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Abstract: Depression is highly prevalent in patients suffering from chronic inflammatory diseases. Dysregulated neuroinflammation and concomitant activated micorglia play a pivotal role in the pathogenesis of depression. Paricalcitol (Pari), a vitamin D2 analogue, has been demonstrated to exert anti-inflammative effects on renal and cardiovascular diseases. In this study, mice were pretreated with Pari before being induced to acute depression-like behaviors by systemic lipopolysaccharide (LPS) injection. To determine the therapeutic effects of Pari, alterations in acute body weight, sucrose preference, forced swimming and tail suspension tests were assessed. Then, alterations of pro-inflammation cytokine IL1-β level and microglia activity in the hypothalamus, which are involved in the pathophysiology of depression, were examined. The results showed that Pari significantly alleviated systemic LPS injection induced depressive-like behaviors as shown by increased sucrose preference and decreased TST and FST immobility. Pari could specifically regulate microglia-mediated neuroinflammation process and local activity of renin-angiotensin system to exert its anti-depressant effects. This study demonstrated a potential for paricalcitol in treating depressive symptoms induced by systemic inflammation, particularly in patients with chronic hypertension.

Paricalcitol alleviates lipopolysaccharide-induced depressive-like behavior by suppressing hypothalamic microglia activation and neuroinflammation

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Abstract

Depression is highly prevalent in patients suffering from chronic inflammatory diseases. Dysregulated neuroinflammation and concomitant activated micorglia play a pivotal role in the pathogenesis of depression. Paricalcitol (Pari), a vitamin D2 analogue, has been demonstrated to exert anti-inflammative effects on renal and cardiovascular diseases. In this study, mice were pretreated with Pari before being induced to acute depression-like behaviors by systemic lipopolysaccharide (LPS) injection. To determine the therapeutic effects of Pari, alterations in acute body weight, sucrose preference, forced swimming and tail suspension tests were assessed. Then, alterations of pro-inflammation cytokine IL1-β level and microglia activity in the hypothalamus, which are involved in the pathophysiology of depression, were examined. The results showed that Pari significantly alleviated systemic LPS injection induced depressive-like behaviors as shown by increased sucrose preference and decreased TST and FST immobility. Pari could specifically regulate microglia-mediated neuroinflammation process and local activity of renin-angiotensin system to exert its anti-depressant effects. This study demonstrated a potential for paricalcitol in treating depressive symptoms induced by systemic inflammation, particularly in patients with chronic hypertension.

Keywords:

Paricalcitol; microgliosis; neuroinflammation; depression-like behavior; LPS

1. Introduction

Evidences from clinical studies revealed that depression is highly prevalent in patients suffering from chronic inflammatory diseases [1]. Although the precise mechanism remains unclear, mounting evidence have demonstrated that dysregulated neuroinflammation and concomitant activated micorglia play a pivotal role in the pathogenesis of depression [2-5]. The severity of depression is associated with the levels of circulating proinflammatory cytokines, such as IL-1β, IL-6, TNF-α and IFN-γ [6]. Once the barrier functions of the blood-brain barrier (BBB) and choroid plexus compromised, the innate immune system in the central nerve system will be activated [7]. Microglia，which comprise 10-15% of all brain cells, are the resident immune cells of the central nerve system. They are crucial in regulating neurodevelopment and neurological functions [8]. Microglia could be activated when pathologically insulted and exacerbate central inflammatory response with the releasing of pro-inflammatory cytokines [9]. These peripheral and central inflammatory signals are integrated by microglia and ultimately leads to neuronal dysfunction, aberrant plasticity, oxidative stress and the suppression of adult neurogenesis, all of which are potential mechanisms of depression [10, 11]. Once the central inflammatory response exceeds the organism's coping capacity, mental disorders will occur [12, 13].

Experimental bacterial endotoxin lipopolysaccharide (LPS) challenge evokes the activation of microglia and acute phase response of inflammation, which induces

depressive symptoms in humans and depressive-like behavior in rodents [11, 14, 15]. Moreover, LPS-induced depressive-like symptoms can be ameliorated by microglial inhibitor minocycline, which suggests that inhibiting microglia activation and neuroinflammation should be regarded as promising therapies for inflammation-induced depression [16].

Recently, several clinical and experimental findings suggested that vitamin D (VD) deficiency are significantly associated with depression [17-19]. VD is a pleiotropic secosteroid concerning many aspects of physiological functions, including regulating calcium and phosphorous balance, influencing cell differentiation and proliferation, as well as modulating the immune and inflammation processes [20]. The inflammatory modulation properties of VD indicate that it has the potential to ameliorate inflammation-induced depressive-like behavior. Paricalcitol (Pari), a vitamin D2 analogue, has been demonstrated to exert anti-inflammative effects on renal and cardiovascular disease via modulating nuclear factor (NF)-κB signaling [21, 22]. However, no attampt has been made to determine the beneficial effect of Pari on mental disease. Thus, we hypothesized that Pari might have the potential to alleviate systemic-inflammation induced depressive-like behavior.

