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LPS-induced cortical kynurenic acid and neurogranin-NFAT signaling is associated with deficits in stimulus processing during Pavlovian conditioning

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Abstract

The N-Methyl-D-Aspartate receptor (NMDAR) antagonist kynurenic acid (KYNA) and the post-synaptic calmodulin binding protein neurogranin (Nrgn) have been implicated in neurological and neuropsychiatric conditions including Alzheimer's disease and schizophrenia. This study indicates that systemic dual-lipopolysaccharide (LPS) injections increases KYNA in the medial prefrontal cortex (mPFC), which is accompanied with increased phosphorylation of nuclear factor kappa chain of activated B cells (NFκB) and activation of the nuclear factor of activated T- cells (NFAT). Our results also indicate that dual-LPS increases Nrgn phosphorylation and concomitantly reduces phosphorylation of calmodulin kinase-II (CaMKII). We confirmed that systemic blockade of kynurenine-3 monooxygenase in conjunction with kynurenine administration results in significant increases in Nrgn phosphorylation and a significant reduction of CaMKII phosphorylation in the mPFC. Consequently dual-LPS administration induced significant impairments in stimulus processing during Pavlovian conditioning. Taken together, our study indicates that elevations in KYNA in the mPFC can directly regulate NMDA-Nrgn-CaMKII signaling, suggesting that neuroinflammatory conditions affecting this pathway may be associated with cognitive dysfunction.

Graphical abstract

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Keywords

kynurenic-acid; neurogranin; ventral-hippocampus; medial-prefrontal cortex; lipopolysaccharide; NFAT

Introduction

Although the molecular underpinnings of neuropsychiatric pathophysiology remain elusive, recent studies have implicated pro-inflammatory cytokines in schizophrenia (SCZ), bipolar disorder, and depression (Beumer et al., 2012; Khandaker and Dantzer, 2016; Soderlund et al., 2011; Soderlund et al., 2009). In microglia and astrocytes, proinflammatory cytokines induce indoleamine 2, 3 dioxygenase (IDO-1) and tryptophan 2, 3 dioxygenase (TDO2), thus stimulating tryptophan degradation along the kynurenine pathway (Campbell et al., 2014; Sellgren et al., 2015). Activation of this pathway increases formation of kynurenic acid (KYNA), a terminal metabolite shown to be elevated in the cerebrospinal fluid (CSF) and post mortem brain of patients with bipolar disorder and schizophrenia (Erhardt et al., 2001; Schwarcz et al., 2001; Sellgren et al., 2015). Interestingly, the kynurenine pathway also produces the excitotoxic N-Methyl-D-Aspartate receptor (NMDAR) agonist quinolinic acid (QUIN), which has been shown to be elevated in the CSF of suicide attempters (Brundin et al., 2016; Erhardt et al., 2013; Lugo-Huitron et al., 2013). KYNA blocks the glycine site of the NMDAR as well as the cholinergic $\alpha 7$ nicotinic receptor (Hilmas et al., 2001; Schwarcz et al., 2012). Investigation of NMDAR dependent signaling has recently identified associations between patients with SCZ and risk variants on the neurogranin (Nrgn) gene, which codes for a calmodulin binding protein functionally situated upstream of calcineurin and downstream of the NMDAR (Broadbelt et al., 2006; Ruano et al., 2008; Thong et al., 2013). Additional studies have shown that Nrgn variants are clinically associated with deficits in hippocampal activity during contextual learning (Pohlack et al., 2011), while constitutive deletion of this gene in mice indicate that it is necessary for hippocampal spatial memory function (Huang et al., 2006; Pak et al., 2000). However, the role of Nrgn in neuropsychiatric conditions remains unclear, as this protein has been proposed to be a neurodegenerative biomarker which accumulates in the CSF of patients with Alzheimer's disease (AD) (Portelius et al., 2015).

Here we investigate the effects of dual-lipopolysaccharide (dual-LPS) administration on KYNA generation and signaling alterations downstream of the NMDAR in the cortex and

hippocampus, two critical brain regions associated with neurodegenerative and neuropsychiatric disease, including SCZ and AD (Campbell et al., 2014; Lipska, 2004). Compared to a single LPS injection, dual-LPS administration significantly elevates kynurenine and KYNA in whole brain (Larsson et al., 2016). Using this paradigm, we examined KYNA in the medial prefrontal cortex (mPFC) and ventral hippocampus (vHip) and detected significant elevations of this metabolite. We investigated effectors downstream of the NMDAR and found that dual-LPS differentially affected calmodulin associated signaling molecules including Nrgn, calmodulin kinase-II (CaMKII) and the neuroinflammatory mediator nuclear factor of activated T- cells (NFAT) in the mPFC without significantly affecting the vHip. We also demonstrate that pharmacological inhibition of kynurenine-3 monooxygenase (KMO) by Ro-61-8048, which is known to increase KYNA, similarly affected Nrgn and CaMKII phosphorylation in the mPFC. We found that dual-LPS induced stimulus processing deficits during Pavlovian conditioning, which is consistent with studies showing that KYNA elevations impair conditioned stimulus (CS+) processing in rodents (Chess and Bucci, 2006), and patients with SCZ (Vaitl et al., 2002). Taken together, the dysregulation of kynurenine-mediated NMDAR signaling on Nrgn and CaMKII provides novel biological mechanisms underlying neurocognitive disorders.

2. Materials and Methods

2.1. Animals

Male C57BL/6N and C57BL/6J mice utilized for this study were bred at the Karolinska Institutet (Stockholm, Sweden) Department of Physiology & Pharmacology, or were acquired from Jackson Laboratories (Bar Harbor, ME USA), respectively. All mice utilized in this study were age matched at 2-to-4 months of age and were cared for as previously described (Larsson et al., 2016; Nam et al., 2013). Water and food was available *ad libitum*, although food was restricted only in control mice that underwent Pavlovian conditioning. Animals were housed in groups of 3-5 individuals on a 12-h lights on/off cycle (lights on at 06:00 h). Temperature was maintained at 25 °C and humidity between 40 – 60%. All efforts were made to minimize the number of animals used. Experiments were approved by and performed in accordance with the guidelines of the Ethical Committee of Northern Stockholm, Sweden, and the Mayo Clinic Institutional Animal Care and Use Committee in accordance with NIH guidelines.

