Original Article

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Lipopolysaccharide-induced serotonin transporter up-regulation involves PKG-I and p38MAPK activation partially through A3 adenosine receptor

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Summary **Serotonin transporter (SERT) is a critical determinant of synaptic serotonin (5-hydroxytryptamine, 5-HT) inactivation which plays a critical role in the pathology of depression and other mood disorders. Lipopolysaccharide (LPS), a potent activator of the inflammatory system, has been reported to cause depression symptoms by the modulation of SERT *in vivo* and *in vitro*. This study is aimed to investigate the underlying mechanism of LPS-induced SERT modulation. The 4-(4-(dimethylamino) styryl)-N-methylpyridinium iodide (ASP) assay was used to detect dynamic 5-HT uptake as read out of SERT activities in RBL-2H3 cells, and cytosol Ca^{2+} concentrations ($[Ca^{2+}]i$) and nitric oxide (NO) were examined. Using specific cyclic GMP-dependent protein kinase type I (PKG-I), p38 mitogen-activated protein kinases (p38MAPK) and A3 adenosine receptor (A3AR) inhibitors, SERT expression was evaluated by western blot and immunofluorescence analysis. Results showed that 24 h treatment with LPS stimulated 5-HT transport and up-regulate plasma membrane distribution of SERT in RBL-2H3 cells. LPS treatment increased NO and $[Ca^{2+}]i$, and led to significant increases in levels of phosphorylated calcium/calmodulin-dependent protein kinase type II (CaMK-II), inducible NOS (iNOS) and PKG-I as well as active p38 MAPK. Moreover, PKG-I inhibitor KT5823 or p38MAPK inhibitor SB203580 respectively impaired SERT activation and transposition to plasma membrane by LPS. Notably, A3 adenosine receptor inhibitor MRS1191 also hindered SERT stimulation by LPS. In conclusion, LPS-induced 5-HT uptake and transposition to plasma membrane of SERT in RBL-2H3 cells involves CaMK-II/iNOS/PKG-I and p38 MAPK activation, which may be partially mediated by A3 adenosine receptor activation. This finding provides a novel insight into the interrelationship between LPS and depression.**

Keywords: Serotonin transport (SERT), lipopolysaccharide, cyclic GMP-dependent protein kinase type I (PKG-I), p38 mitogen-activated protein kinases (p38MAPK), A3 adenosine receptor

1. Introduction

Serotonin (5-hydroxytryptamine, 5-HT) is a neurotransmitter which plays a critical role in the pathology of depression following its binding to specific 5-HT receptors and their downstream signalling cascade (*1*). The serotonin transporter (SERT) is a critical determinant of synaptic 5-HT inactivation and an important target molecule for the antidepressant drugs including selective serotonin reuptake inhibitors (SSRIs) (*2,3*). When these drugs bind to the SERT, they inhibit its function, thereby blocking 5-HT uptake from the synapse and consequently enhancing synaptic 5-HT concentration. Their delayed effect in remission of patients suggests that it is not simple rapid blockade *per se*, but rather additional mechanisms regulating

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SERT function that may underlie the eventual clinical improvement after prolonged exposure to antidepressant drugs (*4*). Although the entire mechanism responsible has not been elucidated, SERT is subject to multiple posttranslational regulations that can rapidly alter 5-HT uptake and clearance rates. Specific cell surface receptors as well as pathways activating protein kinase C (PKC), protein kinase G (PKG) and p38 mitogen activated protein kinase (p38MAPK) regulating SERT trafficking and catalytic function are now well established and received greater attention (*5*). Zhu *et al*. revealed two PKG-dependent pathways supporting rapid SERT regulation by A3 adenosine receptor (A3AR), one leading to enhanced SERT surface trafficking, and a separate, p38 MAPK-dependent process augmenting SERT intrinsic activity, demonstrating mechanistic links between the A3AR and SERT (*6*). Remarkably, disease-associated alterations in SERT not only implicate SERT activity but also impact SERT regulatory pathways.

Although the etiology of depression is complex and remains unknown, there is a growing body of evidence that depressed subjects display an elevation of pro-inflammatory cytokines, and inflammation plays an important role in the development of depression (*7*). Viral and bacterial infections that stimulate the production of pro-inflammatory cytokines can produce symptoms of depression (*8*). The antidepressant-sensitive SERT can be regulated by pro-inflammatory cytokine signaling. Zhu and co-workers have shown that the inflammatory cytokines interleukin-1β (IL-1β) and tumor necrosis factor-α (TNF-α) produce rapid catalytic activation of SERT in cultured 5-HT neurons-derived RN46A cells and mouse nerve terminal preparations *in vitro* (*9*). Lipopolysaccharide (LPS) is a component of Gram-negative bacteria outer membrane, which acts as a potent activator of the inflammatory system. It is reported that LPS induces depression symptoms associated with elevations of serum IL-1β in subjects without a psychiatric history (*10*). It has been demonstrated that LPS binds to toll-like receptor 4 (TRL4) leading to the rapid systemic release of pro-inflammatory cytokines and induces anhedonia in rats and mice (*11*). Most remarkably, Zhu *et al*. found that peripheral activation of the innate immune system with LPS leads to a rapid (1 h) stimulation of central nervous system (CNS) SERT activity, accompanied by an acceleration of 5-HT clearance rate and alterations in SERT-dependent behaviors (*12*). In contrast, another study on the human enterocyte-like Caco-2 cell line have shown that LPS treatment diminished SERT activity and SERT protein level on brush border membrane, and the LPS effect might be due to an alteration of the intracellular traffic of SERT partially mediated by protein kinase C (PKC) activation (*13*). Therefore, more investigation is needed to provide more information about the effects of LPS on SERT activity and expression.

Using cells derived from rodent mast cells RBL-2H3, the aim of this work was to study the effect of LPS on SERT activity and expression and to determine the intracellular mechanism underlying this effect. Many studies have demonstrated RBL-2H3 cells expressing serotonin synthesis and transporter systems as an ideal model for elucidating the regulatory mechanisms relating to SERT (*14-16*). Consistent with previous reports, results obtained in our laboratory have shown that the fluorescent styryl dye ASP assay enables the detection of dynamic transport activities of SERT and is amenable for measuring 5-HT uptake (*17-21*).The results obtained from the present study showed that LPS treatment enhances 5-HT uptake in RBL-2H3 cells in a dose-dependent manner. LPS-induced up-regulation of 5-HT uptake results from the increase in surface-expressed SERT protein, which might be partially mediated by PKG-I and p38MAPK activation following A3 adenosine receptor activation. This finding may contribute to a better understanding of the involvement of the LPS in the pathology and modeling of depression.