In the present study, we first determined whether Pari could alleviate depressive-like behavior in mice after systemic LPS challenge. Then, alterations of pro-inflammation cytokine IL1-β and microglia activity in the hypothalamus, which involved in the pathophysiology of depression,were examined. Finally, we attempted

to demonstrate the possible mechanism that could account for the antidepressant effect of Pari.

2. Materials and methods

2.1. Animals

Totally thirty eight-week-old male C57BL/6J mice were purchased from the Slac Laboratory Animal (Shanghai, China). Mice were group housed until behavioral tests beginning and provided ad libitum access to maintenance diet and water in a temperature (25 ℃) and humidity (45–55%)-controlled environment with a 12-hour light/dark cycle (lights off at 8:00 pm). Mice were handled daily for 1 week before experimentation. All animal procedures were performed strictly according to NIH Guide for Care and Use of Laboratory Animals. The animal study protocol was approved by the Animal Care and Use Committee of Shanghai University of Traditional Chinese Medicine.

2.2. Drugs and Administration

Animals in the Pari (Paricalcitol) group were treated with paricalcitol at 0.4 μg/kg (dissolved in propylene glycol:ethanol_90:10, i.p. injection three times per week) (provided by Prof. Yan-Chun Li, University of Chicago) for fourteen days prior LPS injection. Meanwhile, animals in the control and LPS group were daily i.p. administrated with equal dose volume of vehicle. To avoid additional handling-induced unexpected behavioural alteration, mice in the Pari group received

daily i.p. injection of equal dose volume of vehicle as well during the days without Pari pre-treatment.

To induce an inflammatory reaction, a dose of 1mg/kg of LPS (Escherichia coli, 0127:B8, Sigma-Aldrich, Lyon, France) was intraperitoneally injected (ten mice in each group were employed) between 9 and 11 am. One milligram LPS were diluted in 5 mL sterile endotoxin-free saline (NaCl 0.9%) before injection. LPS is a component of the cell wall of Gram-negative bacteria and represents a useful model for investigating changes that accompany brain inflammation. Control mice received an injection of endotoxin-free injectable saline solution (0.9%). Body weight was measured 24 hours after LPS injection.

2.3. Sucrose preference test

The sucrose preference test was employed to evaluate anhedonia. Before testing, all mice were acclimated to drinking water and 2% sucrose solution for 3 days. On the day of testing, drinking water and 2% sucrose solution were placed in the home cage for 23 hours. At the end of the testing, fluid content was measured and sucrose preference was calculated using the following equation: Sucrose preference (%) = sucrose intake/(sucrose intake + water intake) × 100.

2.4. Tail suspension test (TST)

The TST was conducted at 23h post treatment. Mice were suspended by adhesive tape that was positioned about 2.5 cm from the tail-tip with the head 40 cm above the floor. The trial was carried out for 6 min and the mice were video-recorded for analysis. The duration of immobility was manually recorded by two trained blinded observers during the final 5 min interval of the test. Mice were considered immobile when they hung passively and motionlessly.

2.5. Forced swimming test (FST)

At 24h after treatment, mice were placed individually into glass cylinders (height: 30 cm, diameter: 16 cm) containing 25 cm water maintained at 23–25 ℃. Water was renewed before test. FST lasted for 6 min and mice were immediately returned to their home cage. The total time spent immobile was scored by two well trained observers who were blinded to the treatments. A mouse was judged to be immobile when it floated in an upright position, and made only small movements to keep its head above water. The duration of immobility was recorded during the last 5 min of the testing period.

2.6. Sample collection

Immediately after behavioral testing, animals were sacrificed by terminal anesthesia (pentobarbital; IP, 70mg kg^{-1}). Blood was collected by cardiac puncture and brains were removed after perfusion with sterile NS. The hypothalamus were collected.

Tissues from one hemisphere were used for ELISA assay while tissues from the contralateral hemisphere were employed for qPCR test.

2.7. ELISA for IL1-β

Plasma cytokine IL-1βwere assessed using commercially available enzyme-linked immunosorbent assays (ELISAs) kits (Excell, Shanghai, China) according to manufacturer's instructions. Samples were diluted 1:10 (v:v). Assays were sensitive with lower limits of quantification at 10 pg/ml; inter and intra-assay coefficients of variation were less than 10%.

2.8. Quantitative Real-time-PCR

The transcription levels of IL-1β in the hypothalamus was determined with quantitative real-time-PCR. Total RNA was isolated using RNA-Solv Reagent (OMEGA, Georgia, USA). Reverse transcription was performed with 2 μg RNA using Rever Tra Ace (TOYOBO, Osaka, Japan) and Oligo(dT)18 (TaKaRa, Shiga, Japan). qRT-PCR was carried on SYBR Premix Ex Taq (TaKaRa, Shiga, Japan) using ViiA 7 Real-Time PCR System. Reaction procedures were as follow: an initial step at 95°C for 5min, 40 cycles of 94°C for 15s, 60°C for 34s. The primers used were as the following : IL-1β, fwd 5'-AAT GCC TCG TGC TGT CTG ACC-3', rev 5'-TTG TCG TTG CTT GTC TCT CCT TG-3'; β-actin, fwd 5'-TCT GGC ACC ACA CCT TCT A-3', rev 5'-AGG CAT ACA GGG ACA GCA C-3'.

