Reduced acetylcholinesterase activity downregulates peripheral and central inflammation during glucocorticoid resistance induced by chronic restraint stress and systemic lipopolysaccharide challenge in male mice

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Stress sensitizes the neuroinflammatory response to immunogenic challenge and associated behavioral changes in rodents. Glucocorticoids (GCs) have been well known for their immunosuppressive and anti-inflammatory properties. However, recent advances have uncovered situations wherein they have opposite effects, especially when activated immune cells show resistance to circulating GCs. Under these circumstances, studying the role of the recently described ‘cholinergic anti-inflammatory pathway’ was of considerable interest. In this study, we investigated the level of serum C-reactive protein (CRP), cytokines and reactive oxygen and nitrogen species and antioxidant enzyme activities in the liver, brain and adrenal gland following LPS administration in stressed mice. Hypothalamic acetyl cholinesterase (AChE) enzyme activity and the expression of heat shock proteins 70 and 90, superoxide dismutase-1 and cyclooxygenase-2 proteins in the hypothalamus were estimated by immunoblotting. Behavioural changes were observed on an elevated plus maze and in an open field. Our results suggest that there exists a synergistic effect between inflammation and stress only when the stress exposure is acute in nature. Immune activation following chronic stress, downregulated inflammation, in spite of the resistant endocrine response to inflammation, via the newly described cholinergic anti-inflammatory pathway. Thus, it indicates that acute immune activation during chronic stress may be beneficial for the host to maintain homeostasis.

Keywords: Antioxidant enzyme activities, Behaviour, C-reactive protein (CRP), Cholinergic anti-inflammatory pathway, Cytokines, Homeostasis, Hypothalamic AChE activity, Hypothalamic-pituitary-adrenal (HPA) axis, Reactive oxygen and nitrogen species

The impact of systemic inflammation on the pathogenesis of chronic brain diseases, such as neurodegenerative diseases, is an emerging area of biomedical research. The mediators of systemic inflammation have effects on both immune and nervous systems, and are reported to be key players of inflammation which may contribute to changes in the brain during physiological and pathological processes. The hypothalamic-pituitary-adrenal (HPA) axis has long been known to play an important role in regulation of stress induced changes in cellular and biochemical factors of stress response and associated changes in behaviour of humans and animals. Anomalies in the function of the HPA axis have been described in patients with psychiatric illness, such as depression, bipolar disorder, schizophrenia, cognitive disorders including Alzheimer’s disease and others.

Systemically administered lipopolysaccharide (LPS) activates microglia, known to be the principal inflammatory cells in the brain. In addition, inflammatory cells of the blood stream, activated by systemically administered LPS, may enter the brain by increasing the permeability of the blood-brain-barrier (BBB) to infiltrating leukocytes and these pro-inflammatory leukocytes participates in neuroinflammation and BBB disruption. Several studies have reported that acute or chronic stress sensitizes the neuroinflammatory response to both peripheral and central immunologic challenges. In particular, the effect of chronic stress or chronic glucocorticoid (GC) administration on the immune response in the brain is surprisingly different from the classic picture of suppression in the periphery. Moreover, depending upon the brain region, chronic exposure to GCs is often not anti-inflammatory, and in greatest contrast to dogma, can actually exacerbate various aspects of inflammation. This is in stark contrast to the effects of high concentrations of GCs in the periphery.
After the discovery of the anti-inflammatory role of the vagus nerve in an animal model of endotoxemia, the circuit for neural inhibition of inflammation has been reported. The autonomic regulation of local and systemic inflammation through the 'cholinergic anti-inflammatory pathway'—a mechanism consisting of the vagus nerve and its major neurotransmitter, acetylcholine (ACh) as well as its regulation by acetylcholinesterase (AChE) as a means to attenuate inflammation has emerged to be of considerable importance. AChE is an essential hydrolytic enzyme in the cholinergic nervous system, and is responsible for catalyzing the degradation of ACh into acetate and choline. It has been reported that inhibition of AChE activity by a drug rivastigmine, upregulates cholinergic activity thereby suppressing neuroinflammation (reduced demyelination, microglia activation and axonal damage). Increased cholinergic activity was also able to decrease the production of pro-inflammatory cytokines (TNF-α, IFN-γ and IL-6) without affecting IL-10 production. Recently, it has been reported that ACh producing natural killer (NK) cells was able to attenuate inflammation in the CNS via modulating the infiltration of monocytes/macrophages.

Hence, we hypothesized that since restraint stress mediated activation of the HPA axis potentiates neuroinflammation and neurodegeneration, a subsequent acute immunogenic systemic challenge following such exposure would either exacerbate the neuroinflammatory process going on or would lead to the triggering of neurohormonal anti-inflammatory pathway in order to maintain homeostasis. This study, thus would indirectly help us to understand the existence of possible link between the hormonal and neural control of inflammation at the periphery and at the CNS. This link may also throw light on the changes in cognitive behaviour that has been impaired due to stress. Moreover, whether activation of the cholinergic anti-inflammatory pathway when the GC concentration is high, particularly during GC resistance or insensitivity as observed during chronic stress, would be of any benefit for the host to maintain homeostasis needs to be elucidated.

Central and peripheral cytokine compartments are integrated, but differentially regulated. Stress or peripheral events such as systemic LPS administration causing inflammation, can activate a cascade of cytokines and related molecules such as nitric oxide (NO), affecting outcomes such as behaviour. Stress induces changes in expressive behavior and anxiety like state, which are associated with oxidative damage, hormone and neurotransmitter and cytokine level. Moreover, it has been reported that restraint stress increases iNOS, COX-2 expression and produces an accumulation of lipid peroxidation products. Activities of 3β hydroxy steroid dehydrogenase (3βHSD) and 17β hydroxy steroid dehydrogenase (17βHSD) enzymes play an important role in the regulation of intracellular levels of biologically active steroid hormones in the adrenal glands. Hence, the role of these enzymes in production of the endogenous corticosterone following acute and chronic restraint stress and their alteration on acute exposure to LPS following stress may also add some important information related to the ongoing inflammatory processes in the brain and at the periphery.

