Effects of immune activation on the retrieval of spatial memory

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Abstract: Objective It has been shown that there are extensive interactions between the central nervous system and the immune system. The present study focused on the effects of lipopolysaccharide (LPS) on memory retrieval, to explore the interaction between immune activation and memory. **Methods** C57BL/6J mice (8 weeks old) were first trained in the Morris water maze to reach asymptotic performance. Then mice were tested 24 h after the last training session and LPS was administered (1.25 mg/kg, i.p.) 4 h prior to the testing. The retrieval of spatial memory was tested by probe trial, and the time spent in the target quadrant and the number of platform location crosses were recorded. ELISA was performed to detect interleukin-1 β (IL-1 β) protein level in the hippocampus of mice tested in the water maze. **Results** Although LPS induced overt sickness behavior and a significant increase in the level of IL-1 β in the hippocampus of mice, there was no significant difference in the time spent in the target quadrant or in the number of platform location crosses between LPS-treated and control groups in the probe trial testing. **Conclusion** Immune activation induced by LPS does not impair the retrieval of spatial memory.

Keywords: lipopolysaccharide; immune activation; sickness behavior; hippocampus; interleukin-1 β ; Morris water maze; spatial memory; memory retrieval

1 Introduction

It has been recognized that there are extensive communications between the central nervous system (CNS) and the immune system. Cytokines are thought to be the bridge linking these 2 systems^[1]. The initial response of organisms to infection is called acute response or “sickness”^[2]. Many responses to infectious agents, such as fever, anorexia, malaise, hypersomnia, depressed activity and reduced social exploration (together termed ‘sickness behavior’), are orchestrated by immune products called pro-inflammatory cytokines,

which are released in response to the exogenous pathogens. The produced peripheral pro-inflammatory cytokines can communicate with the CNS by different routes. One involves the sensory afferents of the organ nerve which can communicate with neuronal populations within the brain^[3]. The peripheral pro-inflammatory cytokines can also enter the brain through choroids plexus or circumventricular organs which are deficient in blood-brain barrier^[4,5]. Furthermore, the findings that the pro-inflammatory cytokines exist in the CNS and respond to peripheral immune activation or brain injury indicate that besides orchestrating the sickness behavior, the pro-inflammatory cytokines may also influence the brain structures that mediate cognition^[1]. Among the pro-inflammatory cytokines, interleukin-1 β (IL-1 β) is the most extensively studied one. IL-1 prohormone, IL-1-converting enzyme, IL-1 β , and receptors for IL-1 β have all been found in the

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brain^[6,7], especially at a high density in the hippocampus^[8]. There has been evidence suggesting that IL-1 β is required for normal physiological regulation of hippocampal plasticity and memory processes. Studies reveal that long-term potentiation (LTP) in the hippocampus is accompanied by a long-lasting increase in IL-1 β gene expression, and exposure to IL-1 receptor antagonist (IL-1ra) impairs the maintenance of LTP^[9]. Moreover, IL-1 receptor type I (IL-1R1)-knockout mice exhibit no LTP, either *in vivo* or *in vitro*, but severe impairment in hippocampus-dependent memory, while normal hippocampus-independent memory is intact^[10].

Many studies have described that immune activation, either by lipopolysaccharide (LPS) or by IL-1 β , causes deficits in learning and memory. The idea that systemic inflammation impairs learning and memory appears to have become a dogma in the psychoneuroimmunology field. However, there are also some studies showing no effect of immune activation on learning and memory. The relationship between immune activation and cognitive impairment has not yet been clearly elucidated. LPS, a large molecule found in the outer membrane of Gram-negative bacteria, is an endotoxin stimulating immune system in animals and human. IL-1 β has been consistently detected in the CNS after peripheral immune activation. For example, both mRNA^[11-13] and protein^[14] of IL-1 β have been found in the brain after peripheral administration of LPS. Several studies indicate that LPS can cause learning deficits in Morris water maze (MWM) test. It has been reported that LPS treatment could induce deterioration in spatial learning^[15] and memory impairment in passive avoidance and water maze tests^[16] in mice. Moreover, LPS is also implicated in the impairment of spatial learning in rats, as revealed by another version of water maze with a fixed start location^[17]. However, there are also some reports showing little effect of LPS on learning and memory in the MWM test. It has been reported that LPS mainly induces performance impairment, particularly in older animals, rather than learning deficits in the water maze^[18]. Another study employed 2 versions of water maze to examine the effects of LPS on learning and memory^[19], including the spatial learning in which a submerged platform was used and the cued learning in which a visual platform was used. The authors conclude that the LPS-

