Brain Research Bulletin 120 (2016) 25–33

Contents lists available at ScienceDirect

Brain Research Bulletin

journal homepage: www.elsevier.com/locate/brainresbull

Research report

Neonatal BCG vaccination of mice improves neurogenesis and behavior in early life

Junhua Yang[1], Fangfang Qi[1], Huaiyu Gu, Juntao Zou, Yang Yang, Qunfang Yuan, Zhibin Yao*

Department of Anatomy and Neurobiology, Zhongshan School of Medicine, Sun Yat-Sen University, #74, Zhongshan No. 2 Road, Guangzhou 510080, PR China

ARTICLE INFO

Article history:
Received 26 August 2015
Received in revised form 14 October 2015
Accepted 27 October 2015
Available online 1 November 2015

Keywords:
Vaccine
Th bias
Cytokine
Neurotrophin
Hippocampus
Microglia

ABSTRACT

Bacillus Calmette–Guérin (BCG) is administered to neonates worldwide, but it is still unknown whether this neonatal vaccination affects brain development during early postnatal life, despite the close association of the immune system with the brain. Newborn C57BL/6 mice were injected subcutaneously with BCG or phosphate-buffered saline (PBS) and their mood status and spatial cognition were observed at four and eight weeks (w) old. The mice were also subjected to tests at 2 and 6 w to examine BCG's effects on neurogenesis, the hippocampal microglia phenotype and number, and the expression of hippocampal neuroimmune molecules and peripheral cytokines. The BCG-injected mice showed better behavioral performances at 4 w. We observed elevated neurogenesis, M2 microglial activation and a neurotrophic profile of neuroimmune molecules [more interferon (IFN)-γ, interleukin (IL)-4, transforming growth factor (TGF)-β, brain-derived neurotrophic factor (BDNF) and insulin-like growth factor (IGF)-1 and less tumor necrosis factor (TNF)-α and IL-1β] in the hippocampus of the 2-w-old BCG-mice. In the periphery, BCG induced a T helper (Th)-1 serum response. At the individual level, there were positive correlations between the serum IFN-γ/IL-4 ratio and the levels of neurotrophins and neurogenesis in the hippocampus. These findings suggest that neonatal BCG vaccination improved neurogenesis and mouse behavior in early life by affecting the neuroimmune milieu in the brain, which may be associated with a systemic Th1 bias.

1. Introduction

BCG is administered to neonates and children worldwide (Centers for Disease and Prevention, 2013). According to the theory of perinatal programming (Barker et al.,1995; Karrow, 2006; Dinel et al., 2014), the brain has been shown to be susceptible to external factors, such as immune activation, which modulates the course of normal brain development. Accumulating evidence shows that early postnatal *Escherichia coli* infection or LPS exposure leads to increased anxiety, disease susceptibility, and a vulnerability to cognitive impairment in later life (Walker et al., 2004; Bilbo et al., 2005b; Spencer et al., 2005). Nonetheless, it has been reported that neonatal exposure to a bacterial infection made the rats more resistant to stressor-induced depression (Bilbo et al., 2008b). It has been reported by our and others' studies that prenatal immune activation influences neurogenesis and behavioral function in offspring (French et al., 2013; Xia et al., 2014a). We have also verified that neonatal BCG vaccination promoted the dendritic development of rat hippocampal neurons (Li et al., 2015).

It is verified that the Th1/Th2 balance serves as a mediator for immune activation by affecting the central nervous system (CNS). A Th1 bias is regarded to be neurobeneficial and a Th2 bias is neurodetrimental. It was reported that cognitive deficits were related to a decreased systemic Th1/Th2 balance and could be reversed when the balance was restored (Palumbo et al., 2012; Baruch et al., 2013; He et al., 2014). We have reported that influenza vaccines administered during pregnancy induced a systemic Th1 bias and

Abbreviations: BCG, Bacillus Calmette–Guérin; BDNF, brain-derived neurotrophic factor; BrdU, 5-bromo-2-deoxy uridine; CNS, central nervous system; Dcx, doublecortin; DG, dentate gyrus; EPM, elevated plus maze; GFAP, glial fibrillary acidic protein; Iba-1, ionized calcium-binding adaptor molecule 1; IFN-γ, interferon-γ; IGF-1, insulin-like growth factor-1; IL-1β, interleukin-1β; IL-4, interleukin-4; IL-6, interleukin-6; MWM, Morris water maze; NeuN, neuronal nuclei; OFT, open field test; P0, postnatal day 0; PBS, phosphate-buffered saline; RM-ANOVA, repeated measures analysis of variance; SYSU, Sun Yat-Sen University; TGF-β, transforming growth factor-β; Th, T helper; TNF-α, tumor necrosis factor-α and w, weeks.

* Corresponding author. Fax: +86 20 87330709.
E-mail address: yao.zb@163.com (Z. Yao).

[1] Junhua Yang and Fangfang Qi contributed equally to this study and share first authorship.

http://dx.doi.org/10.1016/j.brainresbull.2015.10.012
0361-9230/

increased hippocampal neurogenesis and neurotrophin levels in both the dams and their offspring (Xia et al., 2014a; Xia et al., 2014b).

Recently, it has been found that BCG vaccination exerted neuroprotection in several CNS diseases models (Lee et al., 2008; Yong et al., 2011; Laćan et al., 2013; Bourdette and Naismith, 2014). However, it is not known whether neonatal BCG vaccination, which induces a strong Th1 immune response, can influence neurogenesis and behavior. The present study was conducted to address this issue.

2. Experimental procedures

2.1. Animals and study design

Newborn litters of C57BL/6 mice were obtained from the SYSU Laboratory Animal Center (Guangzhou, China) and were housed in a specific pathogen-free facility. This study consisted of many different tests, each of which used two groups of mice (BCG group and CON group). All experiments in the study were started on postnatal day 0 (P0).