Typically, LPS stimulated leukocytes produce pro-inflammatory cytokines, which trigger reactive oxygen species (ROS) production in the tissues through NADPH oxidase activation. Thus, the level of antioxidant enzymes or their activity displays the intracellular complex mechanisms of host defense. Nitric oxide (NO) plays a complex role in free radical mediated injury in the brain during exposure to stressful condition and/or during immunogenic stimulation. Production of NO correlates with the level of expression of the iNOS gene which has been directly correlated with production of COX-2 during stress and inflammation which may be partly regulated by the NO pathway. Heat shock proteins (HSPs) are also synthesized in significant quantity to protect eukaryotic cells from various insults during periods of stress caused by infection, inflammation or similar events. SOD-1 expression in the brain correlates with the production of the enzyme superoxide dismutase that helps to convert harmful superoxide anion to hydrogen peroxide and its subsequent degradation to water and oxygen by the enzyme catalase.

As reduced locomotion is a common sickness behaviour and an adaptive response to illness and infection due to replicating pathogen or their products, locomotor activity and exploration in the open field test provides an excellent index for the assessment of both the effects of and responses to LPS treatment in animals exposed to short and long-term stressors.
neuroinflammatory markers evoked by LPS exposure in mice and study the mechanisms that underlie the stress-illness interaction. Further, this study may also explain the anti-inflammatory role played by ACh released in circulation due to peripheral activation of afferent vagal nerve fibers by circulating proinflammatory cytokines produced during stress and inflammation.

Materials and Methods

Animals
All experiments involving animals were conducted according to the protocols that had been approved by the Institutional Animal Ethics Committee (IAEC), Department of Physiology, University of Calcutta, under the guidance of Committee for the purpose of control and supervision of experiments on animal (CPCSEA) [Approval Number: 820/04/ac/CPCSEA-2010 dated: 16.11.2011], Ministry of Environment and Forest, Govt. of India. This study did not involve any invasive study using human subjects. Male BALB/c (6-8 weeks) mice were obtained from a registered breeder in our department and were used for all studies. All animals were maintained and utilized in accordance with recommendations from the IAEC and were provided with food and water ad libitum. Mice were housed 4-5 per cage and maintained on a 12 h light:dark cycle (lights on at 08.00 am) in a temperature controlled room (22±2°C).

Restraint stress
In order to compare stress responses involving the production of glucocorticoids hormone, which is affected by various hormones, such as growth hormone or sex hormones, 5-7 months old male mice with a stable growth phase without sex cycle, were selected. Due to inherent correlation of the circadian rhythm with corticosterone production, mice were exposed to restraint stress each day during a fixed time period, from 09.00-15.00 h (6 h each day). Restraint was applied in a separate room to eliminate the possible effects of vocalizations or pheromones on the control (non-stressed) mice. Non restrained mice were left in their home cages in a noise-free environment, with food and water during the restraint period. Mice (n=6) per group were restrained each day according to procedures described in previous reports. Briefly, following the acclimation period, individual animals were randomly assigned to six groups. Restraint stress was performed in well ventilated 50 mL polystyrene tubes and food and water were not provided during the restraint period. Restraint animals were also allowed to move freely in their cages until the next restraint cycle. An restraint period once was considered as acute stress where as the similar period of stress for 3 weeks (21 days) on each day was considered as chronic stress. Animals consisted of six groups (n=6 for each group) which were as follows: Group I: control (non-stressed); Group II: acute stress; Group III: chronic stress; Group IV: non-stressed LPS treated; Group V: acute stress + LPS challenged; and Group VI: chronic stress + LPS challenged. LPS (derived from Escherichia coli 055:B5; Sigma Chemical) at a dose of 250 µg/kg body wt. of mice, was administered intravenously, a single dose, 4 h after releasing the animals from the stressed condition for acute stress group. Chronic stressed animals were also challenged with a single, intravenous dose of LPS 4h after completion of the final stress period on the 21st day. 2h after LPS challenge animals were observed for their behavioural changes in an open-field and over an elevated plus-maze and 24 h after LPS treatment animals were sacrificed under ether anesthesia. Group I, II and III were administered equal volume of sterile 0.9% saline on that day. Non-stressed LPS treated group were administered a single dose (250 µg/kg body weight) of LPS (i.v.) via the tail vein and were also sacrificed 24 h post-LPS administration. Animals from all the groups were sacrificed on the same day to avoid inter-day variation in the tested parameters. Behavioural activities in an open field and on an elevated plus maze were recorded consecutively, 2 h after LPS challenge for all the LPS treated group of animals.

Determination of level of corticosterone in serum
Blood samples were collected in anticoagulant free tubes between 10:00 a.m. and 12:00 noon and centrifuged at 1000 g for 10 min. Serum obtained was distributed in separate tubes. Serum for cytokine measurement were stored at −70°C until use and that for determining the concentration of corticosterone using a corticosterone EIA kit from Cayman Chemical as per the manufacturer’s instructions, was done on the same day of blood collection. For each study, corticosterone levels were determined, in duplicate; in a single run to avoid inter-assay variability, and intra-assay variability was less than 10%. The minimum detectable limit for corticosterone was 8.2 pg/mL.
Estimation of hydroxy steroid dehydrogenase (HSD) enzyme activity

Adrenal glands were homogenized separately in 20% spectroscopic glycerol containing 5 mM potassium phosphate and 1 mM ethylene diamine tetra acetic acid (EDTA) at a tissue concentration of 100 mg/mL of homogenizing mixture. It was centrifuged at 4°C at 10000xg for 30 min. 3βHSD and 17βHSD activities were estimated by methods as described in earlier studies 17.

C-reactive protein (CRP) assay

Serum C-reactive protein (CRP) levels were determined using a mouse high sensitive CRP ELISA kit (GenWay Biotech, Inc, San Diego, CA) according to the manufacturer’s protocol. Absorbance were interpolated from a standard curve produced from CRP standards (0-25 ng/mL) supplied within the kit. The minimum detectable limit for CRP was 0.78 ng/mL.

Cytokine ELISA

Serum TNF-α, IFN-γ, IL-6 and IL-10 concentrations was estimated by Sandwich ELISA and calculated based on the standard curve. Serum cytokine levels were expressed in pg/mL of serum analyzed, following manufacturer’s protocol. (Ray Biotech, Inc. USA) For each study, cytokine levels were determined, in duplicate, in a single run to avoid inter-assay variability, and intra-assay variability was lower than 10-12%. The minimum detectable dose of the cytokines for IL-6, TNF-α, IL-10 and IFN-γ were 2, 60, 45and 5 pg/mL, respectively.