induced impairment in spatial learning could be mainly explained by alterations in performance rather than in learning, although some mild learning decrements begin to appear on later days. Moreover, in the cued learning, animals exhibit no learning deficits but the performance impairment during early testing days, primarily in terms of swimming speed.

Memory is considered to be a complex process that has multiple stages, including acquisition, consolidation, and retrieval. Memory retrieval is fundamental in our daily life^[20] and provides the only means for evaluating memory^[20,21]. In the present study, LPS was administered after the acquisition of spatial learning, and the effects of immune activation on memory retrieval process were investigated.

2 Materials and methods

2.1 Animals Female C57BL/6J mice (8 weeks old) were purchased from Shanghai Slack Experimental Animal Center. Animals were housed at the ambient temperature of 22-23 °C under a 12:12 h light/dark cycle, with access to food and water *ad libitum*. Animals were acclimatized to laboratory conditions for one week before all the tests. Animal care and all experiments were approved by the guidelines of the Animal Care and Use Committee of Guangzhou Biomedical and Health Institute, Chinese Academy of Sciences, and were in accordance with the National Institutes of Health Guide for Care and Use of Laboratory Animals (Publication No. 85-23, revised in 1985).

2.2 Behavior testing apparatus and procedure The water maze was a circular pool (120 cm in diameter and 60 cm in depth) with water temperature adjusted to (22 $\pm$ 1) °C. The maze was bisected by 2 crossing principal axes, and the ends of axes demarcate 4 cardinal points: North (N), South (S), East (E), and West (W). Extra-maze cues consisted of highly-visible geometric shapes, curtains, and shelves in the room, and they all remained unchanged throughout the experiments. Intra-maze cues were not employed here. A circular platform (15 cm in diameter) was placed at a fixed location in the center of the NW quadrant, submerged 1 cm below the water surface. The platform was painted with the same color as the maze wall and floor^[22], to avoid the need for addition of agents to render the water opaque. Besides, whether the platform was

still visible was tested by closing the curtains to eliminate spatial cues. Mice trained in this maze did not successfully find the platform at each entry into the pool, indicating that the platform was invisible to mice. For each mouse, 4 trials were conducted in each day, starting at 14:00. In each trial, the mouse was placed in the pool at one of the 4 cardinal points according to a semi-random sequence. A different starting location was used in each trial such that each starting position was used once within the 4 trials. The maximum duration of each swimming trial was 60 s. If the mouse found the platform within the 60-s period, it was allowed to stay on the platform for 15 s. If it did not find the platform during the allowed time, it was then gently guided onto the platform and stayed for 15 s before being placed back into its holding cage. Animals were trained in squads of 10 and warmed under a heating lamp until the next trial. All mice were trained to reach asymptotic performance. In our lab, some mice reached asymptote in 5 d, and most reached asymptotic performance in 6 d. Thus in the present training session, all mice were trained for 6 d. On the last day of training, a probe trial (mouse swam freely in the water maze for 60 s without the platform) was performed immediately after the last training session for each mouse. Testing was conducted 24 h later. The swimming patterns of mice were recorded by a video camera mounted on the ceiling.

2.3 LPS and scopolamine treatment LPS (obtained from *Salmonella enterica*, serotype Typhimurium; Sigma, St. Louis, MO, USA) was dissolved in 0.9% sterile saline, and intraperitoneally injected into mice (0.25, 1.25 or 5 mg/kg). Sterile saline was injected as a control. Sickness behavior was observed 4 h after injection. For the water maze test, LPS (1.25 mg/kg) was administered 4 h prior to the testing, since this provided enough time for the development of overt sickness behavior and also coincided with the central IL-1 β gene expression after intraperitoneal injection of LPS^[23-26].