2.9. Western Blotting Analysis

Tissues were lysed with a lysis buffer containing protease inhibitor cocktails (Roche, Mannheim, Germany). The protein concentration was determined using a BCA protein assay kit (Thermo scientific, Masschusetts, USA). Then, protein extracts were separated by electrophoresis on SDS-PAGE gels and transferred onto polyvinylidene fluoride (PVDF) membranes. The membranes were sequentially incubated with primary antibodies and secondary antibodies, and enhanced chemiluminescence (ECL) solution and followed by autoradiography. The intensity of the blots was analyzed using Image Pro plus 6.0.

Primary antibody of goat anti-Iba-1 was purchased from Abcam (Abcam, Cambrige, UK), rabbit anti-NF-κB p65 was purchased from Sigma-Aldrich (Sigma-Aldrich, St Louis, USA), rabbit anti-NLRP-3 (Abcam, Cambrige, UK), rabbit anti-caspase-1 (Cell Signaling Technology, Boston, USA), rabbit anti-renin (Abcam, Cambrige, UK) and rabbit anti-Ang-II (Abcam, Cambrige, UK). Horseradish peroxidase secondary antibody was from Cell signaling technology (Cell Signaling Technology, Boston, USA).

2.10. Immunohistochemistry and proportional area analyses

Immediately after finishing behavioral tesets, mice were perfused with 4 % paraformaldehyde (PFA) and post-fixed overnight before being dehydrated. Ten

micrometer coronal brain sections were prepared with a Leica CM1950 cryostat (Leica Biosystems). Sections were rinsed and blocked with 0.3 % triton and 5 % bovine serum for 1.5 h at room temperature. The sections were incubated with rabbit polyclonal antibody (pAb) Iba-1 (1:200, Abcam, Cambrige, UK), NeuN (1:200, Millipore, Darmstadt, Germany), NLRP-3 (1:200, Abcam, Cambrige, UK), Ang- II (1:200, Santa Cruz, Dallas, USA) and Renin (1:200, Santa Cruz, Dallas, USA) for 24 h at 4 °C in dark, and then incubated with secondary antibodies at room temperature for 1 h. Images were captured using a Leica TCS SP5MP confocal fluorescence microscope.

To quantify the phenotypic changes of microglia, proportional area analyses of Iba-1 labeling was conducted as previously described in detail [23]. Six bilateral representative images were taken from the LHA at 20X magnification. Apositive staining threshold was determined for each image and the threshold targets was processed by densitometric scanning with NIH ImageJ software. Proportional area was displayd as the average positive labelling area for all representative pictures.

2.11. Statistical analysis

All analyses were performed using SPSS version 23.0 (IBM, USA). Data are represented as the means ± SEM. All measures were analyzed using one-way analysis of variance (ANOVA) followed with LSD post hoc multiple comparisons. The analysis results were only reported when a significant difference was observed.

Statistical significance was defined as $P < 0.05$.

3. Results

3.1. Pari alleviated LPS induced depression-like behaviors

As illustrated in the experimental schem (Fig.1 A), we first determined whether Pari treatment could alleviate LPS induced depression-like behavior in mice. LPS administration resulted in a significant decrease in 24 hours body weight, in compared to the control group (see Fig. 1B). However, mice with 2 weeks of prior Pari administration (LPS+Pari) significantly reduced body weight decrease. In depressive-like behavior tests, mice in the LPS group exerted decreased sucrose preference, increased immobility in FST and TST when compared to the control group. These behavioral changes were all reversed in Pari pre-treated group (Fig. 1C-E).

3.2. Pari suppressed LPS induced production of pro-inflammatory cytokine IL1-β and activation of microglia in the hypothalamus

As predicted, LPS administration elevated IL-1β contentin serum and hypothalamus, which indicated that LPS induced systemic and central inflammtion (Fig.2 A and B). Pari pre-treatment blocked the cytokine increase in serum and hypothalamus.We therefore investigated mRNA levels of IL-1β with RT-PCR in hypothalamus (Fig. 2 C). We found that the transcription level of IL-1β were up-regulated in hypothalamus. However, Pari pre-treatment significantly supressed

IL-1β expression in transcription level. These suggested that Pari pre-treatment could directly lower down the local cytokine production in the brain and circulation.

Microglia are dynamic cells continuously survey the extracellular environments. They response to the envioronment with morphology changes. Activated microglia present an 'amoeboid' appearance with repressed processes and enlarged soma, which can be reflected by elevated ionized calcium-binding adapter molecule-1 (Iba-1) immunoreactivity. As shown in Fig. 2 D - F, we observed ramified microglia in the LHA of mice in the control group. However, microglia in the LHA of mice in the LPS group exerted de-ramified appearance with enlarged soma. Pari pretreatment obviously reversed the aberrant morphological changes of microglia. Moreover, as shown in Fig. 2 G, LPS challenge induced significant microgliosis in the hypothalamus of mice. Pari preteatment could significantly inhibit the microgliasis in the hypothalmus.

