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# Signaling function of Na,K-ATPase induced by ouabain against LPS as an inflammation model in hippocampus

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## Abstract

**Background:** Ouabain (OUA) is a newly recognized hormone that is synthesized in the adrenal cortex and hypothalamus. Low doses of OUA can activate a signaling pathway by interaction with Na,K-ATPase, which is protective against a number of insults. OUA has central and peripheral anti-inflammatory effects. Lipopolysaccharide (LPS), via toll-like receptor 4 activation, is a widely used model to induce systemic inflammation. This study used a low OUA dose to evaluate its effects on inflammation induced by LPS injection in rats.

**Methods:** Adult male Wistar rats received acute intraperitoneal (ip) OUA (1.8 µg/kg) or saline 20 minutes before LPS (200 µg/kg, ip) or saline injection. Some of the animals had their femoral artery catheterized in order to assess arterial blood pressure values before and after OUA administration. Na,K-ATPase activity, cytokine mRNA levels, apoptosis-related proteins, NF-κB activation brain-derived neurotrophic factor BDNF, corticosterone and TNF-α levels were measured.

**Results:** OUA pretreatment decreased mRNA levels of the pro-inflammatory cytokines, inducible nitric oxide synthase (iNOS) and IL-1β, which are activated by LPS in the hippocampus, but with no effect on serum measures of these factors. None of these OUA effects were linked to Na,K-ATPase activity. The involvement of the inflammatory transcription factor NF-κB in the OUA effect was indicated by its prevention of LPS-induced nuclear translocation of the NF-κB subunit, RELA (p65), as well as the decreased cytosol levels of the NF-κB inhibitor, IκB, in the hippocampus. OUA pretreatment reversed the LPS-induced glial fibrillary acidic protein (GFAP) activation and associated inflammation in the dentate gyrus. OUA also prevented LPS-induced increases in the hippocampal *Bax/Bcl2* ratio suggesting an anti-apoptotic action in the brain.

**Conclusion:** Our results suggest that a low dose of OUA has an important anti-inflammatory effect in the rat hippocampus. This effect was associated with decreased GFAP induction by LPS in the dentate gyrus, a brain area linked to adult neurogenesis.

**Keywords:** Ouabain, Na,K-ATPase, TNF-α, NF-κB, Inflammation, LPS

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## Introduction

Na,K-ATPase (NKA) is a membrane protein that is essential for the survival of the organism. This enzyme is expressed in all the cells of the human body, having many functions including the maintenance of osmotic balance, cell volume, pH and membrane potential. This occurs by the hydrolysis of an adenosine triphosphate (ATP) molecule leading to the export of three sodium ions and the import of two potassium ions into the cell, which is fundamental for neuronal excitability and cell maintenance [1,2].

NKA is constituted of three subunits:  $\alpha$ ,  $\beta$  and  $\gamma$  [3], with each subunit having a number of isoforms that provide functional versatility across different cell types, in turn highlighting the different roles and responses produced by NKA activation across cell types [4-8]. However, the  $\gamma$  (gamma) subunit, is not present in all the cells, with the other subunits being required for NKA to be functional [9].

In the adult brain,  $\alpha_1$  is expressed in all cells, with  $\alpha_2$  being expressed primarily in astrocytes and  $\alpha_3$  in neurons [10,11]. Mutations in the  $\alpha_2$ - and  $\alpha_3$ -isoform genes are involved in neurological disorders, such as familial hemiplegic migraine type-2 [12], rapid-onset dystonia [13], alternating hemiplegia of childhood [14] and cerebellar ataxia, areflexia, pes cavus, optic atrophy and sensorineural hearing loss (CAPOS) [15], with genetic variations in NKA also associating with bipolar disorder, suggesting a role for this enzyme in the etiology of this disease [16]. The NKA  $\alpha$ -isoform plays a critical role in the modulation of learning and memory, in turn regulating susceptibility to Alzheimer's disease [17]. Several works show NKA to operate as a receptor and not only as a pump, with a number of intracellular pathway activations driving its effects [18,19].

Ouabain (OUA) is synthesized by the adrenal gland and hypothalamus [20,21] and is likely to have important physiological roles in both the central and peripheral nervous systems [22,23]. OUA binds to NKA in hippocampal astrocytes, activating inositol trisphosphate receptor (InsP3R), which generates calcium oscillations, thereby activating NF- $\kappa$ B [23]. Xie and Askari [24] also showed OUA to act as a signal transducer, by binding to NKA and thereby activating the Ras-Raf-MAPK signaling cascade by the epidermal growth factor receptor (EGFR).

OUA has a dual role, given its dose-dependent response curve effects. A high concentration of OUA can cause cell death, driving neuronal necrosis via NKA inhibition, leading to potassium ion depletion and thereby increasing intracellular sodium and calcium ions [25]. Conversely, low concentrations of OUA (0.01 nM) are protective against kainic acid-induced lesions in the rat striatum, where it reduces apoptosis by increasing Bcl-2 [26]. Similarly, OUA affords protection in rat kidney primary

cultures against Shiga toxin [27]. As such, OUA can afford protection both peripherally and centrally.

NF- $\kappa$ B is a nuclear transcription factor, which is commonly induced following danger or inflammatory signaling, including by lipopolysaccharide (LPS) [28]. NF- $\kappa$ B comprises homo- and heterodimers via the combination of the subunits p65 (RELA), p50, p52, c-REL and REL of the REL/NF- $\kappa$ B family of proteins [28]. Different dimer combinations can activate or block distinct gene transcriptions, exemplified by the inhibitory homodimers p50/p50 and p52/p52 [29]. This transcription factor is evolutionarily conserved, driving a wide range of effects that depend on the specific activating stimulus, particular cell type and the cellular phenomenon that it regulates.

When it is not stimulated, NF- $\kappa$ B resides in the cytoplasm in association with the inhibitory protein, I $\kappa$ B (IKB) [30]. When IKB is phosphorylated by IKK (I $\kappa$ B kinase), IKB is degraded by proteasome 26S [31], freeing NF- $\kappa$ B, which can then translocate to the nucleus and bind to the regulatory sequence close to the promoter region, thereby regulating the expression of target genes such as, the pro-apoptotic *Bax*, anti-apoptotic *Bcl2*, inducible nitric oxide synthase (iNOS), several cytokines, manganese-superoxide dismutase (MnSOD) and brain-derived neurotrophic factor (BDNF) [29]. BDNF is an important trophic protein, including in the central nervous system (CNS), where it plays important roles in brain development and plasticity, with its malfunction being involved in many CNS diseases [32]. In the CNS, NF- $\kappa$ B plays a dual role in neurodegenerative diseases, enhancing survival in neurons, whilst driving pathological glial inflammatory processes [33].

