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Neonatal vaccination with bacillus Calmette–Guérin and hepatitis B vaccines modulates hippocampal synaptic plasticity in rats

Qingqing Li, Fangfang Qi, Junhua Yang, Luwen Zhang, Huaiyu Gu, Juntao Zou, Qunfang Yuan, Zhibin Yao *

Department of Anatomy and Neurobiology, Zhongshan School of Medicine, Sun Yat-Sen University, Guangzhou 510080, China

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ABSTRACT

Immune activation can exert multiple effects on synaptic transmission. Our study demonstrates the influence of neonatal vaccination on hippocampal synaptic plasticity in rats under normal physiological conditions. The results revealed that neonatal BCG vaccination enhanced synaptic plasticity. In contrast, HBV hampered it. Furthermore, we found that the cytokine balance shifted in favour of the T helper type 1/T helper type 2 immune response in BCG/HBV-vaccinated rats in the periphery. The peripheral IFN-γ:IL-4 ratio was positively correlated with BDNF and IGF-1 in the hippocampus. BCG raised IFN-γ, IL-4, BDNF and IGF-1 and reduced IL-1β, IL-6, and TNF-α in the hippocampus, whereas, HBV triggered the opposite effects.

1. Introduction

The immune system plays a pivotal role in modulating nerve injury, regeneration, and learning and memory, which has been firmly established over the past two decades (Kohman & Rhodes, 2013; Perry, 2004; Yirmiya & Goshen, 2011). Immune activation early in life can significantly affect the development of neural processes (Bitanihirwe et al., 2010; Chen et al., 2013; Chugh et al., 2013; Ito et al., 2010). Global coverage with bovis bacillus Calmette–Guérin (BCG) and hepatitis B virus (HBV) vaccinations has reached more than 80% for neonates. It has been reported that neonatal vaccination with BCG inhibited allergic airway inflammation in mice and may protect against tuberculous meningitis (Freyne & Curtis, 2014; Shen et al., 2008). In addition to BCG, the association between neonatal vaccination with HBV and autism has also been researched (Gallagher & Goodman, 2010). However, the related reports regarding the role of neonatal vaccination have almost all been derived from studies conducted under pathological conditions. The safety and side effects of neonatal vaccination are controversial and need to be evaluated in association with physiological status (Demirjian & Levy, 2009; Hodgins & Shewen, 2012).

According to these reports, the correlation between neonatal vaccination and neural developmental processes warrants investigation. Although the related reports demonstrate that early-life immune activation affects brain development and is related to neurodegenerative diseases, there has been no study reporting whether neonatal vaccination could influence brain development in a physiological manner. Synaptic plasticity is the primary and sensitive model for investigating brain development. Therefore, the purpose of this study was to investigate the changes in hippocampal synaptic plasticity induced by neonatal vaccination.

Previous studies have demonstrated that BCG/HBV vaccination can induce Th1/Th2 serum cytokine bias (Libraty et al., 2014; Ota et al., 2002, 2004). The Th1/Th2 cytokine balance could modulate the expression of neurotrophins in the central nervous system (CNS) (Besser & Wank, 1999). Based on previous reports, we suggested the hypothesis that early-life vaccination may alter this normal developmental trajectory of synapses via regulation of the expression of cytokines and neurotrophins. Hippocampal synaptic plasticity was measured because it is particularly sensitive to neuroinflammation (Min et al., 2009). In this study, neonatal vaccination with BCG/HBV modulated hippocampal synaptic plasticity, including long-term potentiation (LTP), spine density and morphology, and the protein expression of synapses. Interestingly, two opposite alterations of synaptic plasticity were observed to be induced by BCG or HBV vaccination.

2. Materials and methods

2.1. Subjects

Adult male and female Sprague Dawley (SD) rats (70 days) were purchased from the Sun Yat-Sen University laboratory animal centre

Abbreviations: LTP, long-term potentiation; DG, dentate gyrus; BCG, bacillus Calmette–Guérin; HBV, hepatitis B vaccination; CNS, central nervous system; BDNF, brain derived neurotrophic factor; IGF-1, insulin-like growth factor 1; EPSP, excitatory postsynaptic potential.

