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Dual Effect of Central Injection of Recombinant Rat Interleukin-4 on Lipopolysaccharide-Induced Sickness Behavior in Rats

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Systemic administration of the bacterial endotoxin lipopolysaccharide (LPS) has profound depressive effects on behavior that are mediated by the inducible expression of pro-inflammatory cytokines, such as interleukin-1 (IL-1), IL-6 and tumor necrosis factor α (TNF), in the brain. To assess the regulatory effects of the anti-inflammatory cytokine IL-4 on LPS-induced sickness behavior, rats injected intra-peritoneally (i.p.) with LPS were administered intracerebroventricularly (i.c.v.) with IL-4. IL-4 (30 and 300 ng) potentiated the behavioral effects of LPS (175 μg/1000 g) when both molecules were co-injected. However, when IL-4 (30 ng) was injected 12 h prior to LPS, it blocked the depressing effects of LPS on social exploration. These results indicate that the regulation of cytokine-induced sickness behavior by IL-4 can be either inhibitory or stimulatory depending on the sequencing of IL-4 and LPS treatments.

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KEY WORDS: *Cytokine; Interleukin-4; Lipopolysaccharide; Social behavior; Rat; Brain*

When an organism becomes sick during the course of an infection, several changes occur, which are mediated by the central nervous system (CNS). These changes include regulated increases in body temperature, sleep, activation of the hypothalamo-pituitary-adrenocortical (HPA) axis, decreases in locomotor activity, feeding, drinking, and social interactions, and alterations in brain neurotransmitters. They are due to the release of pro-inflammatory cytokines such as interleukin-1 (IL-1), IL-6 and tumor necrosis factor-alpha (TNF-α) by activated monocytes and macrophages (Dantzer et al. 2001; Dinarello 2000; Gabay and Kushner 1999; Krueger et al. 1999; Mulla and Buckingham 1999; Rothwell 1997; Turrin and Plata-Salaman 2000). The same effects can be obtained by systemic administration of the cytokine-inducer lipopolysaccharide (LPS), a component of the cell wall of Gram-negative bacteria (Cabrera et al. 2000; Francis et al. 2000; Lacosta et al. 1999; Ma et al. 2000; Mohan-Kumar et al. 2000; Roth and de Souza 2001; Swiergel and Dunn 1999; Yirmiya 1996; Yirmiya et al. 2001). Peripherally released cytokines act on the brain by inducing the expression and release of cytokines in the central nervous system (Eriksson et al. 2000; Konsman et al. 1999; Layé et al. 2000; Quan et al. 2000). LPS induces the expression of not only pro-inflammatory but also anti-inflammatory cytokines such as IL-10 and IL-13 in the brain (Wong et al. 1997). Another important anti-inflammatory cytokine is IL-4. Although it is not syn-

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thesized in the normal brain (Lovett-Racke et al. 2000; Schluter et al. 1997), it is strongly expressed during brain injury, infection, experimental autoimmune encephalomyelitis, and neurodegenerative processes (Arsenijevic et al. 1998; Laman et al. 1998; Liedtke et al. 1998; Woodroofe and Cuzner 1993). Anti-inflammatory cytokines have the ability to suppress the synthesis of IL-1, TNF, and other cytokines in peripheral immune and non-immune cells (Dinarello 1997; Marie and Cavaillon 1997).

There has been a recent surge of interest in the behavioral effects of cytokines in neuropsychopharmacology. Several reasons account for that, including the fact that cytokine-induced sickness behavior is not the result of weakness and physical debilitation but appears to be the expression of a previously unrecognized motivational state that is triggered by peripheral immune stimuli and reorganizes the organism's priorities (Dantzer 2001). In addition, the possibility of an intersection between sickness behavior and depression (Dantzer et al. 1999) has raised new and important issues in psychopathology.