2.2. Experimental Design

Mice utilized for this study weighed between 25 – 30 g and were weighed immediately prior to administration (*i.p.*) of saline vehicle (0.9%) or lipopolysaccharide (LPS; *Escherichia coli*, Sigma-Aldrich, St. Louis MO, USA) prepared immediately prior to T = 0 injection in 0.9% saline. Adult C57BL/6N mice were divided into four groups that separately received either vehicle or dual-LPS (serotype O111:B4) at 0.83 mg/kg (0.1 ml/10g body weight). As shown in Supplementary Fig. 1A, vehicle and LPS were initially administered at T = 0, with a second injection given 16 h later (T = 16) as previously described (Larsson et al., 2016). This dose was selected because single administration of LPS has been reported to induce depressive-like symptomatology as well as have an effect on conditioning behavior

(Mormede et al., 2004; O'Connor et al., 2009). Following dual-LPS injections, C57BL/6N mice were sacrificed at 24h for High Performance Liquid Chromatography (HPLC) analysis. A separate cohort of C57BL/6J mice were used for western blot and behavior testing at 24 h, 48 h, or 72 h following T = 0 LPS (serotype O55:B5) or vehicle administration (Supplementary Fig.1A). Animals decreasing more than 15 % of body weight or showed excess signs of sickness due to LPS administration were excluded from the study. To determine the relationship between KYNA and Nrgn signaling, C57BL/6J mice were administered vehicle alone, L-Kynurenine alone (100 mg/kg, *i.p.*, Sigma), or the kynurenine monooxygenase inhibitor (KMO-I) Ro-61-8048 (100 mg/kg, *i.p.*, EMD Millipore, MA USA) plus L-Kynurenine (100 mg/kg, *i.p.*). L-Kynurenine was administered 30 min post vehicle or Ro-61-8048 injections (Supplementary Fig. 1B). Injection volumes were delivered at 0.1 ml/10 g body weight. L-Kynurenine was dissolved in 15% DMSO (Sigma) in 0.9% saline, while Ro-61-8048 was dissolved with 15% DMSO and 15% Cremophor (Sigma) in 0.9% saline.

2.3. Tissue Extraction

All mice were subjected to rapid isoflurane inhalation to induce unconsciousness, followed by decapitation and subsequent harvesting of brain for manual isolation of the mPFC (AP: +1.70 mm from Bregma) and vHip (AP: -2.92 mm from Bregma) from both hemispheres under a surgical microscope. The extracted tissue was weighed and snap-frozen on dry ice for storage at -80°C until processing for HPLC or western blot.

2.4. HPLC analysis of L-Kynurenine and kynurenic acid

The mPFC and vHip (separately from each mouse) was combined with a volume (1: 5) of 0.4M perchloric acid (PCA), 0.1% sodium metabisulfite ($\text{Na}_2\text{S}_2\text{O}_5$) and 0.05% EDTA. Each brain sample was homogenized in a Storm 24 magnetic Bullet Blender for 3 min at a speed setting of 4 (Next Advance Inc., Averill Park NY, USA), with 0.5 mm zirconium oxide beads. The samples were then centrifuged ($21,000 \times g$) for 5 min at 4°C and the supernatants were collected. The supernatants were mixed with 10% volume of 70% PCA and centrifuged ($21,000 \times g$) for an additional 5 min at 4°C. The resulting supernatant was transferred to a new Eppendorf tube and stored at -20°C until ready for HPLC analysis.

Using an isocratic reversed-phase HPLC system coupled to a UV detector (Shimadzu SPD-10A, 360 nm wavelength) followed by a fluorescence detector (FP-2020 Plus, Jasco Ltd., Hachioji, Japan; 344 nm excitation wavelength, 398 nm emission wavelength, 18 nm bandwidth), samples (50 μl) were injected to measure kynurenine and KYNA concentrations in the mPFC of mice treated with vehicle ($n = 5$) or dual-LPS at 0.83mg/kg ($n = 5$). Similarly, we determined kynurenine and KYNA concentrations in the vHip of mice treated with vehicle ($n = 6$) or dual-LPS at 0.83 mg/kg ($n = 5$). A mobile phase consisting of 50 mM sodium acetate and 7% acetonitrile (pH set to 6.2 using acetic acid) was pumped through a ReproSil-Pur C18 column (4 $\times$ 150 mm, Dr. Maisch GmbH, Ammerbuch, Germany). A second mobile phase containing 0.5 M Zinc acetate in dH_2O was delivered post-columnar, before the fluorescence detector, by a Pharmacia P-500 (GE Healthcare; Uppsala, Sweden) at a flow rate of 10 ml/h. Signals from the detectors were transferred to a computer for

analysis with Datalys Azur (Grenoble, France). Retention times for kynurenine and KYNA were 4 and 7 minutes, respectively.

2.5. Sample preparation for cytosolic proteins and enrichment of synaptosome fractions

2.5.1. Sample preparation for non-synaptosome enriched cytosolic proteins—

To confirm whether systemic dual-LPS administration induced signaling alterations in the mPFC and vHip, protein expression of inflammatory mediator NF κ B was examined. This tissue preparation method differed from sample preparation for synaptosome enriched fractions (described below). In this non-behavior tested cohort, the mPFC (n = 4/treatment) and vHip (n = 3/treatment) of C57BL/6J mice treated with LPS (0.83 mg/kg) or vehicle were harvested at 24 h post T = 0 injection as described in *Section 2.3* (Supplementary Fig. 1A). Each brain region was homogenized in a Storm 24 magnetic Bullet Blender for 4 min at a speed setting of 4 (Next Advance Inc., Averill Park NY, USA), with 0.5 mm zirconium oxide beads in combination with 50–70 μ l of Cell-lytic MT mammalian tissue extraction reagent (Sigma-Aldrich) containing 50 mM Tris buffer (pH 7.4), 2 mM EDTA, 5 mM EGTA, and 0.1% SDS. The homogenization buffer contained Complete (Roche) protease inhibitor cocktail and phosphatase inhibitor cocktails Type II and III (Sigma-Aldrich). Homogenates were then centrifuged at 16,400 rpm (4°C) for 15 min and supernatants were collected for subsequent SDS PAGE and western blot analysis, as described below (*Section 2.5.3*).

