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Indoleamine 2,3-dioxygenase inhibition attenuates lipopolysaccharide induced persistent microglial activation and depressive-like complications in fractalkine receptor (CX_3CR1)-deficient mice

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Abstract

An impaired ability to regulate the activation of microglia by fractalkine (CX_3CL1) leads to persistent neuroinflammation and behavioral alterations following lipopolysaccharide (LPS) challenge. While these responses are usually transient, LPS injection caused prolonged depressive-like behavior in fractalkine receptor deficient mice ($CX_3CR1^{-/-}$) that was associated with exaggerated microglial activation and induction of the tryptophan (TRP) degrading enzyme indoleamine 2,3-dioxygenase (IDO). IDO activation and subsequent generation of neuroactive kynurenine metabolites may have a pivotal role in the development of depression. Therefore, the purpose of this study was to determine the extent to which LPS-induced depressive-like behavior in $CX_3CR1^{-/-}$ mice was dependent on IDO activation. $CX_3CR1^{-/-}$ mice were implanted prior to LPS challenge with a slow release pellet of 1-methyl-tryptophan (1-MT), a competitive inhibitor of IDO. Here we show that the depressive-like behavior evident in $CX_3CR1^{-/-}$ mice 72 h after LPS injection was abrogated by inhibition of IDO. LPS also decreased body weight and locomotor activity in $CX_3CR1^{-/-}$ mice, but these effects were independent of 1-MT. Consistent with the increased metabolism of TRP by IDO, the ratio of 3-hydroxykynurenine (3-HK) to TRP was

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increased in the brain 72 h after LPS. Increased serotonin (5-HT) turnover was also evident in the brain. The LPS-associated increases in both 3-HK:TRP and 5-HIAA:5-HT ratios were prevented by the inhibition of IDO. Last, IDO blockade attenuated microglial activation in the prefrontal cortex and hippocampus 72 h after LPS. Collectively these data indicate that LPS-induced IDO activation contributes to persistent microglial activation and depressive-like behavior in $CX_3CR1^{-/-}$ mice.

Keywords

Microglia; Depression; Indoleamine 2; 3-dioxygenase; Kynurenine; fractalkine receptor; 1-methyl-tryptophan

1. Introduction

Microglia are resident innate immune cells of the CNS that are important in immune surveillance (Davalos et al., 2005; Nimmerjahn et al., 2005) and help to interpret and propagate inflammatory signals in the brain. For example, activated microglia produce pro-inflammatory cytokines and secondary messengers that promote physiological and behavioral responses including sickness behavior (Dantzer et al., 2008). The cytokine mediated sickness response is normally adaptive, but amplified or prolonged microglial activation is associated with behavioral and cognitive complications (Corona et al., 2011). For this reason it is critical that the activation of microglia is tightly regulated. There are several key regulatory systems within the brain that modulate microglia activation including fractalkine (CX_3CL1) production by neurons.

Complementary expression of CX_3CL1 on neurons and fractalkine receptor (CX_3CR1) on microglia (Hughes et al., 2002; Maciejewski-Lenoir et al., 1999; Nishiyori et al., 1998; Pan et al., 1997) establishes a unique communication system whereby neurons constitutively express CX_3CL1 to regulate the activation of microglia (Cardona et al., 2006). In support of this notion, CX_3CR1 deletion ($CX_3CR1^{-/-}$) increased the inflammatory profile of the brain and was associated with decreased neurogenesis, reduced long term potentiation, and deficits in motor and spatial learning (Bachstetter et al., 2011; Rogers et al., 2012). Moreover, several rodent models indicate that CX_3CL1-CX_3CR1 interactions are impaired with normal aging (Bachstetter et al., 2011; Lyons et al., 2009; Wynne et al., 2010).

The interactions between fractalkine (CX_3CL1) and the corresponding fractalkine receptor (CX_3CR1) are particularly important when microglia become activated by an inflammatory challenge (Cardona et al., 2006; Mizuno et al., 2003; Zujovic et al., 2001; Zujovic and Taupin, 2003). In aged mice, peripheral injection of LPS caused protracted down-regulation of CX_3CR1 expression on microglia that corresponded with amplified IL-1β mRNA levels compared to adult mice (Wynne et al., 2010). Similar to previous studies in older mice (Godbout et al., 2005; Godbout et al., 2008), $CX_3CR1^{-/-}$ mice had prolonged sickness behavior (24 h) and depressive complications (72 h) following a single i.p. injection of LPS (Corona et al., 2010). Furthermore, the LPS-induced prolonged depressive-like behavior and microglial activation in $CX_3CR1^{-/-}$ mice was associated with an amplified induction of indoleamine 2,3-dioxygenase (IDO) and kynurenine monooxygenase (KMO) mRNA (Corona et al., 2010). These two enzymes are involved in the degradation of tryptophan into several neuroactive metabolites that influence the inflammatory state of the CNS and affect behavioral responses (Haroon et al., 2012).

IDO is activated by inflammatory cytokines (i.e., IFNα, IFNγ, IL-1, TNFα) and provides the initial enzymatic activity in the degradation of tryptophan into several metabolites that

have the potential to disrupt several key neurotransmitter systems in the CNS. In the brain, active IDO in microglia converts tryptophan (TRP) to kynurenine (KYN) (Guillemin et al., 2005) and then KYN is further processed by KMO to produce the neuroactive metabolites, 3-hydroxykynurenine (3-HK) and quinolinic acid (QUIN). Because the rate liming step in the synthesis of serotonin (5-HT) involves the catabolism of TRP by tryptophan hydroxylase, the reduction in TRP may affect the concentrations of 5-HT. In addition, increased levels of KYN-related metabolites (3-HK and QUIN) are associated with increased lipid peroxidation, agonism of NMDA glutamate receptors and disruption of dopamine signaling (Dantzer et al., 2011; Haroon et al., 2012; Muller and Schwarz, 2007). Because these neurotransmitters are affected, activation of the IDO pathway has been linked to depressive-like complications in rodents (O'Connor et al., 2009a; O'Connor et al., 2009b; O'Connor et al., 2009c) and humans (Capuron et al., 2011; Raison et al., 2010; Steiner et al., 2011). For example, Bacille Calmette-Guerin (BCG) infection or LPS injection in CD-1 mice caused depressive like behavior that was blocked by 1-methyl-tryptophan (1-MT), a tryptophan analog and competitive inhibitor of IDO (O'Connor et al., 2009b; O'Connor et al., 2009c). In humans, interferon (IFN)-α therapy for hepatitis C patients is associated with depressive complications (Haroon et al., 2012). IFN-α increases the KYN concentration in the blood and the cerebrospinal fluid (CSF) (Capuron et al., 2011; Raison et al., 2010) and the increased levels of KYN in the CSF of depressed patients positively correlated with depressive symptoms (Raison et al., 2010).

Based on these data, we hypothesized that an impaired ability to regulate microglia by CX_3CR1 causes prolonged IDO activation in response to LPS and the subsequent downstream processing of KYN, leading to the development and persistence of depressive-like behavior. Here we show that inhibition of IDO with 1-MT attenuated microglial activation in the pre-frontal cortex and hippocampus and blocked depressive-like behavior induced in $CX_3CR1^{-/-}$ mice by LPS injection.

