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Paroxetine differentially modulates LPS-induced TNF α and IL-6 production in mouse macrophages

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Abstract

Paroxetine is a selective serotonin reuptake inhibitor (SSRI) that is clinically used for the treatment of depression in human patients. Because of recent reports on the role of serotonin in modulating inflammation and the link between inflammation and depression, we sought to test the effect of paroxetine directly on macrophage response to an inflammatory stimulus. Lipopolysaccharide (LPS) treatment of mouse macrophages significantly enhanced TNF α and IL-6 production. Paroxetine treatment of macrophages however, significantly inhibited LPS-induced IL-6 production. In contrast, paroxetine enhanced LPS-induced TNF α production in macrophages. These effects of paroxetine were mimicked by fluoxetine, another SSRI. To determine if the effects of paroxetine are mediated via modulation of the 5-HT system, we treated macrophages with 5-HT or 5-HT receptor antagonist (LY215840) in the presence of LPS and/or paroxetine. 5-HT treatment by itself did not affect LPS-induced cytokine production. LY215840 however, reversed paroxetine's effect on LPS-induced TNF α production but not IL-6. To understand the signaling mechanisms, we examined paroxetine's effect on MAPK and NF κ B pathways. While paroxetine inhibited LPS-induced I κ B α phosphorylation, MAPK pathways were mostly unaffected. Together these data demonstrate that paroxetine has critical but differential effects on IL-6 and TNF α production in macrophages and that it likely regulates these cytokines via distinct mechanisms.

Introduction

Paroxetine is an FDA approved drug for treating depression in humans. It belongs to the class of “Selective Serotonin Reuptake Inhibitors” (SSRIs). In addition to paroxetine (Paxil, Pexeva), other members of the SSRIs include fluoxetine (Prozac), citalopram (Celexa), escitalopram (Lexapro), and sertraline (Zoloft)[1,2]. SSRIs are prescribed mainly for major depressive disorders but are also used in the treatment of anxiety, panic and eating disorders and occasionally for post-traumatic stress disorder [3]. Paroxetine and other members of the SSRI class were identified based on their ability to inhibit reuptake of serotonin by blocking

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serotonin transporters (SERT) that are present on the cell surface of the pre-synaptic neuron [1]. Serotonin (5-Hydroxy Tryptamine, 5-HT) is a monoamine neurotransmitter primarily synthesized in the gastrointestinal (GI) tract and the central nervous system. In addition to its effects on the GI tract and the neuronal system, nonneuronal serotonin modulates other physiological processes including inflammation.

Recent studies have established neurogenic inflammation as the likely cause of depression in humans[4]. Studies have also shown that increased pro-inflammatory cytokine (IL-6, and TNF α) levels, both in periphery and in the brain, precipitate development of depression (reviewed in [5]). Although anti-depressants in clinical use are effective in ameliorating the symptoms, there is considerable interest in identifying novel antidepressants and understanding the mechanisms of action of existing antidepressants. In this regard, SSRIs have been reported to have anti-inflammatory properties in not only neuronal tissues but also in non-neuronal cells [6,7]. In animal models of disease, SSRIs are able to effectively modulate neuronal as well as non-neuronal inflammatory diseases [8-10]. Given that paroxetine and fluoxetine are already FDA approved, there is also interest in repurposing these drugs possibly as anti-inflammatory therapeutics for diseases such as arthritis and colitis [11,12]. In spite of these studies in animal models, the role of paroxetine on lipopolysaccharide-induced IL-6 and TNF α production in macrophages and the mechanisms of regulation are not well known. Here we provide evidence that paroxetine significantly modulates LPS-induced IL-6 and TNF α in mouse macrophages and that its effects on these two cytokines are differentially regulated.