Acetyl cholinesterase (AChE) assay in brain tissue

On the day of sacrifice, stressed (acute and chronic) and unstressed control mice were given mild anesthesia with anaesthetic ether. The whole intact brain was then removed carefully and placed in the petri dish, over ice for 15 min. The brain was washed with ice-chilled normal saline repeatedly to clean. A 10% (w/v) homogenate of brain samples was prepared first by homogenizing in a glass homogenizer at a speed of 9500 rpm using a sodium phosphate buffer (30 mM, pH 7.0). The assay of AChE in the above mentioned supernatant was performed by the modified Ellman’s method 27, using acetyltiocholine iodide as substrate at a final concentration of 1 mmol/L. A kinetic profile of the enzyme activity was studied spectrophotometrically at 412 nm at an interval of 15 s. The assay for each sample was run in duplicate and each experiment was performed thrice. Protein was estimated in the range 0.01-0.1 mg/mL in the brain samples by the Folin–Lowry method, using bovine serum albumin (BSA) as standard at a concentration of 1 mg/mL.

Assay of antioxidant enzymes from liver, brain and adrenal gland

The liver, brain and adrenal tissues were separately homogenized in 10 volumes of 50 mM phosphate buffer (pH 7.4) on ice for 30 s using a power driven polytron homogenizer. The homogenate was transferred into centrifuge tubes and centrifuged at 9000xg at 4°C for 20 min. The supernatant was utilized to measure activity of antioxidant enzymes and the amount of protein present.

Measurement of reduced glutathione level (GSH)

Reduced glutathione content (as acid soluble sulfhydryl) was estimated by its reaction with DTNB (Ellman’s reagent) following the method of Sedlak & Lindsay 28. Values were expressed as nanomoles of GSH per mg protein.

Catalase (CAT) activity

Decomposition of H2O2 due to CAT activity was assayed by the decrease in absorbance of H2O2 at 240 nm. Catalase activity in cell free homogenate was determined spectrophotometrically by measuring the decrease in H2O2 concentration at 240 nm. At time zero, 1.8 mL of each homogenate was combined with 0.2 mL of a phosphate buffer containing 10 mmol H2O2. One mL of the mixture was immediately added to a cuvette and placed into a spectrophotometer. CAT activity was observed via degradation of H2O2 as determined by a reduction in UV light absorbance over time. Measurement of absorbance was taken at 15 s interval after addition of the homogenate to hydrogen peroxide buffer. Units of CAT activity present in 1 mL of homogenate were calculated 29.

Superoxide dismutase (SOD) activity

Tissue homogenate (100 µL) was mixed with 1.5 mL of a Tris-EDTA-HCl buffer (pH 8.5), then 100 µL of 7.2 mM pyrogallol was added and the reaction mixture was incubated at 25°C for 10 min. The reaction was terminated by the addition of 50 µL of 1M HCl and measured at 420 nm. One unit was determined as the amount of enzyme that inhibited the oxidation of pyrogallol by 50%. The activity was expressed as U/mg protein 29.
Estimation of nitric oxide (NO) production

The concentration of nitrite in the brain tissues was measured as an index for NO production. Equal weights of the brains of control, stressed and LPS treated mice were homogenized in sterile PBS (1 mL). Supernatants were collected, and analyzed for NO production by modified Greiss method as described earlier. Briefly, nitrate was converted to nitrites with β-nicotinamide adenine dinucleotide phosphate (NADPH; 1.25 mg/mL) and nitrate reductase followed by addition of the Griess reagent. The reaction mixture was incubated at room temperature (25°C) for 20 min followed by the addition of TCA. Samples were centrifuged, clear supernatants were collected, and optical density was recorded at 550 nm. The amounts of NO produced were determined by calibrating a standard curve using sodium nitrite.

Determination of blood brain barrier (BBB) leakage

The permeability of the BBB was quantitatively evaluated by detection of extravasated Evans blue dye. Briefly, 2% Evans blue dye in saline was injected intraperitoneally to 3 mice from each group, on the day of sacrifice (24 h post LPS administration) and after 4 h mice were deeply anesthetized with Nembutal and transcardially perfused until colorless perfusion fluid was obtained from the right atrium. After decapitation, brain tissue was removed, weighed, and homogenized. The supernatant was obtained by centrifugation, and protein concentration was determined. Evans blue intensity was determined by a microplate reader at 550 nm. Calculations were based on external standards dissolved in the same solvent. The amount of extravasated Evans blue dye was quantified as micrograms per milligram protein.

Brain IL-6 and TNF-α

Frozen brain samples (100 mg tissue/mL buffer) were homogenized in cold lysis buffer (20 mM Tris, 0.25 M sucrose, 2 mM EDTA, 10 mM EGTA, 1% Triton X-100) in the presence of 1 unit of protease inhibitor cocktail/10 mL of lysis buffer. Homogenates were centrifuged at 100000×g for 40 min. Supernatant was collected, and protein levels determined using the Pierce™ BCA Protein Assay Kit (Thermo Scientific, USA). The levels of IL-6 and TNF-α in the brain were measured with commercial enzyme-linked immunosorbent assay (ELISA) kits from Ray Biotech, Inc. For each study, cytokine levels were determined, in duplicate, in a solo run to avoid inter-assay variability, and intra-assay variability was less than 10-12%. The minimum detectable doses of cytokines for IL-6 and TNF-α were 2 and 60 pg/mL, respectively.

Expression of HSP 90, HSP 70, SOD1 and COX-2 in hypothalamic tissue

Expression of cyclooxygenase-2 (COX-2), HSP 90, HSP 70, SOD1 in hypothalamic tissues was determined by immunoblotting. Estimation of these protein levels in the brain tissue was determined by immunoblotting after estimating the protein level in the tissue homogenates by the Bradford method. 20 μg of each sample was electrophoresed on polyacrylamide gel and transferred onto a nitrocellulose membrane. After blocking with 7% skimmed milk, the blots were incubated overnight at 4°C with primary antibodies against COX-2 (1:1000; Chemicon, USA), HSP 90, HSP 70, SOD1 (1:1000; Santa Cruz Biotechnology, USA). After extensive washes with PBS-Tween, blots were incubated with appropriate secondary antibodies conjugated with peroxidase (Vector, Laboratories, USA). The blots were again washed in PBS-Tween and processed for development using chemiluminiscence reagent (Millipore. USA). The images were captured and analyzed using Chemigenius, biomaging system (Syngene, Cambridge, UK). The blots were then stripped (30 min at 50°C in 62.5 mM Tris-HCl, pH 6.8, 2% sodium dodecyl sulphate, and 100 mM β-mercaptoethanol) and reprobed with anti-β tubulin (Santa Cruz Biotechnology, USA) to determine equivalent loading of samples.