2. Materials and methods

2.1. Animals

Adult (3–6 mo) C57BL/6 fractalkine receptor deficient mice ($CX_3CR1^{-/-}$) from our in-house specific pathogen free colony were used (Corona et al., 2010). Mice were housed in polypropylene cages and maintained at 25°C under a reverse-phase, 12 h light/12 h dark cycle with *ad libitum* access to water and rodent chow. Mice were individually housed 2-weeks prior to implantation with placebo or 1-MT pellets and remained individually housed for the duration of the experimental procedures. All procedures were in accordance with the National Institute of Health Guidelines for the Care and Use of Laboratory Animals and were approved by The Ohio State University Institutional Laboratory Animal Care and Use Committee.

2.2. Experimental Design

In these experiments, adult $CX_3CR1^{-/-}$ mice were implanted subcutaneously (s.c) with a slow release pellet filled with either placebo or 1-methyl-DL-tryptophan (1-MT; Sigma) as previously described (O'Connor et al., 2009b; O'Connor et al., 2009c). Pellets were purchased pre-filled from Innovative Research of America (Sarasota, FL) and were designed to deliver 5 mg/day of drug or placebo for 7 days (O'Connor et al., 2009b; O'Connor et al., 2009c). After 3 days of 1-MT or placebo pretreatment, mice were injected intraperitoneally (i.p.) with saline or LPS (0.5 mg/kg; serotype 0127:B8, Sigma, St. Louis, MO). This LPS dosage was selected because it elicits a pro-inflammatory cytokine response in the brain resulting in a transient sickness response in wild type and $CX_3CR1^{+/-}$ mice (Corona et al., 2010). Locomotor activity, body weight, and food intake were determined 0, 24, 48, and 72

h after injections (n=8). At 72 h after injections, depressive-like behavior was determined using the tail suspension test (n=8–10). Within 2 h of the completion of behavioral testing, a subset of mice were sacrificed and the brain was collected to determine 1-MT, TRP, KYN, 3-HK, 5-HT and 5-hydroxyindoleacetic acid (HIAA) levels (n=4–8). A second cohort of mice were transcardially perfused with sterile phosphate-buffered saline (PBS, pH 7.4 w/ EDTA) followed by fixation with 4% formaldehyde. Brains were removed, processed, and labeled with an anti-Iba-1 antibody (n=4).

2.3. Behavior

Locomotor activity and depressive-like behavior were determined as previously described (Godbout et al., 2005; Godbout et al., 2008; O'Connor et al., 2009c). In brief, mice were acclimated to routine handling for 5 days before experimentation. Behavioral tests were conducted during the dark phase (between 1200 and 2400) of the photoperiod under infrared lighting in the same room in which the mice were normally housed. Behavior was scored by a trained observer who was blind to the experimental treatments.

For locomotor activity, mice were maintained in their home cage with a floor area of 26 × 20 cm, and activity was video recorded for 3 minutes. On the video records, cages were divided into 6 identical virtual rectangles and the number of line crossings was determined.

For depressive-like behavior, the tail suspension test (TST) was used. Mice were suspended by their tail in a 32 × 33 × 33 cm box and the duration of immobility was determined over a 10 min period (Corona et al., 2010; Godbout et al., 2008; O'Connor et al., 2009c). Time spent immobile was determined by a trained observer who was blind to the experimental treatments. The first 2 minutes were omitted to allow the mice to acclimate to the testing procedures. Results are expressed as the total immobility for the last 8 minutes of the test.

2.4. Neurochemistry

Plasma and brain levels of KYN, TRP, and 1-MT were determined as previously described (O'Connor et al., 2009c). For the plasma samples, 50 μl of plasma was deproteinated with 10% sulfosalicylic acid (5:1, v:v). For the brain samples, whole brain was weighed and then mechanically homogenized in 0.1N $HClO_4$ + 25 μM ascorbate using an ultrasonic tissue disruptor (Sonics & Materials, Inc., Newtown, CT). For brain and plasma samples, supernatants were extracted and loaded into a Costar Spin-X centrifuge tube filter (0.22μM Nylon Part #8169 Corning Incorporated) and centrifuged at 12,000 x g for 6 minutes at 4°C. Samples were collected and diluted in 0.02 N $HClO_4$ and KYN, TRP, and 1-MT were determined by HPLC using an ESA Coulochem III detector with a 5041 Enhanced Analytical cell containing a glassy carbon electrode (+600 mV). Mobile phase (pH = 4.6) consisted of 75 mM NaH_2PO_4, 25 μM EDTA (disodium salt), and 100μl/L triethylamine in acetonitrile:water (6:94 v:v)

5-hydroxytryptamine (5-HT), 5-hydroxyindoleacetic acid (5-HIAA), and 3-hydroxykynurenine (3-HK) were analyzed in whole brain using an ESA Coulochem III detector with a 5041 Enhanced Analytical cell containing a glassy carbon electrode (+320 mV) as previously described (O'Connor et al., 2009c). The mobile phase (pH = 3.0) consisted of 75 mM NaH_2PO_4, 25 μM EDTA (disodium salt), 0.45 mM octanesulfonic acid, and 100μl/L triethylamine in acetonitrile:water (6:94 v:v). The chromatograms were integrated and quantified using ESA EZ Chrom SI software (ESA Inc., Chelmsford, MA).

2.5. Immunohistochemistry and digital image analysis

Mice were deeply anesthetized and transcardially perfused with sterile PBS followed by 4% formaldehyde. Brains were post-fixed in 4% formaldehyde for 24 h and then cryoprotected

in 20% sucrose for an additional 24 h. Preserved brains were frozen using dry-ice cooled isopentane (−165°C) and then sectioned (20 μm) using a Microm HM550 cryostat. Brain sections were identified by reference markers in accordance with the stereotaxic mouse brain atlas (Paxinos and Franklin, 2004). Sections were mounted on microscope slides and stored at −20°C prior to staining. Iba-1 staining was performed as previously described (Corona et al., 2010). In brief, sections were blocked and then incubated with rabbit anti-mouse Iba-1 antibody (Wako Pure Chemical Industries, Ltd., VA) overnight at 4°C. Next, HRP-conjugated goat anti-rabbit secondary antibody was added for 1 h at room temperature and staining was developed using the Vector VIP kit (Vector laboratories, CA).

Brightfield images were visualized using an epifluorescent Leica DM5000B microscope and were captured using a Leica DFC300 FX camera and imaging software. To quantify the phenotypic changes of microglia, digital image analysis (DIA) of Iba-1 staining was performed (Donnelly et al., 2009) in the hippocampus (−.1.7 to −2.3 mm bregma) and prefrontal cortex (+2.34 to +1.98 mm bregma). To determine Iba-1 phenotype in the hippocampus, five representative images were taken at 20x magnification in the dentate gyrus, CA1, and CA3 regions. For the prefrontal cortex, 6 representative pictures were analyzed. A threshold for positive staining was determined for each image that included all cell bodies and processes, but excluded background staining. Thresholded targets were analyzed with ImageJ software using densitometry. Results were reported as the average percent area in the positive threshold for all representative pictures.