MATERIALS AND METHODS

Reagents

Paroxetine hydrochloride hemihydrate (MW 374.83) and Fluoxetine hydrochloride (MW 345.79) were purchased from Sigma-Aldrich (St Louis, MO). Serotonin hydrochloride (M, 217.18) and LY 215840 (MW 400.04) were obtained from Tocris bioscience (Bristol, UK). RPMI 1640 (Rosewell Park Memorial Institute) media, Fetal Bovine Serum (FBS), Pen-strep (Penicillin Streptomycin mixtures contain 5,000 units of penicillin and 5,000 μ g of streptomycin/ml in saline) and Versene (0.2 g EDTA/liter of PBS) were purchased from Life technologies (Carlsbad, CA). Ultrapure LPS (from 0111:B4 *E. Coli*) was obtained from Invivogen (San Diego, CA).

Antibodies

Antibodies (P-I κ B α , P-ERK, P-JNK, Pp38, Pp105, and tubulin) were purchased from Cell Signaling Technology Inc. (Danvers, MA). Antibodies (GRK2 and ERK2) were obtained from Santa Cruz Biotechnology Inc. (Santa Cruz, CA). Alexa fluor goat anti-rabbit antibody (Invitrogen, Carlsbad, CA) and anti-mouse IgG IRdye 800 conjugated antibody (Rockland Immunochemicals Inc, Gilbertsville, PA) were used with LICOR Odyssey system. Peroxidase conjugated anti-mouse antibody (Vector Laboratories Inc, Burlingame, CA) developed with Immunocruz luminal reagent (Santa Cruz Biotechnology) was used for chemiluminescence.

Animals and collection of primary peritoneal macrophages

Mice (C57BL/6) were obtained from NCI and were housed in groups of 4 to 5 mice per cage in rooms maintained at 22-24 C with 50% humidity with 12-hour light and dark cycle. All animals had access to normal chow and water ad libitum. Experimental procedures involving mice were conducted in accordance to the protocol approved by the Institutional Animal Care and Use Committee at Michigan State University and conformed to NIH guidelines. For thioglycollate-induced peritoneal cell collection, mice were injected intraperitoneally with 1 ml of 4% Brewer's thioglycollate broth (Sigma-Aldrich, St Louis). Peritoneal cells from each mouse were collected 4 days post injection, as previously described [13]. 10^6 cells/well were seeded in 12-well plates for the experiments. Cells were serum starved at least for 1 hour prior to treatment. All treatments were done in serum free media.

Cell culture

Raw 264.7 cells were purchased from ATCC (Manassas, VA) and grown in RPMI media supplemented with 10% Fetal Bovine serum (FBS) along with 1% PenStrep and maintained in 5% CO₂ at 37 C. Cells from 10 to 20 passages were used for the experiments. Note that prior to treatment with various ligands, cells were serum starved for at least 1 hour. Treatments were done with serum free media.

Enzyme-linked immunosorbent assay (ELISA)

Supernatants from the treated cells were collected and cytokine analysis for IL-6, and TNF α performed according to manufacturer's instructions using ELISA Kits (eBioscience, San Diego, CA) and as described before [14]. Optical density measurements were taken at 450 nm in an Infinite M1000 PRO plate reader (Tecan, Mannedorf, Switzerland). Cytokines were quantified using the Magellan data analysis software (Tecan, Mannedorf, Switzerland) and normalized to the total cellular protein. Protein levels were determined by Bradford assay (Biorad, Hercules, CA).

Western blot analysis

Western blot analysis was performed as described before [15]. Briefly, lysates (prepared as described [16]) were subjected to SDS-PAGE electrophoresis, transferred to nitrocellulose and blotted with respective antibodies. Depending on the secondary antibody, blots were analyzed using LICOR's Odyssey (IR-dye secondary) or by chemiluminescence (HRP secondary). Quantitation was done using Odyssey software (IR dye) or Image J (for HRP).

Statistical analysis

All the experiments were repeated 3 to 6 times and each N represents different passages of Raw 264.7 cells or a different mouse in case of peritoneal macrophages. Data is expressed as mean SE. Data was analyzed using GRAPHPAD PRISM software (San Diego, California, USA). Student's unpaired t-test was used to compare 2 groups and ANOVA (with Tukey post-test) was used for comparing more than 2 groups. $P < 0.05$ was considered statistically significant.