In the water maze, Scopolamine Hydrobromide (0.3 mg/mL, obtained from Guangzhou Baiyunshan Ming Xing Pharmaceutical Co., Ltd., China) was intraperitoneally injected 20 min prior to the testing. Mice with 0.9% sterile saline injection served as the control. The injection volume was 10 mL/kg body weight. So the dose of scopolamine was 3 mg/kg.

2.4 Sickness behavior Sick rodents often exhibit characteristic hunched posture, ruffled fur and reluctance to move. Four hours after LPS injection, sickness behavior in animals was assessed using a series of well-established ordinal scales^[27] for a broad array of health and behavioral features. The following sickness-related behaviors and general appearance were observed and assessed. Hunching: Sick rodents often display a hunched posture during periods of quiescence. Hunching was scored as 0 for the absence of hunching, and 1 for the presence of hunching. Piloerection: It is a measure of autonomic instability and a sign of distress. It was scored as 0 for piloerection absence, 2 for slight piloerection, and 4 for marked piloerection. Grooming/fur: Grooming is the ongoing behavior in mice, which occurs sporadically during the day. Sick or distressed animals often stop grooming, and their coats can appear ruffled, yellow, gray and/or sticky. Grooming was scored as 0 for good, 2 for sub-normal and 4 for poor activity. Palpebral closure: It is a measure of autonomic instability and a sign of distress. It was scored as 0 for eyes wide open, 1 for less 1/2 closed, 2 for over 1/2 closed, and 3 for completely closed. Lacrimation: Lacrimation was scored as 0 for absence, and 1 for its presence. Locomotor activity: The level of spatial locomotion in the test cage was scored as 0 for active, 2 for slow, and 4 for absence of spatial locomotion. Body weight: Body weight is usually a sufficient measure of general health. Animals were weighed just before LPS injection and 24 h after LPS injection.

2.5 Tissue dissection and detection of IL-1 β protein Mice were anesthetized with a brief exposure to ether and decapitated. Brain was quickly removed and dissected on an ice-cold frosted glass plate. Tissues including hippocampus and prefrontal cortex were immediately frozen in liquid nitrogen and stored at -80 °C until use. Each tissue was homogenized in 0.4 mL RIPA Lysis Buffer (containing 50 mmol/L Tris, pH 7.4, 150 mmol/L NaCl, 1% Triton X-100, 1% Deo-Na, 1 mmol/L NaF, 2 mmol/L EDTA, 1 mmol/L Na₃O₄V and 0.1% SDS) with cocktail enzyme inhibitor (containing 2 mmol/L PMSF, 5 μ g/mL leupeptin, 160 μ g/mL benzamidine, 10 μ g/mL aprotinin, and 5 μ g/mL pepstatin). Then the tissue homogenate was sonicated for 20 s using an ultrasonic cell disrupter with power setting on 100 watts. Sonicated samples were

centrifuged at 12 000 r/min for 15 min at 4 °C. Supernatant was collected and stored at 4 °C until ELISA analysis. Total protein concentrations were determined with a protein assay kit (Bio-Rad, Richmond, CA). IL-1 β protein level was determined using a commercially available mouse IL-1 β ELISA kit (R&D systems, Minneapolis, MN), according to the manufacturer's instructions. The concentration of IL-1 β protein was expressed as pg/mg total protein.

2.6 Statistics Data were analyzed using one-way analysis of variance (ANOVA). Following a significant omnibus F ratio, *post hoc* testing was performed using the Newman-Keuls method. The ranked data for the sickness behavior were analyzed by a nonparametric test (Kruskal-Wallis Test). $P < 0.05$ was indicated as significantly different.