2.6. Statistical Analysis

To ensure a normal distribution, data were subjected to Shapiro-Wilk test using Statistical Analysis Systems (SAS) software (Cary, NC). Observations greater than 3 interquartile ranges from the first and third quartile were considered outliers and were excluded in the subsequent analysis. Overall, less than 1% of the total observations were determined to be outliers. To determine significant main effects and interactions between main factors, data were analyzed using one- (Pretreatment, Treatment, Time), two- (Pretreatment × Treatment, Treatment × Time) or three- (Pretreatment × Treatment × Time) way ANOVA using the General Linear Model procedures of SAS. When appropriate, differences between treatment means were evaluated by an *F*-protected *t*-test using the Least-Significant Difference procedure of SAS. All data are expressed as treatment means ± standard error of the mean (SEM).

3. Results

3.1. LPS-induced depressive-like behavior in $CX_3CR1^{-/-}$ mice is blocked by 1-MT pretreatment

Our previous report indicates that a single i.p. injection of LPS caused depressive-like complications that persisted longer in $CX_3CR1^{-/-}$ mice compared to $CX_3CR1^{+/-}$ control mice (Corona et al., 2010). Moreover, this protracted depressive-like behavior in $CX_3CR1^{-/-}$ mice was associated with exaggerated microglial activation and higher induction of IDO, KMO, and IL-1β mRNA (Corona et al., 2010). Therefore, the objective of this study was to determine the degree to which LPS-induced depressive-like behavior in $CX_3CR1^{-/-}$ mice was dependent on the activation of the IDO pathway. To address this objective, $CX_3CR1^{-/-}$ mice were implanted (s.c.) with slow-release pellets filled with either placebo or 1-methyl-tryptophan (1-MT), a tryptophan analog and competitive inhibitor of IDO (O'Connor et al., 2009c). Only $CX_3CR1^{-/-}$ mice were used in these 1-MT experiments because our previous data indicates that LPS caused depressive-like behavior in $CX_3CR1^{-/-}$ mice, but not in $CX_3CR1^{+/-}$ mice (Corona et al., 2010).

Three days following implantation with placebo or 1-MT pellets, $CX_3CR1^{-/-}$ mice were injected i.p. with saline or LPS. Locomotor activity and body weight were determined at 0 (baseline), 24, 48 and 72 h later and were expressed as percent of baseline. Fig. 1A shows that LPS injection decreased body weight in a time-dependent manner (LPS × time interaction, $F(3,31)=9.54$, $p<0.001$). There was neither a main effect of 1-MT treatment nor a LPS × 1-MT interaction on body weight. Fig. 1B shows that LPS injection also decreased locomotor activity ($F(1,31)=25.9$, $p<0.0001$) in a time dependent manner (LPS × time interaction; $F(3,31)=6.74$, $p < 0.04$). For instance, LPS-injected mice (Placebo-LPS and 1-MT-LPS groups) had significant reductions in locomotor activity at 24 h ($p < 0.001$) and 48 h ($p < 0.04$), but returned to baseline locomotor activity by 72 h. Similar to the body weight loss data, there was neither a main effect of 1-MT treatment nor a LPS × 1-MT interaction on locomotor activity. Taken together, LPS injection reduced body weight and locomotor activity in $CX_3CR1^{-/-}$ mice independent of IDO activation.

Reductions in locomotor activity can confound the testing for depressive-like behavior. Therefore, immobility in the tail suspension test (TST) was determined 72 h after LPS when mice had returned to baseline locomotor activity (Corona et al., 2010; Frenois et al., 2007; Godbout et al., 2008; O'Connor et al., 2009c). Fig. 1C shows time spent immobile in the TST 72 h after injection of saline or LPS. Immobility was affected by LPS ($F(1,44)= 4.01$, $p<0.05$), 1-MT ($F(1,44)= 6.52$, $p<0.02$), and there tended to be an interaction between LPS and 1-MT ($F(1,44)=3.22$, $p=0.08$). *Post hoc analysis* confirmed that Placebo-LPS mice were significantly more immobile in the TST compared to all other treatment groups ($p< 0.02$) and that the 1-MT-LPS mice were not different than either saline treated groups (Placebo-saline or 1-MT-saline). Taken together, these data indicate that IDO activation contributes to the LPS-induced depressive-like behavior in $CX_3CR1^{-/-}$ mice.

3.2 Tryptophan (TRP) and Kynurenine (KYN) in the plasma and brain 72 h after LPS

Following the completion of behavioral testing at 72 h, blood and whole brains were collected to determine the concentrations of 1-MT, TRP, and KYN in the plasma and brain. As expected, Fig. 2A shows that 1-MT was detected only in the plasma of $CX_3CR1^{-/-}$ mice that were implanted with 1-MT pellets ($F(1,19)=108.9$, $p<0.001$). Moreover, there was a significant reduction in the levels of 1-MT in the plasma of mice that were injected with LPS (1-MT × LPS interaction, $F(1,19)=5.1$, $p < 0.04$). Fig. 2B&C show that 72 h after LPS injection plasma levels of TRP were decreased ($F(1,23)=15.0$, $p<0.001$) and levels of KYN were increased ($F(1,23)=5.0$, $p < 0.04$). The corresponding ratio of KYN to TRP in the plasma is shown in Fig. 2D. While LPS injection increased the ratio of KYN to TRP in the plasma ($F(1,23)=40.8$, $p < 0.0001$), this increase was unaffected by 1-MT pretreatment.

The concentrations of 1-MT, TRP, and KYN were also determined in the whole brain (pg/mg of wet brain weight). Similar to the results obtained in the plasma, Fig. 3A shows that 1-MT was only detected in mice implanted with 1-MT pellets ($F(1,31)=201.6$, $p < 0.0001$). Moreover, the levels of 1-MT in the brain were also reduced by LPS injection ($F(1,31)=9.9$, $p<0.005$). In the brain, 1-MT tended to decrease the levels of TRP (Fig. 3B, $F(1,23)= 3.8$, $p=0.07$). *Post hoc analysis* indicated that the highest levels of TRP in the brain were in the placebo-treated mice injected with saline (Placebo-Saline) ($p < 0.05$). Levels of KYN in the brain were not significantly affected by either LPS or 1-MT (Fig. 3C). The corresponding ratio of KYN to TRP in the brain is shown in Fig. 3D. These data indicate that the ratio of KYN to TRP in the brain was increased by 1-MT ($F(1,31)=5.21$, $p<0.04$) and tended to be increased by LPS ($F(1,31)=3.2$, $p=0.09$). There was not a significant interaction between 1-MT and LPS on the KYN to TRP ratio.