Activity and exploratory behavior in open-field test

The open field was constructed as described elsewhere. A central square was drawn in the middle of the receptive field and was used to measure exploration. The central square is used because some mouse strains have high locomotor activity and cross the lines of the test chamber many times during a test session. The central square has ample space surrounding it to give meaning to the central location as being distinct from the outer locations. A digital camera was fixed to the ceiling 2.1 m above the apparatus and was utilized to measure line crosses. Activity and exploratory behaviour of mice were observed in the open field for a 5-min period, and the following behavioural activities were scored: TNSCP, total number of squares crossed in periphery; DTP, distance traveled in the periphery; LRC, latency to reach centre; DI, duration of restraint; TNR, total...
number of rearing; URC, unable to reach the central region of the open field; TNG, total number of grooming; and TNF, total number of freezing.

**Anxiety test-elevated plus maze (EPM)**

One of the most popular tests of anxiety-like behaviour in mice is the EPM, in which the decreased number of entries or time spent in the open arms of the EPM suggests the operation of anxiety-like processes. The elevated plus maze was built according to the instruction described elsewhere\(^29\). A camera was attached to the ceiling 2.1 m above the apparatus to measure entries and duration in the center, open and closed zones. We measured: (A) TSC-total time spent at centre; (B) TSCA-the time spent in the closed arms; (C) TSOA-time spent in open arm; (D) NRCA- number of rearing in closed arms; and (E) NGCA- number of grooming in closed arms during the 5-min test period. An entry was meant by all four paws in the arm. The elevated plus-maze was thoroughly cleaned with 70% ethanol and air dried following the testing of each animal to avoid promising biasing effects due to odour clues left by previous mice. The total number of open arms entered, as well as the total number of closed arms entered was used as indexes of general locomotor activity.

**Statistical analysis**

All the data are represented as mean ± SD. The results of the experiment were analyzed by a 3 × 2 analysis of variance (ANOVA), and post hoc comparisons of mean were done by Tukey HSD (honest significant difference) tests using the statistical software Origin 8. Factors were identified as follows: stress regimen, which was composed of three levels (non-stressed, acute restraint stress and chronic restraint stress), and systemic treatment, consisted of two levels (intravenous vehicle or intravenous LPS). Threshold for statistical significance was set at \(\alpha=0.05\).

**Results**

**Effect of LPS administration on the serum corticosterone concentration following acute and chronic restraint stress**

Serum corticosterone levels for non-stressed (control), acute and chronic restraint stressed groups administered either LPS or saline (vehicle) are shown in Fig. 1(A). Low levels of corticosterone were detected in the serum in the non-stressed saline administered group whereas there was a significant variation in the corticosterone level in acute and chronic restraint stress groups, \(F(2, 30) = 114.52, P<0.05\). Administration of LPS was also able to increase the serum corticosterone level in the non-stressed group and the more importantly, the effect was significantly more profound in the group of animals exposed to stressor prior to LPS challenge, \(F(1, 30) = 148.45, P<0.05\). But, a two-way ANOVA revealed no significant interaction between stress condition and LPS administration on the increase in serum corticosterone level, \(F(2, 30) = 1.24, p= 0.32\).

**Effects of LPS administration on steroidogenic enzyme (3βHSD and 17βHSD) activities following acute and chronic restraint stress in mouse adrenal gland**

There was a significant effect of stress regimen on 3β hydroxy steroid dehydrogenase (3β HSD) and 17β HSD level in the adrenal gland, \(F(2, 30) = 46.77, P<0.05\), and \(F(2, 30) = 92.66, P<0.05\), respectively. LPS administration produced significant effect on these enzyme levels, \(F(1, 30) = 195.92, P<0.05\), and \(F(1, 30) = 165.47, P<0.05\), respectively. However, although we observed a significant interaction

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**Fig. 1** — Effect of LPS challenge on (A) the serum corticosterone concentration post exposure; (B) on steroidogenic enzyme (3βHSD) activity; (C) on steroidogenic enzyme (17βHSD) activity; and (D) on the brain acetylcholine esterase (AChE) activity in acute and chronic restraint stress. [Corticosterone level (pg/mL) in serum of acute and chronic stressed mice (n=6/group) treated with intravenous LPS (250 µg/Kg) or saline have been expressed as mean ± SD. B & C: enzyme activity (Units/min. mg protein) in the adrenal gland. Bars represents mean (n=6/group) ± standard deviations. * Significant difference (\(P<0.05\)) compared to saline treated group; #, significant difference (\(P<0.05\)) compared to non-stressed group and ^, significant difference (\(P<0.05\)) compared to acute stress group that had received comparable LPS treatment]
between the two factors in case of 3β HSD, $F(2, 30) = 9.15, P < 0.05$, but we failed to observe a significant interaction between stress and LPS administration in case of 17β HSD enzyme activity, $F(2, 30) = 2.88, P < 0.095$, see Fig. 1 (B) and (C).

**Effect of LPS administration on the brain AChE activity in acute and chronic restraint stressed mice**

Acetylcholinesterase activity was significantly influenced by restraint stress exposure, $F(2, 30) = 16.50, P < 0.05$. AChE also varied significantly due to treatment with LPS following stress exposure, $F(1, 30) = 100.70, P < 0.05$. A significant interaction was observed (Fig. 1D) between duration of exposure to stress and type of systemic treatment (Saline or LPS) on brain AChE activity, $F(2, 30) = 67.68, P < 0.05$.

**Effect of LPS on the serum C-reactive protein (CRP) level following acute and chronic restraint stress**

Circulating CRP concentration in serum was significantly influenced by restraint stress, $F(2, 30) = 16.37, P < 0.05$. CRP levels also varied significantly due to treatment with LPS following stress exposure, $F(1, 30) = 73.96, P < 0.05$. There was also a significant interaction between stress duration and type of systemic treatment (Saline or LPS) on the serum CRP level, $F(2, 30) = 39.08, P < 0.05$. However, as seen in Fig. 2 (A), the conjoint effect of stress exposure prior to LPS challenge was primarily evident at the 250 µg dose of LPS administered once, intravenously.