2.5.2. Sample preparation for synaptosome enriched fractions—

To examine protein expression from cytosolic and enriched synaptosomal fractions, a separate group of C57BL/6J mice were administered dual-LPS (n = 3) or vehicle (n = 3). The mPFC and vHip were harvested at 24 h post T = 0 injection as described in *Section 2.3* (Supplementary Fig. 1A). In addition, as described in *Section 2.2* and depicted in Supplementary Fig. 1B, protein expression of synaptosomes was assessed in the mPFC and vHip from C57BL/6J mice that were either treated with vehicle alone (n = 3–4), L-Kynurenine alone (n = 4–7), or the KMO inhibitor Ro-61-8048 plus L-Kynurenine (n = 4–6). The mPFC and vHip were harvested 90 min following initial vehicle or Ro-61-8048 treatment (Supplementary Fig. 1B). Each brain region was homogenized for 2 min at a speed setting of 2 (Storm 24 Bullet Blender, 0.5 mm ZrO₂ beads) in combination with Syn-PER (Thermo Scientific) synaptic protein extraction reagent (1 mg tissue: 5 μ l buffer) containing Complete (Roche) protease inhibitor cocktail and phosphatase inhibitor cocktails Type II and III (Sigma-Aldrich). The homogenized tissue (including beads) was centrifuged at 1,200 $\times$ g at (4°C) for 10 min. The resulting main homogenate (MH) was transferred to a pre-cooled 1.5 mL tube. A 10 μ l aliquot of the MH was saved from each replicate for subsequent western blot analysis. The MH was then centrifuged for 15 min at 15,000 $\times$ g at (4°C), and the resulting supernatant (cytosolic fraction) was saved for western blot analysis. The resulting synaptosome pellet was resuspended at a ratio of 1 g starting tissue: 2,000 μ l of Syn-PER reagent. Protein concentration was determined for each individual biological replicate of the mPFC from each step of the enrichment process (MH, cytosolic fraction, synaptosomes). Due to the low quantity of mPFC starting material, each individual biological replicate was combined into a pooled master suspension. To make certain that this pooled master suspension contained equal amounts of protein from every biological replicate, the lowest biological replicate

concentration was selected as the standard. This standard concentration was then aliquoted from the remaining individual mPFC biological replicates into a master suspension. Protein concentration for the master suspension was then assessed. This was done for every fraction (MH, cytosolic fraction, synaptosomes). In contrast to the mPFC, the starting material for each individual vHip biological replicate was sufficient and it was not necessary to pool these biological replicates into a master suspension. Protein concentrations for individual biological replicates and master suspensions from each fraction were determined via the Bradford protein assay (Bio-Rad, Hercules CA, USA).

2.5.3. SDS PAGE and western blot analysis—For western blot analysis, resuspended synaptosome homogenates were loaded at 15 µg for the mPFC or 20 µg for the vHip. To validate enriched synaptosome fractions from groups that were administered vehicle alone, L-Kynurenine alone or KMO-I plus L-Kynurenine, we loaded a representative cytosolic fraction in a single lane as a control. These bands were not quantified. Brain sample replicates were separated on a 4-12% Nu-Page Bis-Tris gel in MOPS buffer (Invitrogen, Carlsbad, CA) at 140 V for 2 hr, followed by transfer to a PVDF membrane (Invitrogen) at 30 V for 1 hr. Samples were immunoblotted overnight at 4°C (5% BSA in 1× TBST) with antibodies specific to phospho-NFκB (1:500; Cell Signaling Technology Inc., Danvers MA, USA), NFκB (1:500; Cell Signaling), phospho-Nrgn (1:500; Upstate Cell Signaling, Lake Placid NY, USA), Nrgn (1:1000; Millipore), phospho-PKCγ (1:500; Cell Signaling Technology), PKCγ (1:500; Cell Signaling Technology), NFAT1 (1:500; Abcam, Cambridge, UK), phospho-CaMKII (1:500; Cell Signaling Technology), CaMKII (1:500; Cell Signaling Technology), GAPDH (1:1000; Millipore) and β-actin (1:1000; Santa Cruz Biotechnology, Dallas TX, USA). Following three 10 min washes (1× TBST), immunoblots were incubated (5% BSA) for 1 hr at room temperature with respective anti-rabbit and anti-mouse secondary antibodies (1:1000, Millipore). Blots were visualized with the Super-Signal West Dura Chemiluminescent Substrate (Thermo Scientific, Rockford, IL), developed on a Kodak Image Station 4000R scanner (New Haven, CT), and band optical density quantification was performed using NIH ImageJ software.

2.6. Behavior Testing

2.6.1. Prepulse inhibition—To determine the effects of dual-LPS treatment on sensorimotor gating, mice were administered injection #1 of vehicle or LPS (0.83 mg/kg) at T = 0 and prepulse inhibition (PPI) was measured immediately prior to injection #2 at T = 16 and again at T = 24. PPI was examined for 7 vehicle and 15 LPS treated mice at T = 16. For PPI measurement at T = 24, an additional 9 vehicle and 7 LPS treated mice were added, however PPI measurement was not performed for this group at T = 16. All mice received the same dual injection protocol (Supplementary Fig. 1A). PPI was tested as previously described (Oliveros et al., 2010). Briefly, PPI was measured in sound-attenuating cubicles (SR-LAB, San Diego Instruments, San Diego CA, USA) that were equipped with a house light and a loudspeaker. Each chamber contained a cylindrical plexiglass animal enclosure that rested on a platform with a piezoelectric accelerometer mounted below. The piezoelectric accelerometer converted vibrations of the mouse in the enclosure to analog signals that were stored on a computer. At the onset of the startle stimulus, 65 readings were recorded at 1 ms interval to capture maximum startle amplitude. Each session began with a