3.3 The LPS-induced increases in the 5-HIAA to 5-HT and 3-HK to TRP ratios in the brain are reduced by 1-MT

Active IDO degrades TRP, which is the rate limiting amino acid for serotonin (5-HT) biosynthesis (Haroon et al., 2012). In addition, IDO also degrades 5′hydroxytryptophan (5-HTP) and 5-HT to 5-hydroxykynuramine (Fujiwara et al., 1979; Shimizu et al., 1978) (Fig. 6). Moreover, peripheral injection of LPS increases serotonin turnover (e.g., increased ratio of 5-HIAA to 5-HT) in the brain (Corona et al., 2010; Godbout et al., 2008). Therefore, the levels of 5-HT and 5-HIAA were determined in the brain 72 h after saline or LPS injection. Table 1 shows the values for 5-HT and 5-HIAA in the brain. Fig. 4A shows that the 5-HIAA:5-HT ratio was decreased by 1-MT ($F(1,23)=18.05$, $p<0.004$) and increased by LPS ($F(1,23)= 4.29$, $p<0.05$). The *post hoc* analysis confirmed that Placebo-LPS mice had the highest ratio of 3-HIAA:5-HT in the brain compared to all other treatment groups ($p<0.03$). Moreover, the increased 5-HT turnover in the brain 72 h after LPS injection was blocked by 1-MT ($p<0.005$, Fig. 3A).

Because KYN produced by IDO can be subsequently processed to 3-HK by KMO (Okamoto and Hayaishi, 1967; Saito et al., 1957), 3-HK was determined in the brain 72 h after saline or LPS injection. The values for 3-HK and TRP are shown in Table 1 and the ratio of 3-HK to TRP is shown in Fig. 4B. LPS increased the 3-HK:TRP ratio in the brain ($F(1,23)= 7.2$, $p < 0.02$). In addition, the LPS-associated increase in the 3-HK:TRP ratio tended to be reduced by 1-MT treatment (1-MT × LPS interaction, $(1,23)=2.2$, $p=0.10$). The *post hoc* analysis confirmed that Placebo-LPS mice had the highest ratio of 3-HK to TRP in the brain compared to all other treatment groups ($p < 0.03$). Taken together, the LPS associated increases in 5-HIAA:5-HT and 3-HK:TRP were attenuated by 1-MT treatment.

3.4. LPS-induced microglial activation in the hippocampus and prefrontal cortex of $CX_3CR1^{-/-}$ mice is attenuated by 1-MT

Because LPS caused exaggerated and persistent microglial activation in $CX_3CR1^{-/-}$ mice compared to $CX_3CR1^{+/-}$ controls (Corona et al., 2010), ionized calcium binding adapter molecule 1 (Iba-1) staining was determined in the prefrontal cortex and hippocampus (CA1, CA2, and dentate gyrus) 72 h after saline or LPS injection. The prefrontal cortex (PFC) and hippocampus (HPC) were selected because these regions are associated with the control of mood and behavior (Drevets et al., 2008). Representative images of Iba-1 positive microglia from the PFC and the CA1 region of the HPC of $CX_3CR1^{-/-}$ mice are shown in Fig. 5.

Fig. 5 shows that there was increased Iba-1 immunoreactivity in the PFC (top panels) and HPC (bottom panels) 72 h after LPS injection in $CX_3CR1^{-/-}$ mice (Placebo-LPS) compared to all other treatment groups. Microglia from the brain of LPS injected mice had larger cell bodies with thicker and more condensed processes (Fig. 5A). Moreover, the activated morphology of the microglia after LPS injection (Placebo-LPS) was reduced in mice implanted with 1-MT pellets prior to LPS (1-MT-LPS).

Proportional analysis of Iba-1 (Wohleb et al., 2011) staining confirmed that LPS injection increased Iba-1 immunoreactivity in the PFC ($F(1,17)=48.8$, $p< 0.0001$, Fig. 5B). Moreover, microglial activation 72 h following LPS injection in the PFC was attenuated by 1-MT pretreatment (1-MT × LPS interaction, $F(1,17)= 6.3$, $p < 0.03$). A similar effect was also present in the HPC. Fig. 5C shows that there was increased Iba-1 immunoreactivity 72 h after LPS injection in the HPC ($F(1,16)=227.6$, $p < 0.0001$) that was reduced by 1-MT (1-MT × LPS interaction, $F(1,16)=8.4$, $p< 0.01$). Taken together, these data indicate that IDO plays a role in the LPS-associated microglia activation in the PFC and HPC of $CX_3CR1^{-/-}$ mice.

4. Discussion

In our previous studies, impaired regulation of microglia by CX_3CR1 was associated with prolonged and exaggerated microglial activation, sickness behavior, and depressive-like behavior following peripheral LPS challenge in both aged BALB/c mice (Wynne et al., 2010) and adult $CX_3CR1^{-/-}$ mice (Corona et al., 2010). An important finding of the current study is that IDO inhibition with 1-MT blocked the depressive complications in $CX_3CR1^{-/-}$ mice associated with i.p LPS injection (Fig. 1C). The presence of depressive-like behavior in $CX_3CR1^{-/-}$ mice 72 h after LPS injection is consistent with the results of our previous work showing that depressive complications developed and persisted in $CX_3CR1^{-/-}$ mice compared to LPS injected $CX_3CR1^{+/-}$ controls (Corona et al., 2010). Also, similar to previous reports using LPS injection or BCG infection in CD-1 mice (O'Connor et al., 2009b; O'Connor et al., 2009c), 1-MT did not affect the LPS-induced reduction in body weight (Fig. 1A), locomotor activity (Fig. 1B) or food intake (data not shown). These results are consistent with previous reports that immobility in motivational-based tests (e.g., forced swimming and tail suspension tests) are distinct from generalized lethargy induced by i.p. LPS injection (Dantzer et al., 2011). Indeed, the current study (Fig. 1B) and our previous study (Corona et al., 2010) indicate that $CX_3CR1^{-/-}$ mice do not return to baseline locomotor activity and food intake until 72 h after LPS injection. The key point is that when the $CX_3CR1^{-/-}$ mice returned to baseline locomotor activity 72 h after LPS, immobility in the tail suspension test was evident in the Placebo-LPS mice, but not the 1-MT-LPS mice (Fig. 1C).

Several studies have used 1-MT to prevent depressive-like behavior associated with peripheral and central inflammatory challenges. For example, 1-MT blocked depressive-like behavior in CD-1 mice associated with acute LPS injection and chronic infection with BCG (O'Connor et al., 2009b; O'Connor et al., 2009c). 1-MT was also effective in blocking depressive complications associated with i.c.v injection of LPS (Dobos et al., 2012). The current findings extend these previous findings because they indicate that a targeted reduction of IDO activity was effective in limiting depressive-like behavior in a model where microglia regulation is impaired (Cardona et al., 2006; Corona et al., 2010). Taken together, blocking IDO, a downstream mediator of cytokine and inflammatory signals, provided significant protection against the development of depressive-like behavior in $CX_3CR1^{-/-}$ mice.