RESULTS

Differential regulation of LPS-induced cytokines by paroxetine in macrophages

LPS induces a number of cell signaling and cytokine responses in macrophages including IL-6, and TNF α . We focused on these cytokines because of their established roles in many inflammatory diseases[17,18]. Raw264.7 macrophages and primary peritoneal macrophages were untreated or treated with LPS in the absence or presence of paroxetine for 6 and 24-hour time points. Concentrations of paroxetine used were based on previous studies [6,19-21]. At the end of the treatments, culture supernatants were subjected to ELISAs for IL-6, and TNF α as shown. Interestingly, paroxetine significantly inhibited LPS-induced IL-6 levels in both Raw264.7 cells and peritoneal macrophages (Fig 1). TNF α levels however were significantly enhanced with combined treatment of LPS and paroxetine when compared to LPS alone, in both Raw264.7 cells and in primary peritoneal macrophages (Fig 1). These data suggest that paroxetine-sensitive pathway(s) positively mediates LPS-induced IL-6 and but negatively regulates LPS-induced TNF α production in macrophages.

Effect of fluoxetine on LPS-induced cytokines in macrophages

Because paroxetine belongs to the class of SSRIs we wanted to test the specificity of paroxetine's effect as an inhibitor of SERT. For this we used fluoxetine, another member of the SSRI and tested its effects on LPS-induced cytokine production in macrophages. It is important to note that even though fluoxetine is a member of the SSRIs it is structurally distinct from paroxetine [19,22]. Interestingly, as shown in Fig 2, fluoxetine also significantly modulated LPS-induced cytokines in a manner very similar to that of paroxetine. Importantly these effects of fluoxetine were reproducible in primary peritoneal macrophages demonstrating the physiological relevance of the inhibitor's effect on LPS-induced cytokine production (Fig 2).

Role of serotonin in paroxetine-mediated responses in macrophages

Both paroxetine and fluoxetine had similar effects on LPS-induced cytokine production and both of these compounds act by inhibiting the serotonin re-uptake thereby enhancing serotonin's effect. Therefore we examined the role of direct treatment of serotonin on LPS-induced cytokine production. As shown in Figure 3, serotonin treatment had no effect on LPS-induced cytokine production in Raw264.7 macrophages at any of the concentrations tested (Fig 3). This result posed two possibilities: 1. LPS-induces an increase in extracellular serotonin levels and that the levels are already high enough that addition of extracellular serotonin does not have additional effects; 2. SSRIs affect LPS-induced cytokine production independent of the 5-HT system. If the former were true, then LPS treatment would induce serotonin production and the extracellular serotonin remains in the extracellular environment in the presence of SSRIs in macrophages. This endogenous released extracellular serotonin via its receptor(s) might mediate further effects on LPS-induced cytokines. In this case, treatment of cells with serotonin receptor antagonists in the presence of LPS+SSRI should reverse the effect of SSRI on LPS-induced TNF α , and IL-6. To first test if macrophages are able to produce 5-HT (as suggested by previous studies [23]), we measured 5-HT levels as well as that of 5-HIAA levels (5-HT catabolized to 5-HIAA) using HPLC[24] to assess the presence of 5-HT and its turnover. Note that this was done in the absence of any serum,

since media containing fetal bovine serum has been shown to have high concentrations of 5-HT [25,26]. As shown in Figure 4, both 5-HT and 5-HIAA levels were detected under basal conditions but the levels were mostly similar in the different treatment groups, except for the paroxetine+LPS group at 24 hours that showed a modest increase. Interestingly, turnover of 5-HT (as assessed by the ratio of 5-HIAA to 5-HT) showed a modest decrease with paroxetine+LPS treatment compared to basal at 24 hours. Although the levels of 5-HT are not as high as our treatment conditions in Fig 3, the concentrations (0.3357 0.01559 ng/ml) are in the low nanomolar range sufficient to activate the 5-HT receptors. To test this, we pretreated macrophages with LY215840 (a 5-HT₂/5-HT₇ receptor antagonist) [27] prior to treatment with LPS and paroxetine. We focused on 5-HT₂/7 because these subtypes are preferentially expressed at high levels in macrophages [28]. At the end of the treatment, the supernatants were measured for levels of TNF α , and IL-6. Interestingly, LY215840 by itself significantly suppressed LPS-induced IL-6 production. However, paroxetine's effect on LPS-induced IL-6 production was further enhanced by LY215840 (Fig 5), suggesting that paroxetine's effect on IL-6 is unlikely to be mediated via 5-HT. In contrast to the effects on IL-6 however, paroxetine's effect on LPS-induced TNF α was suppressed by LY215840. LY215840 by itself however, had no significant effect on LPS-induced TNF α production. Together, these results suggest that paroxetine affects multiple signaling pathways likely via multiple mechanisms that are dependent and independent of 5-HT₂/7.