5-min acclimation period followed by four successive 120 dB stimulus alone trials. These four initial trials were excluded from the analysis. Four different trial types were then presented randomly: “no stimulus” (background, 65 dB), “startle pulse alone” (120 dB; 40 ms), “prepulse alone” (4, 8 or 16 dB above background; 20 ms) or “prepulse + startle pulse” (4, 8 or 16 dB prepulse given 100 ms before 120 dB startle pulse). The intertrial intervals varied randomly from 5 to 15 s. All trials were presented 5 times except for the “no stimulus” and “startle pulse alone” trials, which occurred 10 times. The average maximum amplitude vibrations from the “no stimulus” trials were subtracted from all startle response values to account for baseline movement in the chambers. The percentage of acoustic PPI was calculated as: $\% \text{ PPI} = 1 - [(\text{prepulse} + \text{startle pulse}) / (\text{startle pulse alone})] \times 100$. For calculation of the startle response, we used the maximum startle amplitude (v-max) within each PPI session to determine the magnitude of this reflex.

2.6.2. Pavlovian conditioning—Mice were allowed 30 min acclimation to the testing room followed by baseline measurement of general activity inside standard mouse operant chambers (MED-307A-B2, Med-Associates Inc., St. Albans VT, USA) where they were allowed to freely explore the operant chamber for 30 min with the cubicle fan (CF) and the house light (HL) turned on. This baseline analysis occurred prior to dual-LPS or vehicle administration. Total session time, right and left nose pokes (inactive-holes), and magazine head-entries (magazine-entries) were recorded (ENV-302HD head entry detector, Med-Associates). CS+ processing is impaired in rats subjected to pharmacological elevations of kynurenine (Chess and Bucci, 2006) as well as SCZ patients (Vaitl et al., 2002). Therefore, we investigated whether dual-LPS administration affected acquisition of Pavlovian conditioning (Keller and Schoenfeld, 1950; Mead and Stephens, 2003). To match the weight decrease induced by dual-LPS, vehicle treated mice were food restricted and weights were monitored daily during conditioning (Boules et al., 2007). During conditioning, mice were presented with a random series of CS+ tone cues (65dB and 0.25 s in duration; ENV-323HAM, 4500 Hz Sonalert, Med-Associates), that were paired with delivery of reward (10µl/sucrose reward). This established a tone-reward contingency (Rescorla, 1967). During conditioning, each CS+ was spaced by random time intervals ranging between 2 s and 60 s, so that on average a reward was delivered every 15 s. Acquisition of Pavlovian conditioning was determined by measuring the latency between a CS+ and the next-immediate CS+ evoked magazine entry (CS+ reaction time), thus giving a measure of stimulus induced attentional processing. Session termination was determined by reaching a pre-defined number of CS+ presentations, and signaled by the HL and CF shutting off. The resulting latencies were summed and then divided by the number of CS+ to determine an average reaction time following CS+ presentations.

The effects of vehicle or dual-LPS administration on conditioning were investigated on two separate cohorts of mice. In Pavlovian conditioning *Experiment 1*, activity in the conditioning chambers was measured during baseline as described above, prior to T = 0 vehicle or dual-LPS administration. Pavlovian conditioning was performed at 48 h and 72 h post T = 0 injections (n = 7-8/treatment). Conditioning sessions terminated after 65 CS+ presentations. In Pavlovian conditioning *Experiment 2*, behavior parameters were similar to *Experiment 1* except that a group of mice were removed following conditioning testing at 24

h post T = 0 injections, in order to harvest and save the mPFC and vHip for western blot analysis. For *Experiment 2*, baseline activity was measured prior to T = 0 vehicle or dual-LPS treatment and Pavlovian conditioning was performed at 24 h (n = 7-8/treatment). The remaining mice underwent Pavlovian conditioning at 48 h and 72 h post T = 0 injections (n = 3-4/treatment). Conditioning sessions for this experiment were terminated after 50 CS+ presentations.

2.6.3. Accelerated Rotarod—To evaluate the effects of dual-LPS treatment on motor function, a standard mouse Rotarod treadmill (UGO Basile, Verese, Italy) was used. The rotarod was programmed to gradually accelerate from 2 rpm to 40 rpm over a period of 300 seconds. Latency to fall from the treadmill was used as a measure of motor coordination. Each group of mice (n = 7-8/treatment) underwent Rotarod testing at T = 0 h, 16 h and 24 h. For the T = 24 h time point, mice were rotarod tested immediately prior to Pavlovian conditioning (Supplementary Fig. 1A). For the remaining mice that underwent conditioning at 48 h and 72 h (n = 3-4/treatment), they were also tested on the rotarod immediately prior to Pavlovian conditioning (Supplementary Fig. 1A).

2.7. Data and statistical analysis

For western blot analysis, each biological replicate was normalized to its respective GAPDH or β -actin protein expression, where appropriate. We utilized an unpaired Student's *t*-test for all western blot comparisons. Images are representative of 2-3 experiments. For statistical analysis of kynurenine and KYNA in the mPFC and vHip, we used a two-tailed Mann Whitney U-test. For PPI, pre-pulse intensities were examined with repeated measures two-way ANOVA (RM two-way ANOVA). Startle response analysis was conducted with an unpaired two-tailed Student's *t*-test. Two-way ANOVA was utilized to compare weights following vehicle and dual-LPS treatment and during Pavlovian conditioning across time points. To compare accelerated rotarod performance following vehicle and dual-LPS treatment across time points, we used two-way ANOVA. For our Pavlovian conditioning experiments, CS+ reaction times, magazine entries, and inactive-hole entries were combined for *Experiment 1* and *Experiment 2* and analyzed with two-way ANOVA. Statistical significances reported for all ANOVA results were followed with Bonferroni's multiple comparisons (Sigma Plot 12.0, Systat Software San Jose CA, USA). For all data reported, results were considered statistically significant when $p < 0.05$ and are presented as mean and $\pm$ SEM (GraphPad Prism, La Jolla CA, USA).