3.3. Pari exerted anti-inflammatory effects by regulating NF-κB and NLRP-3

We examined if Nuclear factor kappa-light-chain-enhancer of activated B cells (NF- κB) signaling is involved in Pari effects of suppressing neuroinflammation. We found increased nuclear NF-κB expression level in the hypothalamus, which was prevented by Pari pretreatment (Fig. 3 A). Moreover, Pari treatment could obviously reduce the expression of NLRP-3 and Caspase-1 (Fig. 3 B and C). These findings indicated that Pari's anti-inflammatory effects might contribute to the inhibition of NF-κB and NLRP-3 signaling.

3.4. Pari suppressed RAS activation to alleviate inflammatory responses

We further asked if LPS-induced depressive behavior is partly associated with RAS activity. Therefore we examined the expression levels of renin and Ang- II in the hypothalamus. Interestingly, we found that LPS treatment led to obvious elevation of renin and Ang- II expression in the hypothalamus, which was reversed by Pari pretreatment (see Fig. 4 A-C).

4. Discussion

The inflammatory hypothesis of depression proposed that depression is a byproduct of the immune systems resisting infection [11, 24]. LPS is a pro-inflammation mediator which can trigger the production of pro-inflammatory cytokine and activate NF-κB, the central regulator of inflammation, in the central nerve system [25]. The activated central inflammatory response results in depressive-like behavior that develops over a background of "sickness" similar to those observed in depressed patients [26, 27]. Nonetheless, depressive-like behavior was present without the confounding effects of sickness twenty-four hours after LPS challenge [11, 16]. As shown in Fig.1, in line with findings from literature, systemic LPS injection significantly evoked characteristic sickness and depressive-like behavior [28]. Our findings demonstrated that pretreatment with Pari was able to remit pro-inflammatory cytokine-induced depressive-like behaviors. Generally, to avoid hypocalcemia, Pari is pretreated three times per week for two weeks to exert therapeutic effects [29, 30].

Since there still lacks evidence to elucidate the pharmacokinetic processes of Pari in rodent, we could empirically infer that the effective concentration of Pari has been sustained during LPS insults using this dosage regimen.

We noticed that the severity of depressive symptoms of mice was closely associated with the levels of inflammation-related mediator interleukin-1β (IL1-β) in circulation and hypothalamus. The stress-reactive brain regions, especially the HPA axis, are vulnerable to inflammatory insult [31]. The hypothalamus is a critical componant of the hypothalamic-pituitary-adrenal (HPA) axis, the major endocrinological stress axis responding to stress stimulation and implicated in the pathogenesis of depression [32, 33]. It rapidly responds to the release of proinflammatory cytokines and the neuroendocrine responses peaks off in hours [34, 35]. Consistently, our previous findings demonstrated that LPS challenge resulted in depressive-like behavior in mice without affecting stress hormone secretion in the hypothalamus after 24 hours, which indicated that neuroinflammation mainly contributed to the behavioral changes under such circumstances [36]. Numerous genetically distinct neuron populations in the lateral hypothalamic area (LHA) involve in orchestrating feeding, rewardand other motivated behaviors [37-39]. Thus, activated neuroinflammatory response in this brain area is critical in the induction of depressive-like behavior. Nonetheless, the possibility that Pari could ameliorate the neurovegetative symptoms, such as reduced locomotor activity, decreased food intake and loss of bodyweight, during the “sickness” phase via normalizing the hyperactivity of HPA axis, which requires further determination in our future research, could not be

ruled out.

Microglia are targets and sources of pro-inflammatory cytokines, which are either local synthesized in the brain or derived from peripheral circulation. We observed that microglia in the LHA exerted deramified appearance with enlarged soma in the LPS group. Resting microglia are highly ramified cells which are acutely sensitive to brain homeostasis. They continuously survey the local environment with their fine and motile processes and respond to stimulus to maintain homeostasis [40, 41]. Activated microglia present an ‘amoeboid’ appearance with repressed processes and enlarged soma [42]. The morphological changes of microglia are correlate with pathological transformation. Pari pretreatment significantly restored microglia to ramified with normal soma size. Besides monitoring the local environment, microglia continuously contact neurons, axons, and dendritic spines with their fine and motile processes. They reciprocally communicate with neurons and influence neuronal functions by releasing neuromodulators including cytokines [43, 44]. Over-activated and dysregulated microglia together with increased IL-1β expression amplifies neuroinflammation and leads to pathological and neurobehavioral changes [45, 46]. We observed a significant microgliosis and increased secretion of IL1-β in the hypothalamus of mice receiving LPS challenge. As expected, Pari pretreatment significantly supressed microglia proliferation and IL1-β secretion in the hypothalamus. The results suggested that surpressing the activation of microglia and the production of pro-inflammatory cytokine could contribute to the remission of depressive symptoms.