LPS is a bacterial Gram-negative membrane element which is released in a free form or in aggregates. LPS is classically utilized as a model of inflammation and sepsis, with effects via toll-like receptor 4 (TLR4) [34,35]. Peripheral LPS administration induces NF- $\kappa$ B activation in different brain areas [36], as well as *de novo* synthesis of *Ikb* mRNA (an NF- $\kappa$ B activation index), in the brain parenchyma [37]. Peripheral LPS can, therefore, activate pro-inflammatory CNS genes [38]. The present work aims to evaluate the alterations induced by a low dose of OUA in the rat hippocampus, in an inflammation model induced by intraperitoneal (ip) LPS injection.

## Methods

### Animal and tissue preparation

Three-month old male Wistar rats (Biomedical Sciences Institute, University of São Paulo) were kept under 12 hour light/dark cycle (lights on at 7:00 am) and allowed free access to food and water. Animals were treated with OUA (1.8  $\mu$ g/kg, ip) or saline 20 minutes before the injection LPS (200  $\mu$ g/kg, ip) or saline and euthanized 2 hours after the administration (between

9:00 and 11:00 am) following procedures approved by the Biomedical College of Animal Experimentation (COBEA). All procedures were also approved by the Ethical Committee for Animal Research (CEEA) of the Biomedical Sciences Institute of the University of São Paulo. The brain was immediately removed and immersed in cold PBS. Each hippocampus was rapidly dissected, quickly immersed in liquid nitrogen, and stored at  $-80^{\circ}\text{C}$  for later use.

#### Hemodynamic parameters

Male Wistar rats ( $n = 4$ ) were anesthetized with a ketamine, xylazine and acepromazine mixture (64.9, 3.2 and 0.78 mg/kg). The left femoral artery was cannulated with a polyethylene catheter (PE-10 connected to PE-50 filled with heparinized saline, (BD, New Jersey, NY, USA) that was exteriorized in the mid-scapular region. After 24 hours, arterial pressure and heart rate were measured in conscious animals by a pressure transducer (model DT-100, Utah Medical Products, Midvale, UT, USA) and recorded using an interface and software for computer data acquisition (Power Lab 4/25, AD Instruments, Sydney, Australia). Heart rate was determined from the intra-beat intervals. The effects of OUA (1.8  $\mu\text{g}/\text{kg}$ , ip) on the arterial blood pressure and heart rate, was evaluated before and 1 hour after OUA administration in conscious animals. Results are expressed as mean  $\pm$  SE.

#### Chemicals and kits

Routine reagents, OUA and LPS from *Escherichia coli* O111:B4 were purchased from Sigma Chemicals (St. Louis, MO, USA); Bio-Rad protein assay kit was purchased from Bio-Rad (Hercules, CA, USA). Corticosterone, TNF- $\alpha$  and BDNF immunoassay kits were purchased from Enzo Life Sciences, Inc. (Farmingdale, NY, USA), eBioscience (San Diego, CA, USA) and Promega (Fitchburg, WI, USA), respectively. The kits were utilized according to manufacturer instructions. All solutions were prepared immediately before use.

#### Semiquantitative RT-PCR determination of inducible nitric oxide synthase (*iNos*), *Il-1 $\beta$* , *Bax* and *Bcl2* mRNA levels

The effect of OUA on LPS-modulated gene expression in the hippocampus of rats was measured. Total RNA was isolated with Trizol reagent (Invitrogen, Carlsbad, CA, USA) from the hippocampus according to the manufacturer's instructions. Semiquantitative reverse transcription-PCR (RT-PCR) amplification was performed using the ThermoScript RT kit (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions. The primer sequences were: *iNos* (GenBank access number 012611.3, 651 bp) 5'-GTGCTAATGCGGAAGGTCATA-3' (sense) and 5'CCAAATGTGCTTGTCACCACA-3' (antisense); *Bax* (GenBank access number 017059.1, 260 bp),

5'-TGAAGTGGACAACAACATGGAGC-3' (sense) and 5'-GGTCTTGGATCCAGACAAACAGC-3' (antisense); *Bcl2* (GenBank access number 016993.1, 271 bp), 5'-GGAGGATTGTGGCCTTCTTTGAG-3' (sense) and 5'-TATGCACCCAGAGTGATGCAGGC-3' (antisense); *Il-1 $\beta$*  (GenBank access number 031512.2, 1,339 bp), 5'-ATGCTCAGCAGTCAAGTGCC-3' (sense) and 5'-AGCCTT CCTTCGTGTAACCC-3' (antisense).

The PCR conditions consisted of 5 minutes at  $94^{\circ}\text{C}$ , 33 cycles of  $94^{\circ}\text{C}$  for 45 seconds,  $63^{\circ}\text{C}$  for 45 seconds, and  $72^{\circ}\text{C}$  for 1 minute and 30 seconds and a final extension at  $72^{\circ}\text{C}$  for 10 minutes. Glyceraldehyde-3-phosphate dehydrogenase (*Gapdh*; GenBank access number 017008.3, 264 bp) was also amplified as an internal PCR control using the following primers: 5'-CGGGAAGCTTGTGATCAATGG-3' (sense) and 5'-GGCAGTGATGCCATGGACTG-3' (antisense). In this case, the temperature cycling conditions were as follows: 5 minutes at  $94^{\circ}\text{C}$ , 20 cycles of  $94^{\circ}\text{C}$  for 45 seconds,  $63^{\circ}\text{C}$  for 45 seconds, and  $72^{\circ}\text{C}$  for 1 minute and 30 seconds and a final extension at  $72^{\circ}\text{C}$  for 10 minutes. Gel electrophoresis of the PCR product was performed using an ethidium bromide-containing agarose gel (2%), and resulting bands were visualized under UV light.

The photographs were captured by photo documentation system DP-001-FDC (VilberLourmat, Torcy, France), and the optical density of the bands was determined using NIH ImageJ software (<http://rsb.info.nih.gov/ij>). Results were expressed in relation to the intensity of *Gapdh* mRNA levels.