2. Materials and Methods

2.1. *Reagents and antibodies*

ASP (4-(4-(dimethylamino) styryl)-N-methylpyridinium iodide) and Fluo-3/AM were obtained from Invitrogen (Carlsbad, CA, USA); SB203580 and KT5823 were obtained from the Beyotime Institute of Biotechnology (Jiangsu, China). LPS and MRS1191 were obtained from Sigma Chemical Company (St Louis, MO, USA). Primary antibodies against SERT and PKG-I were purchased from Santa Cruz Biotechnology Inc. (Santa Cruz, CA, USA), and those against phosphor-p38MAPK, iNOS were purchased from Cell Signaling Technology (Danvers, MA, USA).

2.2. *Cell culture*

RBL-2H3 cells were purchased from the China Center for Type Culture Collection (CCTCC; Wuhan, China). RBL-2H3 cells were maintained in nucleoside free Minimum Essential Medium (MEM) α containing 15% fetal bovine serum and were grown into monolayers. Cells were plated on polystyrene tissue culture dishes at densities of 3-10 × 10^5 cells/mL. After 12 h incubation, cells were replaced with fresh serum-free media. After 4 h, RBL-2H3 cells were incubated in fresh media containing 0.75, 1.5, 3 μg/mL LPS or 0.1% DMSO (as vehicle control) with or without pre-treatment with MRS1191, SB203580 or KT5823.

Primary neuron cultures were prepared from E18 Sprague Dawley rat pups. Animals were treated in accordance with the National Institute of Health Guidelines for the Care and Use of Laboratory Animals. Briefly, tissues were harvested on ice and were washed in D-Hanks Balanced Salt Solution containing 100

IU/mL penicillin, and 100 μg/mL streptomycin, and then mechanically dissociated using trituration and trypsinization. Cells were then seeded onto poly-D-lysine-coated 6-well plates at 2×10^6 cells/well in DMEM/F12 media containing B27, L-glutamine, 100 IU/mL penicillin, and 100 μg/mL streptomycin. Neuron cultures were typically grown for 7-10 days.

2.3. *Assay for 5-HT uptake*

5-HT transport activities were measured using ASP assay on RBL-2H3 cells. Briefly, cells from 80% confluent culture flasks were harvested using 0.25% trypsin and were seeded (35,000 cells per well) into 96-well plates and cultured for 24 h in nucleoside free MEM α containing 15% fetal bovine serum. After 20 h, the media was replaced with serum free media and cells were cultured for 4 h prior to assays. ASP (Invitrogen) dissolved in DMSO was added (final concentration, 1 mM) and incubated in the dark at 37°C for 60 min. Unincorporated ASP was removed by washing (3 times with PBS) and 5-HT transporter activity was quantified according to fluorescence intensities (λex, 475 nm; λem, 605 nm) using a SpectraMax M5 Microplate Reader (Sunnyvale, CA, USA).

2.4. *Detection of cytosol Ca^{2+} using Fluo-3/AM*

Detection of intracellular calcium in RBL-2H3 cells was performed using the fluorescent indicator Fluo-3/AM. All images were acquired on a Leica TCS SP5 imaging system mounted on a microscope and an argon ion laser (458-514 nm). Image processing and quantification was carried out using the mean fluorescence intensities of sections acquired.

2.5. *NO assay*

NO levels were measured with the Griess method using a nitrite detection kit (Beyotime, Jiangsu, China) according to the manufacturer's instructions. Briefly, 100 μL of medium or standard $NaNO_2$ was mixed with 100 μL of Griess reagent in a 96-well plate. After 15 min, optical density was read using a SpectraMax M5 Microplate Reader at 540 nm. Results were calculated for statistical analysis by a standard curve using $NaNO_2$.

2.6. *RT-PCR*

Total RNA was extracted from cells cultured under different experimental conditions. The extracted RNA (1 μg) was used as a template for first-strand cDNA synthesis using oligo (dT) primers and a modified M-MLV reverse transcriptase (Carlsbad, CA, Invitrogen). Negative amplification control was performed in the absence of reverse transcriptase. One-tenth of the resultant cDNA was used for human TLR4 and human SERT PCR amplification with human β-actin as an internal control. Real-time quantitative polymerase chain reaction (RT-PCR) was performed with the Bio-rad CFX real-time PCR system and β-actin was used as the reference gene. Gene expression was determined using the $2^{-\Delta\Delta CT}$ method with the following custom designed primers: for β-actin, 5'-AGATCCTGACCGAGCGTGGC-3' and 5'-CCAGGGAGGAAGAGGATGCG-3'; for SERT, 5'-GGTGTGGGTAGATGCCGCCG-3' and 5'-GCTGGGGCCTGCGTCTTTGG-3'.

2.7. *Western blot analysis*

For surface-localized SERT, the Cell Surface Protein Isolation Kit (Thermo Scientific, Pierce, #89881, Waltham, MA, USA) was used for biotinylation and the isolation of cell surface proteins for western blot analysis. Equal amounts of protein (30 μg) were separated on 12% polyacrylamide gels. Proteins were then transferred to 0.45-μm polyvinylidene difluoride (PVDF) membranes using a Bio-Rad Laboratories Mini Protein system. After treating PVDF membranes with blocking buffer TBS/0.1% Tween20 (TBST) containing 3% BSA for 2 h at 37°C, membranes were incubated overnight at 4°C in TBS buffer containing 3% BSA and primary antibodies against active p38MAPK (1:1000), SERT (1:100), PKG-I (1:100), iNOS (1:1000). Membranes were washed 3 times for 10 min in TBST, and were then incubated for 1 h at 37°C with a secondary horseradish peroxidase (HRP)-conjugated antibody at a dilution of 1:1000 in blocking buffer. Finally, membranes were washed extensively in TBST and photographic images were taken after illumination using an enhanced chemiluminescence (ECL) detection reagent. The densitometric analyses of autoradiograms were performed using quantity one software (Bio-Rad Laboratories Inc., Hercules, CA, USA), and data were expressed relative to internal control protein expression.

2.8. *Immunofluorescence staining of SERT*

Cells were seeded at 5,000 cells per well into 96-well black wall clear bottom plates to receive different experimental treatments. Cells were then fixed in 1× PBS containing 4% paraformaldehyde for 30 min, and were subsequently washed three times with 1× PBS. Fixed cells were then blocked in 1× PBS containing 3% BSA for 10 min. Primary antibodies against SERT and phospho-p38MAPK were applied overnight at 4°C. Cells were then washed three times in PBS and were incubated with fluorescent secondary antibodies at 37°C for 2 h in the dark. Finally, Hoechst 33342 (Molecular Probes) was added (final concentration, 1 μg/mL) for the final 10 min, cells were washed three times in PBS, and then PBS containing 50% glycerol was added. Images were acquired and analyzed using a Cellomics ArrayScan®

VTI Imaging Platform (Thermo Fisher Scientific Inc., Waltham, MA, USA).