These findings may be clinically relevant to elderly populations who are more susceptible to depression (Penninx et al., 1999; Penninx et al., 2003) associated with increased inflammatory status (Thomas et al., 2005) and increased TRP metabolism (Capuron et al., 2011). Increased IDO activation and susceptibility to depressive-like behavior following LPS challenge was also apparent in aged (20–24 mo) BALB/c mice (Godbout et al., 2008). Associated with a maladaptive sickness response to i.p. LPS challenge was that aged BALB/c mice had amplified microglia activation, lipid peroxidation, 5-HT turnover, IDO activation, and cytokine production compared to adult controls (Godbout et al., 2005; Godbout et al., 2008; Henry et al., 2008; Henry et al., 2009). A potential connection with the current studies to previous aging work is that the older BALB/c mice also had impaired CX_3CL1/CX_3CR1 interactions. For example, CX_3CL1 protein in the brain was decreased with age and surface expression of CX_3CR1 on microglia from aged mice was decreased after LPS for an extended period compared to adult controls (Wynne et al., 2010). Taken together, these data indicate that there are significant behavioral consequences of an impaired ability to regulate microglia by CX_3CL1/CX_3CR1 interactions and that blocking IDO prevents the development of depressive-like behavior.

A previous study showed that LPS injection increased the ratio of KYN to TRP in the brain and plasma and that these LPS-induced increases were reversed by 1-MT (O'Connor et al., 2009c). In a similar manner, we have previously reported that the KYN to TRP ratio was increased 24 h after LPS in both $CX_3CR1^{+/-}$ and $CX_3CR1^{-/-}$ mice (Corona et al., 2010). Therefore, we expected a similar effect of 1-MT in the current study, but this was not the case. We report that the KYN to TRP ratio tended to be increased in the brain and plasma 72 h after LPS (0.5 mg/kg), but there was not a statistically significant interaction between 1-MT and LPS (Fig. 2D and 3D). It is notable that the concentration of 1-MT in the plasma and brain was decreased in LPS-injected mice (1-MT-LPS) compared to saline-injected mice (1-MT-Saline) (Fig. 2A and 3A, respectively). We interpret these results indicate that increased activity of IDO in the LPS-injected mice led to increased binding of 1-MT to IDO and that the 1-MT bound to IDO may not be detectable as free 1-MT. So while 1-MT is present at this time, we did not detect a significant interaction between 1-MT and LPS on the KYN: TRP ratios. It is possible that the current results are related to timing and that 1-MT blocked IDO activation during a critical window of time after LPS (4–24 h later). By 72 h after LPS, however, most of the direct evidence of IDO activation has dissipated. Adding to the complexity, the higher ratio of KYN to TRP in LPS treated mice at 72 h may reflect an increase in tryptophan 2,3-dioxygenase (TDO) dependent degradation of TRP into KYN, which is not inhibited by 1-MT (Suzuki et al., 2001). Nonetheless, 1-MT intervention was still beneficial because it reduced the LPS induced increase in depressive-like behavior (Fig. 1C), the 5-HT turnover (Fig. 4A), the 3-HK to TRP ratio (Fig. 4B) and Iba-1 reactivity in the brain (Fig. 5).

An interesting finding from this study was that the LPS-associated increase in 5-HT turnover was attenuated by 1-MT (Fig. 3A). This is relevant because increased 5-HT turnover may reflect a reduction in 5-HT bioavailability (Haroon et al., 2012). These results are similar to our previous study showing that the 5-HIAA to 5-HT ratio was increased 24 h after LPS (Corona et al., 2010). This increased 5-HT turnover may be a direct result of IDO activation because active IDO can use TRP, 5-hydroxytryptophan (5-HTP) and 5-HT as substrates (Fujiwara et al., 1979; Shimizu et al., 1978) (Fig. 6). Whatever the attractiveness of the argument, a previous report showed that 1-MT intervention blocked depressive-like behavior, but had no effect on 5-HT turnover in CD-1 mice 24 h after the LPS injection (O'Connor et al., 2009c). Plausible explanations for this discrepancy include different mouse strains, genotypes, and time points of analysis. It may also be that increased KYN production and its subsequent downstream processing to 3-HK and QUIN has a more direct role in the development in depressive-like behavior than decreases in TRP or 5-HT (Dantzer et al., 2011). For instance, IFNα-induced depression in humans was not associated with a reduction in TRP availability, but rather increased levels of KYN and QUIN in the CSF (Raison et al., 2010). In addition, i.p. injection of KYN alone caused depressive-like behavior in mice in a dose-dependent manner (O'Connor et al., 2009c). Thus, increased 5-HT turnover in the brain of $CX_3CR1^{-/-}$ mice may not directly cause depressive-like behavior, but it may be indicative of increased cytokine exposure. In support of this premise, LPS-induced neuroinflammation was associated with higher 5-HT turnover in several models (Corona et al., 2010; Godbout et al., 2008) and LPS-induced 5-HT turnover was reduced by minocycline, which is an anti-inflammatory agent and microglial-related inhibitor (O'Connor et al., 2009c).

Our data support the idea that the IDO initiated pathway progressed in the brain 72 h after LPS. For instance, there was increased 3-HK, 3-HK to TRP, and increased 5-HT turnover in the brain 72 h after LPS. Moreover, these LPS-associated events detected in the brain were prevented by 1-MT (Fig. 4). The increase in 3-HK is consistent with the activation of IDO with the excess KYN being directed towards 3-HK production by KMO (Fig. 6). KYN can also be further processed by kyunrenine aminotransferase (KAT). While the KMO pathway

shifts the processing of KYN towards 3-HK and QUIN by microglia (Guillemin et al., 2005), the KAT pathway shifts the processing of KYN towards the production of kynurenic acid (KYNA) by astrocytes (Du et al., 1992; Mason, 1954). A higher 3-HK to TRP ratio is relevant because 3-HK is a pro-oxidant and a precursor of QUIN, which is also a pro-oxidant and a glutamate agonist (Haroon et al., 2012; Stone and Perkins, 1981). Furthermore, a reduced ratio of KYNA to KYN is associated with depression in humans (Myint et al., 2007). Moreover, a recent study using post mortem immunohistochemical analysis showed increased QUIN positive microglia in the anterior cingulate gyrus of severely depressed and suicidal patients compared to controls (Steiner et al., 2011). Although neither QUIN nor KYNA were determined in this study, the increased 3-HK:TRP ratio 72 h after LPS injection in $CX_3CR1^{-/-}$ mice (Fig. 4B) indicates that increased IDO activation is associated with a shift towards the production of QUIN over KYNA. This idea is supported by our previous finding of increased KMO mRNA levels in microglia isolated from the brain of $CX_3CR1^{-/-}$ mice 24 h after LPS injection (Corona et al., 2010).

A novel aspect of the 1-MT treatment was the attenuation of microglial activation in the prefrontal cortex and hippocampus (Fig. 5). This is relevant because abnormalities in these regions are associated with mood disorders in humans (Drevets, 2000; Drevets et al., 2008). In addition, the cortex and hippocampus also had the highest expression of CX_3CL1 compared to other brain regions in rodents (Nishiyori et al., 1998; Tarozzo et al., 2003). Thus, these regions may be particularly vulnerable to the loss of CX_3CR1 mediated regulation of microglia and corresponding behavioral complications (Corona et al., 2011). While enhanced Iba-1 immunoreactivity was apparent at 72 h after LPS in the Placebo-LPS group, mRNA expression of inflammatory cytokines was no longer detected at this time (data not shown). In our previous report, LPS injection caused amplified and extended IL-1β mRNA expression that was detectable 24 h later in $CX_3CR1^{-/-}$ mice (Corona et al., 2010). Based on other studies, even if cytokines were detected at this time, they would not have been affected by 1-MT (O'Connor et al., 2009b; O'Connor et al., 2009c). This is because IDO activation is downstream of cytokines (Dantzer et al., 2011; Henry et al., 2008; O'Connor et al., 2009c). One possible explanation for an activated morphology (72 h after LPS) in $CX_3CR1^{-/-}$ mice in the absence of cytokines is increased oxidative stress. In support of this notion, 3-HK is a pro-oxidant and 3-HK levels were highest in the Placebo-LPS mice compared to all other treatment groups (Fig. 4). Therefore, limiting enzymes like IDO may be an effective strategy to limit damage caused by oxidative stress.