3 Results

3.1 LPS dose selection and sickness behavior Previous studies have indicated that the common dose of LPS used for studies involving behavioral measures is 0.25 mg/kg^[18, 28-30]. However, endotoxin from different bacteria strains, even different subtypes may have diverse activities, and different animal strains show different degrees of tolerance to the endotoxin (C57BL/6J strain is more resistant to LPS than certain other strains). Higher doses of LPS, such as 0.6 mg/kg^[15], 2 mg/kg^[29], 4 mg/kg^[31], and 5 mg/kg^[32] had also been used to induce sickness behavior in rodents. Here we examined the effects of 3 doses (0.25, 1.25 and 5 mg/kg) of LPS on the sickness behavior and on the IL-1 β level in the brain in C57BL/6J mice. As shown in Fig. 1, LPS induced an increase in IL-1 β level in the hippocampus (Fig. 1A) and the prefrontal cortex (Fig. 1B) in a dose-dependent way. Besides, as shown in Fig. 1C, at the dose of 0.25 mg/kg, LPS did not consistently produce obvious sickness behavior in 8-week-old C57BL/6J mice. However, at the dose of 5 mg/kg, LPS induced too severe sickness behavior. With very few exceptions, at the dose of 1.25 mg/kg, LPS reliably induced obvious sickness behavior in the C57BL/6J mice. As shown in Fig. 1D, all 3 doses of LPS led to a significant reduction in body weight of mice. Therefore, the dose of 1.25 mg/kg was used in the subsequent experiment.

3.2 LPS does not impair the retrieval of spatial memory in

mice As shown in Fig. 2A and B, before LPS injection, mice in both groups acquired the spatial learning very well in the training sessions. Testing was performed 24 h after the last training session. LPS was injected before the testing to examine its effects on the retrieval of spatial memory. Data from the animals that exhibited no evidence of sickness-related changes after LPS injection were excluded from statistical analysis. In our experiment, one mouse failed to show any indications of sickness behavior after LPS injection. As shown in Fig. 2C and D, there was no significant difference in the time spent in the target quadrant [$F(3, 34) = 0.32, P = 0.81$; Fig. 2C] or in the number of platform location crosses [$F(3, 34) = 0.26, P = 0.85$; Fig. 2D] between control and LPS groups, suggesting that LPS did not affect the retrieval of spatial memory in mice.

In order to make sure that our model setting had no problem, scopolamine, which had been shown to impair memory retrieval, was used as a positive control. As shown in Fig. 2E, one-way ANOVA revealed a significant effect of scopolamine on the time spent in the target quadrant in the probe trial testing [$F(3, 27) = 5.62, P < 0.01$]. Newman-Keuls *post hoc* analysis showed a significant difference between control and scopolamine groups ($P < 0.01$). In addition, scopolamine-treated mice also spent less time in the target quadrant in the testing than in the training ($P < 0.01$). Similar results were obtained from the cross times of the platform location [$F(3, 27) = 9.16, P < 0.001$; Fig. 2F]. As shown in Fig. 2G, the swimming paths during the testing (lower panel) differed among groups. Mice in control and LPS groups searched predominately in the target quadrant, like in the training session. However, scopolamine-treated mice searched predominately in another quadrant.

3.3 Effects of LPS on swimming speed and hippocampal IL-1 β level in the mice tested in the MWM Although LPS treatment reduced the locomotor activity of mice and decreased the swimming speed in the water maze, the swimming speed was not significantly changed as shown in Fig. 3A [$F(3, 34) = 0.19, P = 0.90$]. In order to confirm the elevation of IL-1 β level in the hippocampus of mice tested in the MWM, some mice were decapitated after the testing and the hippocampal IL-1 β level was measured. As shown in Fig. 3B, LPS

Fig. 1 Brain IL-1 β protein production and sickness behavior induced by different doses of LPS. IL-1 β protein levels in hippocampus (A) and prefrontal cortex (B) were measured 4 h after peripheral i.p. injection of vehicle or different doses of LPS ($n=5$ in each group). C: Sickness-related behaviors and general appearance were assessed 4 h after peripheral administration of LPS. Each animal was ranked using a series of well-established ordinal scales ($n=8$ in each group). D: Effect of LPS on body weight. Body weight was measured just before LPS injection and 24 h after LPS administration ($n=8$ in each group). Data were presented as mean $\pm$ SD. N.D., not detected. HIP: hippocampus. PFC: prefrontal cortex. LPS: lipopolysaccharide. The means not sharing a common letter were significantly different between groups.

induced a significant elevation of IL-1 β level in the hippocampus of mice.