If sickness behavior is the ineluctable result of the brain action of those proinflammatory cytokines that are released at the periphery during the course of an innate immune response or even in response to exteroceptive stressors, it becomes important to find out how this behavior is regulated. The production and action of proinflammatory cytokines at the periphery is primarily regulated by the so-called anti-inflammatory cytokines that include IL-10, IL-4 and IL-13. Little is know yet concerning the ability of these latter cytokines to oppose the development of sickness behavior. Using social withdrawal as a measure of sickness behavior, we have shown earlier that central administration of the anti-inflammatory cytokine IL-10 attenuates the development of sickness behavior in rats injected with LPS at the periphery (Bluthé et al. 1999). The same model was used in the present study to assess the anti-inflammatory effect of IL-4. However, IL-4 blocked the depressing effect of LPS only when injected 12 h before the endotoxin treatment whereas it potentiated sickness behavior when injected at the same time than LPS. These results show that IL-4 can have pro- or anti-inflammatory effects depending on the sequencing of events.

MATERIALS AND METHODS

Subjects

Male Wistar Crl: (WI) IGS BR rats (125 –150 g) were obtained from Charles River, St-Aubin-lès-Elbeuf, France. After arrival at the facility, they were housed in groups of five in polypropylene cages and acclimated to the laboratory for at least two weeks. Room temperature was maintained at 23 ± 1°C, and lighting was maintained on a 12-hr light/dark cycle (lights on at 2100). Food (Extralabo, Provins, France) and water were available *ad libitum.* Juveniles of the same strain served as stimulus animals and were housed in groups of 10 in a different room. The investigators adhered to the guidelines published in the NIH Guide for the Care and the Use of Laboratory Animals.

Surgery

For central injections, a stainless steel guide cannula (23-gauge, 7 mm length) was implanted unilaterally 1 mm above the lateral ventricle. For this surgery, rats were anesthetized intraperitoneally (i.p.) with a mixture of ketamine (Imalgene 1000, Rhône Mérieux, Lyon, France) and xylazine (Bayer Pharma, Puteaux, France), at doses of 61 mg and 9 mg/1000 g, respectively, and secured in a Kopf stereotaxic instrument (Tujunga, CA, USA). Coordinates were with toothbars 5 mm above the interaural zero, 0.6 mm posterior to the bregma, 1.5 mm lateral and 3.2 mm below the skull surface, at the point of entry (Paxinos and Watson 1986). At this point, the body weight was 160–200 g. Rats were allowed a 2-week recovery period before behavioral testing.

Chemicals

Recombinant rat interleukin-4 (IL-4) preparation: Messenger RNA (mRNA) was extracted from endotoxin-stimulated rat monocytes using the QuickPrep mRNA Purification Kit (Pharmacia Biotech, 23 Grosvenor Road, St Albans, Hertfordshire AL1 3LW, UK). cDNA was transcribed using the First Strand cDNA synthesis kit (Pharmacia Biotech). PCR primers were designed to engineer the restriction sites Bgl II at the 5′ end and Xho I at the 3′ end of the rat IL-4 coding sequence respectively. Resultant PCR products were ligated into the vector pMT.BiP (Invitrogen, 3985B Sorrento Valley Boulevard, San Diego, CA 92121, USA) downstream of the metallothionein promoter (MT) and the immunoglobulin binding chaperone protein (BiP) Drosophila signal sequence. The pMT.BiP/rat IL-4 vector was transformed into *Escherichia coli* strain JM109, where the construct was checked by restriction mapping followed by DNA sequencing. Drosophila S2 cells were stably co-transfected with pMT.BiP/rat IL-4 and the vector pCoHYGRO at a ratio of 19:1. Stable cell lines were grown in complete DES medium containing 800 μg/ml hygromycin-B (Invitrogen), hygromycin-resistant co-transfected cell lines took approximately three weeks to grow through, with a viability of >95%. One cell line was expanded into shake cultures (24°C, shaking 115 rpm) in complete DES containing 300 μg/ml hygromycin-B and 0.05% Pluronic F-68 to prevent cell shearing. Cells were grown to a density of 10×10^6 cells/ml, centrifuged at 1000 × g for five minutes, the cell pellets washed twice with serum-free

medium (containing 50 units/l penicillin G, 50 μg/l streptomycin sulphate, 2mM L-glutamine and 0.05% Pluronic F-68), then re-suspended at a cell density of 6×10^6 cells/ml and induced by the addition of $CuSO_4$ to a final concentration of 500 μM. Cells were grown on for 8 days, 24°C, shaking 115 rpm, then the medium harvested by centrifugation at 1000 × g for 10 minutes at 4°C.

