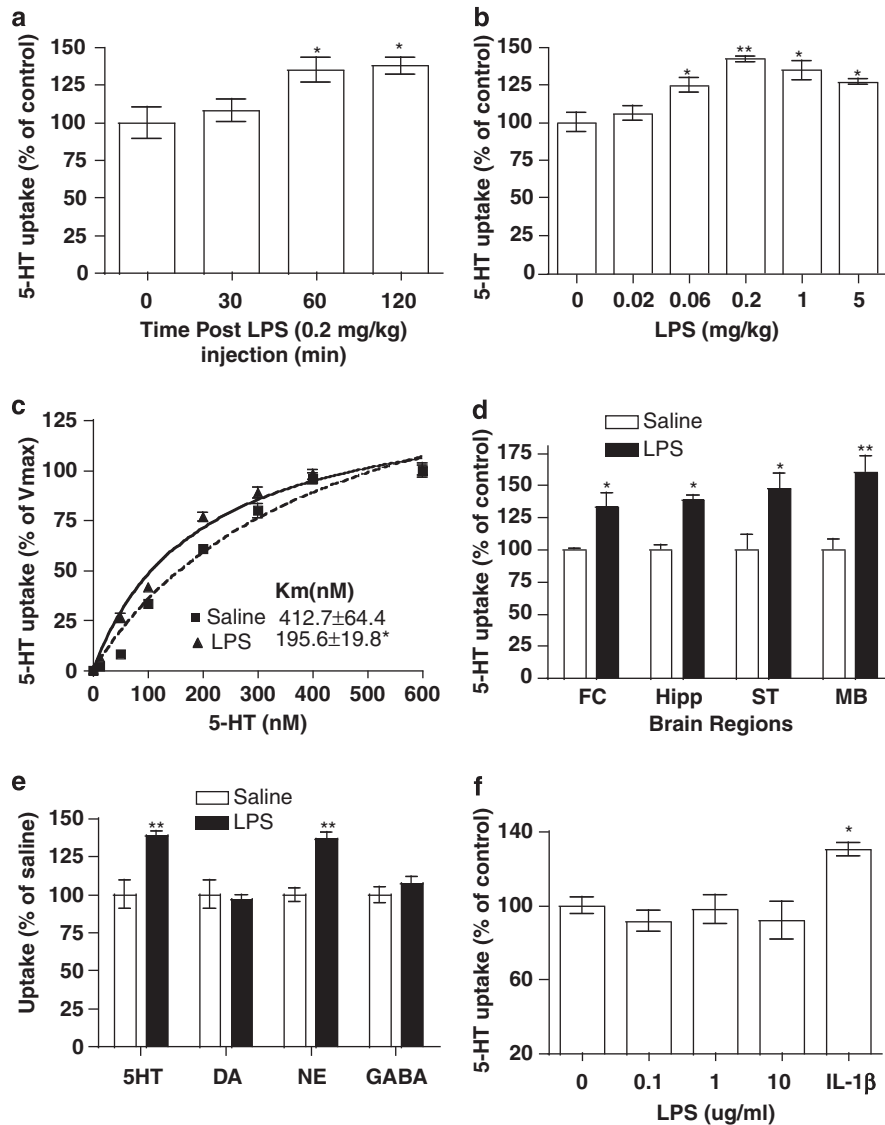


Figure 1 Peripheral injection of LPS induces 5-HT uptake in mouse brain. (a) Course of time of LPS effects on midbrain 5-HT uptake. At the indicated times following i.p. injection of 0.2 mg/kg LPS, midbrain synaptosomes from C57BL/6 mice ($n = 3$ for each time point) were assessed for 5-HT uptake as described in Materials and methods. (b) Dose-response of LPS on midbrain 5-HT uptake, assayed 60 min after i.p. LPS at the indicated doses ($n = 3$ for each dose). (c) Saturation kinetic analysis of LPS stimulation. C57BL/6 mice were injected with saline or LPS (0.2 mg/kg i.p.) and 5-HT uptake was conducted 1 h after treatment ($n = 6$). V_{max} for saline- and LPS-treated animals were 48.9 ± 4.1 and 44.4 ± 1.8 fmol/min per mg protein, respectively, and the K_m for saline and LPS treated mice were 412.7 ± 64.4 and 195.6 ± 19.8 nM, respectively. * $p < 0.05$ saline vs LPS. (d) Effect of LPS on 5-HT uptake in different brain regions, assayed 60 min after LPS (0.2 mg/kg i.p.). FC = frontal cortex, Hipp = hippocampus, ST = striatum, MB = midbrain ($n = 3$ for each condition). (e) Neurotransmitter transport specificity of peripheral LPS assayed using midbrain synaptosomes ($n = 3$ for each condition). (f) Lack of effect of direct application of LPS on midbrain 5-HT transport. IL-1 β (10 ng/ml) or LPS (0.1–10 μ g/ml) was incubated for 10 min at 37°C before 5-HT transport assays ($n = 3$). * $p < 0.05$; ** $p < 0.01$ vs vehicle control.