* Corresponding author at: Department of Anatomy and Neurobiology, Sun Yat-Sen University, #74, Zhongshan No. 2 Road, Guangzhou 510080, China.
E-mail address: yao.zb@163.com (Z. Yao).

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(Guangzhou, China) and were raised in same-sex pairs in a specific pathogen-free facility. The colony was maintained under controlled temperature (22 ± 2 °C) and artificial light under a 12-h cycle, with water and food available *ad libitum*. After acclimation to breeding conditions, males and females were paired into breeders. All experimental protocols were approved by the Institutional Research Ethics Committee at Sun Yat-Sen University.

2.2. Experimental design

The present study included four sets of newborns. The results of hippocampal LTP in this study were based on set one; set two confirmed morphology findings; synaptic protein levels were investigated in set three; and set four was used for cytokine expression analysis. Each set was included in three experiments, and every experiment was conducted at the age of 2, 4, and 8 weeks. These experimental time points were chosen because they are distributed across the important age span when rats grow from juvenile into adults.

Newborn pups were randomly assigned to three experimental groups and three matched control groups. They were administered BCG, HBV, or a combination of BCG and HBV and were named the BCG group, HBV group, and BH group, respectively. The control groups received injections of sterile phosphate-buffered saline (PBS) following the same protocol as that used in the matched experimental group. Every experiment included six groups (three experimental groups and three matched control groups) and the four sets of newborns were conducted at the age of 2, 4, and 8 w, respectively.

2.3. Vaccination

Female rats were visually checked for confirmation of pregnancy, and male rats were removed from cages before the birth of pups [postnatal day 0 (P0)]. Female pups were culled to 12 pups per litter on P0, retaining two females and as many males as possible (Bilbo et al., 2005). All subjects were males to avoid effects of hormonal variation in females (Cui et al., 2009).

The BCG and HBV vaccination procedures imitated those used for human infant vaccinations (Marchant et al., 1999). BCG was administered in a single dose at P0, and HBV was administered in a 3-dose series at P0, P7, and P21. Freeze-dried living Bacillus Calmette–Guérin (D2-BP302 strain, Biological Institute of Shanghai, Shanghai, China) was suspended in sterile PBS. Newborn pups were injected intradermally in the back with 50 μl/rat of BCG suspension containing 10^5 colony forming units (CFU) or 50 μl of sterile PBS according to a previously described procedure (Kiros et al., 2010). The dose was originally chosen because it successfully induced an immune response and cytokine production in the periphery. In the HBV group and matched CON group, newborn pups were intraperitoneally injected with a total volume of 100 μl/rat of HBsAg-aluminium-vaccine (20 μg/ml, yeast-derived, Kangtai Biological Pharmaceutical Company, China) containing approximately 2 μg HBsAg and an equal amount of PBS. The doses of HBV (2 μg/rat) and BCG (10^5 CFU/rat) were chosen based on our pilot experiments because they were effective without causing obvious body weight changes (Table S1 and Table S2). Newborn pups in the BH group were vaccinated with both the BCG and HBV procedures mentioned above. The newborn pups in the CON groups matched to the BH group received PBS injections with the same methods as those used in the BH group. All male pups that underwent synaptic analyses were weaned on P21 and caged separately in sibling pairs; the remaining female pups were culled.