In search for additional mechanisms of action we focused on G-protein coupled receptor kinase-2 (GRK2). Recently, paroxetine was shown to be a potent inhibitor of GRK-2, independent of SERT inhibition [19]. To test if any of the effects of paroxetine are indeed mediated via inhibition of GRK2, we transfected Raw264.7 macrophages with control or GRK2 siRNA, and then performed experiments as before to test the effect of paroxetine on LPS-mediated cytokine production. As expected, in control siRNA transfected cells paroxetine significantly inhibited LPS-induced IL-6, and markedly enhanced TNF α production. In GRK2 knock down cells, however, paroxetine's effects on LPS-induced IL-6 and TNF α production were similar to that of control siRNA transfected cells suggesting that these effects are likely independent of GRK2 (Fig 6).

Signaling mechanisms of paroxetine's effect in macrophages

To further delineate the signaling mechanisms by which paroxetine could be regulating LPS-induced cytokine production, we examined the effect of paroxetine treatment on LPS-induced MAPK and NF κ B signaling pathways. For this, we pretreated Raw264.7 macrophages with paroxetine followed by treatment with LPS for 30 or 60 minutes. As shown in Fig 7, even though paroxetine did not affect LPS-induced MAPK pathways at either 30 or 60 min after LPS treatment, I κ B α phosphorylation was significantly attenuated by paroxetine at 30 min after LPS treatment (Fig 7). Since pI κ B α level is associated with NF κ B activation, this effect of paroxetine on NF κ B pathway might be translated to LPS-induced IL-6 production. However, since the effect appears to be modest, it is likely that paroxetine's effects on LPS-induced cytokines are likely to be mediated via additional signaling mechanisms.

Discussion

Serotonin (5-HT) is an important neurotransmitter that has myriad effects in many cell types and modulates neuronal and extra-neuronal functions including gastric motility, epithelial cell secretion, vasodilation as well as inflammation [12]. Because of these various functions, the serotonergic system has been considered a therapeutic target for a number of different diseases. The most noteworthy of these is the use of therapeutic drugs to target this system for treating depression. 5-HT is synthesized from L-Tryptophan via a rate-limiting enzymatic step catalyzed by tryptophan hydroxylases (TPH). Extra-neuronally, 5-HT is synthesized by enterochromaffin cells. 5-HT released into the blood stream is taken up and stored by platelets to a larger extent but also by other immune cells to a smaller extent[29]. 5-HT acts via G-protein coupled receptors (5-HT₁ to 5-HT₇). While 5-HT system can be modulated at the level of TPH as well as at the level of the receptor, controlling re-uptake of 5-HT via the SERT transporters has also proven to be a clinically relevant target. In this regard, SERT inhibitors (selective serotonin reuptake inhibitors, SSRIs) have been shown to inhibit 5-HT reuptake thereby enhancing 5-HT actions. This in fact is of high clinical relevance since selective serotonin reuptake inhibitors (SSRIs) are widely used in the treatment of depression primarily due to their effects in enhancing serotonin effects. Paroxetine and other SSRIs are indeed the first line anti-depressants in clinical use. In addition to their anti-depressant activity, a number of SSRIs including paroxetine, fluoxetine, sertraline, venflaxine, and fluoxamine have been attributed anti-inflammatory properties. However, most studies looking at the role of SSRIs especially in immune cells have not confirmed that the effects are indeed mediated via modulating the 5-HT system.