significant SERT stimulation at doses of 0.06 mg/kg and above, with a maximal effect detected at 50% above saline control levels (Figure 1b, $p < 0.05$, one-way ANOVA). Similar time and dose-response findings were obtained with the outbred line, CD1 (Supplementary Figure S1A and B). Kinetic analysis revealed that these increases in SERT activity were due to a decrease in K_m . The V_{max} was not significantly changed (Figure 1c). Our binding studies confirmed that the LPS-induced SERT activity was not associated with increases in total or plasma membrane-associated SERT, as neither total, nor 5-HT displaceable (measured at 4°C) specific [3H]-paroxetine binding in synaptosomes was altered by the systemic LPS treatment (Supplementary Figure S2).

Using the optimal conditions established above for LPS stimulation of midbrain SERT (0.2 mg/kg, 60 min), we evaluated the regional dependence and the neurotransmitter specificity of LPS effects. We detected significantly enhanced SERT activity in synaptosomes from all the brain regions tested (frontal cortex, hippocampus, striatum, and midbrain, Figure 1d). To evaluate whether transporter stimulation by LPS is unique to SERT, we evaluated the transport of radiolabeled NE, DA, and GABA in parallel with 5-HT in midbrain synaptosomes. No stimulation of DA or GABA transport was observed after LPS treatment (Figure 1e). Interestingly, NE transporter (NET) activity was stimulated to a degree comparable to that of SERT (Figure 1e). These findings demonstrate neurotransmitter specificity for LPS effects, and argue against a global alteration in membrane potential or ion gradients. LPS is also unlikely to exert its effects on SERT directly, as we found that midbrain synaptosomes treated with LPS (0.1–10 μ g/ml, 10 min) demonstrated no effect on 5-HT uptake (Figure 1f), while the inflammatory cytokine IL-1 β (10 ng/ml, 10 min) produced significant SERT activation ($p < 0.05$, one-way ANOVA), as previously reported (Zhu *et al*, 2006). Finally, as 5-HT uptake can be supported by other mechanisms (eg, OCT3, Schmitt *et al*, 2003; Baganz *et al*, 2008), we tested whether midbrain synaptosomes derived from SERT knockout mice demonstrate an increase in 5-HT uptake following peripheral LPS injections. As expected, no alteration in 5-HT uptake was seen (Figure 2a), whereas a significant increase in NE uptake was detected ($p < 0.05$, one-way ANOVA), similar to that seen in wild-type animals.

p38 MAPK and IL-1 Receptors Have an Essential Role in LPS Stimulation of SERT

Previously, we demonstrated that IL-1 β and TNF- α stimulation of synaptosomal SERT activity *in vitro* is mediated through the activation of p38 MAPK (Zhu *et al*, 2006). As LPS induces these proinflammatory cytokines, we examined the requirement for p38 MAPK in SERT modulation using systemic and synaptosomal treatments with SB203580. In initial studies, C57BL/6 mice were injected with SB203580 (50–1350 μ g/kg i.p.) to establish a dose of the drug that lacked basal effects in midbrain synaptosomal 5-HT transport assays 60 min post injection. As shown in Figure 2b, SB203580 at 50 μ g/kg i.p. had no effect on basal 5-HT transport. Only at doses of 150 μ g/kg and above was the SERT activity inhibited in a dose-

dependent manner. When next we preinjected (30 min) LPS-treated animals with SB203580 at 50 μ g/kg, we detected a full suppression of LPS-induced SERT stimulation (Figure 2c). Remarkably, when synaptosomes derived from LPS-injected animals were directly treated with SB203580 at a dose without any effects on baseline SERT uptake (2 μ M, 15 min), the LPS-induced SERT activation was lost (Figure 2d), suggesting a requirement for continued p38 MAPK activation in maintaining an elevated SERT activity. Similar findings were obtained using synaptosomes prepared from CD-1 mice (Supplementary Figure S3A and B).