Recombinant rat IL-4 was purified using ion-exchange chromatography on SP-Trisacryl in 0.01M sodium phosphate, pH 6.0 (Sigma). Recombinant rat IL-4 was eluted with 0.05M sodium phosphate, pH 7.0 containing 1.0M NaCl. IL-4–containing fractions were pooled, trehalose added to 1 mg/ml and the preparation lyophilized. The lyophilized preparation was reconstituted in 2.5 ml of Dulbecco's phosphate buffered saline (PBS) and large contaminating proteins removed by gel filtration on a Sephadex G75 column (450 ml bed volume). Recombinant IL-4 eluted at V/Vt = 0.64–0.85, IL-4 containing fractions were pooled and concentrated through a stirred cell membrane concentrator (Amicon). Endotoxin levels of recombinant rat IL-4 preparations ranged from 120–240 EU/ml (10 EU = 1 ng).

For central injections, recombinant rat IL-4 dissolved in 0.9% pyrogen free saline (SAL) was first injected in a volume of 2 μl with a 30-gauge stainless-steel cannula, over a 30-sec period during which the rat was placed in a circular bowl. Control animals received SAL in the same conditions. Then, SAL or lipopolysaccharide (LPS, from *Escherichia coli*, Serotype 0127: B8, phenol extract, Sigma, St-Louis, MO, USA, L3129 – Horan et al. 1989) was injected into the peritoneal cavity (i.p.) at a dose of 175 μg/1000 g of body weight diluted in 2 ml of sterile, pyrogen-free saline (SAL). At the conclusion of the experiment, cannula placement was verified by injecting a solution of India ink into the guide cannula and then slicing the brain after removal from the skull.

Experimental Procedure

Behavioral observations were carried out during the dark phase, between 9 A.M. and 5 P.M., using a video camera under red light illumination. Rats were isolated 24 hours before the experiment in transparent plastic cages (30 × 45 × 19 cm). Sickness behavior induced by LPS was assessed by decreases in duration of the injected adult's social exploration of a conspecific juvenile introduced into the home cage of the test animal. The observation procedure had been previously validated (Bluthé et al. 1992). One day before the experiment, baseline social exploration was assessed. The time spent by the experimental rat in social exploration, consisting of ano-genital sniffing, body sniffing and grooming of the juvenile, was measured during a 4-min period by using pre-set keys on the keyboard of an Apple IIe computer. The day after, rats were first injected, then tested with different juveniles on repetitions of the behavioral test at different time intervals. Two different series of experiments were carried out to assess the effects of IL-4 on LPS-induced sickness behavior.

Experiment 1: Effect of Co-injections of i.c.v. IL-4 and i.p. LPS

Forty rats were allocated to six treatment groups matched for mean baseline social exploration time and body weight: (i) i.c.v. SAL + i.p. SAL (n = 7); (ii) i.c.v. SAL + i.p. LPS (n = 8); (iii) i.c.v. IL-4 (30 ng) + i.p. SAL (n = 6); (iv) i.c.v. IL-4 (30 ng) + i.p. LPS (n = 7); (v) i.c.v. IL-4 (300 ng) + i.p. SAL (n = 5); (vi) i.c.v. IL-4 (300 ng) + i.p. LPS (n = 7). The dose of LPS used (175 μg/1000 g) was selected so as to produce a decrease in social exploration which was not maximum (Bluthé, unpublished data). This dose caused obvious sickness symptoms, including immobility, shivering, and pilo-erection.

Experiment 2: Effect of i.c.v. IL-4 Injected 12 Hours Prior i.p. LPS

27 rats divided into three groups (matched for mean baseline social exploration time and body weight) were used in this experiment, and received (i) i.c.v. SAL + i.p. SAL (n = 11) or (ii) i.c.v. SAL + i.p. LPS (n = 7) or (iii) i.c.v. IL-4 (30 ng) + i.p. LPS (n = 9). The dose of IL-4 was the one that proved to be active in experiment 1. Despite the fact that the dose of LPS was the same as in experiment 1, it produced a stronger effect in Experiment 2, which can happen when working on different batches of rats.

For all the experiments, each rat received only one treatment combination. Behavior was monitored 2, 4, 6, and 24 hours after treatments.