In some early studies, paroxetine was shown not to have any immunomodulatory effects particularly related to the humoral immune response as well as macrophage phagocytosis [30]. Macrophage cytokine response was however, not assessed in those studies. In the present study, we sought to determine the effects of paroxetine and fluoxetine on LPS-induced cytokine production in macrophages. We focused on IL-6, and TNF α because of the role of these cytokines in various inflammatory disease processes[17,18]. Interestingly, our results reveal that paroxetine (and fluoxetine) are potent inhibitors of LPS-induced IL-6 production. Incidentally, in a Japanese study involving midlife women during menopausal transition, treatment with paroxetine was shown to decrease in IL-6 levels in the serum [31]. Even though role of paroxetine on macrophage cytokine responses has not been examined, studies have looked at the role of other SSRIs, especially fluoxetine on cytokine responses in macrophages as well as macrophage-like cells. In this regard, fluoxetine has been shown to inhibit LPS-induced pro-inflammatory cytokines in human monocytes as well as in microglial cells [6,32]. More recent studies have also shown that fluoxetine is a potent modulator of macrophage function and that it is able to transition macrophages from M1 to M2 phenotype. These effects were shown to be due to direct actions of the drug on macrophages, as well as indirectly via neuroendocrine mechanisms [33]. Because of these various effects of SSRIs on inflammation, studies have also looked at the role of SSRIs in mouse models of experimental colitis [9], arthritis[11], periodontitis [10] and also in facilitating wound healing [34]. More recently, paroxetine has also been shown to possess

anti-inflammatory properties in neuro-inflammation models relevant in Parkinson's disease [8].

Even though the effect of paroxetine on LPS-induced IL-6 in the present study is consistent with the reported anti-inflammatory effects of SSRIs, our studies uncovered unique role of paroxetine and fluoxetine on LPS-induced TNF α production. Our results clearly show both SSRIs enhance LPS-induced TNF α secretion, opposite to the effects on IL-6. Although the molecular mechanism of this effect on TNF α remains to be determined, a recent study reported (while this manuscript was in preparation) that fluoxetine is indeed capable of enhancing TNF α secretion from macrophages infected with *Mycobacterium tuberculosis*. Fluoxetine was further shown to enhance autophagy in the infected macrophages [35]. Together it is clear that SSRIs are capable of modulating macrophage inflammatory response in a differential manner.

Macrophages express serotonin transporter SERT and are thus amenable to inhibition by SSRIs [36]. In addition, macrophages have been shown to synthesize and release 5-HT in culture [23] and 5-HT has been shown to modulate IL-6 and TNF α in human immune cells depending on the concentration and source of the agonist (endogenous versus exogenous) [37]. Therefore we tested whether the effects of paroxetine in our system were mediated via the 5-HT system. Pre-treatment of macrophages with 5-HT prior to LPS did not alter LPS response in terms of TNF α , and IL-6 production. This was in contrast to a recent study showing that in M2-skewed human macrophages, LPS-induced TNF α was decreased by 5-HT treatment [28]. Because we didn't see any effect of direct 5-HT treatment, we determined 5-HT levels from macrophages in culture. Our results demonstrate that these cells are indeed capable of producing 5-HT and the 5-HT produced is amenable to catabolism to 5-HIAA. Interestingly, none of our treatment conditions including paroxetine, enhanced 5-HT or altered its turnover, except for LPS+paroxetine at 24 hour treatment. Whether the modest change observed with LPS+paroxetine is sufficient for the observed effects on cytokines is not clear. It is however reasonable to conclude that the effect of paroxetine on LPS-induced IL-6 production is likely independent of 5-HT since the 5-HT receptor antagonist inhibited LPS-induced IL-6 production both in the presence and absence of paroxetine. The same antagonist however, reversed the effect of LPS+paroxetine on TNF α , suggesting that this effect of paroxetine is mediated via 5-HT. Whether the modest effect of LPS+paroxetine on 5-HT levels is sufficient to alter receptor activation is not clear and will be the subject of future studies. Other recent studies have shown that SSRIs have targets distinct from serotonin transporter. In one such study paroxetine but not fluoxetine was shown to be a potent GRK2 inhibitor [19]. Our results using GRK2 knockdown cells and the similarity of effects between fluoxetine and paroxetine suggest that GRK2 inhibition cannot explain the effect of paroxetine on IL-6. Since effect of paroxetine on IL-6 was neither mediated via 5-HT receptor nor by GRK2 inhibition, other mechanisms are likely at play. In this regard, a recent study showed that fluoxetine's inhibitory effect on LPS-induced iNOS/NO release and COX-2/PGE2 production are dependent on GSK3 β in macrophages[38].