In conclusion, this study indicates that the LPS-induced prolonged neuroinflammation and depressive-like behavior in $CX_3CR1^{-/-}$ mice is related to IDO mediated processing of TRP into several downstream metabolites including 3-HK. Targeting the IDO pathway with 1-MT attenuated LPS-induced IDO activation, persistent microglia activation, and depressive-like behavior. Therefore, a potential consequence of impaired CX_3CL1-CX_3CR1 signaling in the brain is amplified microglia activation, increased IDO activation, and prolonged depressive-like symptoms following an acute inflammatory stimulus.

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Research Highlight

The IDO inhibitor 1-methyl-tryptophan attenuates microglia activation and depressive behavior in fractalkine receptor deficient mice challenged with LPS.

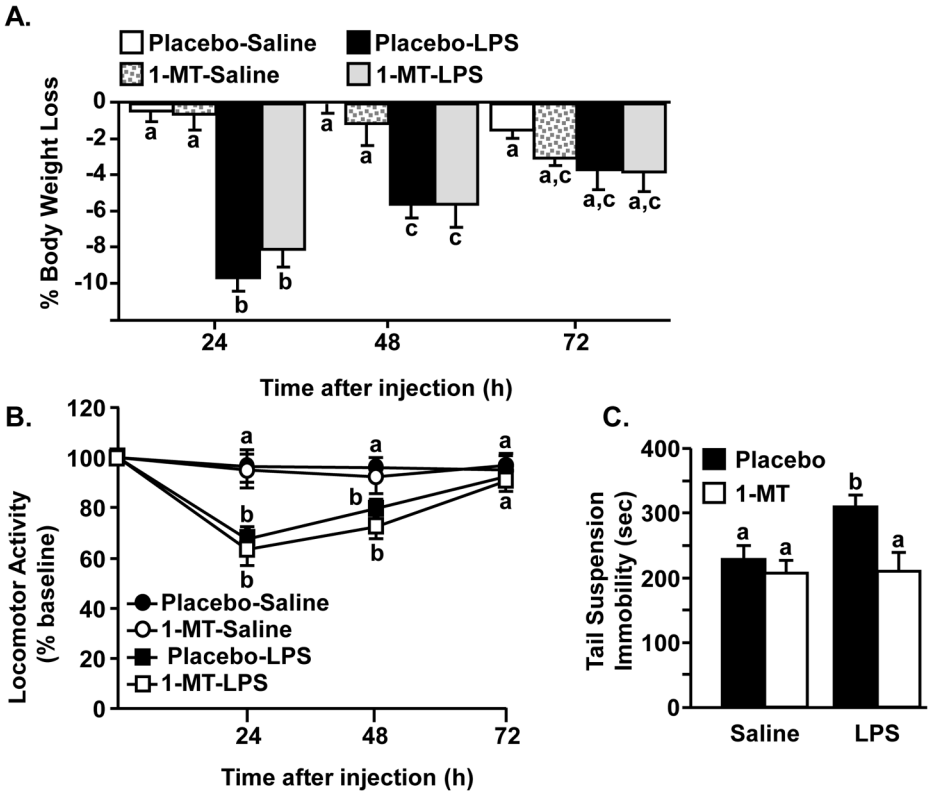

Figure 1. LPS-induced depressive-like behavior in $CX_3CR1^{-/-}$ mice is blocked by 1-MT pretreatment

$CX_3CR1^{-/-}$ mice were implanted s.c. with either placebo or 1-MT filled pellets three days prior to i.p. injection of saline or LPS (0.5mg/kg). A) Body weight (% change) and B) locomotor activity were determined at 0 (baseline), 24, 48 and 72 h after i.p. injections. C) Depressive-like behavior was determined 72 h after LPS injection. Bars represent the mean ± SEM. Means with different letters (a, b or c) are significantly different ($p < 0.05$) from each other.

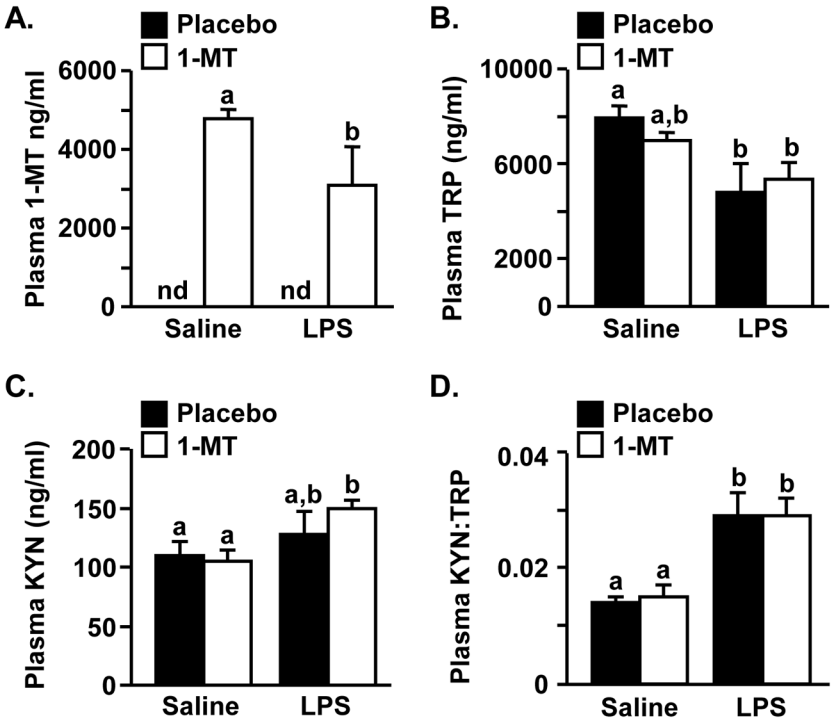

Figure 2. LPS increased the ratio of KYN to TRP in the plasma independent of 1-MT

$CX_3CR1^{-/-}$ mice were implanted s.c. with either placebo or 1-MT filled pellets three days prior to i.p. injection of saline or LPS (0.5 mg/kg). Plasma was collected 72 h later and concentrations of (A) 1-MT, (B) KYN, and (C) TRP were determined. (D) The ratio of KYN to TRP in the plasma is shown. Means with different letters (a or b) are significantly different ($p < 0.05$) from each other.