2.9. *Statistical analysis*

All experiments were replicated a minimum of three times. Subsequently, raw data was normalized and exported into GraphPad Prism (GraphPad Software Inc. San Diego, CA, USA) to generate figures. Differences were identified using one- and two-way analysis of variance (ANOVA) with subsequent Dunnett's comparisons and Student *t*-test. Differences were considered significant when $p < 0.05$.

3. Results

3.1. *LPS enhanced 5-HT uptake in RBL-2H3 cells in a dose-dependant manner*

To examine the effects of LPS on 5-HT uptake, the fluorescent styryl dye ASP was used to detect dynamic transport activities of SERT, and ASP fluorescent intensities in RBL-2H3 cells were monitored after 24 h treatment with LPS. As shown, treatment with 1.5, 3 μg/mL LPS significantly elevated 5-HT transport indicated by increased ASP fluorescence, as readout of SERT activity, in RBL-2H3 cells (Figure 1A), suggesting that

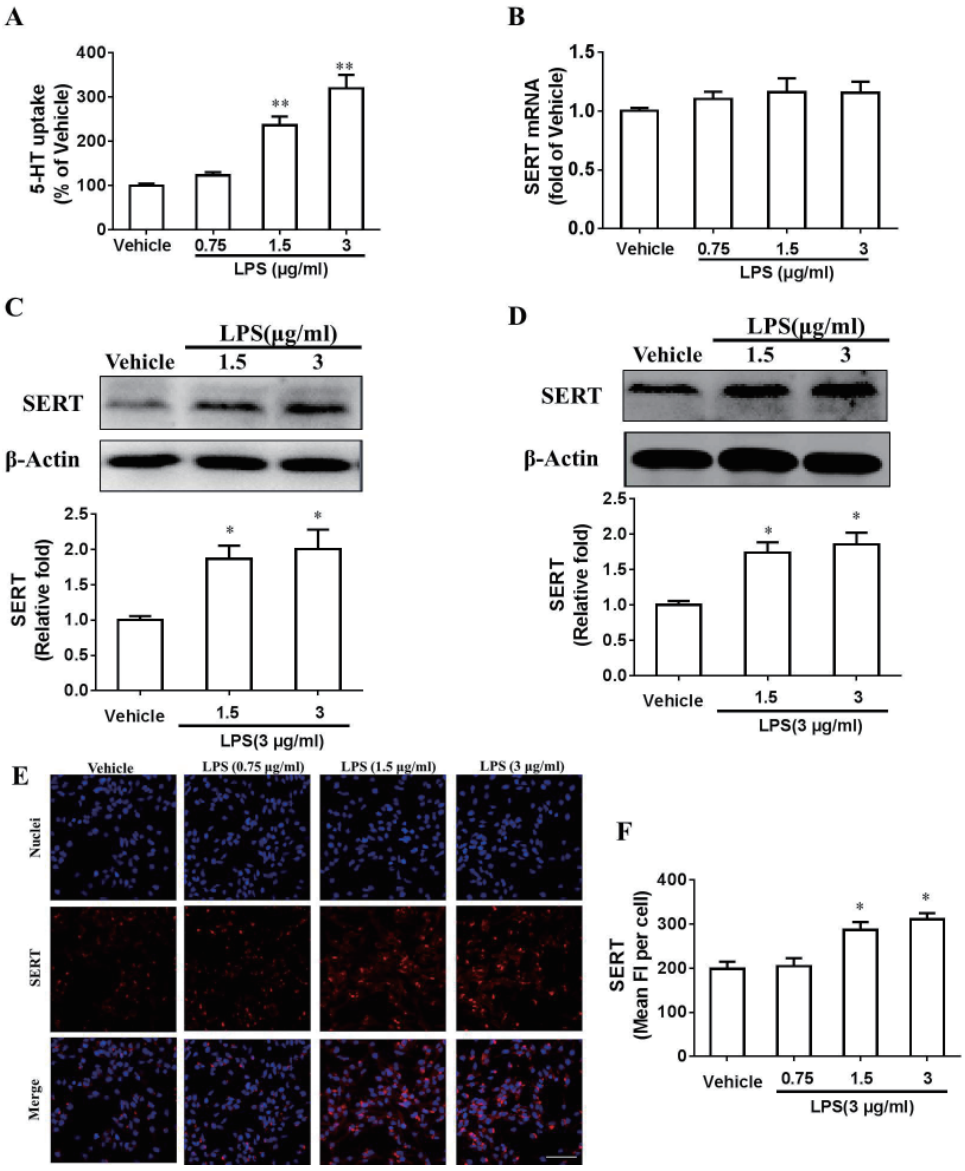

Figure 1. LPS enhanced 5-HT uptake and the level of surface-expressed SERT. (A) RBL-2H3 cells (3.5×10^5 cells/well) were seeded in 96-well microplate and treated with LPS at the indicated concentrations for 24 h before examination. LPS enhanced 5-HT uptake indicated by ASP fluorescence intensity monitored. **(B)** The mRNA level of SERT was analyzed by real-time PCR following LPS treatment. **(C)** The protein content of SERT in RBL-2H3 cells was analyzed following LPS treatment. A Thermo Scientific Pierce Cell Surface Protein Isolation Kit (#89881) was used to biotinylate and isolate cell surface proteins for western blot analysis. **(D)** The protein content of SERT in isolated E18 primary rat cortical neurons was analyzed by western blot following 24 h LPS treatment. **(E, F)** The protein content of SERT in RBL-2H3 cells was confirmed by immunofluorescence analyse following 24 h LPS treatment. Scale bar, 40 μm. Values are expressed as the mean of at least three experiments ± S.E.M; $^*p < 0.05$, $^{**}p < 0.01$ compared to vehicle.

LPS caused dramatic alteration in 5-HT uptake into RBL-2H3 cells in a dose-dependent manner.