Statistical Analysis

Experiment 1: Effects of Simultaneously Injected i.c.v. IL-4 and i.p. LPS. Duration of social exploration (sec) was analyzed by a two-way ANOVA with IL-4 (three levels: 0, 30 and 300 ng/rat) and LPS (two levels: 0 and 175 μg/1000 g) as between subject factors, and time (five levels: 0, 2, 4, 6 and 24 hours after treatment for social exploration) as a within subject factor. *Post-hoc* comparisons of individual group means were carried out by the protected least significant difference test (LSD).

Experiment 2: Effects of i.c.v. IL-4 Injected 12 Hours Prior to i.p. LPS. Duration of social exploration (sec) was analyzed by a two-way ANOVA with IL-4 (2 levels : 0 and 30 ng/rat) and LPS (two levels : 0 and 175 μg/1000 g) as between-subjects factors, and time (five levels: 0, 2, 4, 6 and 24 hours after treatment for social exploration) as a within-subject factor. *Post-hoc* compari-

sons of individual group means were carried out by the protected least significant difference test (LSD).

RESULTS

Experiment 1: Effects of Simultaneously Injected i.c.v. IL-4 and i.p. LPS on Behavior (Figure 1)

A two-way ANOVA (treatment X time) on duration of social exploration revealed significant effects of the main factors and their interaction [$F(5,34) = 5.7$, $p < .001$; $F(4,136) = 16.3$, $p < .001$; $F(20,136) = 3.0$, $p < .001$, respectively]. *Post-hoc* comparisons of individual means by the LSD test revealed that i.c.v. IL-4 (30 ng) potentiated LPS-induced decrease in social exploration 2–6 hours after injection, and recovery was complete after 24 hours. In the same manner, i.c.v. IL-4 (300 ng) potentiated LPS-induced decrease in social exploration 2–4 hours after injection, and recovery was complete after 24 hours. However, the effect of IL-4 (30 ng) was more potent than that of IL-4 (300 ng) 2 h after injection on LPS-induced sickness behavior. Whatever the dose, IL-4 had no effect of its own on the duration of social exploration in saline-treated animals (Figure 1).

Experiment 2: Effects of i.c.v. IL-4 Injected 12 Hours Prior to i.p. LPS (Figure 2)

A two-way ANOVA (treatment X time) on duration of social exploration revealed significant effects of the main factors and their interaction [$F(2,24) = 9.3$, $p < .001$; $F(4,96) = 36.9$, $p < .001$; $F(8,96) = 16.8$, $p < .001$, respectively]. *Post-hoc* comparisons of individual means by the LSD test revealed that i.c.v. IL-4 (30 ng) blocked LPS-induced decrease in social exploration 2–4 hours after injection (Figure 2).

DISCUSSION

The results of the present study show that centrally administered IL-4 potentiates sickness behavior induced by systemic LPS when IL-4 is co-injected with LPS, whereas it attenuates the effects of LPS when administered 12 h before.

Peripherally administered LPS has been shown to induce the synthesis and release of proinflammatory cytokines in the brain (see Dantzer 2001 for a recent review). This local production of cytokines is responsible for the development and maintenance of sickness behavior, certainly by altering the metabolism of neurotransmitters including neuropeptides, although the exact mechanisms are still elusive (Dunn et al. 1999). The possibility that IL-4 is involved in the regulation of cytokine-induced sickness behavior has not yet been examined.