As an IL-1 receptor (IL-1R) antagonist blocks p38 MAPK-dependent SERT stimulation induced by IL-1 β *in vitro* (Zhu *et al*, 2006), we asked whether peripheral LPS stimulation of CNS SERT is lost in IL-1R knockout mice (C57BL/6 background). We found that not only was IL-1 β ineffective in stimulating SERT in midbrain synaptosomes of IL-1 R knockout animals *in vitro* (Figure 2f), but the systemic LPS administration also showed no ability to stimulate SERT activity in these knockout mice (Figure 2e). Together, these findings reveal an essential requirement for IL-1R and p38 MAPK signaling pathways in the modulation of CNS SERT by LPS.

LPS Enhances Hippocampal 5-HT Clearance *in vivo*

To establish the ability of LPS to modulate CNS SERT activity *in vivo*, we monitored SERT-mediated 5-HT clearance before and after i.p. LPS injection via chronoamperometric recordings in the CA3 region of hippocampus of anesthetized mice (Daws and Toney, 2007). As shown in Figures 3a–c, i.p. injection of saline was without a significant effect on 5-HT clearance time (T_{80}) across the 85-min recording period. In contrast, LPS injection reduced the T_{80} (faster clearance) in a time-dependent manner, first becoming significant at 25 min post injection. The onset of LPS-enhanced-5-HT clearance occurred more rapidly than that observed with synaptosomes and may reflect the less invasive nature of the *in vivo* analyses. Regardless, these data provide critical support for the contention that peripheral immune system activation can effect a significant and relatively rapid enhancement of brain 5-HT clearance *in vivo*.

Immobility Effects of LPS Require p38 MAPK, IL-1R, and SERT Expression

Inflammatory cytokines and their inducers produce depression-like states in animals (Maes, 1999; Raison *et al*, 2006; Dantzer *et al*, 2008). We therefore sought to assess the SERT dependence of LPS-induced behavioral effects. The tail suspension test (TST) and forced swim test (FST) are models where cessation of struggling (immobility) is analogized to 'behavioral despair'. These tests are sensitive to pharmacological manipulations, providing a screen for antidepressant medications (Cryan *et al*, 2005; Dunn and Swiergiel, 2005). They can be assessed over the same time frame as our observations of SERT stimulation. As the TST and FST paradigms are dependent on a motor readout, drug treatments that compromise the overall motor activity confound interpretations (Cryan *et al*, 2005). Therefore, we first obtained the dose-response relationship for LPS treatment in C57BL/6 mice using an open-field assessment