3.2. *LPS elevated the level of surface-expressed SERT in RBL-2H3 cells and isolated E18 primary rat cortical neurons*

The observation of increased 5-HT uptake by LPS treatment in RBL-2H3 cells prompted us to investigate whether LPS induced the alteration in the level of SERT expression. Thus, the transcript level of SERT was determined using quantitative PCR analysis, and the results showed that treatment with LPS did not affect SERT mRNA level compared to vehicle treatment (Figure 1B). Furthermore, western blot analysis on the whole lysate of RBL-2H3 cells demonstrated that LPS did not influence total protein level of SERT, however, level of SERT protein in plasma membrane subfraction prepared from RBL-2H3 cells remarkably increased (Figure 1C). Immunofluorescence analysis using specific SERT antibody showed the protein content of plasma membrane-localized SERT in RBL-2H3 cells significantly increased with mean FI per cell of 286 ± 31 and 311± 46 following 24 h LPS treatment compared to 198 ± 23 of vehicle treatment (Figures 1E and 1F). Moreover, we isolated E18 primary rat cortical neurons to further examine the increase in SERT protein level induced by LPS. Representative blot images

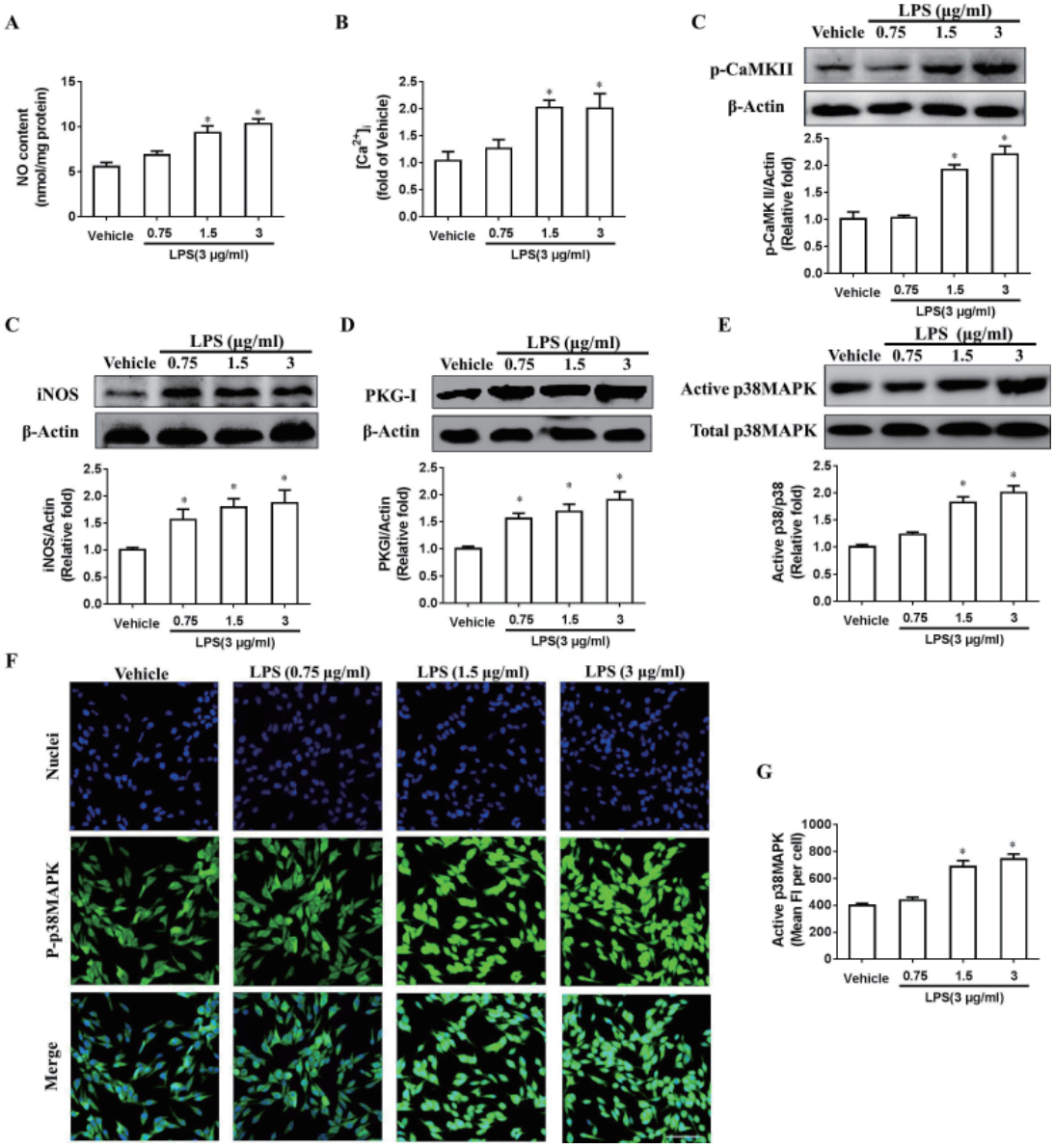

Figure 2. LPS elevated NO content and the level of cytosol calcium, and stimulated CaMK-II/iNOS/PKG-I signaling pathway and p38MAPK in RBL-2H3 cells. RBL-2H3 cells (3.5×10^5 cells/well) were seeded in 96-well microplate and treated with LPS at the indicated concentrations for 24 h before examination. **(A)** LPS elevated NO content examined by Griess method. **(B)** The level of cytosol calcium was determined using Fluo-3/AM following LPS treatment. The phosphorylation of CaMK-II **(C)**, protein level of iNOS **(D)**, PKG-I **(E)** and active p38MAPK **(F)** in RBL-2H3 cells was analyzed by western blot following 24 h LPS treatment. **(G, H)** The activation of p38MAPK in RBL-2H3 cells was examined by immunofluorescence analysis following 24 h LPS treatment. Scale bar, 40 μm. Values are expressed as the mean of at least three experiments ± S.E.M; $^{*}p < 0.05$, $^{**}p < 0.01$ compared to vehicle.

demonstrated that 1.5 and 3 μg/mL LPS treatment significantly boosted the expression of surface-expressed protein SERT (Figure 1D). Taken together, these data indicate that although LPS did not affect the total SERT expression, but notably induced the elevation in the plasma membrane distribution of SERT.

3.3. *LPS Increased Ca^{2+} and NO followed by PKG-I/p38MAPK pathway activation*

Increasing evidence indicates that SERT-mediated 5-HT clearance is controlled by multiple pathways that regulate both plasma membrane expression and catalytic activity of SERT (*18*). Recent studies demonstrate that the up-regulation of SERT activity by serine/threonine kinases, PKG-I and p38MAPK-linked pathways contains increased SERT activation and membrane distribution (*22*). Thus, Ca^{2+}-CaMK- II/iNOS-NO/PKG-I and p38MAPK pathway received more attention as one of the most widely studied regulatory mechanisms. In the current study, NO level was firstly determined using Griess method after 24 h treatment with LPS.