#### Protein extraction

Nuclear extract of each hippocampus was prepared as previously described [39]. Briefly, hippocampal structures were homogenized using a Dounce homogenizer in cold PBS supplemented with 0.5 mM dithiothreitol (DTT), 0.5 mM phenylmethylsulfonyl fluoride (PMSF), 2  $\mu\text{g}/\text{ml}$  leupeptin, 2  $\mu\text{g}/\text{ml}$  antipain, and 3 mM sodium orthovanadate and centrifuged at  $4^{\circ}\text{C}$  for 30 seconds at 12,000 g. Supernatant was discarded and pellet was resuspended in lysis buffer (10 mM HEPES, pH 7.9, 1.5 mM  $\text{MgCl}_2$ , 10 mM KCl, 0.1 mM ethylenediaminetetraacetic acid (EDTA), 0.5 mM DTT, 0.5 mM PMSF, 2  $\mu\text{g}/\text{ml}$  leupeptin, 2  $\mu\text{g}/\text{ml}$  antipain, and 3 mM sodium orthovanadate) and incubated on ice for 10 minutes. After addition of NP-40 (10%), samples were vigorously mixed and centrifuged for 30 seconds at 12,000 g. The supernatant was reserved for Western blotting and enzymatic assays (cytosolic fraction), and the pellet was resuspended in lysis buffer (10 mM HEPES, pH 7.9, 1.5 mM  $\text{MgCl}_2$ , 10 mM KCl, 0.1 mM EDTA, 0.5 mM DTT, 0.5 mM PMSF, 2  $\mu\text{g}/\text{ml}$  leupeptin, 2  $\mu\text{g}/\text{ml}$  antipain, and 3 mM sodium orthovanadate) and incubated on ice for 10 minutes. After addition of NP-40 (10%), samples were vigorously mixed and centrifuged for

30 seconds at 12,000 g. Supernatant was discarded, and the pellet was resuspended in extraction buffer (20 mM HEPES, pH 7.9, 25% glycerol, 1.5 mM  $MgCl_2$ , 300 mM NaCl, 0.25 mM EDTA, 0.5 mM DTT, 0.5 mM PMSE, 2  $\mu$ g/ml leupeptin, 2  $\mu$ g/ml antipain, and 3 mM sodium orthovanadate), incubated for 20 minutes on ice, and centrifuged for 20 minutes at 12,000 g at 4°C. The resulting supernatants containing nuclear proteins were stored at -80°C and used to test the expression of RELA. Protein concentration was determined using the Bio-Rad protein reagent (Hercules, CA, USA).

#### Western blotting

Electrophoresis was performed using 10% polyacrylamide gel and the Bio-Rad mini-Protean III apparatus (Bio-Rad, Hercules, CA, USA). In brief, the proteins present in the hippocampal cytosolic and nuclear fractions were size-separated in 10% SDS-PAGE (90 V). The proteins were blotted onto a nitrocellulose membrane (Bio-Rad, Hercules, CA, USA) and incubated with the specific antibody RELA (p65) (sc-0372; Santa Cruz Biotechnology, Santa Cruz, CA, USA) and IKB (sc-0371; Santa Cruz Biotechnology, Santa Cruz, CA, USA). The Ponceau method to immunoblot was used to ensure equal protein loading [40]. Proteins recognized by antibodies were revealed by an electrochemiluminescence (ECL) technique, following the manufacturer's instructions (Amersham Biosciences, Amersham, UK). To standardize and quantify the immunoblots, we used the photo documentation system DP-001-FDC (VilberLourmat, Torcy, France) and NIH Image software (<http://rsb.info.nih.gov/ij>). Several exposure times were analyzed to ensure the linearity of the band intensities.  $\beta$ -ACTIN antibody (sc-1616; Santa Cruz Biotechnology, Santa Cruz, CA, USA) was used as an internal experimental control, with results expressed in relation to  $\beta$ -ACTIN intensity.

#### Measurement of Na, K-ATPase activity

The NKA activity was determined by assaying Pi released from ATP hydrolysis. This inorganic compound forms a complex with molybdate which can be read spectrophotometrically at 700 nm [41]. For this colorimetric ATP assay, hippocampus supernatant (S1) was centrifuged (12,000 g for 15 minutes at 4°C). The particulate fraction was resuspended in a buffer containing: 0.32 M sucrose, 20 mM HEPES, 1 mM EDTA, 1 mM DTT, and 1 mM PMSE, pH 7.4.

Protein content was determined in particulate samples by colorimetric assay (Bio-Rad, Hercules, CA, USA) [42]. NKA activity was tested by adding 10  $\mu$ g of the particulate fraction (in 40  $\mu$ l of buffer) to 360  $\mu$ l of buffer containing: 3 mM ATP, 120 mM NaCl, 2 mM KCl, 3 mM  $MgCl_2$ , and 30 mM histidine (pH 7.2), with or without OUA (3  $\mu$ M or 3 mM). After 20 minutes of

incubation at 37°C, NKA activity was measured. The reaction was terminated by the addition of a quenching solution (0.6 ml) containing 1 N  $H_2SO_4$  and 0.5% ammonium molybdate. Formation of a phosphomolybdate complex was determined spectrophotometrically at 700 nm [43].

The total ATPase, Mg-ATPase, NKA  $\alpha_1$ - and  $\alpha_{2/3}$ -isoform activities were linearly related up to 20 minutes. In rodents, the NKA  $\alpha_1$ -isoform is 1,000 times less sensitive to the cardiac glycoside than the NKA  $\alpha_{2/3}$ -isoform measured from the difference between OUA-untreated and OUA-treated samples.

The high-affinity  $\alpha_{2/3}$ -isoform fraction was calculated by subtracting the activity obtained with 3  $\mu$ M OUA from total-ATPase activity. To determine the low-affinity fraction ( $\alpha_1$  subunit-associated NKA activity), the values obtained in the presence of 3  $\mu$ M OUA were subtracted from those obtained in the presence of 3 mM OUA. The NKA activity was expressed as nmol/mg protein x minutes.

#### Immunofluorescence in the hippocampus

The brain was fixed with paraformaldehyde and kept in 30% sucrose for 48 hours. Hippocampal coronal sections (30  $\mu$ m) were cut in the microtome and the tissues collected were kept in 0.1 M PBS. The sections were incubated with 2% normal donkey serum for 2 hours. For immunofluorescence reactions, the sections were incubated overnight with primary antibody (glial fibrillary acidic protein (GFAP), Sigma-Aldrich, St. Louis, MO, USA) and 4',6-diamidino-2-phenylindole; DAPI), followed by 2-hours incubation with secondary antibody (Alexa fluor 488 donkey anti-rabbit for GFAP). Six slices were placed in each slide and mounted with a coverslip. The sections were examined by fluorescence microscopy (Nikon Eclipse 80i with DXM 1200C digital camera; Nikon, Tokyo, Japan).