**Diminished NO production in the brain of mice**

As shown in Fig. 2 (B), there was a significant effect of stress duration on the production of nitric oxide (NO) in the brain tissue, $F(2, 30) = 62.02, P < 0.05$. A similar significant effect was also observed on administration of systemic LPS or saline following stress, $F(1, 30) = 41.61, P < 0.05$. There was a significant interaction between stress duration and type of systemic treatment on brain NO level, $F(2, 30) = 48.29, P < 0.05$.

**Blood brain barrier (BBB) leakage**

There was no significant effect of stress duration on BBB leakage, $F(2, 30) = 1.73, P = 0.22$. Similarly, administration of LPS or saline to stressed (acute and chronic) and non-stressed animals, showed no effect of treatment type on extravasations of the Evan’s blue dye through the BBB, $F(1, 30) = 0.39, P = 0.54$. But, a significant interactive effect between stress and LPS was observed, $F(2, 30) = 101.99, P < 0.05$, (Fig. 2C).

**Effect of LPS administration on serum IL-6, IL-10, TNF-α and IFN-γ level following acute and chronic restraint stress**

Serum IL-6, IL-10, TNF-α and IFN-γ levels were significantly influenced by exposure to restraint stress, $F(2, 30) = 14.48, 255.84, 176.86$ and $60.78, P < 0.05$, respectively, see Fig. 3 (A) - IL-6; (B) - IL-10; (C) - TNF-α and (D) - IFN-γ. Except IL-6, the other cytokine levels also varied significantly as a function of LPS administration, $F(1, 30) = 0.02, p = 0.89$ and $F(1, 30) = 191.31, 712.98$ and $294.50, P < 0.05$, respectively. Moreover, in each case the interaction between the LPS treatment and restraint stress exposure was statistically significant, $F(2, 30) = 266.04, 6.67, 16.05$, and $30.92, P < 0.05$, respectively.

Fig. 2 — Effect of LPS administration on (A) serum C-reactive protein (CRP) level (µg/mL) in acute and chronic restraint stressed mice; (B & C) nitric oxide (NO) production and blood brain barrier leakage (BBB) in mouse brain following restraint stress. [Results are expressed as mean (n=6/group) ± SD. *, Significant difference ($P < 0.05$) compared to saline treated group; #, significant difference ($P < 0.05$) compared to non-stressed group and ^, significant difference ($P < 0.05$) compared to acute stress group that had received comparable LPS treatment]
of the animals exposed to restraint stress and were administered LPS following the stress exposure. There was a significant influence of both, the stress duration and the type of treatment, on the production of these pro-inflammatory cytokines in the brain, $F (2, 30) = 38.19$ and $F (1, 30) = 99.77$, $P < 0.05$. A significant interaction, $F (2, 30) = 24.04$, $P < 0.05$, was also reflected when the stress duration $\times$ LPS treatment was analyzed by a two-way analysis of variance. Significant difference in mean between the saline and LPS treated, the non-stressed and the stressed group were confirmed by Tukey’s HSD post hoc comparison of means.

Effect of LPS administration on the catalase, GSH and SOD activity of liver, brain and adrenal tissues in acute and chronic restraint stressed mice

There was a significant influence of stress regimen on the catalase activity in liver (Fig. 5A),

Estimation of cytokines in the brain

A similar response in the production of IL-6, Fig. 4 (A) and TNF-α (B) was observed in the brain
brain (Fig. 5D) and adrenal gland (Fig. 5G), F (2, 30) = 4.059, 371.05 and 17.83, P < 0.05, respectively. Excepting liver, F (1, 30) = 0.127; p = 0.72, administration of saline or LPS also had significant effect on brain and adrenal gland catalase activity, F (1, 30) = 36.99 and 5.44, P < 0.05, respectively. Most importantly, there was a significant interaction of stress x LPS on the catalase activity in all these tissues, F (2, 30) = 88.19, 33.49 and 65.32, P < 0.05, respectively.

There was a significant influence of stress regimen on the GSH content in liver (Fig. 5B), brain (Fig. 5E) and adrenal gland (Fig. 5H), F (2, 30) = 158.08, 137.05 and 327.64, P < 0.05, respectively. There was also a significant influence of LPS administration on the GSH content in liver, brain and adrenal gland, F (1, 30) = 37.11, 226.87 and 712.64, P < 0.05, respectively. There was a significant interaction of stress x LPS on the GSH content in all these tissues, F (2, 30) = 88.19, 33.49 and 65.32, P < 0.05, respectively.

Significant variation in SOD activity due to stress was observed in liver (Fig. 5C), brain (Fig. 5F) and adrenal gland (Fig. 5I), F (2, 30) = 47.06, 35.25 and 75.27, P < 0.05, respectively. The enzyme activity varied significantly due to LPS or saline administration in liver and brain, F (1, 30) = 54.23 and 25.19, respectively, P < 0.05, but not in the adrenal gland, F (2, 30) = 2.91, p=0.113. There was no significant interaction of stress and LPS on the SOD activity in the liver and brain, F (2, 30) = 3.20; p = 0.076 and 0.598; p = 0.56, respectively, though significant interaction was observed in the adrenal gland, F (2, 30) = 10.84, P < 0.05.

Expression of heat shock proteins (HSPs), SOD1 and COX-2 in hypothalamic tissue

Densitometrical analysis of immunoblots (Fig. 6A), normalized to equal protein content, showed a significant influence in the expression of HSPs 90 (Fig. 6B) and 70 (Fig. 6C) in the hypothalamus due to stress, F (2, 30) = 78.14 and 130.68, P < 0.05, respectively. There was a significant effect of LPS administration on the expression of HSP 90, F (1, 30) = 25.03, P < 0.05; but not on HSP 70, F (1, 30) = 0.16, p = 0.69. However, there was a significant interaction of both the factors on the expression of both of the HSPs tested in the hypothalamic tissue, F (2, 30) = 176.39 and 152.89, P < 0.05, respectively.
There was a significant effect of stress regimen on the expression of COX-2 (Fig. 6D), $F(2, 30) = 10.02$, $P < 0.05$; but there was no significant effect of systemic LPS challenge on the expression of this protein level in the hypothalamus, $F(1, 30) = 0.09$, $p = 0.77$. Interestingly, there was a significant interaction between the factors (stress × LPS) on the expression of this protein, $F(2, 30) = 100.61$, $P < 0.05$.