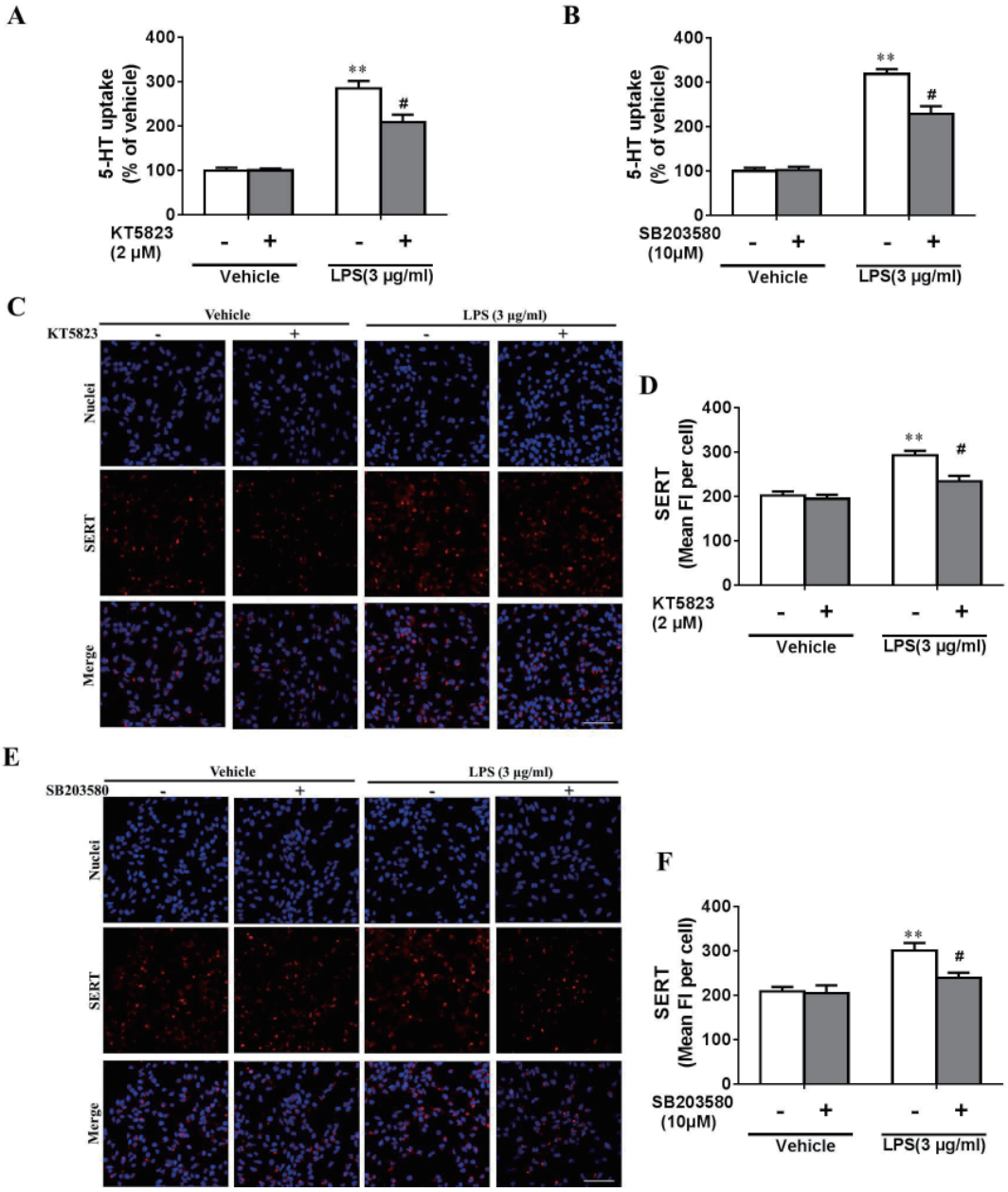

Figure 3. LPS-induced 5-HT uptake and surface-expressed SERT in RBL-2H3 cells was hindered by inhibition of PKG-I or p38MAPK. RBL-2H3 cells (3.5×10^5 cells/well) were seeded in 96-well microplate and treated with 3 μg/mL LPS for 24 h following 6 h pre-treatment with PKG-I inhibitor KT5823 (2 μM) treatment or p38MAPK inhibitor SB203580 (10 μM). **(A-B)** Change in 5-HT uptake was determined using ASP assay. **(C-D)** Representative immunofluorescence images and quantification of SERT expression following 24 h LPS treatment in the presence of PKG-I inhibitor KT5823. **(E-F)** Representative immunofluorescence images and quantification of SERT expression following 24 h LPS treatment in the presence of p38MAPK inhibitor SB203580. Scale bar, 40 μm. Values are expressed as the mean of at least three experiments ± S.E.M; $^{*}p < 0.05$, $^{**}p < 0.01$ compared to vehicle; $^{\#}p < 0.05$ compared to LPS 3 μg/mL treatment.