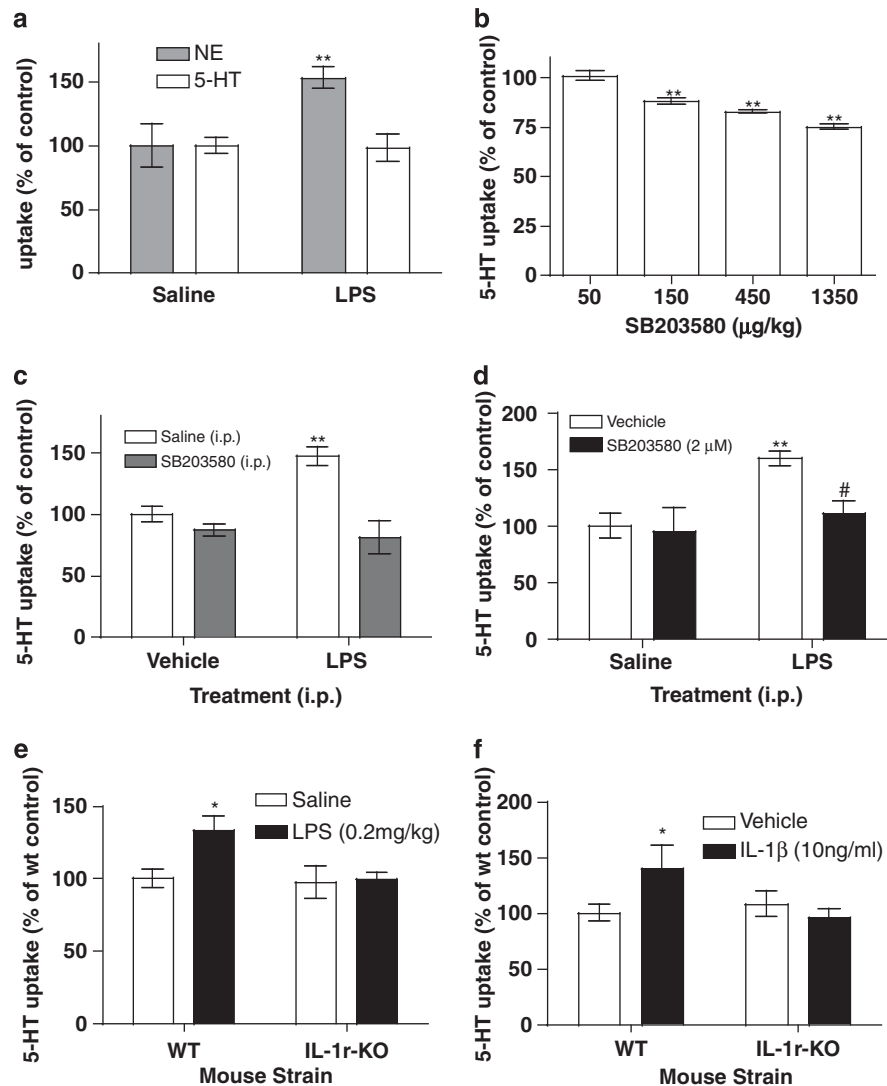


Figure 2 Evaluation of p38 MAPK and IL-1R in basal and LPS-stimulated 5-HT transport. (a) Loss of LPS (0.2 mg/kg, 60 min) effect on 5-HT, but not NE uptake in SERT knockout mice ($n=3$ for each condition). (b) SB203580 (doses as indicated) was injected i.p. in C57BL/6 mice followed by midbrain synaptosomal 5-HT uptake assays 60 min after injection ($n=3$). (c) Vehicle or SB203580 (50 µg/kg i.p.) was administered to mice 30 min before LPS injection. 5-HT transport was monitored 60 min after LPS (0.2 mg/kg) treatment ($n=3$). (d) Synaptosomes from LPS-treated C57BL/6 mice were preincubated with SB203580 (2 µM) for 15 min followed by a 5-min 5-HT uptake assay ($n=3$ for each condition). (e) Wild-type (C57BL/6) and IL-1R knockout mice were injected with vehicle or LPS (0.2 mg/kg). Midbrain synaptosomal 5-HT uptake assay was conducted 1 h after injection ($n=3$ for each). (f) Synaptosomes were prepared from midbrains of wild-type or IL-1R knockout mice. Vehicle or IL-1β (10 ng/ml) was incubated with synaptosomes for 10 min at 37°C, followed by a 5-min ^3H -5HT uptake assay ($n=3$). * $p<0.05$; ** $p<0.01$ vs vehicle control; # $p<0.05$ vs LPS.

of spontaneous locomotion. Compared with saline treatment, no significant impact on open-field activity (both horizontal and vertical movement) was observed at LPS doses at or below 0.2 mg/kg within a 240-min post injection testing interval (Figure 4a; data on vertical activity not shown), whereas higher doses produced a significant reduction in the locomotor activity that persisted up to 48 h after administration. Similarly, 0.2 mg/kg LPS induced no significant changes in body temperature (Supplementary Figure S4A). Consistent with the findings of others (Dunn and Swiergiel, 2005; Swiergiel and Dunn, 2007), when C57BL/6 mice (or CD1 mice, Supplementary Figure S4B–F) were treated with LPS (0.2 mg/kg i.p.), they showed significantly increased immobility (reduced struggling) relative to saline-injected animals by 60 min post LPS injection (Figures 4b and f). As with stimulation of SERT,

the systemically injected p38 MAPK inhibitor, SB203580, blocked the ability of LPS to promote immobility (Figures 4c and f). Finally, as the ability of LPS to alter midbrain 5-HT transport activity is lost in the IL-1R and SERT knockout mice, we tested these mice for their behavioral sensitivity to LPS. Neither the IL-1R knockout (Figure 4d) nor the SERT knockout animals (Figure 4e) showed changes in TST immobility at a dose (0.2 mg/kg, 60 min) of LPS that significantly increased immobility in wild-type mice.