Interestingly, there was a significant effect of stress and LPS administration on the expression of SOD-1 protein level (Fig. 6E) in the hypothalamus of mice brain, $F(2, 30) = 33.52$ and $F(1, 30) = 218.65$, $P < 0.05$. A significant interaction was also found to exist between these factors on the expression of this protein level, $F(2, 30) = 24.23$, $P < 0.05$.

Locomotor activity and exploratory behaviour

In the open field test it was observed that the total number of squares crossed by the mice in the periphery, along with distance traveled in periphery was increased, whereas the total time of restraint in periphery, total number of rearing and total number of freezing was decreased, in the mice chronically stressed and subsequently challenged with LPS when compared with the stressed plus saline treated group, indicating improvement of exploratory behavior when stressed mice were treated with LPS (Table 1). The effect of LPS administration on chronically stressed mice showed a prominent increase in these locomotor and behavioral activities compared to the acute stressed group that had received comparable LPS treatment. Moreover, the chronic stress plus LPS treated animals were also able to reach the central region of the open field, similar to the non-stressed saline treated group of animals, compared to the non-stressed or acute stress plus LPS challenged group ($P < 0.05$).

Anxiety test-elevated plus maze (EPM)

When tested in EPM, it was observed that the time spent in central area increased significantly only in case of acute stressed mice challenged with LPS when compared to the acute stress plus saline treated group or to the non-stressed LPS treated group, though no significant changes in the behaviour was observed in chronic condition. The time spent in closed arm decreased significantly, time spent in the unsafe open arms of the maze increased significantly, rearing behaviour in closed arm was decreased in the group of
Difference (NRCA) compared to non-P33 compared to acute stress in response to systemic LPS significantly increased the corticosterone level. Prior exposure to chronic restraint stress or to lethal LPS challenge augmented the inflammatory process. Moreover, restraint stress sensitizes the HPA axis to immune challenge. In addition, the activity of the enzymes \(3\beta\) and \(17\beta\) HSD, in the adrenal gland also increased significantly in both acute and chronic restraint stress group in response to immune stimulation. These results suggest that prior exposure to acute or chronic restraint stress sensitizes the HPA axis to immune activation that makes the subsequent response greater.

Acute phase protein like CRP has been considered as a representative marker of the inflammatory response and has been shown to protect mice from lethal LPS challenge. From our study, it was observed that LPS challenge or stressor exposure or both increased the serum CRP level than the control mice. Acute stress and/or LPS challenge also augmented the inflammatory process. Moreover, elevated corticosterone level following chronic stress or following exposure to both (stressor plus immune

Table 1 — Effects of LPS administration on the locomotor activity IS mice in the open field test

<table>
<thead>
<tr>
<th>Groups</th>
<th>TNSCP (Mean±SD)</th>
<th>DTP (cm) (Mean±SD)</th>
<th>LRC (s) (Mean±SD)</th>
<th>DI (s) (Mean±SD)</th>
<th>TNR (Mean±SD)</th>
<th>TNG (Mean±SD)</th>
<th>TNF (Mean±SD)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Non-stressed + saline</td>
<td>287±39.08</td>
<td>2296±552.69</td>
<td>178±61.48</td>
<td>48.66±17.01</td>
<td>32±5</td>
<td>17.66±4.72</td>
<td>13.67±2.51</td>
</tr>
<tr>
<td>Acute stress + saline</td>
<td>16±5</td>
<td>128±40</td>
<td>282±8.18</td>
<td>15.3±2.1</td>
<td>4±1</td>
<td>2±1</td>
<td></td>
</tr>
<tr>
<td>Chronic stress + saline</td>
<td>85.3±27.15</td>
<td>680.66±67.23</td>
<td>246.3±22.47</td>
<td>6±1</td>
<td>9±1</td>
<td>7.3±1.15</td>
<td></td>
</tr>
<tr>
<td>Non-stressed + LPS</td>
<td>216±14.26</td>
<td>1728±128.36</td>
<td>142±33.37</td>
<td>67.7±10.08</td>
<td>21±4</td>
<td>12±4</td>
<td>5.67±1.33*</td>
</tr>
<tr>
<td>Acute stress + LPS</td>
<td>51±7.21*</td>
<td>408±27.68*</td>
<td>226.66±12.66</td>
<td>1.66±0.57</td>
<td>9±1</td>
<td>1.3±0.57</td>
<td></td>
</tr>
<tr>
<td>Chronic stress + LPS</td>
<td>179±20.22**</td>
<td>1432±121.78**/#</td>
<td>128±12.08*</td>
<td>38.3±6.25**/#</td>
<td>4.66±2.08</td>
<td>4.33±2.08**</td>
<td>12±2.64**</td>
</tr>
</tbody>
</table>

[Activity and exploratory behavior of mice were observed in open field for a 5-min period, and the following behavioral activities were scored. TNSCP- Total number of squares crossed in periphery, DTP- distance traveled in the periphery, LRC- latency to reach centre, DI- duration of immobilization, TNR- total number of rearing, URC- unable to reach the central region of the field, TNG- total number of grooming, TNF- total number of freezing. Each value represent the mean ± SD (n=6 mice/group) of counts in 5 min open field test. *, Significant difference (P < 0.05) compared to saline treated group; #, significant difference (P < 0.05) compared to non-stressed group and ^, significant difference (P < 0.05) compared to acute stress group that had received comparable LPS treatment]

Table 2 — Elevated plus maze (EPM) test to observe the effect of LPS challenge on stressed mice