#### Statistical analysis

Results are expressed as mean  $\pm$  SEM of the indicated number of experiments. Statistical comparisons for OUA-induced changes in mRNA levels, protein expression, ELISA kits and NKA activities were performed by one-way analysis of variance (ANOVA), followed by the Newman-Keuls post-test. All analyses were performed using a Prism 6 software package (GraphPad Software, San Diego, CA, USA). *P*-values < 0.05 were considered to reflect a statistically significant difference.

#### Results

##### OUA (1.8 $\mu$ g/kg) does not induce any effect in blood pressure and NKA activity

One-hour OUA administration (1.8  $\mu$ g/kg; ip) did not change mean arterial pressure (Before:  $113 \pm 3.16$  versus After OUA:  $112 \pm 4.71$  mmHg; *P* > 0.05) or heart rate (Before:  $323 \pm 21.35$  versus After OUA:  $331 \pm 15.46$  bpm;

$P > 0.05$ ). In addition, this OUA dose does not change total ATPase, Mg-ATPase,  $\alpha_1$ -NKA and  $\alpha_{2/3}$ -NKA activities (Figure 1) in the rat hippocampus, suggesting the absence of OUA effects on blood pressure and central NKA activity.

#### Pretreatment with OUA reduced *iNos*, *Il-1 $\beta$* , and the *Bax/Bcl2* ratio mRNA levels activated by LPS

*iNos* mRNA levels, an important indicator of inflammation, increased in the LPS group in comparison with the control group as expected. OUA pretreatment decreased the LPS induction of *iNos* mRNA activation, returning levels to that of controls. OUA alone had no impact on *iNos* mRNA level (Figure 2A). *Il-1 $\beta$*  mRNA also increased in the LPS-treated group, which OUA pretreatment attenuated. OUA itself had no impact on *Il-1 $\beta$*  mRNA level (Figure 2B).

We also analyzed changes induced by LPS in the presence or absence of OUA on the ratio between the pro-apoptotic protein, *Bax*, and the anti-apoptotic protein, *Bcl2*. LPS increased the hippocampal *Bax/Bcl2* ratio, which OUA pretreatment prevented, suggesting an anti-apoptotic effect of OUA against LPS. Together, these data suggest that OUA has anti-inflammatory and anti-apoptotic effects.

#### Pretreatment with OUA can block LPS-induced NF- $\kappa$ B translocation

Western blotting assays showed that LPS (ip) administration increases both nuclear RELA (p65) translocation and

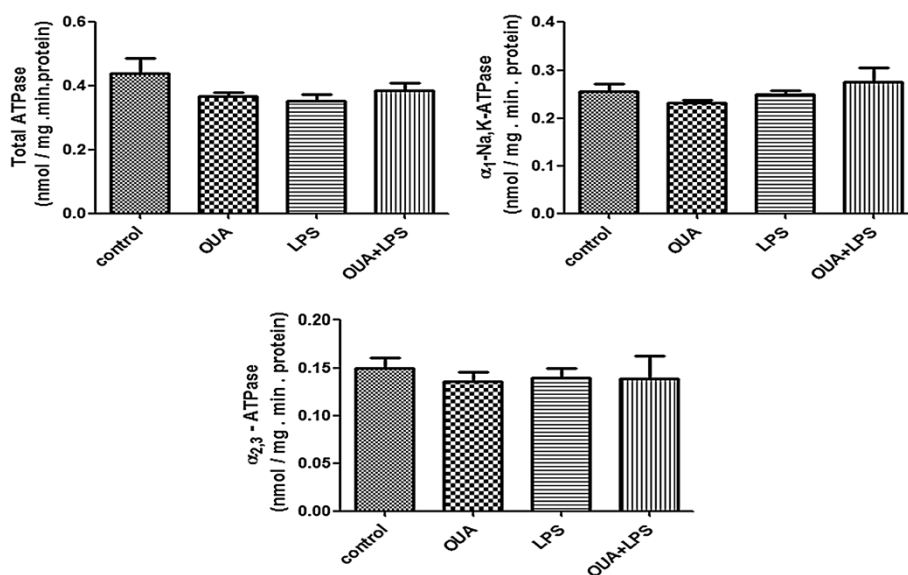
cytosolic I $\kappa$ B degradation, which could lead to NF- $\kappa$ B activation in the rat hippocampus. Although OUA itself did not change either nuclear p65 translocation or cytosolic I $\kappa$ B, it did block LPS-induced RELA (p65) and I $\kappa$ B effects in the rat hippocampus (Figure 3A and B). The inhibition of LPS-induced NF- $\kappa$ B translocation by OUA likely underlies the OUA inhibition of LPS-induced *iNos*, *Il-1 $\beta$*  and increased *Bax/Bcl-2* ratio.