DISCUSSION

Alterations of the native immune system have been proposed as potential risk factors for multiple neuropsychiatric

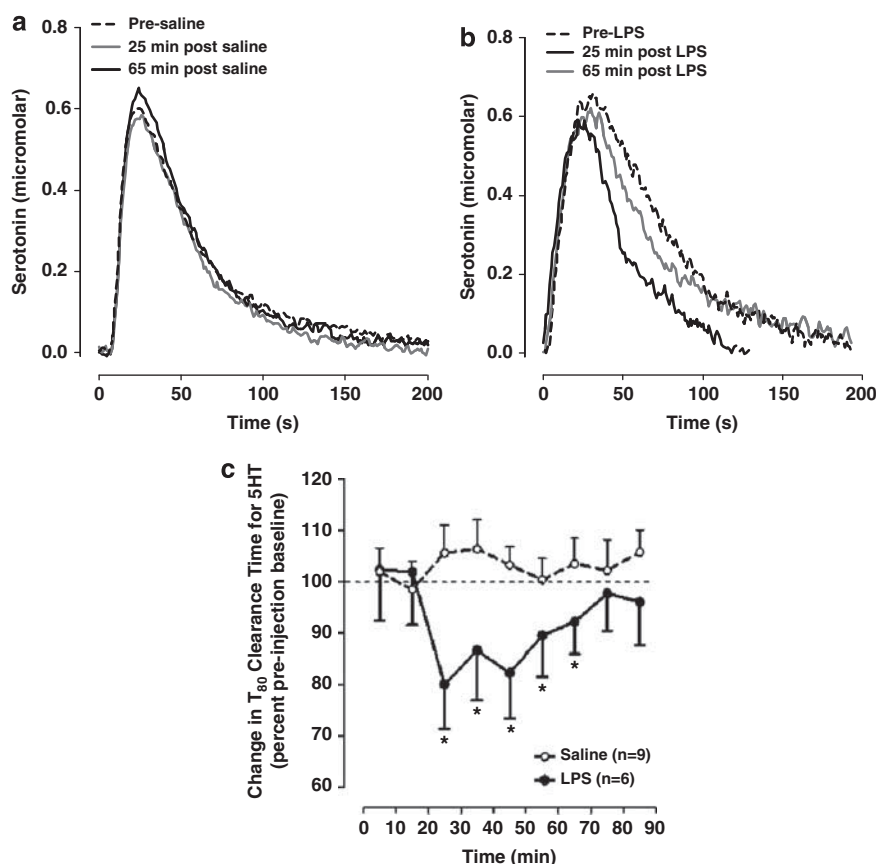


Figure 3 Effect of LPS (0.2 mg/kg) on 5-HT clearance time in mouse hippocampus. (a) Representative recording of hippocampal 5-HT clearance pre saline and post saline injection (i.p.). (b) Representative recording of hippocampal 5-HT clearance pre and post LPS injection (0.2 mg/kg, i.p.). (c) Time dependence of saline or LPS (0.2 mg/kg i. p.) effects on hippocampal 5-HT clearance. Points represent mean \pm SEM of results from nine animals (saline) and six animals (LPS). * $p < 0.05$ vs saline.

disorders (Dantzer *et al*, 2008; Anisman, 2009; Maes, 1999). The mechanisms by which peripheral inflammatory processes modulate behavior are only beginning to be revealed (Dantzer *et al*, 2008; Raison *et al*, 2006). Behavioral changes triggered by immune system activation in humans overlap with the features of mood disorders linked to disrupted 5-HT signaling, and are treatable with SSRI medications (Yirmiya *et al*, 2001; Castanon *et al*, 2003). In humans, injection of LPS or interferons produces behavioral changes that resemble aspects of major depression (Dantzer *et al*, 2008; van den Biggelaar *et al*, 2007). In animal models, such treatments elevate both the circulating and CNS levels of proinflammatory cytokines, including IL-1 β (van den Biggelaar *et al*, 2007; Qin *et al*, 2008), and produce a suite of behavioral alterations often termed as 'sickness syndrome' (Dantzer *et al*, 2008). The degree to which altered 5-HT signaling and/or SERT contributes to these behaviors is unknown. Based on our previous findings that *in vitro* treatments of synaptosomes and raphe cells with IL-1 β and TNF- α rapidly stimulates SERT activity (Zhu *et al*, 2006), we sought to determine whether changes in brain SERT activity could arise from an inflammatory process of peripheral origin.