3. Results

3.1. LPS-induced NF κ B phosphorylation in the medial prefrontal cortex and ventralhippocampus

We first assessed whether systemic dual-LPS treatment activates neuroinflammatory signaling in the mPFC and vHip (Shih et al., 2015; Viatour et al., 2005). In the mPFC, compared to vehicle, dual-LPS treatment significantly increased Ser-536 phosphorylation of NF κ B (p65/RelA) at 24 h post T = 0 injection ($t_6 = 7.53$, $p < 0.01$) (Fig. 1 and Supplementary Fig. 2A). A similar increase in phospho-NF κ B was detected in the vHip ($t_4 = 2.99$, $p < 0.05$) at 24 h post T = 0 injection (Fig. 1 and Supplementary Fig. 2C).

3.2. LPS-induced activation of kynurenine metabolism in the medial pre-frontal cortex and ventral hippocampus

Next, we examined whether dual-LPS activates kynurenine metabolism in the mPFC and vHip. Our results show a significant increase in kynurenine ($U_{15, 40} = 0.0$, $p = 0.011$) and KYNA ($U_{15, 40} = 0.0$, $p = 0.007$) at 24 h post T = 0 injection in the mPFC (Fig. 2A-B). Likewise, we detected a significant increase in kynurenine ($U_{21, 45} = 0.0$, $p = 0.007$) and KYNA ($U_{21, 45} = 0.0$, $p = 0.004$) in the vHip at 24 h post T = 0 injection compared to the vehicle treated group (Fig. 2C-D).

3.3. Neurogranin-CaMKII signaling in the medial pre-frontal cortex

To determine whether dual-LPS administration alters Nrgn signaling, synaptosomal fractions containing the molecular contents of presynaptic terminals and post synaptic densities were isolated from the mPFC (Bai and Witzmann, 2007). Our results demonstrate that at 24 h post T = 0 injection, dual-LPS significantly increased phospho-Nrgn (Ser-36) in synaptosomal ($t_4 = 3.86$, $p < 0.05$) and cytosolic fractions ($t_4 = 3.21$, $p < 0.05$) of the mPFC compared to vehicle treated controls (Fig. 3A-B and Supplementary Fig. 2B). No differences were detected for Nrgn between the treatment groups (Fig. 3A-B and Supplementary Fig. 2B). However, dual-LPS significantly decreased phospho-CaMKII in the synaptosomal fraction ($t_4 = 10.95$, $p < 0.001$) but not in the cytosolic fraction of the mPFC (Fig. 3A-B and Supplementary Fig. 2B). Total CaMKII levels were decreased in the cytosolic fraction ($t_4 = 3.99$, $p < 0.05$), but not in the synaptosomal fraction (Fig. 3A-B and Supplementary Fig. 2B).

Since increased phospho-Nrgn expression in response to dual-LPS was incongruent with decreased phospho-CaMKII, we hypothesized that activated Nrgn could alternatively promote calmodulin-NFAT signaling in the mPFC (Sama et al., 2008). This analysis revealed that the active form of NFAT1 (phospho-NFAT1) was decreased in both synaptosomal ($t_4 = 3.95$, $p < 0.05$) and cytosolic ($t_4 = 3.43$, $p < 0.05$) fractions (Fig. 3A-B and Supplementary Fig. 2B), suggesting that dual-LPS promotes inflammatory signaling through activation of the Nrgn-calmodulin-NFAT pathway. Next, to confirm whether increased conversion of kynurenine into KYNA can directly regulate phospho-Nrgn levels, we examined the effect of KMO inhibitor Ro-61-8048 and L-Kynurenine on phospho-Nrgn levels in the mPFC (Supplementary Fig. 1B). We found that synaptosomal phospho-Nrgn ($t_{11} = 3.07$, $p < 0.05$) along with total Nrgn ($t_{11} = 2.71$, $p < 0.05$) was increased in the KMO-I plus L-Kynurenine group compared to L-Kynurenine alone, but not compared to the vehicle-treated group (Fig. 3C-D and Supplementary Fig. 3A-B). In addition, we examined the activated form of protein kinase C (PKC γ), which is known to phosphorylate Ser-36 of Nrgn (Kim et al., 2010; Watson et al., 1992). As shown in Fig. 3C-D and Supplementary Fig. 3A, synaptosomal phospho-PKC γ was increased in the KMO-I plus L-Kynurenine group in comparison to L-Kynurenine alone ($t_6 = 4.51$, $p < 0.01$) or to the vehicle treated group ($t_6 = 3.46$, $p < 0.05$). We also detected a significant increase in total PKC γ expression in the L-Kynurenine alone group compared to the vehicle treated group ($t_6 = 2.71$, $p < 0.05$) without detectable differences among the remaining treatment groups (Supplementary Fig. 3A-B).

We then investigated the effects of KMO-I inhibition on CaMKII signaling in the mPFC (Fig. 3C-D and Supplementary Fig. 3A-B). Our results showed a significant decrease in synaptosomal phospho-CaMKII in the KMO-I plus L-Kynurenine group in comparison to L-Kynurenine alone ($t_8 = 2.99$, $p < 0.05$) or to the vehicle treated group ($t_7 = 7.10$, $p < 0.001$). Examination of total CaMKII levels revealed an increase in the KMO-I plus L-Kynurenine group compared to L-Kynurenine alone ($t_{11} = 2.21$, $p < 0.05$) or vehicle alone ($t_7 = 3.80$, $p < 0.01$) groups (Supplementary Fig. 3A-B).

3.4. Neurogranin-CaMKII signaling in the ventral hippocampus

Next, we assessed the effect of dual-LPS and KMO inhibition on Nrgn and CaMKII signaling in the vHip. In contrast to the mPFC, we did not detect significant differences in expression of phospho-Nrgn, phospho-CaMKII, phospho-NFAT1, and total Nrgn in the vHip, between mice administered vehicle and dual-LPS (Supplementary Fig. 2D). However, we did detect increased synaptosomal total CaMKII expression as a result of dual-LPS administration in comparison to vehicle administration ($t_6 = 4.97$, $p < 0.01$) (Supplementary Fig. 2D).