4 Discussion

When studying the effects of immune activation on learning and memory, there are some potential confounding factors such as sickness, decreased motivation, and increased stress. Recently, Cunningham *et al.* have reviewed the major potential confounding factors such as state-dependent effects, alternative search strategies induced by stress and

locomotor activity decrements when studying the effects of LPS or IL-1 β on learning and memory in the water maze^[33]. Firstly, learning experience is always accompanied with contextual stimuli. Although contextual cues typically refer to external stimuli, the internal state of the animal may also become a contextual cue. The state of illness would alter the way by which the animal encodes the learning experience. It is well known that in human, the context or state in which learning occurs plays an important role in subsequent retrieval of memory^[34]. Therefore, if LPS is given prior to learning,

Fig. 2 Effects of LPS administration on the retrieval of spatial memory. **A:** The learning curve of mice in control group in the MWM training sessions. **B:** The learning curve of mice in LPS group in the MWM training sessions. Data were the average scores across 4 trials per test day, and presented as mean $\pm$ SEM. $n=10$ in each group. LPS: lipopolysaccharide. There was no significant difference in the time spent in the target quadrant (**C**) or in the number of platform location crosses (**D**) between the control and LPS groups, indicating that LPS did not impair the retrieval of spatial memory ($n=10$ in control group, $n=9$ in LPS group). Moreover, the time spent in the target quadrant and the number of platform location crosses in scopolamine-treated mice were shown in **E** and **F**, respectively. $^{\#}P < 0.01$ vs the control in the testing. $^{*}P < 0.01$ vs the same group in the training. Data were shown as mean $\pm$ SD. $n=8$ in each group. **G:** Representative swimming paths during memory retrieval in the 3 groups were recorded.

Fig. 3 Effects of LPS on swimming speed (A) and hippocampal IL-1 β level (B). The swimming speed of the mice was not significantly changed by LPS ($n=10$ in control group, $n=9$ in LPS group), while the hippocampal IL-1 β level in LPS-treated mice was significantly elevated ($n=5$ in each group). N.D.: not detected, LPS: lipopolysaccharide. Data were shown as mean $\pm$ SD.

it would be difficult to interpret the state-dependent effects on memory. Secondly, it is well described that LPS induces the activation of the hypothalamic-pituitary-adrenal stress axis^[35,36]. In order to reduce the stressful conditions of immune stimulation, stressed animals usually pursue thigmotaxic strategies to avoid spending time in open, anxiogenic areas of mazes^[37,38]. If a mouse does pursue a thigmotaxic strategy (for example, swimming along the walls of the maze) in the water maze, it may just deploy a different learning pattern rather than the possibility that learning is impaired. Thirdly, LPS will result in locomotor hypoactivity^[39]. So the memory impairment of animals indicated by the increased latency to locate the platform may be simply due to the decreased swimming speed in response to locomotor activity decrements. Based on the above considerations, LPS was administered after the acquisition of spatial learning. We did not observe the thigmotaxic strategy employed by the LPS-treated mice, which may be attributed to the training sessions reducing stress in animals in the water maze. Here the swimming speed data indicated that LPS did not cause obvious motor deficits in 8-week-old C57BL/6J mice in the water maze.

The pro-inflammatory factor IL-1 β has been implicated in learning and memory. An inverted U-shaped model for the influence of IL-1 on memory has been proposed^[40]. This model hypothesizes that IL-1 at the physiological or low level is necessary for memory formation, while elevated IL-1 levels have detrimental effects on memory. Indeed, extensive evi-

dence has shown that the elevation of IL-1 β level is involved in memory impairment. First, the detrimental effects of direct peripheral or central administration of IL-1 β on learning and memory have been widely reported^[40]. Moreover, it is believed that IL-1 β is a key factor mediating the impairment of learning and memory induced by activation of immune system and psychological stresses, since administration of IL-1ra can block the impairment^[29,41-43]. However, there is also evidence showing no effects of IL-1 β on learning and memory. Thomson and Sutherland have shown that i.p. injection of IL-1 β (2 μ g/kg) has no effect on learning and memory in the MWM^[44]. In another study, they reported that LPS (2 μ g/kg) could impair the hippocampus-dependent memory consolidation, which could not be replicated by systemic injection of IL-1 β even at a very wide range of doses (0.1, 2, 4, and 100 μ g/kg)^[45]. This suggests that systemic IL-1 β injection is not sufficient to cause memory consolidation deficits. It has been shown that intrahippocampal injection of IL-1 β (10 ng/site) does not impair the working memory of rats in the three-panel runway task, while at the doses of 32 and 100 ng/site, IL-1 β can cause the impairment in working memory^[46]. Another study has reported that intracerebroventricular injection of IL-1 β immediately before training does not impair the rat performance on day 2 in the water maze, while IL-1 β injection 1 h before training does^[47]. When tested in cold water (18 °C), mice treated with IL-1 β do not show any impairment in spatial learning in the water maze, while at 23 °C, the