<table>
<thead>
<tr>
<th>Group</th>
<th>TSC (s) Mean±SD</th>
<th>TSCA (s) Mean±SD</th>
<th>TSOA (s) Mean±SD</th>
<th>NRCA Mean±SD</th>
<th>NGCA Mean±SD</th>
</tr>
</thead>
<tbody>
<tr>
<td>Non-stressed + Saline</td>
<td>9.33±2.06</td>
<td>142.64±13.50</td>
<td>148.03±7.09</td>
<td>16.66±2.08</td>
<td>12±1</td>
</tr>
<tr>
<td>Acute stress + Saline</td>
<td>22.07±5.15</td>
<td>156.67±16.02</td>
<td>121.26±7.51</td>
<td>4±1</td>
<td>2±1</td>
</tr>
<tr>
<td>Chronic stress + Saline</td>
<td>18.66±5.03</td>
<td>193.33±14.04</td>
<td>88.01±5.56</td>
<td>11.3±1.53</td>
<td>8.3±0.57</td>
</tr>
<tr>
<td>Non-stressed + LPS</td>
<td>18.27±4.19^*</td>
<td>184.27±12.47</td>
<td>97.46±8.23*</td>
<td>12.35±2.68</td>
<td>5.67±1.82*</td>
</tr>
<tr>
<td>Acute stress + LPS</td>
<td>33.67±3.05^*</td>
<td>160.67±9.01</td>
<td>105.66±11.37</td>
<td>1.3±0.57</td>
<td>4.3±0.57</td>
</tr>
<tr>
<td>Chronic stress + LPS</td>
<td>24.26±4.89</td>
<td>135.07±12.49^*#</td>
<td>140.67±13.60^*#</td>
<td>7.33±1.15^*#</td>
<td>6.3±0.57</td>
</tr>
</tbody>
</table>

[To test the anxiety-like behavior in mice is the EPM, in which the reduced number of entries or time spent in the open arms of the EPM suggests the operation of anxiety-like processes. Mouse from different groups (n=6 mice/group) was placed individually in the center portion of the plus-maze, facing one of the open arms. The observer measured: (A) TSC-total time spent at centre; (B) TSCA-the time spent in the closed arms; (C) TSOA-time spent in open arm; (D) NRCA- number of rearing in closed arms; and (E) NGCA-number of grooming in closed arms during the 5-min test period. An entry was defined as all four paws in the arm. *, Significant difference compared to saline treated group (P < 0.05); #, significant difference compared to non-stressed group and ^, significant difference (P < 0.05) with respect to acute stress group that had received comparable LPS treatment]
activation) did not showed significant down regulation of this acute phase protein level. Thus, it can be speculated that due to a functional corticosterone resistance during chronic stress, inflammatory process was facilitated upon immune activation post exposure to stress.

The activation of pituitary-dependent adrenal responses after endotoxin administration provided early evidence that inflammatory stimuli can activate anti-inflammatory signals from the central nervous system. A key finding in this study was that exposure to acute or chronic restraint stress and subsequent systemic immune activation elicits systemic and local inflammatory responses, which also leads to neuroinflammation. The pro-inflammatory cytokines like IL-6, TNF-α and IFN-γ levels increased in group of mice that were exposed to stress or LPS. Thus, this study provides evidence on the presence of acute neuroinflammation characterized by release of proinflammatory cytokines IL-6 and TNF-α in the systemic circulation (Fig. 3 A and C) as well as in the brain (Fig. 4A and B) following LPS administration. Interestingly, we found that LPS administration in acute stressed animals showed exaggerated release of these proinflammatory cytokines whereas single intravenous LPS administration in chronic stressed animals did not potentiated this release. Thus, it was evident that during chronic restraint stress and immune activation, some anti-inflammatory mechanism might be responsible for increasing the level of IL-10 and down regulating the production and release of IL-6, TNF-α and IFN-γ in the periphery as well as in the CNS. The increase in the IL-10 level most likely represents a regulatory response to restrict the action of IL-6, including the aggravation of acute phase reactants. Since the balance between pro and anti-inflammatory cytokines affects the outcome of certain diseases such as neurodegenerative disorders, it was essential to study the neuro-inflammatory processes during exposure to both short and long term stresor and immune activation. Moreover, the increase in IL-10 and decrease in IL-6 and TNF-α in chronic stress plus LPS challenged group cannot be solely attributed to the anti-inflammatory properties of GCs due to resistance or insensitivity of this steroid hormone by immune cells during chronic stress paradigm.

Recent findings indicate that neural mechanisms are also involved in limiting inflammatory responses. ACh is a major parasympathetic neurotransmitter and inhibits LPS-induced production of pro-inflammatory cytokines such as TNF-α, IL-6 from macrophages. The levels of ACh are continuously regulated by the hydrolytic enzyme AChE which rapidly degrades ACh both in periphery and in the brain. Therefore, we have measured AChE activity in the hypothalamic region as a marker of cholinergic activity. Systemic LPS administration in acute stress group exacerbated the activity of this enzyme in the hypothalamus where as the activity of this enzyme was down regulated in the chronic stress plus LPS challenged group. This finding shows macrophages and other immune cells activated by systemic LPS challenge releases proinflammatory cytokines to stimulate the afferent vagus nerve, although the corticosterone level was found to be substantially high during chronic stress. This pathway may serve as a bypass mechanism mediated by the cholinergic anti-inflammatory pathway to maintain homeostasis when anti-inflammatory properties of GCs has been disturbed due to GC resistance by immune cells. ACh released attenuates the production of TNF and IL-6. Moreover, ACh does not alter IL-10 release, which indicates a direct inhibitory effect of ACh on pro-inflammatory cytokine production. Moreover, it has been reported that in a model of endotoxemia, electrical stimulation of the cervical vagus nerve significantly reduced serum and liver TNF levels, prevented development of hemodynamic shock and improved survival without significantly altering IL-10 or corticosterone serum levels. However, one important difference between PNS/vagal modulation of inflammation vs. regulation of inflammation by the HPA axis or SNS is that the vagus does not use endocrine signaling mechanisms.

The presence of blood brain barrier (BBB) restricts the movement of soluble mediators (cytokines and chemokines) and leukocytes from the periphery to the CNS. Dysfunction of the BBB precedes immune cell infiltration, but leukocyte migration modifies BBB permeability. It has been reported that immune cells of stressed and LPS challenged animals express inflammatory cytokines, reactive oxygen species (ROS) and reactive nitrogen intermediates (RNS) that can facilitate their migration to the CNS. Since in the periphery, ACh release by the vagus nerve restrains inflammation by inhibiting the activation of leukocytes and suppressing cytokine release by monocytes and macrophages activated due to LPS challenge, reduced AChE activity in the chronic stress plus LPS treated group, but not in case of acute stress
plus LPS treatment, provides evidence for the anti-inflammatory role played by ACh molecules in immunomodulation.