#### Pretreatment with OUA can reverse the activation of astrocytes

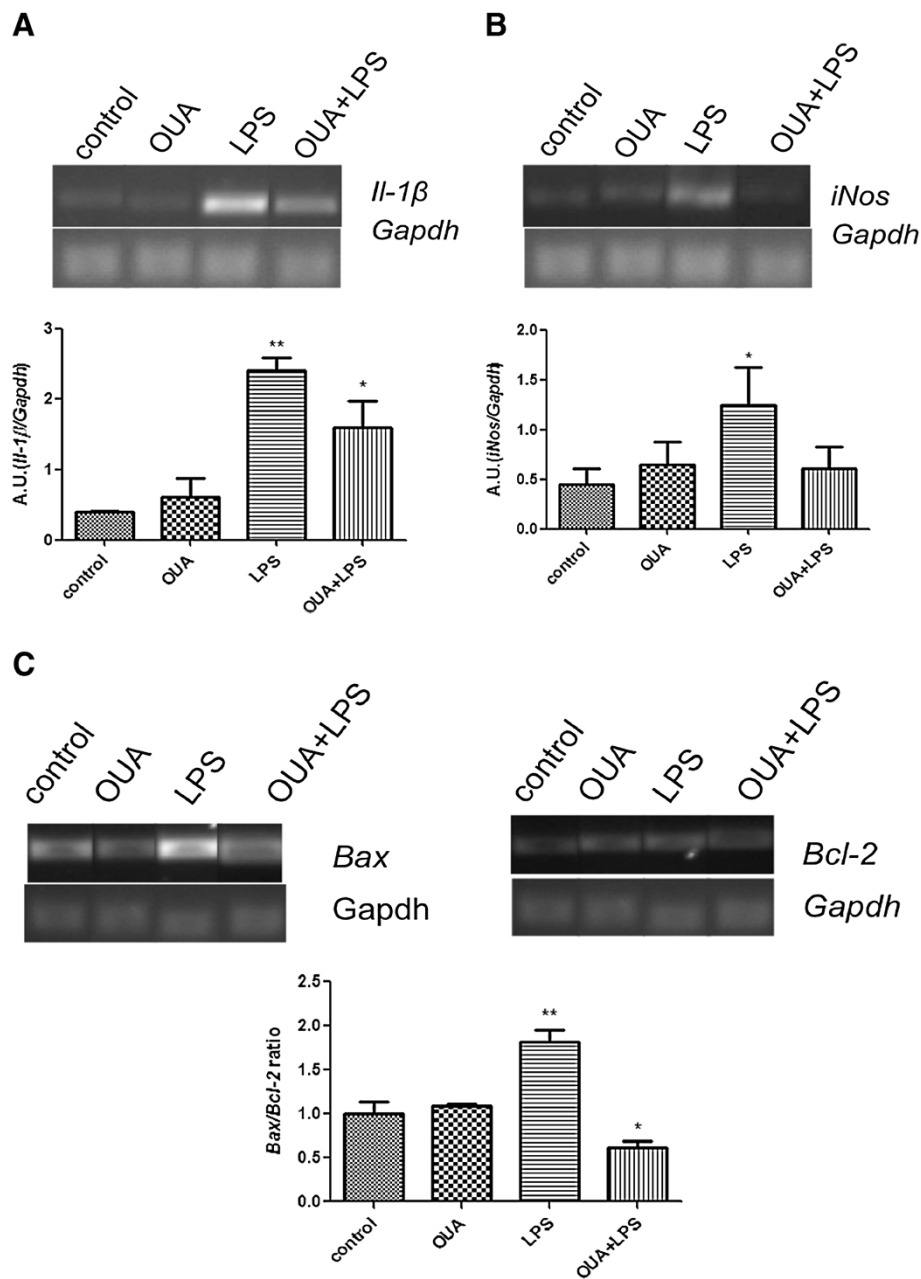
GFAP immunofluorescence of the dentate gyrus region of the hippocampus indicated that LPS induced astrocyte activation, which OUA pretreatment inhibits (Figure 4). Increased GFAP expression indicates astrocyte activation, showing that OUA pretreatment not only has an effect on pro-inflammatory cytokine levels, but also on the activation of pro-inflammatory cells in the dentate gyrus. Such data indicate OUA to be neuroprotective, at least in part by decreasing astrocyte activation.

#### OUA does not reverse LPS-induced increase in serum corticosterone and TNF- $\alpha$ levels

Our data showed that LPS, but not OUA, increased serum levels of corticosterone and TNF- $\alpha$  (Figure 5A and B). In addition, OUA pretreatment was unable to block LPS-induced increases in serum corticosterone and TNF- $\alpha$ .



**Figure 1** Pretreatment of ouabain (OUA) (1.8  $\mu$ g/kg) in lipopolysaccharide (LPS) (200  $\mu$ g/kg) inflammation model induced no changes on Na,K-ATPase (NKA) activity. The total NKA activity was tested by adding 10  $\mu$ g of the particulate fraction from the rat hippocampus obtained 2 hours after OUA (1.8  $\mu$ g/kg, ip) or saline pretreatment + LPS (200  $\mu$ g/kg, ip).  $\alpha_{2/3}$ -NKA activity was measured by subtracting the activity obtained with 3  $\mu$ M OUA (it inhibits just these 2 isoforms) from total NKA activity. To determine the  $\alpha_1$  subunit-associated NKA activity, the total NKA activity was subtracted from the activity obtained with 3 mM OUA (Mg-ATPase). Results are expressed as nmol/mg.min protein (mean + SEM) from five individual experiments. One-way ANOVA followed by Newman-Keuls post-test.



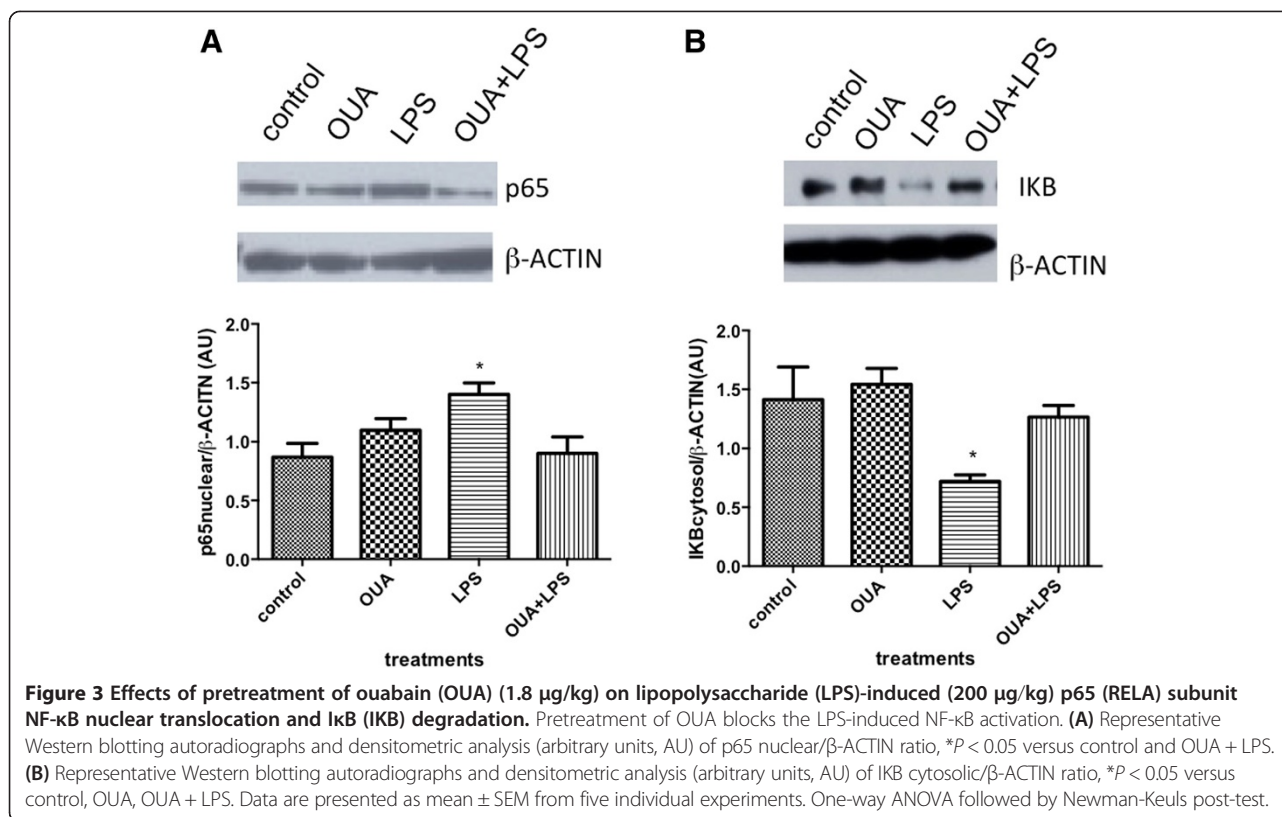
**Figure 2** Effects of pretreatment of ouabain (OUA) (1.8  $\mu\text{g}/\text{kg}$ ) in the lipopolysaccharide (LPS) (200  $\mu\text{g}/\text{kg}$ ) inflammation model on the mRNA levels of *Il-1 $\beta$* , *iNos*, *Bax*, and *Bcl2*. Pretreatment of OUA can decrease the mRNA levels of pro-inflammatory cytokines and pro-apoptotic proteins. Representative semiquantitative RT-PCR photographs and densitometric analysis (arbitrary units, AU) of the specific bands. mRNA levels are presented as ratios of the target gene to *Gapdh* expression. Data are presented as mean  $\pm$  SEM from five individual experiments. One-way ANOVA followed by Newman-Keuls post-test. **(A)** *Il-1 $\beta$*  mRNA, \* $P < 0.05$  versus control and OUA and \*\* $P < 0.01$  versus control and OUA and OUA + LPS. **(B)** *iNos* mRNA, \* $P < 0.05$  versus control and OUA + LPS. **(C)** *Bax/Bcl2* ratio, \*\* $P < 0.001$  versus OUA, control and OUA + LPS and \* $P < 0.05$  versus OUA.