NF-κB regulates the transcription of genes such as chemokines, cytokines, proinflammatory enzymes, adhesion molecules and proinflammatory transcription factors [47]. The translocation of activated NF-κB from cytoplasm to nucleus is essential for both acute and chronic inflammatory responses [48]. Regulation of NF-κB plays a key role in neuroinflammation-associated diseases control [49]. Moreover, activated NF-κB induces the transcription of cytosolic innate immune signalling receptor NOD-, LRR- and pyrin domain-containing 3 (NLRP3), a critical mediator of IL-1β-related CNS inflammation [50-52]. It connects cytokines, psychological stress and depression together [12, 53, 54]. Once activated, NLRP3 nucleates the assembly of an inflammasome, which results in caspase 1-mediated proteolysis of the IL-1β family [50]. The NLRP3 inflammasome coupled with NF-κB activation increased IL-1-β transcription and amplified inflammatory responses [55]. As we mentioned above, Pari exerted anti-inflammative effects in renal and cardiovascular disease via modulating NF-κB signaling [21, 22]. Our findings indicated that Pari might suppress local synthesis of IL1-β in the hypothalamus at transcription level, potentially through inhibiting LPS-induced NF-κB activation and NLRP3 and caspase-1 overexpression. Our results offer the first evidence that Pari treatment prevented LPS-induced depressive behavior via directly regulating NF-κB activity and inflammasome assembly in the hypothalamus.

Intriguingly, we noticed that Pari could significantly reduce LPS-induced expression of renin and angiotensin II (Ang-II) in the hyopothalamus. Renin and Ang-II are essential components of the renin-angiotensin system (RAS) that plays an

important role in both inflammatory process and blood pressure regulation [56]. RAS activation disrupts BBB permeability, which exacerbates the infiltration of peripheral pro-inflammatory cytokines into the CNS, and increases pro-inflammatory cytokine production in the hypothalamus via NF-κB signaling [57, 58]. Peripheral inflammation and RAS activity seem to share a common mechanism to modulate central inflammatory process [59]. Therefore, it is plausible that blocking renin-angiotensin activity could not only contribute to enhance the anti-inflammatory effect of Pari, but also prevent the leakage of circulating cytokines to the CNS.

In conclusion, Pari significantly alleviated the depressive-like behavior induced by systemic LPS injection. Pari could specifically regulate microglia-mediated neuroinflammation process and RAS activity to exert its anti-depressant effects. Our findings suggested that Pari has a good potential in treating systemic inflammation induced depressive symptoms, particularly in patients with comorbidity of chronic hypertension.

Abbreviations

BBB: Blood-brain barrier; FST: Forced swimming test; LPS: Lipopolysaccharide; NF-κB: Nuclear factor kappa light chain enhancer of activated B cells; Pari: Paricalcitol; RAS: Renin-angiotensin system; SPT: Sucrose preference test; TST: Tail suspension test; VD: Vitamin D.

Competing interests

The authors declare that they have no competing interests.

Authors' contributions

Z. Shi, T. Yuan, Y. Wang and Y. Zhang designed the study and wrote the draft together; M. He, N. Sha, N. Chen, S. Peng, D. Liao, X. Dong and M. Wong performed the study and analyzed the results, and all authors approved the final version.

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Figure legend

Figure 1. (**a**) Experimental manipulation of drug treatment and behavioral tests. (**b**) LPS administration induced acutely decrease in 24 hours body weight , in compared to the control group. The pre-treatment of Pari for 2 weeks prevented LPS-induced body weight decrease. (**c, d, e**) In behavioral tests, LPS challenge significantly decreased sucrose preference, increased immobility in FST and TST in the LPS group when compared to the control group. These behaviors were all reversed by Pari pre-treatement. * suggests for $P<0.05$, ** for $P<0.01$ and *** for $P<0.001$ in compared to LPS group.

Figure 2. (**a**) In serum, LPS induced increased levels of IL-1βin LPS group. (**b**) LPS administration induced neuroinflammation in the hypothalamus, with increased levels of IL-1β. Pari pre-treatment blocked IL-1β increase in circulation and hypothalamus. (**c**) LPS induced transcription increase of IL-1β in the hypothalamus. Pari pre-treatment inhibited IL-1β release in transcriptional level. β-actin is employed as the internal control. **(d)** Representative images of Iba-1 and NeuN labeling in LHA of mice are shown (scale bar = 100μm). **(e)** showed enlarged confocal image of Iba-1 labeling microglia (scale bar = 5μm). **(f)** showed the average Iba-1 proportional area in LHA. **(g)** LPS increased Iba-1 proliferation in hypothalamus, which were

suppressed by Pari pretreatment. * suggests for $P<0.05$, ** for $P<0.01$ and *** for $P<0.001$ in compared to LPS group.

Figure 3. (**a**) LPS increased nuclear NF-kappa B p65 in hypothalamus, which were prevented by Pari pretreatment. **(b)** Pari treatment decreased LPS-induced NLRP-3 and Caspase-1 expression. **(c)** Representative images of Iba-1 and NLRP-3 labeling in LHA of mice were shown (scale bar = 50 μm). * suggests for $P<0.05$, ** for $P<0.01$ and *** for $P<0.001$ in compared to LPS group.

Figure 4. (**a**) Representative immunohistochemistry images for renin in LHA (scale bar = 20 μm). **(b)** showed the mean density of renin positive areas in the LHA. **(c)** relative renin and Ang-II expression levels in hypothalamus . * suggests for $P<0.05$, ** for $P<0.01$ and *** for $P<0.001$ in compared to LPS group.