Peripheral injections of LPS induced a dose- and time-dependent alteration in SERT activity, measured *ex vivo* in synaptosomes from both C57BL/6 (inbred) and CD-1 (outbred) mice. The fact that LPS can stimulate SERT in

the outbred strain suggests that the effect is robust over a wide variety of mouse haplotypes. LPS treatment appears to increase one or more aspects of SERT catalytic functioning, as we observed no alterations in the total or surface SERT-specific binding in synaptosomes from LPS-treated animals (see also Zhu *et al*, 2005, 2006). These results are consistent with our findings that *in vitro* treatment of synaptosomes with IL-1 β increases the apparent affinity of SERT for 5-HT (Zhu *et al*, 2006), but does not induce SERT trafficking. Although LPS, injected centrally, produces a number of neurochemical and behavioral alterations (Gottschall *et al*, 1992; Plata-Salamán and Borkoski, 1993), no change in SERT activity occurs when LPS is directly applied to synaptosome preparations, consistent with the need for an LPS-induced factor to stimulate SERT activation. Systemic pretreatment with the p38 MAPK inhibitor SB203580 at a dose without any effects on synaptosomal 5-HT uptake abolishes SERT stimulation induced by LPS. In addition, we found that LPS-induced SERT activation was lost in synaptosomes treated with SB203580, demonstrating a need for local, ongoing synaptic p38 MAPK activation to sustain the enhanced serotonin uptake.

We found that LPS-induced uptake was neurotransmitter selective as synaptosomal GABA and DA transport activities were unaffected by LPS treatment, limiting concerns that nonspecific alterations in thermodynamic factors (eg Na⁺ gradient and membrane potential) might be responsible for