### OUA can modulate TNF- $\alpha$ and BDNF

OUA or LPS alone significantly decreased BDNF in comparison to controls, although the decrease induced by LPS was significantly greater. However, OUA pretreatment reversed LPS effects, leading to BDNF levels at that of controls (Figure 6A). Likewise, LPS

or OUA treatment alone increased the inflammatory cytokine, TNF- $\alpha$ , with OUA pretreatment preventing LPS-induced TNF- $\alpha$  increases versus control (Figure 6B).

This suggests protection of the rat hippocampus by OUA pretreatment against the effects of LPS, given that TNF- $\alpha$  is an important early pro-inflammatory cytokine,



and BDNF is a significant neuronal protectant, as well as being a growth factor.

## Discussion

Neuroinflammation can be associated with neurological insults and neurodegenerative disorders. Neuroinflammation is characterized by increased pro-inflammatory cytokines, such as TNF- $\alpha$  and the expression on endothelial cells of adhesion molecules that increase leukocyte infiltration over the blood–brain barrier (BBB). Concurrently, astrocytes and microglia are activated [43].

LPS is an inflammation model that can activate NF- $\kappa$ B, partly mediated by glutamate acting at N-methyl-D-aspartate (NMDA) receptors, in turn increasing calcium-dependent activation of NOS and reactive oxygen species generation; the latter contributing to membrane peroxidation [36]. NF- $\kappa$ B activation induces the expression of many inflammatory cytokines and inflammatory factors that drive the innate immune response [29,44].

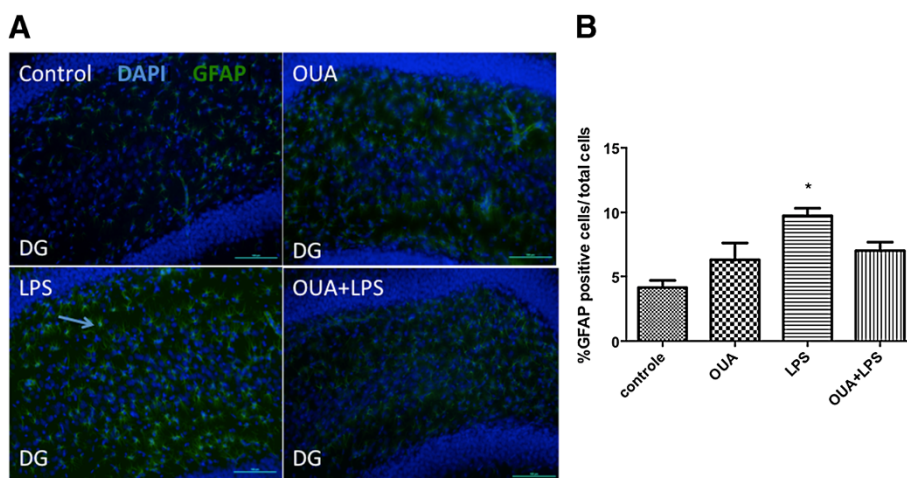
However, it is unpredictable as to whether circulating high concentrations of LPS increase BBB permeability, leading to the entry of LPS and other molecules into the brain parenchyma [36,37]. In the early stages of LPS infection, central areas not protected by the BBB are likely to contribute to the inflammatory process, including the actions of LPS at TLR4 in the pineal gland, which inhibits pineal melatonin production, in turn contributing

to increased BBB permeability and immune system responsivity (reviewed in [45]).

TLR4 is also constitutively expressed in the leptomeninges, choroid plexus, subfornical organ, organum vasculosum of the lamina terminalis, median eminence, and area postrema. LPS effects in these non-BBB protected areas are likely to influence the inflammatory response in BBB protected areas, such as the hippocampus [46]. As such, LPS inflammatory effects may not be dependent on increased BBB permeability. The activation of inflammatory cytokines by LPS in this study confirms the results of previous studies from our laboratory, which showed that most of the transcriptional activity regulated by LPS administration is mediated by the p50/p65 dimer of NF- $\kappa$ B [36,38]. LPS intraperitoneally causes astrocyte activation in the dentate gyrus in aged rats, with this effect can be prolonged for many days [47]. Inflammation in the dentate gyrus can block neurogenesis in adult rats, which can be restored by an anti-inflammatory drug [48].