*Graphical Abstract (for review)

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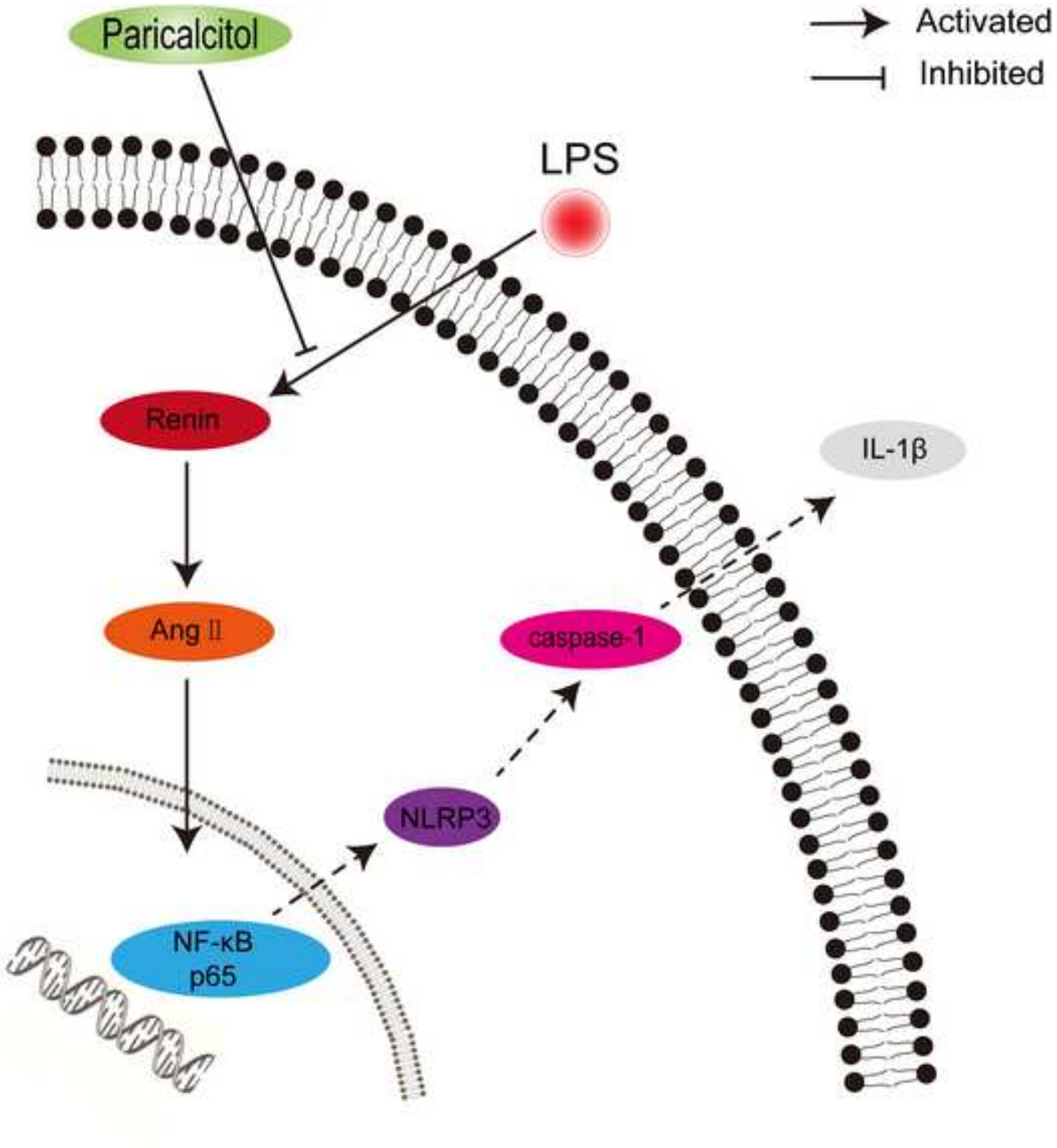