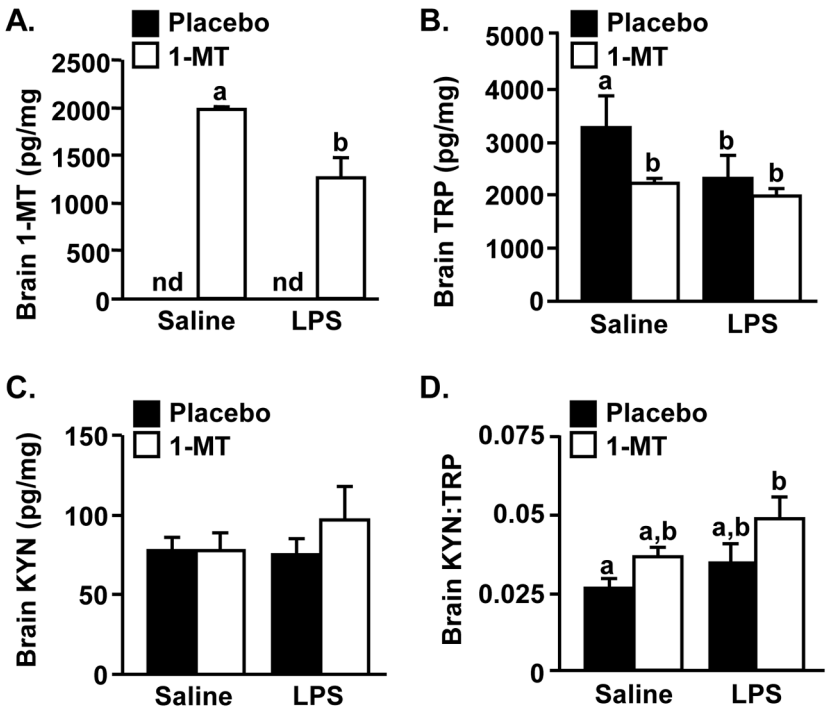

Figure 3. LPS increased the ratio of KYN to TRP in the brain independent of 1-MT

$CX_3CR1^{-/-}$ mice were implanted s.c. with either placebo or 1-MT filled pellets three days prior to i.p. injection of saline or LPS (0.5 mg/kg). Whole brain was collected 72 h later and concentrations of (A) 1-MT, (B) KYN, and (C) TRP were determined. (D) The ratio of KYN to TRP in the brain is shown. Means with different letters (a or b) are significantly different ($p < 0.05$) from each other.

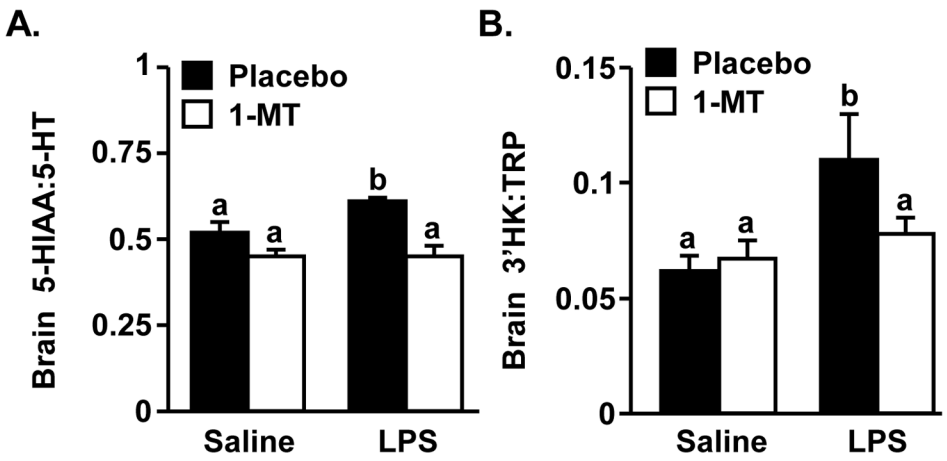

Figure 4. LPS-induced increase in the ratios of 5-HIAA to 5-HT and 3-HK to TRP in the brain were reduced by 1-MT

$CX_3CR1^{-/-}$ mice were implanted s.c. with either placebo or 1-MT filled pellets three days prior to i.p. injection of saline or LPS (0.5mg/kg). Brains were collected and TRP and several other TRP-related metabolites were determined 72 h later. A) 5-HIAA:5-HT and B) 3-HK:TRP ratios are shown. Bars represent the mean ± SEM. Means with different letters (a or b) are significantly different ($p < 0.05$) from each other.

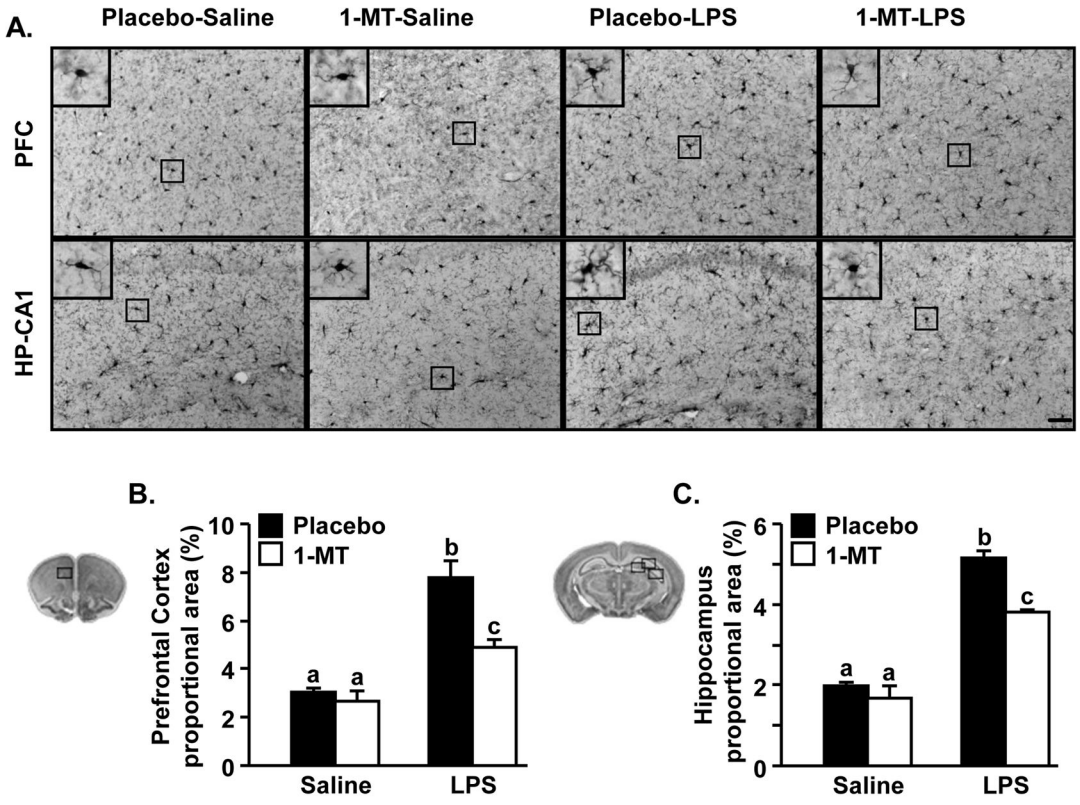

Figure 5. LPS-induced microglial activation in the prefrontal cortex (PFC) and hippocampus (HPC) of $CX_3CR1^{-/-}$ mice was attenuated by 1-MT

$CX_3CR1^{-/-}$ mice were implanted s.c. with either placebo or 1-MT filled pellets three days prior to i.p. injection of saline or LPS (0.5 mg/kg). Brains were collected, fixed, and processed 72 h later A) Representative images of Iba-1 staining in the PFC (top panels) and in the CA1 region of the hippocampus (bottom panels) are shown. Scale bars represent 50 μm. For each region there are 40x images that are inset (top left corner) to show selected microglia. Proportional area of Iba-1 staining in the B) PFC and C) HPC are shown. Bars represent the mean ± SEM. Means with different letters (a, b, or c) are significantly different ($p < 0.05$) from each other.