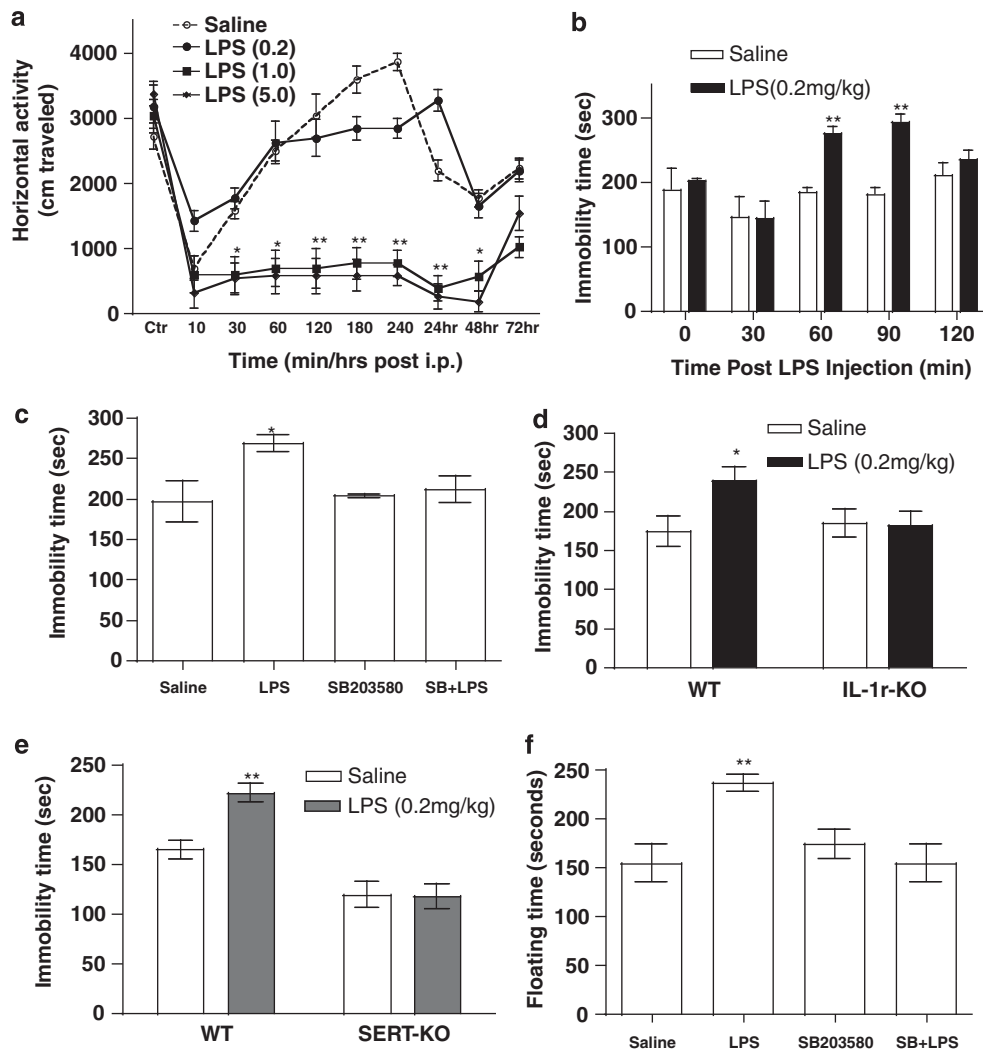


Figure 4 SERT, IL-1R, and p38 MAPK-dependent behavioral effects of LPS. (a) C57BL/6 mice were injected with saline ($n=8$) or LPS (0.2, 1.0, and 5.0 mg/kg i.p., $n=6$ for each dose). Total activity was monitored during the indicated time. Total activity of mice tested 20 min before treatment served as the control condition. Total activity at 24, 48, and 72 h time points was measured for 20 min. * $p<0.05$; ** $p<0.01$ vs saline control. (b) Time-response of LPS in TST. Saline or LPS (0.2 mg/kg i.p.) was injected to C57BL/6 mice and the TST was conducted at the indicated time points ($n=6$ for each condition). (c) C57BL/6 mice were injected with saline, LPS (0.2 mg/kg), SB203580 (50 μ g/kg), or SB203580 (50 μ g/kg) 10 min before LPS (0.2 mg/kg). TST was conducted 60 min after the last injection ($n=6-10$). (d) C57BL/6 wild-type or IL-1R intact or IL-1R knockout mice (all with C57BL/6 background) were administered saline or LPS (0.2 mg/kg i.p.). TST was conducted 60 min after the last injection ($n=6$ for each condition). (e) C57BL/6 wild-type or SERT knockout (KO) mice were administered saline or LPS (0.2 mg/kg i.p.). TST was conducted 60 min after the last injection ($n=6$ for each condition). (f) C57BL/6 mice were injected with saline, LPS (0.2 mg/kg), SB203580 (50 μ g/kg), or SB203580 (50 μ g/kg) 10 min before LPS (0.2 mg/kg). FST was conducted 60 min after the last injection ($n=6$ for each condition).

the elevated transport function. Interestingly, we found midbrain NET activity was also stimulated by peripheral LPS. NET, like SERT, can be stimulated in a trafficking-independent manner by p38 MAPK-linked pathways (Apparsundaram *et al*, 2001; Zhu *et al*, 2005). Peripheral LPS is also known to produce an activation of CNS noradrenergic pathways, and adrenergic receptors (Guo *et al*, 1996) have been implicated by an induction of fever from LPS (Bencsics *et al*, 1995). At the dose of LPS used in our studies (0.2 mg/kg), no induction of fever was evident over the course of time in our studies. Finally, we extended our *in vitro* studies to an *in vivo* demonstration that LPS induces enhanced rates of 5-HT clearance in the hippocampus of anesthetized mice. Consistent with these studies, Katafuchi *et al* (2005) monitored reductions in extracellular

5-HT in the medial prefrontal cortex using microdialysis, beginning 1–2 h after the systemic immune system stimulation with poly I:C, another inflammatory cytokine inducer and viral mimic. Significantly, in the latter study, the reduction in dialysate 5-HT levels could be reversed by local microinjections of the SERT inhibitors imipramine or fluoxetine. In our preliminary studies with poly I:C, we found that this proinflammatory agent, which produces cytokine stimulation through mechanisms distinct from those seen with LPS, demonstrates similar effects on SERT activity.

Our findings of SERT regulation can be readily integrated into the growing body of literature that links the immune system to 5-HT signaling. For example, peripheral LPS rapidly induces c-fos activation in CNS serotonergic

neurons (Hollis *et al*, 2006), and alters the 5-HT release and turnover (Lavicky and Dunn, 1994). With chronic LPS treatments, indoleamine 2,3 dioxygenase (IDO), an enzyme that metabolizes the 5-HT precursor tryptophan, is induced (O'Connor *et al*, 2009). SSRIs block the behavioral effects of immune system activation in humans and animal models (Yirmiya *et al*, 2001; Castanon *et al*, 2003). Interestingly, SERT and 5-HT receptors also contribute to immune system activation (Guo *et al*, 1996), reinforcing the idea that 5-HT and immune signaling pathways are reciprocally connected to provide for coordinated responses to environmental challenges.