We also investigated the effects of KMO inhibition in the vHip. In contrast to the mPFC, we found that synaptosomal phospho-PKC γ levels were decreased in the group administered L-Kynurenine alone ($t_6 = 3.10$, $p < 0.05$) and the KMO-I plus L-Kynurenine group ($t_6 = 5.62$, $p < 0.01$) when compared to the vehicle alone group (Supplementary Fig. 3C-D). Likewise, synaptosomal phospho-PKC γ levels were significantly lower in the KMO-I plus L-Kynurenine group when compared to L-Kynurenine alone ($t_6 = 2.58$, $p < 0.05$) (Supplementary Fig. 3C-D). Our results also show that synaptosomal PKC γ expression was significantly decreased in the L-Kynurenine alone group ($t_6 = 3.31$, $p < 0.05$) and the KMO-I plus L-Kynurenine group ($t_6 = 3.80$, $p < 0.01$) in comparison to vehicle alone controls (Supplementary Fig. 3C-D). There were no differences detected between L-Kynurenine alone and KMO-I plus L-Kynurenine treatment groups (Supplementary Fig. 3C-D). Given the role of PKC γ in activation of Nrgn, it was not surprising that this analysis did not detect differences in synaptosomal phospho-Nrgn between vehicle alone and L-Kynurenine alone or between KMO-I plus L-Kynurenine treatment groups. There were also no differences detected in total Nrgn between L-Kynurenine alone or KMO-I plus L-Kynurenine treatment groups in comparison to vehicle (Supplementary Fig. 3C-D).

Similar to the mPFC, our analysis of the vHip did reveal a significant decrease in phospho-CaMKII in response to KMO-I plus L-Kynurenine when compared to vehicle alone ($t_6 = 6.38$, $p < 0.001$) or L-Kynurenine alone ($t_6 = 7.02$, $p < 0.001$) (Supplementary Fig. 3C-D). We also detected an increase in phospho-CaMKII as a result of administration of L-Kynurenine alone when compared to vehicle alone ($t_6 = 3.02$, $p < 0.05$) (Supplementary Fig. 3C-D). Lowered expression of CaMKII was detected in the KMO-I plus L-Kynurenine group when compared to vehicle alone ($t_6 = 3.44$, $p < 0.05$). Taken together, our results demonstrate that elevation of KYNA levels either from dual-LPS or via KMO inhibition can activate Nrgn signaling in the mPFC and decrease CaMKII phosphorylation in both the mPFC and vHip.

3.5. Effects of dual-LPS on Pavlovian conditioning

To assess whether dual-LPS treatment alters acquisition of Pavlovian conditioning, mice underwent this test at 24 h, 48 h, and 72 h post $T = 0$ injection. Our results show a significant main effect of conditioning, as vehicle and dual-LPS treated mice displayed decreased CS+ reaction times across testing sessions [$F_{(2, 53)} = 22.85, p < 0.001$] (Fig. 4A). Dual-LPS treatment impaired reaction times to CS+ when compared to the vehicle treated group as a main effect of treatment [$F_{(1, 53)} = 32.26, p < 0.001$] was detected (Fig. 4A). For magazine entries, we found a significant effect of treatment [$F_{(1, 66)} = 8.03, p < 0.01$] and conditioning [$F_{(3, 66)} = 5.93, p < 0.01$] without a significant interaction between treatment and conditioning across test sessions (Fig. 4B). Although no difference was found in inactive-hole entries during Pavlovian conditioning (Fig. 4C), we found a significant effect of conditioning across test sessions [$F_{(3, 66)} = 17.3, p < 0.001$] and a significant interaction between treatment and conditioning [$F_{(3, 66)} = 3.14, p < 0.05$], revealing lower inactive hole entries in the LPS treated group at 24 h post $T=0$ injection in comparison to the vehicle-treated group ($p < 0.05$; Fig. 4C).

Conditioning weights in the vehicle treated group were similarly matched to the decrease in weight a result of LPS administration [$F_{(1,36)} = 0.14, p = 0.712$] (Supplementary Fig. 4A). This control suggests that the decreased number of magazine entries and impaired CS+ reaction time were due to LPS treatment and not due to differences in weights between the groups.

3.6. Effects of dual-LPS on locomotor function

We examined whether dual-LPS treatment had a significant effect on general gait and motor function by testing each group in the accelerated rotarod. As shown in Supplementary Fig. 4B, we did not detect differences in rotarod performance between vehicle and LPS treated mice up to 72 h following $T = 0$ h injections, or an interaction between treatment and time post $T = 0$ h injections. However, we found that both LPS and vehicle-treated groups showed increased motor coordination performance in a time-dependent manner [$F_{(4,49)} = 6.75, p < 0.001$], implying that the LPS-induced impaired reaction times is not due to a deficit in gait or motor function.

3.7. Effects of dual-LPS on sensorimotor gating

A commonly used behavioral index to measure startle reflex and sensorimotor gating is prepulse inhibition (PPI), which is the ability for a stimulus of sub-threshold intensity (prepulse) to attenuate the startle reflex in response to a loud and unpredictable acoustic stimulus. Deficits in sensorimotor gating has been reported in neurocognitive disorders typified by impairments in impulse control, memory and attention, including SCZ (Braff et al., 1992; Erhardt et al., 2001; Linderholm et al., 2012), obsessive compulsive disorder (Kohl et al., 2013). In addition, these deficits have also been reported for autism spectrum disorders (Kohl et al., 2014; Perry et al., 2007) and potentially in attentional deficit hyperactivity (Schulz-Juergensen et al., 2014; Shibasaki et al., 2015). Therefore, we examined whether mice treated with dual-LPS would display deficits in PPI. Our analysis of sensorimotor gating at 16 h post $T = 0$ injection indicates appropriate sensorimotor gating (Supplementary Fig. 5A), as we observed an improvement in PPI as a result of each prepulse

intensity (4dB, 8dB, and 16 dB) during the test [$F_{(2, 40)} = 48.70$, $p < 0.001$]. This analysis did not detect a significant effect of treatment, nor a significant interaction. Similarly, dual-LPS did not have an effect on PPI at 24 h post T = 0 injection (Supplementary Fig. 5B). Likewise, we did not detect a significant interaction although prepulse intensities did improve PPI across the testing session [$F_{(2, 72)} = 51.98$, $p < 0.001$]. Dual-LPS significantly lowered this reflex at 24 h post T = 0 injection compared to vehicle group ($t_{36} = 3.51$, $p < 0.01$) without having an effect at 16 h post T = 0 injection (Supplementary Fig. 5C).