revised Figure1

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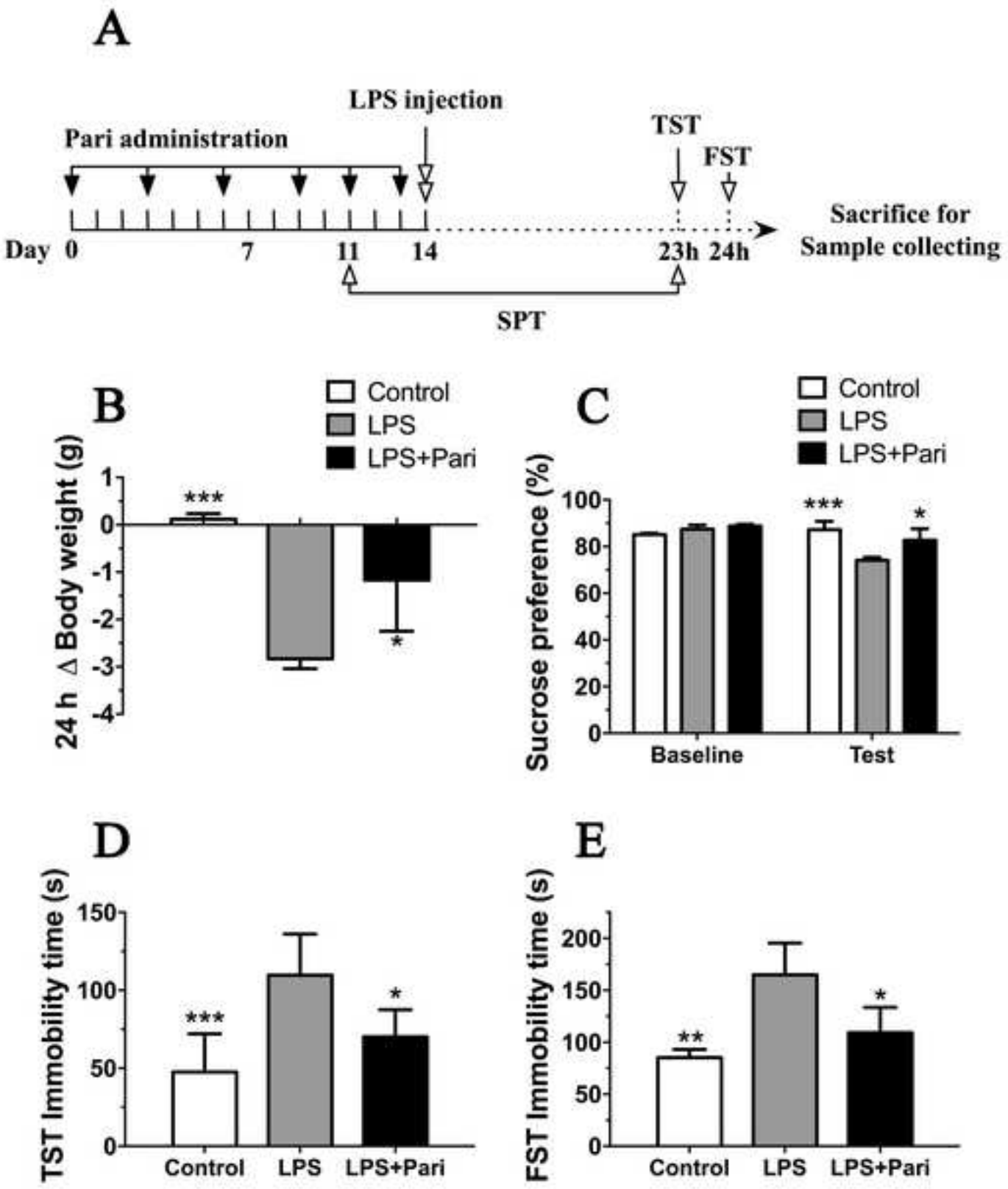

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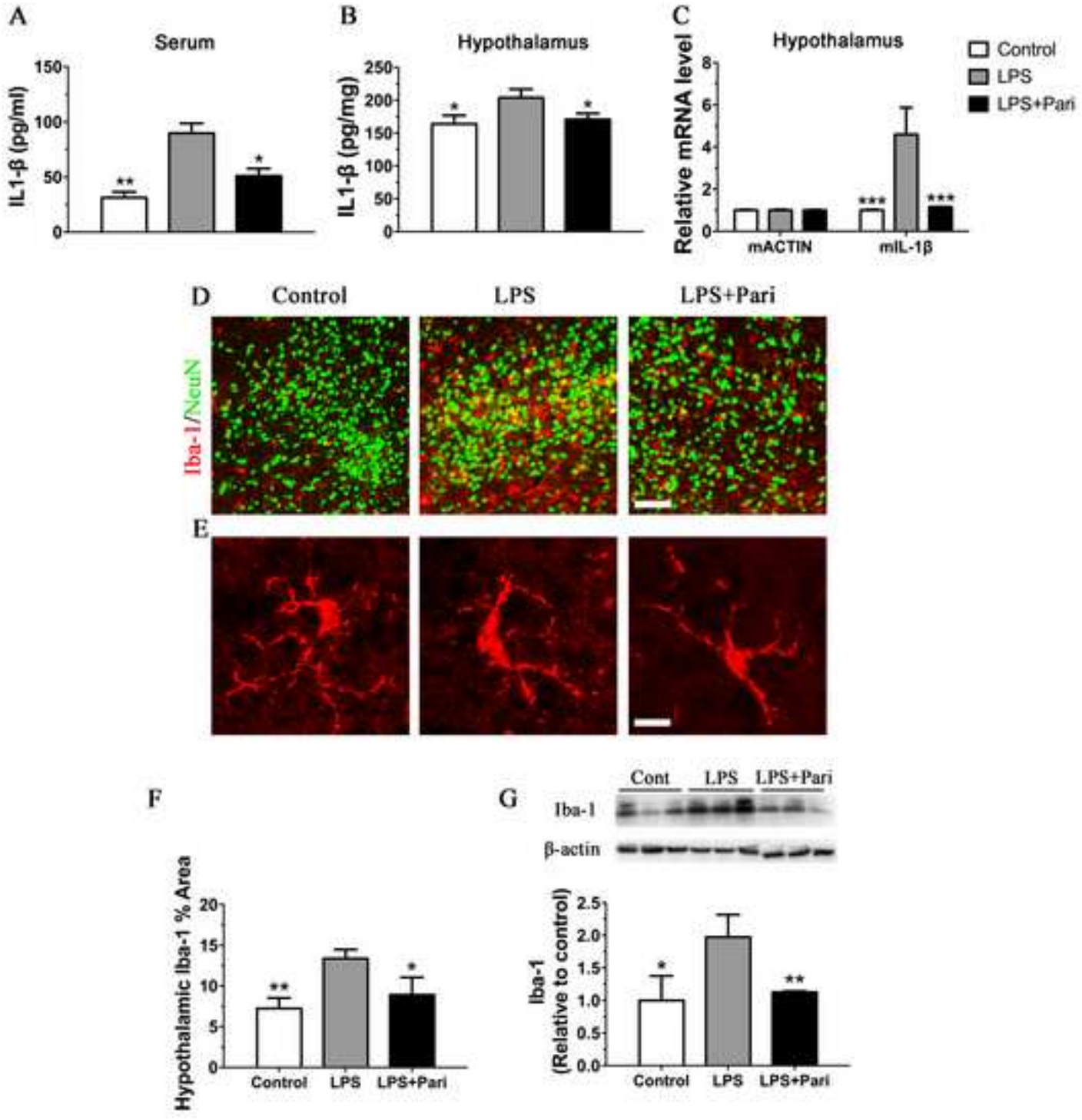