IL-1 β -treated animals show impairment in spatial learning^[48]. Besides, bilateral injection of IL-1 β directly into the hippocampus 48 h after pre-exposure to the context has no effect on fear conditioning in rats, but injection immediately, 3 h, and 24 h after pre-exposure to the context can impair the fear conditioning^[49]. Moreover, injection of *Escherichia coli* (*E. coli*) could impair contextual fear responses and long-term memory in water maze in old (24-month-old), but not in young (3-month-old) rats^[50]. Further studies have revealed that infection with *E. coli* could significantly increase the IL-1 β level in hippocampus in old rats, compared with *E. coli*-treated young ones. Another study also shows that *E. coli* injection 4 d or 8 d prior to the contextual fear conditioning could impair hippocampal memory consolidation in older rats, but not in younger rats^[51]. Correspondingly, older rats, but not the younger rats, continue to show elevated levels of IL-1 β in the hippocampus at days 4 and 8 post infection. These data indicate that aged rats are more vulnerable to disruptions of memory following the injection of *E. coli*. Based on the above discussion, it seems that elevated IL-1 β level is essential for the impairment of learning and memory. However, the induction of impairment in learning and memory also depends on other important factors, such as dose^[46], time^[47,49], environment^[48], and age^[50,51].

It has been suggested that the description of cognitive effects after immune activation should be more cautious and more specific. Indeed, the effects of immune activation on learning or memory, different types of memory (hippocampus-dependent or -independent, short-term or long-term, *etc.*), and different phases of memory (consolidation, storage and retrieval) need to be more specifically elucidated. Using contextual fear conditioning, Pugh *et al.* have reported the detrimental effects of immune activation on memory consolidation^[29, 43, 52]. It has been suggested that the hippocampus, which is important for the encoding and retrieval of spatial memory^[53-56], is one of the important targets through which pro-inflammatory cytokine IL-1 β exerts its effects on learning and memory^[52]. In the present study, although immune activation by LPS induced a significant elevation of IL-1 β level in the hippocampus of mice, it did not impair the retrieval of spatial memory in the MWM, indicating that im-

mune activation after the consolidation of spatial memory does not affect the retrieval of the memory.

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免疫激活对空间记忆提取的影响

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摘要: 目的 神经系统和免疫系统之间存在广泛的相互作用。许多研究表明, 细菌脂多糖(lipopolysaccharide, LPS)或白介素 1 β 引起的免疫激活会造成学习记忆的损伤。但也有一些研究报道免疫激活对学习记忆几乎没有影响。因此, 免疫激活与学习记忆损伤之间的关系尚不十分清楚。本研究旨在检测 LPS 对空间记忆提取的影响, 探讨免疫激活与记忆之间的相互作用。**方法** 先在水迷宫中训练 8 周龄 C57BL/6J 系小鼠, 随后小鼠腹腔注射 LPS 并进行空间定向能力测试。记录小鼠在目标象限游泳的时间和穿过原平台位置的次数, 以判断动物记忆提取再现能力。用酶联免疫法检测小鼠海马中白介素 1 β 的水平。**结果** LPS 能引起小鼠产生明显的病态行为, 并导致海马中白介素 1 β 的水平显著升高。注射 LPS 的小鼠在目标象限游泳的时间以及穿越逃生台位置的次数与对照组小鼠相比没有显著差异。**结论** LPS 引起的免疫激活并不损害空间记忆的提取。

关键词: 细菌脂多糖; 免疫激活; 病态行为; 海马; 白介素 1 β ; 水迷宫; 空间记忆; 记忆提取