To obtain evidence for the role of SERT in LPS-induced behavioral changes, we used two antidepressant-sensitive measures, the TST and FST, that monitor the animal's willingness to sustain struggling in the face of an inescapable stressor. We found that mice injected with a dose of LPS that does not induce overt motor effects or fever produced enhanced immobility in the TST and FST. LPS increased immobility in both the inbred (C57BL/6) and outbred (CD-1) strains. Moreover, the time dependence and dose-response of LPS's effects on TST immobility closely paralleled with those found for SERT activation. SB203580, only at doses that blocked LPS-induced synaptosomal SERT activation, abolished LPS-induced immobility, potentially illuminating a novel pharmacological mechanism by which behavioral despair might be overcome. The effect of LPS on immobility was not seen in SERT KO mice. All of these findings are consistent with the requirement for and involvement of SERT in the observed despair behavior induced by this proinflammatory stimulus. As antidepressants that block SERT have effects on the immune system, one might argue that the SERT KO background limited the ability of LPS to induce CNS cytokines in these animals. However, we observed that these knockout mice displayed an LPS-induced stimulation of midbrain NET activity similar to that observed in wild-type mice, suggesting that the lack of a behavioral effect in the knockout was more likely due to the inability of LPS-stimulated cytokines to increase in SERT activity in the mutant's CNS. In addition, although many antidepressants target both SERT and NET together, or NET alone, the normal response of NET to LPS treatment in these mice would suggest that LPS-induced behavioral despair cannot be induced by reductions in central noradrenergic neurotransmission alone, and points to the changes in 5-HT neurotransmission as a critical event in immune-related depressive behavior.

LPS is known to increase CNS levels of IL-1 β , TNF α , and IL-6, and we have shown that both IL-1 β and TNF α can stimulate synaptosomal SERT activity in a p38 MAPK-dependent manner. In this study, we demonstrate that the LPS-induced increase in SERT activity is dependent on the expression of the IL-1R receptor. Similarly, we found that LPS was unable to induce immobility in the IL-1R knockout mouse at a dose that was effective in the wild-type mouse. Consistent with these findings, Minor *et al* (2006) have reported that i.c.v. administration of the IL-1 receptor antagonists (IL-1ra) blocks LPS-induced immobility in the FST. Additionally, multiple groups have shown that IL-1ra blocks LPS-induced reductions in social interaction tests (Konsman *et al*, 2008; Arakawa *et al*, 2009), mimicking the effects of antidepressant medications in these paradigms

(Yirmiya, 1996; Castanon *et al*, 2001). Taken together, our findings are consistent with the suggestion that LPS was unable to induce midbrain IL-1R-mediated stimulation of SERT in the IL-1R knockouts, and that lack of this central p38 MAPK-dependent SERT activation limited the expression of behavioral despair in response to LPS in these animals.

We do not presume that changes in SERT activity alone are sufficient to induce the full spectrum of depression traits, nor that our animal model can reproduce all the elements of a complex neuropsychiatric disorder. Furthermore, it is clear that activation of IL-1R can be only one component of a complex interaction of neuronal systems involved in the expression of depressive-like behavior in animal models. We focused here on an acute LPS treatment paradigm to constrain the number of variables underlying the biochemical and behavioral changes. Nonetheless, we were able to identify the mechanisms that could be viewed as precipitating events in a chain of circumstances involved in depressive illness. If sustained, augmented, or autonomously engaged, such events could support more profound behavioral changes. Indeed, the relatively rapid changes in SERT activity we observe following immune system activation could serve as triggers for long-lasting, cytokine-related reductions in extracellular 5-HT levels that are later supported by elevations in SERT mRNA and protein expression (Ramamoorthy *et al*, 1995; Katafuchi *et al*, 2006), and depletion in tryptophan levels through induction of metabolic pathways (Capuron and Dantzer, 2003). In this regard, IL-1R activation has been implicated in depressive behavior induced through chronic stress (Koo and Duman, 2008, 2009; Goshen *et al*, 2008) and learned helplessness paradigms (Maier and Watkins, 1995), consistent with the idea that depression can arise as a perturbation of affective CNS networks by endogenous cytokines released in response to uncontrollable stress. Additional studies that focus on the long-term effects of immune system activation on SERT and SERT-dependent behaviors are needed to provide a more complete understanding of the relationships between immune system activation, central IL-1 receptor activation, p38-mediated activation of SERT, and the elaboration of depressive behavior in animal models. In addition, a more comprehensive evaluation of genetic variation in this pathway might suggest sources of genetic risk for affective disorders (Müller and Schwarz, 2007; Koo and Duman, 2009), and lead to improved options for treating human depressive illness.

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DISCLOSURE

The authors declare no conflict of interest.

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