Our attempt at further identifying the signaling mechanisms by which paroxetine regulates LPS-induced cytokines was only partly successful since paroxetine did not affect majority of

the MAPK and NF κ B signaling pathways except for a decrease in I κ B α phosphorylation, consistent with a decrease in IL-6 (which is regulated by I κ B α -NF κ B pathway)[39]. It should be noted that our goal here was to uncover any differential effect of paroxetine on MAPK and NF κ B pathways, even though both of these pathways have been shown to be important regulators of IL-6 and TNF α regulation. It certainly appears from our studies that regulation of IL-6 and TNF α by paroxetine cannot be simply explained by modulation of these two important pathways alone. However, consistent with our studies on the effect of paroxetine on I κ B α phosphorylation, fluoxetine was shown to inhibit TNF α -induced NF κ B activation in intestinal epithelial cells [9]. It is also important to note that the dose of LPS used in our study is the maximal concentration of what is being used in the literature in cell culture studies. While studies have shown that high versus very low concentrations of LPS can have varied effects on signaling pathways especially in immune cells, we did not examine the role of very low dose LPS in SSRI-induced effects. This will be the focus of future studies.

Given that paroxetine is an FDA approved drug, future studies focused on the molecular mechanisms of interaction of paroxetine with the components of transcription and translational machineries might provide more insight into understanding its differential effects. In addition, studies focused on a thorough understanding of the molecular mechanisms and identification of potential targets in the 5-HT signaling pathways might enable the use of FDA-approved SSRIs to treat conditions marked by dysregulated inflammation.

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Highlights

Paroxetine, an FDA approved drug distinctly regulates IL-6 and TNF α in macrophages.

Fluoxetine, a drug belonging to the same class as paroxetine mimicks paroxetine.

Mechanisms by which paroxetine regulates LPS-induced IL-6 and TNF α appear to be distinct.

Raw264.7 cells **Elicited Peritoneal macrophages**

Figure 1. Differential effect of Paroxetine on LPS-induced IL-6 and TNFα production in mouse macrophages

RAW 264.7 (top panel) and thioglycollate-elicited peritoneal macrophages (bottom panel) were treated with or without LPS (1 μg/ml) and paroxetine hydrochloride (at the indicated concentrations) for 6 and 24 hours. ELISA was used to determine IL-6 and TNFα levels in the culture supernatants. Cytokine levels were normalized to total cellular protein, and expressed as percent LPS stimulation (LPS stimulation = 100%). Note that cytokines were not detected in the basal group. LPS-induced IL-6 and TNFα levels were as follows: IL-6-Raw264.7: 1.9 ± 0.5 and 5.7 ± 2 ng/μg protein @ 6 and 24 hours respectively; Peritoneal macrophages: 252 ± 106 and 714 ± 146 pg/μg protein @ 6 and 24 hours respectively. TNFα-Raw264.7: 1.7 ± 0.2 and 2.2 ± 0.4 ng/μg protein @ 6 and 24 hours respectively; Peritoneal macrophages: 407 ± 145 and 287 ± 43 pg/μg protein @ 6 and 24 hours respectively. N=4-6; *P<0.05; **P<0.01; ***P<0.001; ****P<0.0001.