4. Discussion

Immune activation is known to significantly contribute to neuropsychiatric disorders as association studies exploring for biomarkers in patients have identified inflammation related genes in the prefrontal cortex (Fillman et al., 2013) and the hippocampus (Kim et al., 2016). In agreement, prenatal (Borrell et al., 2002) and postnatal intra-hippocampal LPS induced inflammation studies in rodents have modeled SCZ-like phenotypes (Zhu et al., 2014a). Treatments with the antibiotic minocycline and the antipsychotic risperidone have been reported to ameliorate these deficits (Zhu et al., 2014b). In this study, systemic dual-LPS administration markedly increased NF κ B signaling that was accompanied by elevations of kynurenine and KYNA in the mPFC and vHip. Our findings revealed that LPS increased phosphorylation of the calmodulin sequestering protein Nrgn in the mPFC (Li et al., 1999) without affecting expression in the vHip. These results suggest that inflammation may preferentially activate KYNA-Nrgn signaling in the mPFC. Moreover, our study is the first to show that pharmacological inhibition of KMO, which deviates kynurenine conversion toward KYNA (Erhardt et al., 2004; Justinova et al., 2013; Speciale et al., 1996), can effectively increase PKC-Nrgn phosphorylation in the mPFC. Supporting our findings, KMO null mice, which exhibit increased brain KYNA, also upregulates Nrgn mRNA expression (Erhardt et al., 2016). As with LPS administration, no significant changes to Nrgn signaling was detected in the vHip from KMO inhibition. Interestingly, Parrott and colleagues show that peripheral LPS administration primarily promotes kynurenine metabolism in the dorsal hippocampus when compared to the vHip (Parrott et al., 2016). This suggests that the dorsal hippocampus and mPFC may be directly synchronized to regulate neuroimmunological responses and behaviors (O'Leary and Cryan, 2014; Oliveros et al., 2017).

Cortical dysfunction in neuropsychiatric disorders is associated with mood disorders, depression and psychosis (Loonen et al., 2017; Shrestha et al., 2015; Sigurdsson et al., 2010). Genetic variants of Nrgn have been associated with cortical degeneration (Thong et al., 2013). However, the role of Nrgn is less clear in rodent models, as overexpression of Nrgn enhances spatial memory (Chaalal et al., 2014; Huang et al., 2006; Zhong et al., 2015). Controversially, recent studies have shown that elevated CSF Nrgn levels are associated with increased severity of cognitive decline (Hellwig et al., 2015; Janelidze et al., 2016; Kester et al., 2015; Portelius et al., 2015). Increased Nrgn phosphorylation has also been associated with memory impairments (Andrade et al., 2008; Kim et al., 2010; Severino et al., 2011). Although the role of increased Nrgn phosphorylation in relation to cognitive function requires further investigation, our results demonstrate that dual-LPS administration decreases cytosolic and synaptosomal phosphorylation of NFAT1 in the mPFC. This

suggests increased nuclear NFAT activity may seemingly contribute to impaired stimulus processing during conditioning. Our findings are in line with studies showing that cognitive decline is associated with NFAT activation (Abdul et al., 2009; Sama et al., 2008), while other reports indicate that increased CSF KYNA promote deficits in stimulus processing in rats (Chess and Bucci, 2006). However, we cannot rule out the possibility that LPS-induced depressive-like signs can affect the stimulus processing during conditioning since it has been reported that LPS-induces depressive-like behaviors between 24h and 48h following LPS administration (Henry et al., 2008; O'Connor et al., 2009; Salazar et al., 2012).

Disruptions of sensorimotor gating are considered to reflect dysfunctions in the ability to filter out extraneous stimuli and deficits in PPI are frequently observed in patients with SCZ (Braff and Geyer, 1990; Geyer et al., 1990; Lipska, 2004), impulse control disorders and potentially ASD and ADHD (Kohl et al., 2013). Peripheral administration of kynurenine increases brain KYNA, and disrupts PPI in the rat (Erhardt et al., 2004). Although we demonstrate that dual-LPS treatment increases kynurenine and KYNA, this administration protocol was ineffective at disrupting PPI. This discrepancy may be explained the type of species tested or that LPS increases the NMDAR antagonist KYNA and the NMDAR agonist QUIN, both of which may counteract each other resulting in no detectable PPI alterations. Our results are in concordance with other studies that have observed LPS induced reductions in the startle reflex, without affecting PPI (Juszczak et al., 2008; 2009a; Lockey et al., 2009b). Conversely, at least one other study reports acute LPS induced PPI disruptions at 24 h post treatment, although they used a single dose of LPS administration (Custodio et al., 2013).

It has been established that CaMKII inactivation can result from sepsis, LPS, and seizure-related excitotoxicity (Aversa et al., 2013; Osawa et al., 2006; Yamagata et al., 2006; Yamagata and Obata, 2004). Consistently, we found that dual-LPS dampened calmodulin-CaMKII activation while elevating KYNA in the mPFC. Remarkably, pharmacological inhibition of KMO in conjunction with L-Kynurenine administration confirmed these observations as we detected a significant dampening of CaMKII phosphorylation in the mPFC and vHip. Since KMO inhibition is known to increase KYNA (Justinova et al., 2013; Moroni et al., 2005), our results supports the hypothesis that excess levels of KYNA possibly contributes to exacerbations in cognitive malfunction (Erhardt et al., 2016; Heisler and O'Connor, 2015). Furthermore, our novel findings promote the possibility that LPS-driven neuroinflammatory signaling contributes to deficits in stimulus processing preferentially through the mPFC. However, we should note that dual-LPS also increase the excitotoxic NMDAR agonist QUIN (Larsson et al., 2016), raising the alternative possibility that additional kynurenine metabolic products may contribute to cognitive dysfunctions (Parrott and O'Connor, 2015). Additional investigations are warranted to determine how QUIN affects neuro-cognitive function.