It is known that OUA can produce negative effects in a dose-dependent manner, but many studies have shown OUA to afford protection centrally and peripherally. In a serum deprivation model in rat embryonic kidney cell cultures, OUA prevents cell death by the activation of NF- $\kappa$ B [49]. It has also been shown that OUA can protect proximal tubular cells against Shiga toxin by activating the anti-apoptotic Bcl-xL [27].





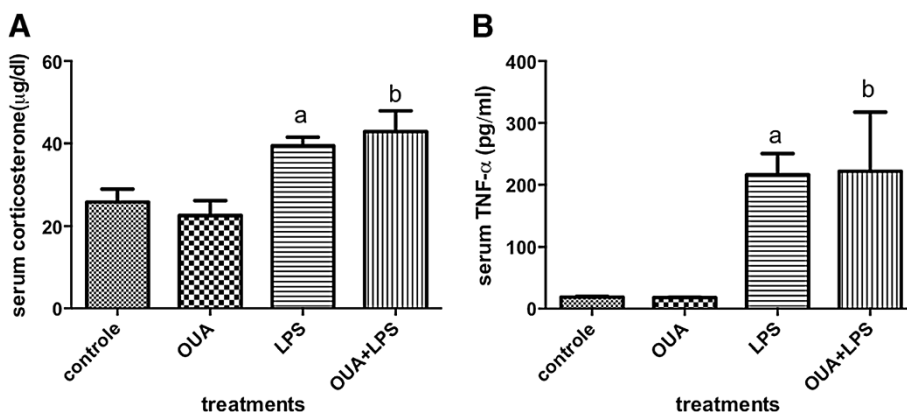
**Figure 4** The pretreatment of ouabain (OUA) (1.8  $\mu\text{g}/\text{kg}$ ) on lipopolysaccharide (LPS)-induced (200  $\mu\text{g}/\text{kg}$ ) astrocyte activation. Astrocyte activity was measured with glial fibrillary acidic protein (GFAP) immunofluorescence in the dentate gyrus. **(A)** Representative photomicrographs showed that LPS increases astrocytic activation, with OUA pretreatment reducing this. The arrow represents an activated astrocyte. **(B)** Densitometric analysis of the % of GFAP-positive cells/total cells. \* $P < 0.05$  versus control, OUA and OUA + LPS. Data are presented as mean  $\pm$  SEM from five individual experiments. One-way ANOVA followed by Newman-Keuls post-test.

Although OUA circulates in blood plasma of resting humans in the subnanomolar-to-nanomolar concentration range, non-inhibitory subnanomolar concentration of OUA might be linked to activation of a signal transducer pathway in rats since the  $K_m$  for OUA inhibition of NKA is 10 to 100  $\mu\text{M}$  for isolated rat vascular smooth muscle, whilst in humans it is 10 to 100 nM [50,51].

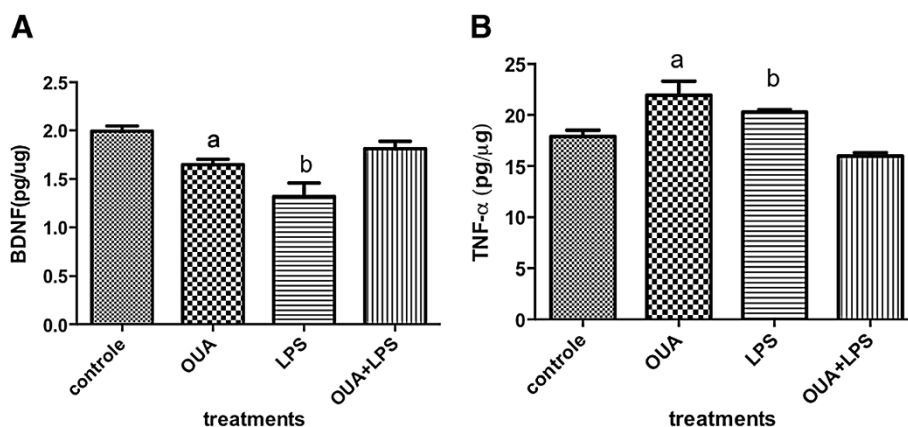
Thus, the OUA concentration (1.8  $\mu\text{g}/\text{kg}$ , ip) used in the present study is likely linked to activation of the NKA signaling cascade, since in this study we used an OUA concentration that had no significant impact on blood pressure and it did not inhibit hippocampal NKA. As such, the results here are not dependent on

NKA inhibition, but are dependent on changes in NKA-related signaling. Our data that showed ip LPS administration increased the mRNA levels of *iNos* and *Il-1 $\beta$* , as well as the *Bax/Bcl2* ratio, whereas OUA alone had no impact on the mRNA levels of these factors. However, we observed a protective effect of OUA, given its decrease in the mRNA levels of *iNos* and *Il-1 $\beta$* , as well as a decrease in the *Bax/Bcl2* ratio induced by LPS in the hippocampus.

This protection is likely to be afforded by OUA blocking LPS-induced nuclear translocation of NF- $\kappa\text{B}$  via the RELA (p65) subunit and by decreasing the degradation of cytosolic I $\kappa\text{B}$ , thereby maintaining I $\kappa\text{B}$  linked to NF- $\kappa\text{B}$  in the cytosol. The major OUA protective



**Figure 5** Effects of ouabain (OUA) pretreatment (1.8  $\mu\text{g}/\text{kg}$ ) on lipopolysaccharide (LPS)-induced (200  $\mu\text{g}/\text{kg}$ ) serum corticosterone and TNF- $\alpha$ . **(A)** The serum levels of corticosterone were elevated in LPS and OUA + LPS in comparison with control and OUA group, <sup>a</sup> $P < 0.01$  versus control and OUA and <sup>b</sup> $P < 0.01$  versus control and OUA. **(B)** The same pattern occurs in TNF- $\alpha$  serum levels, <sup>a</sup> $P < 0.05$  versus control and OUA and <sup>b</sup> $P < 0.05$  versus control and OUA. Data are presented as mean  $\pm$  SEM from five individual experiments. One-way ANOVA followed by Newman-Keuls post-test.