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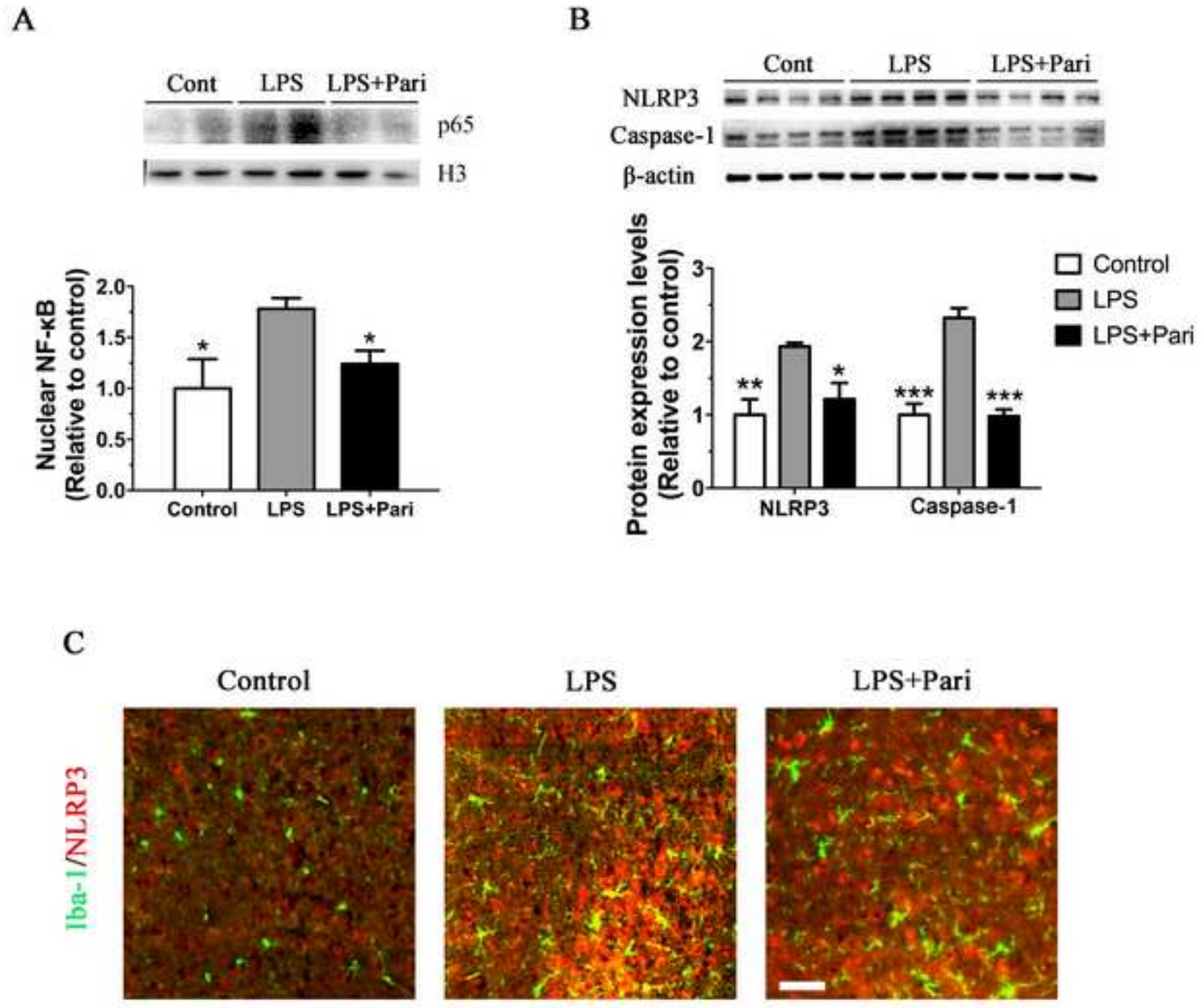

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