Most important changes in the brain of stress-induced animal models are the accumulation of reactive molecules generated due to oxidative stress. Inflammation, infection and administration of bacterial LPS causes oxidative stress, and this entails an increase in tissue concentration of reactive oxygen species, which mediates tissue destruction. Consequently antioxidants such as glutathione (GSH) are foremost determinants of the degree of pathology in models of tissue inflammation. Corticosterone secreted during restraint stress reduces GSH levels in the liver and disrupt anti-oxidant capacity in hepatic and other tissues. From our study, we observed that chronic stress as well as chronic stress plus LPS treatment decreased hepatic GSH levels significantly than acute stress or acute stress plus LPS treated group respectively, but there was an opposite effect in the brain and adrenal gland. Since the quantity of corticosterone remained elevated in chronic stress than in the acute condition, GSH levels in liver decreased after chronic restraint stress compared to acute stress, which was consistent with previous findings. Similar observation was noticed in the LPS administered chronic stress group compared to acute stress ones. But the antagonistic effect observed in case of brain tissue might be due to reduced entry of endogenous corticosterone across the BBB. Moreover a tissue specific variation in GSH activity was observed in the adrenal gland which was not comparable to those observed in liver and brain.

Comparison of catalase (CAT) activities in liver, brain and adrenal gland showed similar response in these tissues, with slight variation particularly in the adrenal gland in response to acute and chronic stress group challenged with LPS. LPS administration post stress resulted in decreased CAT activity in the brain of chronic group whereas increased activity was observed in the liver and adrenal gland. The reduced CAT activity reflected reduced oxidant burden in these tissues. In contrast, the hepatic SOD activity was significantly increased in the LPS challenged acute stressed mice compared to the LPS challenged chronic group. Alternatively, due to less SOD activity, dismutation of superoxide anion to H$_2$O$_2$ would have been limited, leading to accumulation of huge amount of superoxide anion in the tissue and ultimately these accumulated superoxide anion might be responsible for inhibition of hepatic SOD activity which also correlates with the protein expression data. Earlier studies also support the present findings that restraint stress causes robust increase in the production of ROS, depletes GSH and consequently results in oxidative damage and these effects may be governed by differential level of corticosterone in circulation and its time of exposure before immune challenge.

HSPs are produced as a response to various stressors. Earlier studies has shown that neither acute nor chronic stress was sufficient in inducing either HSP90 or HSP70 in brain hypothalamus and that their induction in response to stressors may be independent of glucocorticoids. Few other studies have reported that HSP 90 and 70 remains bound with GC receptor in cytosol of cells when there was no induction of cortisol. Thus, suppressed expression of these protein levels in LPS challenged chronically stressed animals could indicate a gradual decline in corticosterone level in the brain. SOD-1 is considered to be a neuroprotective enzyme, in cases of stress-associated or GC mediated neurotoxicity. Thus, it comes as no surprise when we observe that the expression of this protein was decreased in both post stress LPS challenged groups. COX-2 expression was important in regulating prostaglandin signaling in the brain and both basal and induced expressions of COX-2 were inhibited by GCs. This could partially explain the non-significant difference in COX-2 levels between LPS treated and non-treated groups in both acute and chronic stressed animals even though their levels show a trend similar to that of SOD-1. Moreover, when we performed blot for SOD1, its expression was also decreased in the brain of mice exposed to stress followed by endotoxin challenge, similar to the enzyme activity which was also decreased in the brain tissue. Thus reduced activation of the enzyme pool by less accumulation of superoxide anion among the total brain protein content led to its decreased expression post LPS challenge in acute/chronic stressed mice.

The mechanisms, by which immune signaling to the CNS affect behaviour, have been of considerable interest in recent times. It is well known fact that bacterial endotoxin is a potent stimulator of the synthesis and release of proinflammatory cytokines such as IL-6, TNF-α and IFN-γ, but acute stress has been demonstrated to downregulate these LPS-induced responses. Thus, changes in behaviour of
the animals were correlated with the level of proinflammatory cytokines in the brain.

EPM provides dependable measures of anxiety-related behavior. When tested in EPM, animals with a more anxiogenic phenotype will spend a greater percentage of their time in the closed arm of the maze relative to the open, unsafe arms. Moreover, latency to exit the closed arm of the EPM will be significantly greater than that of animals that is not anxious. It had been reported that chronic stress results in increased anxiety and depressive like behavior. In addition, systemic administration of LPS induces sickness behaviour, as well as alterations of HPA functioning commonly associated with stressors. Our study reveals that acute stress plus acute LPS administration resulted in decreased anxiogenic-like responses than the chronically stressed animals, which may be correlated with decreased serum corticosterone levels. Thus, we can infer that bacterial endotoxin challenge and the ensuing cytokine changes may contribute to anxiety-related behaviour disturbances. Researchers seeking to explain the role of peripheral cytokines in cognitive disorder will have to consider the multiple effects of serum cytokines which may have cognitive processing by way of their effects in the hypothalamic-pituitary axis and various behaviour systems in addition to neurodegeneration.

Locomotor activity provides an admirable index for the assessment of both the effects of and responses to acute endotoxin challenge in acute/chronic stressed animals. Role of TNF-α and IFN-γ in induction of depressive like behaviour in response to Bacillus Calmette-Guerin in mice has been demonstrated. Changes in the open field behaviour have also been documented in animals administered cytokines in isolation, including TNF-α and in animals with relevant cytokine genes deleted or over expressed. The level of cortisol hormone is often elevated in depressed individuals. But in depressed individuals, the negative feedback system for dampening a response does not work well. The problem with CRH not being suppressible in depressed individuals may be related to the inflammatory state in chronic stress. It has been reported that stressors and cytokines may synergistically influence biological and behavioural responses and that these treatments may have long term ramifications through the sensitization of processes associated with stress responses.

**Conclusion**

Taken together with our results, these findings support the notion that cytokines released during immune activation and under the influence of corticosterone can modulate the open field behaviour both in terms of locomotor activity as well as exploration. One of the features observed with chronic stressor was an enhanced ability to suppress inflammation, regardless of the fact that in case of acute stress, LPS was synergistic in mediating inflammation in the brain. These findings in in vivo showed correlated improvement of exploratory behaviour and cognitive functions on administration of LPS post exposure to chronic stressor. Thus, pre-activation of the immune system would enhance survival and recovery from aberrant social behaviours, particularly aggression, suicidal tendency as well as psychomotor agitation in stress related conditions in humans.

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**References**