**Figure 6** Effects of ouabain (OUA) pretreatment (1.8  $\mu\text{g}/\text{kg}$ ) on lipopolysaccharide (LPS)-induced (200  $\mu\text{g}/\text{kg}$ ) regulation of hippocampal brain-derived neurotrophic factor (BDNF) and TNF- $\alpha$  levels. **(A)** The levels of BDNF were decreased in LPS and OUA groups in comparison with control and OUA + LPS group, <sup>a</sup> $P < 0.05$  versus control and LPS and <sup>b</sup> $P < 0.01$  versus control and OUA + LPS and  $P < 0.05$  versus OUA. **(B)** The levels of TNF- $\alpha$  in the hippocampus, <sup>a</sup> $P < 0.01$  versus control and OUA + LPS and <sup>b</sup> $P < 0.05$  versus control and OUA + LPS. Data are presented as mean  $\pm$  SEM from five individual experiments. One-way ANOVA followed by Newman-Keuls test.

effect occurred in the adult neurogenesis-associated dentate gyrus [52].

Previous studies from our laboratory have reported that OUA can increase cytokine levels. Such variability in OUA effects may be due to a number of factors, including testing times and type of administration, as well as different tissue and cell models. In fact, we detected changes in cytokines gene expression 1 hour after injecting OUA directly in to the CA1 region of the hippocampus at a concentration of 10 nM [53], as well as in *in vitro* studies (OUA = 10  $\mu\text{M}$ ) in cerebellar primary culture cells [54].

TLR4 decreases neurogenesis and neuronal differentiation, with consequences for neuronal plasticity and memory [55]. BDNF increases neurogenesis, improves memory acquisition and has anti-apoptotic and anti-inflammatory effects, as well as protecting against hypoxia/ischemia-induced brain injury [56]. OUA pretreatment prevented the decrease in BDNF by LPS, although OUA alone also decreased BDNF. In fact, we cannot rule out that BDNF can also be modulated by other transcription factors that might be activated by OUA. OUA has both toxic [57,58] and protective actions against damage to cells [59,60], including neurons [26]. In the present study, OUA pretreatment reverted LPS-induced decreases in BDNF levels, suggesting a specific action of this compound against the inflammatory signaling response. OUA pretreatment also provided protection against LPS in the rat hippocampus by decreasing the LPS induction of the pro-inflammatory cytokine, TNF- $\alpha$ . OUA alone increased TNF- $\alpha$ , as we found previously [53,54]. This is surprising as another study showed OUA to block the activation of the TNF/NF- $\kappa\text{B}$  pathway by interfering with the interaction between TNF receptor 1 and TRADD in HeLa cell cultures [61]. Further work should clarify the temporal

regulation of TNF- $\alpha$  and/or BDNF by OUA and also its perhaps differential effects in different cell types. In the periphery, our data showed that LPS, but not OUA, increased serum levels of corticosterone and TNF- $\alpha$ , with OUA pretreatment unable to block these changes. This could suggest that the OUA concentration used in the present study may be too low to reverse LPS peripheral activation, although it was high enough to protect against LPS effects in the hippocampus. This would imply that OUA protection could be mediated by its direct action in the brain. Alternatively, the peripheral effect of LPS may be stronger, with the OUA concentration used in the current study unable to reverse the peripheral effects of LPS.

In the peripheral system, mice treated with zymosan and carrageenan leads to paw edema, with OUA decreasing the inflammatory response in these models, suggesting that OUA has peripheral anti-inflammatory effects [62]. However, the OUA concentrations used were higher than in this current study, with the anti-inflammatory effects being mediated by different intracellular pathways, involving the inhibition of prostaglandin E2, bradykinin and mastocyte degranulation [62]. However, it should be noted that other mechanisms may be involved in the anti-inflammatory effects of steroid glycosides, including decreasing IL-6 [63] and inhibition of genes activated by TNF- $\alpha$  [64].

Recent studies have shown that low doses of OUA have anti-inflammatory properties in LPS-treated reactive astrocytes, by reducing IL-1 $\beta$  release. OUA also prevented the LPS-induced downregulation of NKA and restored the actin filaments in reactive astrocytes [65]. This is in contrast to the effects of OUA in LPS-treated microglia, where this compound had no effect on levels of microglial

reactivity [66]. This could suggest that some of the differential effects of OUA on central processing are determined by the relative levels of microglial and astrocyte activation, including at different time points in the inflammatory process.

## Conclusions

Taken together, the present work showed that OUA pretreatment has anti-inflammatory and anti-apoptotic effects in the hippocampus challenged with LPS-induced inflammation. This effect is mediated by NF- $\kappa$ B activation, including in the neurogenesis-associated dentate gyrus. The ability of OUA to suppress the inflammatory process and maintain hippocampal BDNF levels in the face of inflammatory activity suggests that the NKA signaling cascade could be a new therapeutic target in neuroinflammation-associated disorders.

## Abbreviations

ANOVA: analysis of variance; ATP: adenosine triphosphate; BBB: blood-brain barrier; BDNF: brain-derived neurotrophic factor; bp: base pair; CAPOS: cerebellar ataxia, areflexia, pes cavus, optic atrophy and sensorineural hearing loss; CEEA: Ethical Committee for Animal Research; COBEA: Biomedical College of Animal Experimentation; CNS: central nervous system; DAPI: 4',6-diamidino-2-phenylindole; DTT: dithiothreitol; EDTA: ethylenediaminetetraacetic acid; ECL: electrochemiluminescence; EGFR: epidermal growth factor receptor; GAPDH: glyceraldehyde-3-phosphate dehydrogenase; GFAP: glial fibrillary acidic protein; I $\kappa$ B: NF- $\kappa$ B inhibitor protein; IKK: I $\kappa$ B kinase; IL-1 $\beta$ : interleukin 1 beta; iNOS: inducible nitric oxide synthase; ip: intraperitoneal; InsP3R: inositol trisphosphate receptor; Km: Michaelis-Menten equilibrium constant; LPS: lipopolysaccharide; MnSOD: manganese-superoxide dismutase; NF- $\kappa$ B: nuclear factor kappa B; NKA: Na,K-ATPase; NMDA: N-methyl-D-aspartate; OUA: ouabain; PBS: phosphate-buffered saline; PCR: polymerase chain reaction; PMSF: phenylmethylsulfonyl fluoride; TLR4: toll-like receptor 4; TNF- $\alpha$ : tumor necrosis factor alpha.

## Competing interests

The authors declare that they have no competing interests.

## Authors' contributions

PFK, EMK and CS participated in the research design, data analysis and wrote or contributed to the writing of the manuscript. All authors read and approved the final version of the manuscript. PFK performed the experiments. LMY participated in the data analysis and contributed to writing the manuscript. ARV, AMMO and LSL participated in the writing of the manuscript and performed experiments. APDC and LVR participated in the research design, data analysis and wrote the manuscript section dealing with blood pressure.

## Authors' information

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