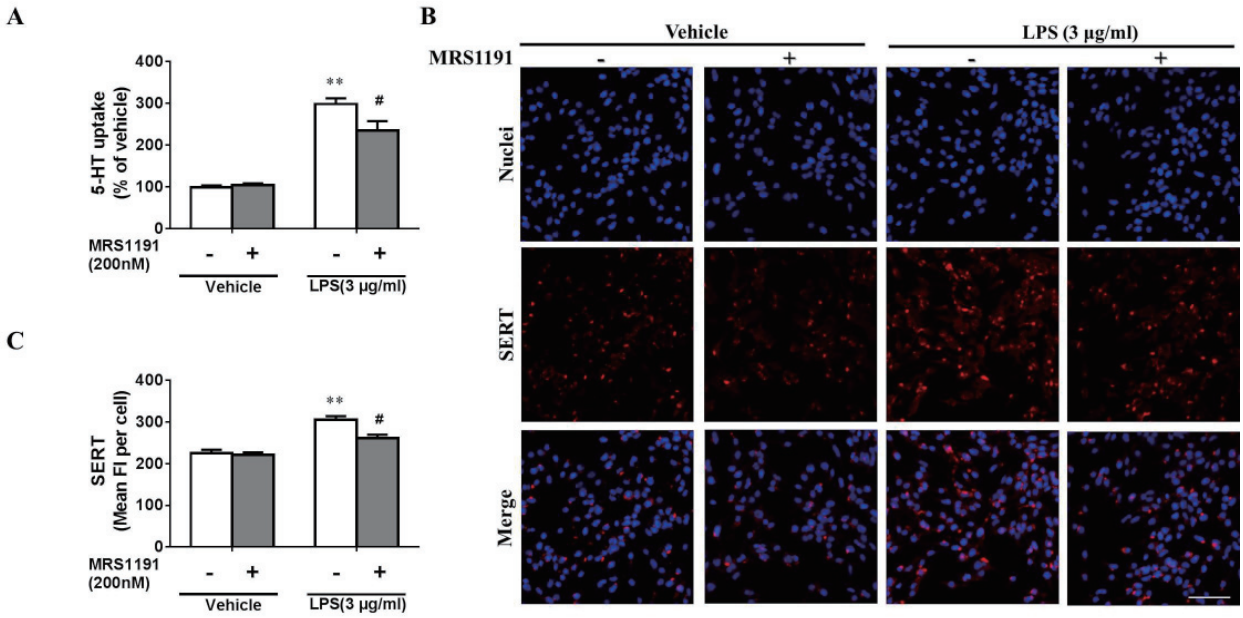

Figure 4. A3AR antagonism inhibited LPS-induced 5-HT uptake and surface-expressed SERT in RBL-2H3 cells. RBL-2H3 cells (3.5×10^5 cells/well) were seeded in 96-well microplate and treated with 3 μg/mL LPS for 24 h in the presence or absence of A3AR antagonist MRS1191 (200 nM). **(A)** Change in 5-HT uptake was determined using ASP assay. **(B-C)** Representative immunofluorescence images and quantification of SERT expression. Scale bar, 40 μm. Values are expressed as the mean of at least three experiments ± S.E.M; $^{*}p < 0.05$, $^{**}p < 0.01$ compared to vehicle; $^{\#}p < 0.05$ compared to LPS 3 μg/mL treatment.

The results showed that NO levels in RBL-2H3 cells significantly increased when treated with 1.5 and 3 μg/mL LPS (nmol/mg protein: 9.36 ± 0.79 and 10.34 ± 0.65) (Figure 2A). Subsequently, the fluorescent staining using Fluo-3/AM demonstrated that intracellular Ca^{2+} concentration ([Ca^{2+}] i) in RBL-2H3 cells was lifted by LPS treatment up to 2-fold of that in vehicle treated cells (Figure 2B). Western blot analysis indicated that phosphorylated CaMK-II, protein level of iNOS and PKG-I as well as active p38MAPK were markedly elevated (Figures 2C-2E). Consistent with the data shown above, immunofluorescence staining using anti-phosphorylated p38MAPK further confirmed the almost 1.6-fold increase in the level of active p38MAPK by LPS treatment (Figures 2F and 2G). Taken together, these data suggest that Ca^{2+}-CaMK-II/iNOS-NO/PKG-I and p38MAPK signaling might be implicated in the up-regulation of SERT by LPS.

3.4. *The Effect of PKG-I or p38MAPK inhibition on LPS-induced 5-HT uptake and SERT expression in RBL-2H3 cells*

To clarify the involvement of PKG-I or p38MAPK in LPS-induced 5-HT uptake and SERT expression, here, PKG-I inhibitor KT5823 or p38MAPK inhibitor SB203580 was respectively pretreated to RBL-2H3 cells to investigate their influences on LPS profile. The assay for 5-HT uptake using ASP transport demonstrated that PKG-I inhibitor KT5823 (2 μM) or p38MAPK inhibitor SB203580 (10 μM) pretreatment did not notably affected the 5-HT uptake, however, 3 μg/mL LPS-induced increase in the 5-HT uptake was remarkably impaired in the presence of the two inhibitors (Figures 3A and 3B). The immunofluorescence assay labeling SERT indicated that as compared to control treatment with just 3 μg/mL LPS, the protein level of SERT in the surface of RBL-2H3 cells markedly declined when treated with 2 μM PKG-I inhibitor KT5823 followed by 3 μg/mL LPS treatment for extra 24 h (Figures 3C and 3D). The fluorescence intensities of surface-localized SERT in RBL-2H3 cells co-treated with 3 μg/mL LPS and 10 μM p38MAPK inhibitor SB203580 also significantly fell into decline. Therefore, these results suggest that PKG-I and p38MAPK activation play a crucial role in the LPS-induced 5-HT uptake and SERT expression in RBL-2H3 cells.

3.5. *Effect of Adenosine 3A Receptor (A3AR) antagonism on LPS-induced 5-HT uptake and SERT expression in RBL-2H3 cells*

It is reported that the activation of A3AR results in increased 5-HT uptake in RBL-2H3 cells and that A3AR regulates serotonin transport *via* NO and cyclic guanosine monophosphate (cGMP) (*22,23*). Thus, it is necessary to dig out the role of A3AR in the LPS-induced 5-HT uptake and SERT expression. RBL-2H3 cells were pre-treated with an A3AR antagonist MRS1191 (200 nM) for 6 h, followed by treatment with 3 μg/mL LPS. After 24 h of incubation, the change in the 5-HT uptake was assessed as described previously, and we observed that the addition of MRS1191 notably disrupted the effects exerted by 3 μg/mL LPS (Figure

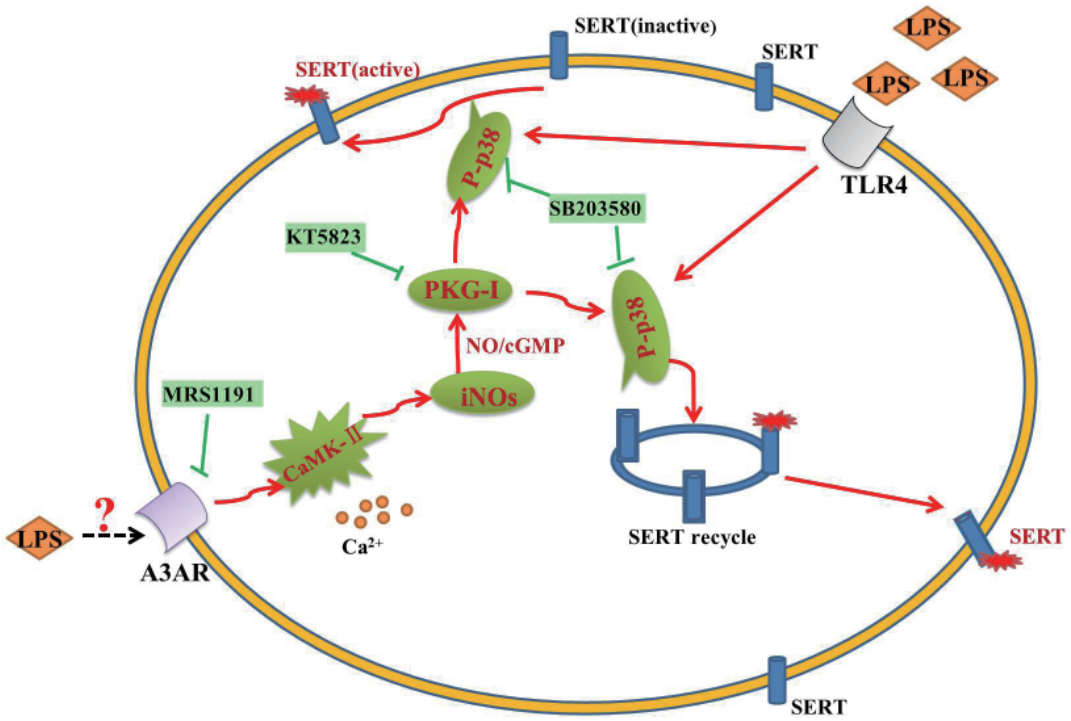

Figure 5. The schematic pathway involved in LPS-induced 5-HT uptake and surface-expressed SERT in RBL-2H3 cells.