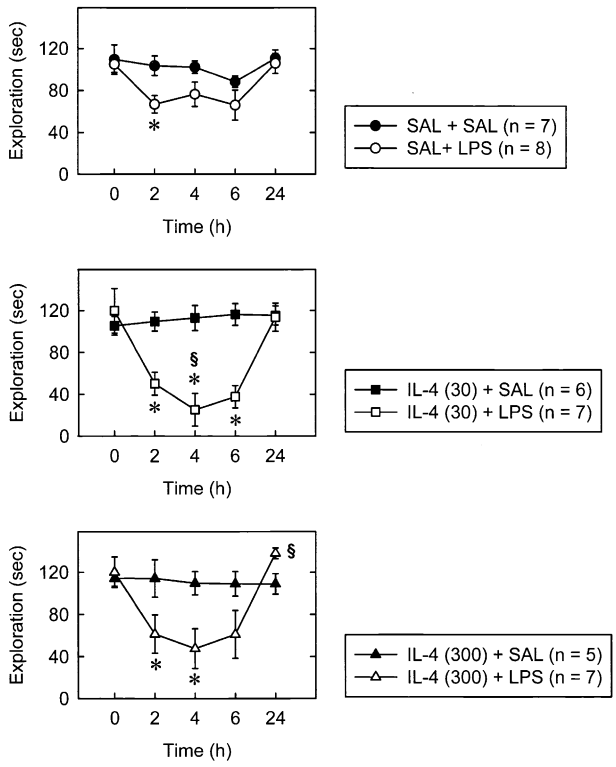

Figure 1. Effects of co-administration of i.c.v. recombinant rat interleukin-4 and i.p. LPS on social exploration. IL-4 (0, 30, or 300 ng/2 μl) was injected i.c.v. just before i.p. LPS (0 or 175 μg/1000 g/2ml). Treatment effects were assessed on the mean duration of social exploration (sec) measured at time 2, 4 , 6 and 24 hours post-injection [*$p < .05$ compared to SAL or IL-4 (30 or 300 ng) + LPS; § $p < .05$, SAL + LPS compared to IL-4 (30 or 300 ng) + LPS)] (mean ± SEM).

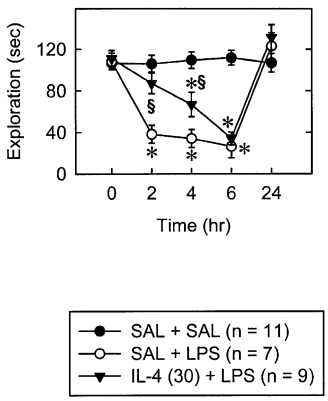

Figure 2. Effects of i.c.v. recombinant rat interleukin-4 injected 12 hours prior to injection of i.p. LPS on social exploration. IL-4 (0 or 30 ng/2 μl) was injected i.c.v. 12 hours before i.p. LPS (0 or 175 μg/1000 g/2ml). Treatment effects were assessed on the mean duration of social exploration (sec) measured at time 2, 4, 6 and 24 hours post-injection [*$p < .05$ compared to SAL or IL-4 (30 ng) + LPS; § $p < .05$, SAL + LPS compared to IL-4 (30 ng) + LPS)] (mean ± SEM).

IL-4 is a pleiotropic cytokine produced by activated helper T cells which exerts multiple biological effects through binding to specific receptors on the cell surface (Mire-Sluis 1998; Nelms et al. 1999). Although IL-4 has initially been described as a B cell growth factor (Farrar et al. 1983), it has since been reported to regulate a wide range of immune responses, including increases in proliferation of B and T cells, enhancement of IgE synthesis, and up-regulation of the expression of class I and II HLA antigens (for reviews see Brown and Hural 1997; Paul 1991). IL-4 exerts its effects on cells of different lineages such as fibroblasts, endothelial cells (Lowenthal et al. 1988), microglia (Chao et al. 1993) and astrocytes (Brodie and Goldreich 1994; Pousset et al. 1999). IL-4 provides support for the development of humoral immune responses through the induction of T-helper type 2 (Th2) cell differentiation (Opal and DePalo 2000).

The anti-inflammatory action of IL-4 is well documented both *in vitro* and *in vivo.* IL-4 attenuates the activation of various immunocompetent cells, including neutrophils, monocytes and macrophages, by limiting the production of pro-inflammatory cytokines (Standiford et al. 1990; te Velde et al. 1990; Wertheim et al. 1993), and it decreases production of PGE_2 (Niiro et al. 1997) and O_2^- (Abramson and Gallin 1990; Maru and Jackson 1996). In a similar way, IL-4 limits the radiation-induced production of inflammatory mediators by endothelial cells (Van der Meeren et al. 1999b) and enhances survival of lethally irradiated mice (Van der Meeren et al. 1999a; Van der Meeren and Lebaron-Jacobs 2001). IL-4 plays an important role in modulating the severity of inflammation, as demonstrated by the use of IL-4-deficient mice. These mice developed a more severe form of allergic encephalomyelitis and displayed a greater expression of pro-inflammatory cytokines in the CNS than their wild-type littermates (Falcone et al. 1998).