Raw264.7 cells **Elicited Peritoneal macrophages**

Figure 2. Differential effect of Fluoxetine on LPS-induced IL-6 and TNFα production in mouse macrophages

RAW 264.7 (top panel) and thioglycollate-elicited peritoneal macrophages (bottom panel) were treated with or without LPS (1 μg/ml) and fluoxetine hydrochloride (at the indicated concentrations) for 6 and 24 hours. ELISA was used to determine IL-6 and TNFα levels in the culture supernatants. Cytokine levels were normalized to total cellular protein, and expressed as percent LPS stimulation (LPS stimulation = 100%). Note that cytokines were not detected in the basal group. LPS-induced IL-6 and TNFα levels were similar to the ones outlined in Fig 1 legend. N=4-6; *P<0.05; **P<0.01; ***P<0.001; ****P<0.0001.

Figure 3. Effect of 5-HT on LPS-induced IL-6 and TNFα production in mouse macrophages

RAW 264.7 cells were treated with LPS (1 μg/ml) and 5-HT at the indicated concentrations for 6 and 24 hours. Levels of IL-6 and TNFα were assessed in the culture supernatants by ELISA. Cytokine levels were normalized to total cellular protein and expressed as ng/ μg of total cellular protein. Note that 5-HT treatment alone did not lead to any detectable levels of cytokines. N=5-6.

Figure 4. Levels of 5-HT and 5-HT turnover (5-HIAA:5HT ratio) in mouse macrophages RAW 264.7 cells were untreated or treated with LPS (1 μ g/ml) $\pm$ paroxetine (20 μ M) for 6 and 24 hours. Cell culture supernatants were then assayed for 5-HT and 5-HIAA concentrations using HPLC coupled with electrochemical detection as described before [24]. 5-HT turnover was determined by calculating the ratio of 5-HIAA:5-HT. N=3.

Figure 5. Effect of LY215840 on LPS and paroxetine-induced IL-6 and TNF α production in macrophages

RAW 264.7 cells were treated with LPS (1 μ g/ml), Paroxetine and LY215840 as indicated for 24 hours and culture supernatants assayed for IL-6 and TNF α by ELISA. Cytokine levels were normalized to total cellular protein and results are expressed as percent LPS stimulation (LPS stimulation=100%). Note that basal levels of cytokines were undetectable. LPS-induced IL-6 and TNF α levels were as follows: IL-6- 0.414 ± 0.1029 and 1.886 ± 0.2723 ng/ μ g protein @ 6 and 24 hours respectively; TNF α - 1.702 ± 0.698 and 3.948 ± 1.512 ng/ μ g protein @ 6 and 24 hours respectively. N=5-6; *P<0.05; ***P<0.001.

Figure 6. Role of GRK2 on paroxetine's effect on LPS-induced IL-6 and TNF α production

RAW 264.7 cells were transfected with either control siRNA or GRK2 siRNA smart pool using amaxa nucleofector (Program- D032). 48 h post transfection, cells were treated with paroxetine (20 μ M) and LPS (1 μ g/ml) as indicated. GRK2 levels were tested by Western blotting of whole cell lysates and shown in A (representative blots in the top, quantitation in the bottom). Culture supernatants from the treatment groups were assayed for IL-6 and TNF α by ELISA and normalized for total protein. Results are expressed as percent LPS stimulation. LPS-induced IL-6 and TNF α levels were as follows: IL-6- 1.988 0.4049 and 1.939 0.482 ng/ μ g protein for control siRNA and GRK2 siRNA transfected cells,

respectively; TNF α - 6.148 $\pm$ 1.345 and 7.55 $\pm$ 1.264 ng/ μ g protein for control siRNA and GRK2 siRNA transfected cells, respectively. N=5. *P<0.05; ***P<0.001; ****P<0.0001.

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Figure 7. Effect of paroxetine on LPS-induced NFκB and MAPK signaling pathways
RAW 264.7 cells were treated with LPS (1 μg/ml) and paroxetine (20 μM) for 30 and 60 minutes as indicated. Whole-cell lysates were subjected to Western blotting as indicated for pIκBα, pP38, pJNKp46/p54, pERK1/2. Quantitation was done after normalizing for loading (by normalizing phospho-blot with tubulin or ERK2 in case of pERK1/2). Results are expressed as percent LPS stimulation (LPS stimulation at 30 min = 100%). Representative blots are shown on the top left. N=3. **P<0.01.