4A). Immunofluorescence assay also showed that there was no remarkable difference between vehicle and MRS1191 treatment, however, level of surface-expressed SERT was significantly higher (316 ± 27) of 3 μg/mL LPS treatment than that of co-treatment with 200 nM MRS1191 and 3 μg/mL LPS (245 ± 31) (Figures 4B and 4C), indicating that the activation of A3AR was implicated in LPS-induced upregulation of 5-HT uptake and SERT expression in RBL-2H3 cells.

4. Discussion

In the present study, we showed that 24 h treatments with LPS significantly increased the 5-HT uptake and plasma membrane expression of SERT in a rat basophilic leukemia cell line RBL-2H3 cell. Moreover, elevation in NO content and intracellular Ca^{2+} concentrations was observed following LPS treatment. Mechanismatic investigation showed that LPS induced CaMK-II/iNOS/PKG-I and p38MAPK signaling pathways, leading to an increase in the activation and plasma membrane distribution of SERT. Furthermore, the effects of LPS on SERT were impaired by the presence of A3AR antagonist MRS1191.

In our experiments, we found that LPS significantly increased the 5-HT uptake and upregulated the protein level of SERT in RBL-2H3 cell, as contrast to the previous report on the human enterocyte-like Caco-2 cell line (*13*). Although Caco-2 expresses the human SERT, this cell line lacks in serotonin synthesis and discretion. Comparatively, rat basophilic mast cells RBL-2H3 express not only transporter systems but also serotonin synthesis, and are regarded more relative to the physiology of 5-HT neurons. Miller and Hoffman first provided evidence of adenosine receptor (AR) regulation of SERT using this cell line (*22*). Kim *et al*. investigated the molecular mechanisms of antidepressant drugs that sertraline up-regulates tryptophan hydroxylase expression and serotonin synthesis in RBL-2H3 cells (*24*). These studies proved RBL-2H3 cells as an ideal model for studying the SERT. In addition, SERT is also widely expressed in the front cortexes of the brain and the hippocampus which are closely associated with the pathogenesis of depression (*23,25*), so we verified the effects of LPS in isolated E18 primary rat cortical neurons and gained the same observation.

It is believed that lipopolysaccharide activity is mediated by TLR 4 activation and a cascade of intracellular reactions which involves different signalling pathways including p38 MAPK, protein kinase A (PKA) and PKC (*26*). It has been reported that interleukin-1β (IL-1β) and TNF-α stimulation of SERT activity *in vitro* is mediated through the activation of p38 MAPK (*9*). Since LPS tightly correlates to these pro-inflammatory cytokines, we examined the requirement for p38 MAPK in SERT modulation exerted by LPS using treatment with p38 MAPK inhibitor SB203580. The observation that active p38 MAPK increased with LPS treatment and the SERT up-regulation by LPS was impaired by treatment with SB203580 indicates that p38 MAPK may act as an activator of SERT function.

Increasing evidences indicate that SERT are tightly controlled by multiple signaling pathways including

G-protein coupled receptors-linked pathways and various kinases as well as their substrates (*27-31*). Recently, Miller *et al*. showed that the activation of A3AR results in increased 5-HT uptake in RBL-2H3 cells and that A3AR regulates serotonin transport *via* NO and cGMP (*22*). Some *in vitro* studies have revealed multiple second messengers or second messenger-linked kinases participate in acute SERT regulation (*32*). Experiments with synaptosomes and cell lines demonstrate that SERT activity decreases after depletion of intracellular Ca^{2+}, treatment with calmodulin inhibitors and phorbol esters (*33,34*). PKA, PKC and PKG activation have also been demonstrated to impose post-translational regulation on SERT by phosphorylation, possibly reflecting the heterologous context of SERT expression. Zhu *et al*. have reported that involvement of A3AR in serotonin transporter trafficking and activation in RBL-2H3 cells and SERT stimulation by A3AR requires activation of PKG by a phospholipase C, Ca^{2+}, NOS and cGMP-dependent mechanism (14,27). In the present study, we provided evidence that Ca^{2+}-CaMK-II/iNOS/PKG-I signaling pathways contribute to up-regulation of SERT in RBL-2H3 cells by LPS. This effect maybe partially mediated through activates A3AR by LPS (Figure 5). Although the potential mechanism by which LPS enhances activates A3AR in RBL-2H3 cells remains unclear, this finding offers a novel insight into the modulation of SERT. Moreover, many researches are still needed to clarify the interaction between A3AR and LPS.

In conclusion, the present experiments demonstrate that LPS enhances the 5-HT uptake and transposition from cytosol to plasma membrane of SERT in RBL-2H3 cells, and the underlying mechanism involves CaMK-II/iNOS/PKG-I and p38 MAPK activation following calcium flux, which may be partially mediated by A3AR activation. LPS-induced alteration possibly provides a powerful tool to model the pathology of depression and the discovery of new antidepressant.