For the open field test (OFT), four to six newborn litters of C57BL/6 mice were used to produce an experimental group and the matched control group. Within each litter, equal numbers of neonates of each sex were obtained and the extra pups of the sex with larger numbers were used in other studies. Next, 24 neonates were selected and divided into two groups (12 pups/group) with an equal background and sex. The pups were weaned on P25. All studies were performed in accordance with the National Institute of Health's Guide for the Care and Use of Laboratory Animals (NIH Publications No. 80-23) that was revised 1996, and approved by the Institutional Animal Care and Use Committee of SYSU. The two groups were prepared similarly for other tests.

2.2. Immunization procedures

BCG was administered to the mice in a single dose on P0, imitating the procedure for human infants. Freeze-dried living BCG (D2-BP302 strain, Biological Institute of Shanghai, China) was suspended in PBS. For the BCG group, the P0 mice were subcutaneously injected in the back with 25 μl/mouse of the BCG suspension (10^5 CFU). The control groups were injected with PBS using the same procedure. The BCG dosage was initially selected based on the previous studies (Shen et al., 2008). The specific dosage (10^5 CFU) was eventually determined by a preliminary study (Table S2 and Fig. S1).

2.3. Selection of the time points for the tests

The ethologic tests were conducted at the ages of four and eight weeks (w). The time points were chosen because they coincide with the important age span when the mice grow from juveniles into adults. Neurogenesis and neurochemicals are involved in the cellular and molecular mechanisms underlying behavior, and thus should be theoretically influenced by immune activation earlier than behavior. Thus, neurogenesis and certain neuroimmune molecules were examined at 2 and 6 w, 2 w earlier than the behavior tests. Notably, these time points for the hippocampal neurogenesis tests are consistent with the timeline for the development of newborn neurons in the hippocampus (Zhao et al., 2008).

2.4. OFT

As described previously (Chandran et al., 2008), the animals were placed in a 40 cm × 40 cm × 38 cm Plexiglas cubicle. The animals' spontaneous motor activities were measured for 30 min using the Flex-Field activity system (San Diego Instruments, CA). Using the Flex-Field software, the numbers of beam breaks, the distance traveled and the time spent in the center zone were quantified for each mouse, and the average numbers were compared between groups. The apparatus was cleaned with 70% ethanol after each trial.

2.5. Elevated plus maze (EPM)

The mice were subjected to the EPM task after the OFT task on the same day. The mice were individually placed in the center facing a constant open arm and were allowed to freely explore the apparatus for 5 min. The behavior of the animal was recorded with a video tracking system (Noldus EthoVision XT, the Netherlands). The time spent in the open arm, the numbers of entries into the open arm and the number of times the mice arrived at the ends of the open arms were analyzed. The apparatus was cleaned as in the OFT.

2.6. Morris water maze (MWM)

Briefly, each mouse was given four successive trials per day for four consecutive days, followed by a single probe trial on the fifth day, which consisted of a 60 s free swim in the pool with the platform removed. For each trial during the first four days, each mouse was given a ceiling time of 60 s to search for the hidden platform. The detailed protocol is in the Supplementary material.

2.7. 5-Bromo-2-deoxy uridine (BrdU) labeling and tissue preparation

At 2- or 6-w-old, the mice received five intraperitoneal injections of BrdU (Sigma–Aldrich, 50 mg/kg) once every 12 h to label the dividing cells. The mice were deeply anaesthetized and transcardially perfused with 4% paraformaldehyde at two, seven and 21 days after the first injection. The brains excised at two days after labeling were stained for the $BrdU^+$ cells, at seven days after labeling for the $BrdU^+$/doublecortin $(Dcx)^+$ cells and at 21 days after labeling for the $BrdU^+$/neuronal nuclei $(NeuN)^+$ cells. The brains were subsequently fixed overnight in 4% PFA at 4 °C and dehydrated with 30% sucrose at 4 °C. Serial coronal sections (40 μm) were collected on a freezing microtome (Leica SM2000R) and were stored in PBS at 4 °C before immunostaining.

2.8. Immunofluorescence and cell quantification

The sections were incubated in 2 N HCl for 30 min at 37 °C and then blocked in PBS containing 1% BSA and 0.25% Triton X-100 (Sigma–Aldrich) at 37 °C for 1 h. The specimens were then incubated with the primary antibodies overnight at 4 °C. For single labeling, the sections were incubated with the rat anti-BrdU (1:500; Oxford Biotechnology) or rabbit anti-Iba-1 (1:1000; Wako Chemicals) antibodies. For double labeling, the sections were incubated with the rat anti-BrdU/goat anti-Dcx (1:100; Santa Cruz Biotechnology), anti-BrdU/mouse anti-NeuN (1:1000; Sigma–Aldrich), rabbit anti-Iba-1/goat anti-IGF-1(1:200; R&D Systems) or mouse anti-NeuN/rabbit anti-arginase (ARG) (1:200, proteintech, Chicago, IL, USA) antibodies. On the next day, the sections were stained with the secondary antibodies, including Alexa Fluor 594-conjugated donkey anti-rat, Alexa Fluor 488-conjugated donkey anti-goat, Alexa Fluor-conjugated goat anti-mouse, Alexa Fluor 488-conjugated goat anti-rabbit and Alexa Fluor 555-conjugated goat anti-rabbit (1:400; Invitrogen) antibodies. The labeled cells in the unilateral DG of each animal were quantified using a stereology system, Stereo Investigator (MicroBrightField, Williston, USA). The numbers of labeled cells in the DG were estimated as previously described