As we summarize in Fig. 5, our results provide novel insights of how a convergence between components of the immune system and the CNS may regulate signaling molecules implicated in cognitive function (Pohlack et al., 2011; Simpson et al., 2011; Ward et al., 2012). Our main finding demonstrates that dual-LPS elevates KYNA, triggers neuro-

inflammatory signaling in the brain and produces stimulus processing deficits that are associated with reduced activity of signaling molecules critical for cognitive function.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Highlights

- LPS increase kynurenic acid (KYNA) in medial prefrontal-cortex (mPFC).
- LPS increase p-neurogranin and lowers p-CaMKII in the mPFC.
- Dysregulated p-CaMKII and Nrgn-NFAT signaling is associated with impaired Pavlovian conditioning.
- Kynurenine monooxygenase inhibition increases p-Nrgn and dampens p-CaMKII in the mPFC.
- Glutamatergic KYNA-Nrgn signaling is associated with cognitive dysfunction.

Fig.1.

Dual-LPS administration increases NFκB phosphorylation in the medial prefrontal cortex (mPFC) and ventral hippocampus (vHip). Protein expression and densitometry quantitation in the mPFC ($n = 4/\text{treatment}$) and vHip ($n = 3/\text{treatment}$) of mice administered repeated LPS injections (*i.p.*) shows an increase in phosphorylation (pNFκB-p65) of the inflammatory mediator NFκB in comparison to vehicle treated mice. $*p < 0.05$ by unpaired two-tailed student's *t*-test. Results are expressed as mean $\pm$ SEM.

Fig. 2.

Dual-LPS administration increases kynurenine pathway activity in the medial prefrontal cortex (mPFC) and ventral hippocampus (vHip). (A) HPLC measurement of kynurenine at 24 h post initial LPS injection detected a significant increase in the mPFC, relative to vehicle. $n = 5/\text{treatment}$. (B) At 24 h post initial LPS injection, KYNA levels were also significantly higher in the mPFC in comparison to vehicle. $n = 5/\text{treatment}$. (C) HPLC measurement of kynurenine at 24 h post initial LPS injection detected a significant increase in the vHip, relative to vehicle. $n = 5-6/\text{treatment}$. (D) At 24 h post initial LPS injection, KYNA levels were also significantly higher in the vHip in comparison to vehicle. $n = 5-6/\text{treatment}$. Results are expressed as mean $\pm$ SEM. * $p < 0.05$ by Mann-Whitney U -test. KYNA, kynurenic acid.

Fig.3.

Dual-LPS administration and the kynurenine mono-oxygenase inhibitor Ro-61-8048 increase phosphorylation of neurogranin (Nrgn) and decreases phosphorylation of CaMKII in the medial prefrontal cortex (mPFC). (A) Representative immunoblots of phospho-NFAT1 (p-NFAT1), phospho-Nrgn (p-Nrgn) and Nrgn, phospho-CaMKII (p-CaMKII) and CaMKII in the mPFC of mice administered dual-LPS injections or vehicle at 24 h post T = 0 h injection. (B) Densitometry quantitation of the synaptosomal (S) fraction (upper graph) in the mPFC of mice administered dual-LPS injections or vehicle show that LPS significantly increased p-Nrgn and significantly decreased p-NFAT1 as well as p-CaMKII. Densitometry quantitation of the cytosolic (C) fraction (lower graph) in the mPFC of mice administered dual-LPS show significantly increased p-Nrgn and significantly lowered expression of p-NFAT1, CaMKII. $n = 3/\text{treatment}$. (C) Representative immunoblots showing the effects in the mPFC of mice that were administered L-Kynurenine (Kyn; 100 mg/kg *i.p.*), the kynurenine mono-oxygenase inhibitor (KMO-I) Ro-61-8048 (100 mg/kg *i.p.*) or vehicle on synaptosomal phospho-PKC γ (p-PKC γ), p-Nrgn, and p-CaMKII. (D) Quantification of p-PKC γ , p-Nrgn, and p-CaMKII in the vHip of mice administered vehicle, L-Kyn or the KMO-I Ro-61-8048. All results shown are representative of 1-3 separate independent western blot experiments. $n = 3-7/\text{treatment}$. * $p < 0.05$ by Student's *t*-test. All results are expressed as mean $\pm$ SEM.

Fig. 4.

Effects of dual-LPS on Pavlovian conditioning. (A) Relative to vehicle, mice administered dual-LPS and tested for Pavlovian conditioning at 24 h, 48 h, and 72 h post T = 0 injection exhibit significantly slower reaction times in response to CS+ presentations. (B) In comparison to vehicle, mice administered dual-LPS display significantly less magazine entries post T = 0 injection. (C) There were no differences detected in inactive hole entries between dual-LPS treated mice relative to vehicle, except at 24 h post T = 0 injection. A-C: n = 14-16/treatment. # $p < 0.05$ by two-way ANOVA. * $p < 0.05$ by Bonferroni's multiple comparisons relative to vehicle treated mice. B, Baseline; CS+, conditioned stimulus; T, time.

Fig. 5.

Dual-LPS or inhibition of the kynurenine mono-oxygenase (KMO) inhibitor Ro-61-8048 alters signaling proteins downstream of the NMDAR in the medial prefrontal cortex (mPFC). Dual-LPS induces an increase in KYNA and phosphorylation of PKC γ (p-PKC γ) and consequently Nrgn (p-Nrgn). Similarly pharmacological inhibition of KMO by systemic Ro-61-8048 increases KYNA and results in phosphorylation of PKC-Nrgn. Increases in p-Nrgn result in free calmodulin (CaM) where it can then potentiate neuroimmunological responses and impair stimulus processing suggestive of cognitive dysfunction. The possibility of neuroinflammation induced cognitive impairments due to elevations in KYNA is supported by LPS or KMO inhibitor mediated decreases in phosphorylation of CaM Kinase-II (p-CaMKII), a well known regulator of learning, synaptic integrity and synaptic function. Ca²⁺, calcium; KYNA, kynurenic acid; Nrgn, neurogranin.