Acknowledgements

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References

1. Sharp T, Cowen PJ. 5-HT and depression: Is the glass half-full. Curr Opin Pharmacol. 2011; 11:45-51.
2. Stahl SM, Lee-Zimmerman C, Cartwright S, Morrissette DA. Serotonergic drugs for depression and beyond. Curr Drug Targets. 2013; 14:578-585.
3. Blier P, de Montigny C. Current advances and trends in the treatment of depression. Trends Pharmacol Sci. 1994; 15:220-226.
4. Lau T, Horschitz S, Berger S, Bartsch D, Schloss P. Antidepressant-induced internalization of the serotonin transporter in serotonergic neurons. FASEB J. 2008; 22:1702-1714.
5. Steiner JA, Carneiro AM, Blakely RD. Going with the flow: Trafficking-dependent and -independent regulation of serotonin transport. Traffic. 2008; 9:1393-1402.
6. Zhu CB, Lindler KM, Campbell NG, Sutcliffe JS, Hewlett WA, Blakely RD. Colocalization and regulated physical association of presynaptic serotonin transporters with A(3) adenosine receptors. Mol Pharmacol. 2011; 80:458-465.
7. Li W, Ling S, Yang Y, Hu Z, Davies H, Fang M. Systematic hypothesis for post-stroke depression caused inflammation and neurotransmission and resultant on possible treatments. Neuro Endocrinol Lett. 2014; 35:104-109.
8. Dantzer R, O'Connor JC, Freund GG, Johnson RW, Kelley KW. From inflammation to sickness and depression: When the immune system subjugates the brain. Nat Rev Neurosci. 2008; 9:46-56.
9. Zhu CB, Blakely RD, Hewlett WA. The proinflammatory cytokines interleukin-1beta and tumor necrosis factor-alpha activate serotonin transporters. Neuropsychopharmacology. 2006; 31:2121-2131.
10. Leonard B, Maes M. Mechanistic explanations how cell-mediated immune activation, inflammation and oxidative and nitrosative stress pathways and their sequels and concomitants play a role in the pathophysiology of unipolar depression. Neurosci Biobehav Rev. 2012; 36:764-785.
11. Van Heesch F, Prins J, Konsman JP, Westphal KG, Olivier B, Kraneveld AD, Korte SM. Lipopolysaccharide-induced anhedonia is abolished in male serotonin transporter knockout rats: An intracranial self-stimulation study. Brain Behav Immun. 2013; 29:98-103.
12. Zhu CB, Lindler KM, Owens AW, Daws LC, Blakely RD, Hewlett WA. Interleukin-1 receptor activation by systemic lipopolysaccharide induces behavioral despair linked to MAPK regulation of CNS serotonin transporters. Neuropsychopharmacology. 2010; 35:2510-2520.
13. Mendoza C, Matheus N, Iceta R, Mesonero JE, Alcalde AI. Lipopolysaccharide induces alteration of serotonin transporter in human intestinal epithelial cells. Innate Immun. 2009; 15:243-250.
14. Zhu CB, Steiner JA, Munn JL, Daws LC, Hewlett WA, Blakely RD. Rapid stimulation of presynaptic serotonin transport by A(3) adenosine receptors. J Pharmacol Exp Ther. 2007; 322:332-340.
15. Harville BA, Dreyfus LA. Release of serotonin from RBL-2H3 cells by the Escherichia coli peptide toxin STb. Peptides. 1996; 17:363-366.
16. Hoffman BJ, Mezey E, Brownstein MJ. Cloning of a serotonin transporter affected by antidepressants. Science. 1991; 254:579-580.
17. Baik SY, Jung KH, Choi MR, Yang BH, Kim SH, Lee JS, Oh DY,Choi IG,Chung H,Chai YG.Fluoxetine-induced up-regulation of 14-3-3zeta and tryptophan hydroxylase levels in RBL-2H3 cells. Neurosci Lett. 2005; 374:53-57.
18. Fowler A, Seifert N, Acker V, Woehrle T, Kilpert C, de Saizieu A. A nonradioactive high-throughput/high-content assay for measurement of the human serotonin reuptake transporter function *in vitro*. J Biomol Screen.

2006; 11:1027-1034.
19. Jorgensen S, Nielsen EO, Peters D, Dyhring T. Validation of a fluorescence-based high-throughput assay for the measurement of neurotransmitter transporter uptake activity. J Neurosci Methods. 2008; 169:168-176.
20. Fowler A, Seifert N, Acker V, Woehrle T, Kilpert C, de Saizieu A. A nonradioactive high-throughput/high-content assay for measurement of the human serotonin reuptake transporter function *in vitro*. J Biomol Screen. 2006; 11:1027-1034.
21. Oz M, Libby T, Kivell B, Jaligam V, Ramamoorthy S, Shippenberg TS. Real-time, spatially resolved analysis of serotonin transporter activity and regulation using the fluorescent substrate, ASP+. J Neurochem. 2010; 114:1019-1029.
22. Miller KJ, Hoffman BJ. Adenosine A3 receptors regulate serotonin transport *via* nitric oxide and cGMP. J Biol Chem. 1994; 269:27351-27356.
23. Ramkumar V, Stiles GL, Beaven MA, Ali H. The A3 adenosine receptor is the unique adenosine receptor which facilitates release of allergic mediators in mast cells. J Biol Chem. 1993; 268:16887-16890.
24. Kim SW, Park SY, Hwang O. Up-regulation of tryptophan hydroxylase expression and serotonin synthesis by sertraline. Mol Pharmacol. 2002; 61:778-785.
25. Hoffman BJ, Hansson SR, Mezey E, Palkovits M. Localization and dynamic regulation of biogenic amine transporters in the mammalian central nervous system. Front Neuroendocrinol. 1998; 19:187-231.
26. MacKenzie CJ, Paul A, Wilson S, de Martin R, Baker AH, Plevin R. Enhancement of lipopolysaccharide-stimulated JNK activity in rat aortic smooth muscle cells by pharmacological and adenovirus-mediated inhibition of inhibitory kappa B kinase signalling. Br J Pharmacol. 2003; 139:1041-1049.
27. Blakely RD, DeFelice LJ, Galli A. Biogenic amine neurotransmitter transporters: Just when you thought you knew them. Physiology (Bethesda). 2005; 20:225-231.
28. Zhu CB, Hewlett WA, Feoktistov I, Biaggioni I, Blakely RD. Adenosine receptor, protein kinase G, and p38 mitogen-activated protein kinase-dependent up-regulation of serotonin transporters involves both transporter trafficking and activation. Mol Pharmacol. 2004; 65:1462-1474.
29. Ramamoorthy S, Blakely RD. Phosphorylation and sequestration of serotonin transporters differentially modulated by psychostimulants. Science. 1999; 285:763-766.
30. Baganz NL, Lindler KM, Zhu CB, Smith JT, Robson MJ, Iwamoto H, Deneris ES,Hewlett WA,Blakely RD. A requirement of serotonergic p38alpha mitogen-activated protein kinase for peripheral immune system activation of CNS serotonin uptake and serotonin-linked behaviors. Transl Psychiatry. 2015; 5:e671.
31. Eaton MJ, Whittemore SR. Adrenocorticotropic hormone activation of adenylate cyclase in raphe neurons: Multiple regulatory pathways control serotonergic neuronal differentiation. J Neurobiol. 1995; 28:465-481.
32. Leenders AG, Sheng ZH. Modulation of neurotransmitter release by the second messenger-activated protein kinases: Implications for presynaptic plasticity. Pharmacol Ther. 2005; 105:69-84.
33. Ramamoorthy S, Giovanetti E, Qian Y, Blakely RD. Phosphorylation and regulation of antidepressant-sensitive serotonin transporters. J Biol Chem. 1998; 273:2458-2466.
34. Ansah TA, Ramamoorthy S, Montanez S, Daws LC, Blakely RD. Calcium-dependent inhibition of synaptosomal serotonin transport by the alpha 2-adrenoceptor agonist 5-bromo-N-[4,5-dihydro-1H-imidazol-2-yl]-6-quinoxalinamine (UK14304). J Pharmacol Exp Ther. 2003; 305:956-965.

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