The attenuating effects of IL-4 on LPS-induced sickness behavior are in accordance with the anti-inflammatory properties attributed to this cytokine. The fact that IL-4 needs to be administered before the inflammatory challenge for its anti-inflammatory activity to show up is not uncommon. For example, i.p. IL-4 improved survival of mice infected systemically with lethal or sublethal inoculation of *Pseudomonas aeruginosa* when IL-4 treatment occurred 24 hours before challenge, and this treatment inhibited serum production of TNF-α (Giampietri et al. 2000). Identical responses were obtained *in vitro* on macrophage cultures. Moreover, the hyperalgesic response to IL-1β was inhibited by 30 to 74% by intraplantar IL-4 when IL-4 was injected 12 h before IL-1β whereas it had no effect when it was administered 30 min before IL-1β (Cunha et al. 1999). Exposure of murine peritoneal macrophages to murine IL-4 for 16 h before stimulation with LPS inhibited LPS-induced production of prostaglandin E_2 (PGE_2) but not of IL-1β. In the early phase of the hyperalgesic response, IL-4 inhibited the production of the hyperalgesic cytokines TNF-α and IL-1, whereas in the later phase of the hyperalgesic response, IL-4 inhibited COX-2 enzymes, since IL-4 given 12 h before IL-1β, inhibited the hyperalgesic effect of IL-1β and the release of PGE_2 by murine macrophages stimulated *in vitro* with LPS (Cunha et al. 1999). The anti-inflammatory effects of IL-4 can be explained by a decrease in the production of pro-inflammatory cytokines, certainly via inhibition of the nuclear transcription factor NFkappaB (Chao et al. 1993; Giampietri et al. 2000; Pousset et al. 1999).

The potentiation of LPS-induced sickness behavior by IL-4 when both molecules were co-administered appears to be at odds with the anti-inflammatory activity of IL-4. Although the effects of LPS were less marked in the co-administration experiment than in the experiment in which LPS was injected 12 h prior to IL-4, this difference is unlikely to account for the present results. Differences in the magnitude of LPS effects are common between different batches of animals and are probably related to minor variations in the sanitary status of the population under test. In accordance with the observation of potentiating effects of IL-4 on LPS-induced sickness behavior, the results of a few reports indicate that IL-4 can exert pro-inflammatory effects. *In vivo* studies have demonstrated both positive and negative effects on the outcome of infectious diseases (Brown and Hural 1997; Jain-Vora et al. 1998). In particular, sublethally infected mice given IL-4 at the time of infection responded to this treatment with increased mortality and overproduction of TNF-α (Giampietri et al. 2000). Moreover, IL-4 administered 30 min before inhibited the hyperalgesia induced by bradykinin and TNF-α but not by IL-1β (Cunha et al. 1999). *In vitro*, IL-4 failed to inhibit LPS-induced production of IL-1β in cocultures of rat microglia and astrocytes (Ledeboer et al. 2000) and IL-6 in mouse primary astrocytes (Pousset et al. 1999), and it was able to enhance TNF-α (Giampietri et al. 2000) and IL-6 production (Pousset et al. 1999) under some conditions. The mechanisms of the potentiating effects of IL-4 on cytokine production and action are still unknown. In the context of the inflammatory response, IL-4 has been shown to upregulate the expression of adhesion molecules on vascular endothelial cells and promote the release of potent chemoattractants such as MCP-1, promoting the early recruitment of mononuclear cells to sites of tissue inflammation (Gundel et al. 1996). This effect could contribute to the potentiation of LPS-induced sickness behavior, by enhancing the normally discrete inflammatory response that develops in the brain in response to systemic LPS (Layé et al. 1994). In view of the importance of IL-1β in LPS-induced sickness behavior, another possibility is that IL-4 counteracts the down-regulatory effects of IL-1 on the expression of its active receptor, the IL-1 type I receptor,

and enhances the up-regulatory effects of IL-1 on the expression of its decoy receptor, the IL-1 type II receptor, as indicated by *in vitro* studies (Pousset et al., in preparation). Whatever the mechanisms, the results of the present study indicate that IL-4, in contrast to IL-10, is unlikely to be a cytokine of choice for down-regulating the expression and action of pro-inflammatory cytokines in the brain.

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