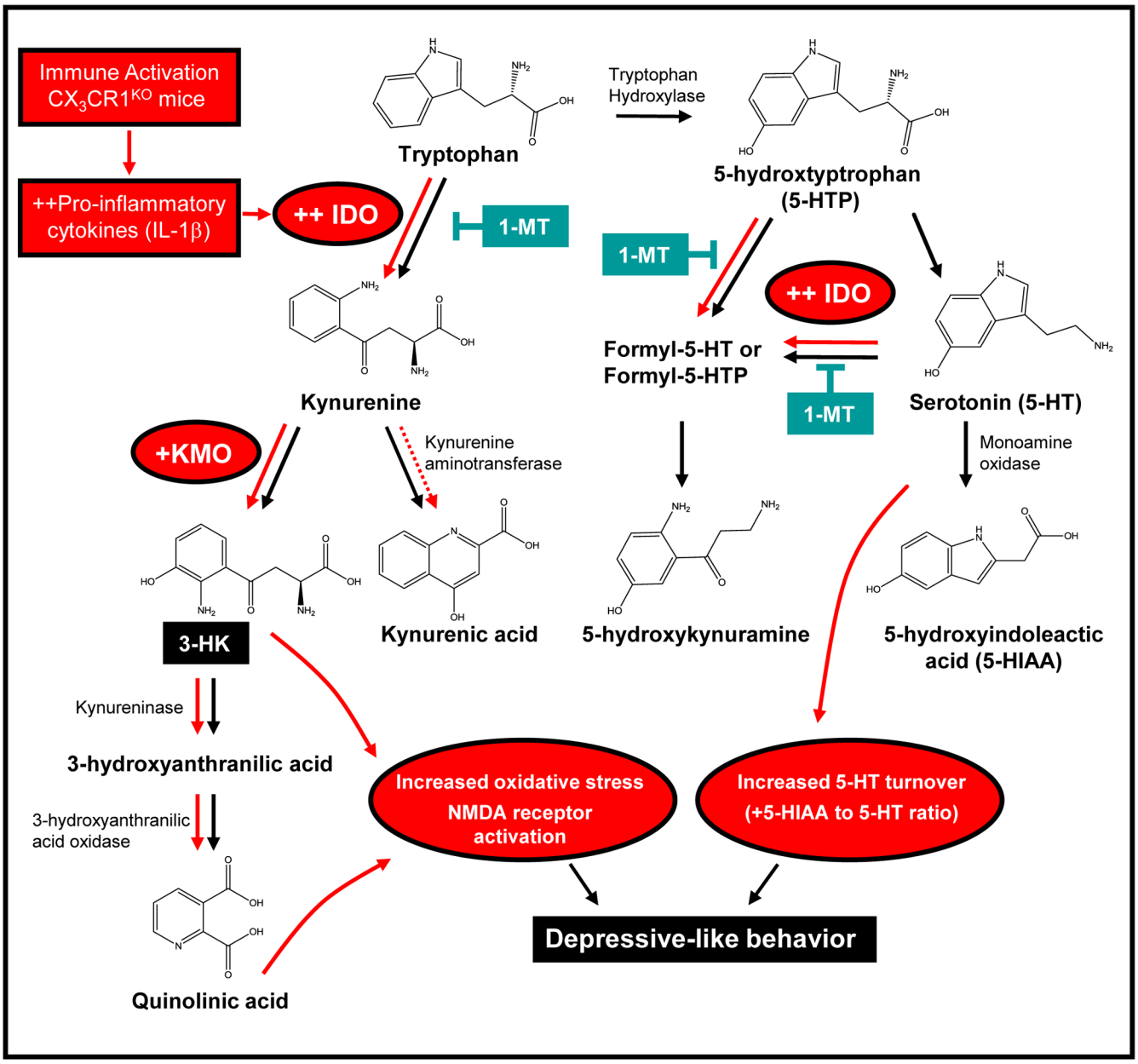

Figure 6. Proposed Role of the IDO pathway in Depressive-like behavior following LPS challenge in $CX_3CR1^{-/-}$ mice

In mice deficient of the fractalkine receptor ($CX_3CR1^{-/-}$), LPS causes amplified microglial activation with prolonged and exaggerated production of pro-inflammatory cytokines. These inflammatory cytokines activate IDO, which in turn, shifts tryptophan metabolism away from the production of serotonin (5-HT) and towards the production of kynurenine (KYN). 5-HT can be degraded to 5-HIAA by monoamine oxidase (MAO) or by IDO to 5-hydroxykynuramine. Active KMO in microglia converts kynurenine into 3-HK, which is a pro-oxidant. This also effectively shifts KYN away from the production of KYNA. 3-HK can then be degraded into quinolinic acid (QUIN) by kynureninase. QUIN is an NMDA receptor agonist and pro-oxidant. The influence of 1-MT on this pathway is also shown. The inflammatory associated changes in TRP metabolism are highlighted using red arrows and boxes. Overall, it is hypothesized that the IDO-initiated increases in tryptophan affect both

serotonergic and glutamatergic neurotransmission and leads to the development of depressive-like behavior.

Table 1

1-MT prevented the LPS induced increases in 3-HK and 5-HIAA. $CX_3CR1^{-/-}$ mice were implanted s.c. with either placebo or 1-MT filled pellets three days prior to i.p. injection of saline or LPS (0.5 mg/kg). Brains were collected 72 h later and concentrations of 5-HT, 5-HIAA, TRP, and 3-HK were determined by HPLC.

Serotonin and Tryptophan Metabolites in Brain				
(pg /mg)	Saline		LPS 72h	
	Placebo	1-MT	Placebo	1-MT
5-HT	1542.4 ± 197.1	1524.4 ± 142.4	1821.6 ± 294.0	1647.± 97.9
5-HIAA	842.6 ± 107.0[a]	651.7 ± 67.2[a]	1115.6 ± 128.5[b]	744.98 ± 53.4[a]
TRP	3275.0 ± 599.0[b]	2219.8 ± 113.6[a]	2320.0 ± 437.3[a]	1990.7 ± 138.8[a]
3-HK	173.7± 19.2[a]	147.2 ± 17.9[a]	242.6 ± 25.4[b]	155.1 ± 10.7[a]

The table represents the mean ± SEM. Means with different letters (a or b) are significantly different ($p < 0.05$) from each other.