2.4. Electrophysiology

Newborn pups (2, 4, 8 w) were anaesthetised with urethane (20% solution, 1.2 g/kg, i.p.) and positioned in a stereotaxic apparatus (Stoelting Instruments, USA). Body temperature was maintained at 37.0 °C via an electric heating pad. A bipolar concentric tungsten electrode (Concentric Bipolar Microelectrode, WPI, USA) was used to stimulate the medial perforant path (coordinates: from bregma, AP, −8.0 mm; ML, 4.4 mm; DV, 2–2.5 mm below the dura) in the left hemisphere. The stimulating electrode was connected to the output of an isolator (ISO-flex, AMPI, Israel) connected with a stimulator (Axon Digidata 1440 A, MDC, USA). A stainless steel, monopolar recording electrode was inserted into the dentate gyrus (DG) granular cell (coordinates: from bregma, AP, 3.5 mm; ML, 2.15 mm; DV, 2.5–3 mm below the dura (Süer et al., 2009)). The depths of the recording electrode was optimised to maximise the excitatory postsynaptic potential (EPSP), and a superimposed negative population spike (PS) was evoked with a 0.1 mm step. Then the depth of stimulating electrodes were adjusted to maximise the PS amplitude in response to the perforant path stimulation. Two screws in the occipital bone were used as the reference and ground.

Evoked field potentials were scored by a population spike (PS). The test stimulation intensity that produced 50% of the maximum amplitude of the PS was chosen, and the measured test pulse for different animals was between 200 and 400 μA. The test stimuli were performed every 30 s. Recordings were allowed to stabilise for 20 min, and the high-frequency stimulation protocol (HFS, 20 trains of 15 pulses at 200 Hz with an inter-train interval of 5 s) was applied to induce LTP. Following the delivery of the tetanic stimuli, application of the test stimuli was continued for up to 60 min at 0.033 Hz. The percentage of the ratio of EPSP slope to the basal value represented the level of synaptic strength. A slope of EPSP change exceeding 20% was defined as a successful induction of LTP (Bliss & Collingridge, 1993). The magnitude of LTP was between 58 and 60 min in the last bin of the recording session and was expressed as the percentage change from the PS baseline. Values from the final 2-min bin were compared between the immunised group and the corresponding control group using Student's *t*-tests.

2.5. DiOlistic labelling

Dendritic spine density and morphology in DG neurons were assessed by quantifying spines in neurons labelled with the fluorescent dye (1-1′-Dioctadecyl-3,3,3′,3′-tetramethylindocarbocyanine perchlorate) DiI (CM-DiI, Sigma-Aldrich, USA) in 2-, 4-, and 8-w-old pups (Erion et al., 2014). Pups were transcardially perfused with 4% paraformaldehyde and postfixed in the same fixative for 1–2 days at 4 °C. Coronal brain slices (200 μm free-floating) were cut on a vibratome (Leica VT 1000S, Bannockburn, IL). Slices were rinsed and stored in 0.1 M phosphate buffer (PB) for DiI delivery. The gene gun bullets were prepared according to a previously described method (Staffend & Meisel, 2011). Briefly, 8 mg of gold particles (1.0 μm in diameter) was mixed with 2 mg of DiI (CM-DiI, Sigma-Aldrich, USA) and dissolved in 300 μl of methylene chloride. After drying, the coated particles were collected in 2 ml of water, vortexed on a sonicator for 5 min, and immediately transferred to gene gun tubing with a 1-mm diameter (Bio-Rad). The tube was held still for 1 h before slowly withdrawing the remaining liquid. Then, we dried the tubing under a constant nitrogen flow for 30 min. The tube was cut into small sections (2 cm in length) and stored in a desiccated container at 4 °C for up to 1 month. For particle delivery, slices were transferred to a Petri dish, and most of the PB was then removed. The DiI-coated particles were delivered using the Helios Gene Gun system (Bio-Rad) at a pressure of 150–180 psi. To prevent clusters of large particles from landing on the tissue, causing non-specific labelling and preventing single-cell resolution, a membrane filter was inserted between the gene gun and the brain sections. After delivery, slices were incubated overnight in 0.1 M PB at 4 °C to allow the diffusion of the dye along the neuronal processes. Finally, the sections were rinsed 3 times with PB before being mounted on slides and coverslipped with 65% glycerine in 0.1 M PB.

2.6. Photography and confocal imaging

Image acquisition and analysis were performed in a systematic manner by individuals who were blinded to the treatment. On the same day, brain sections were imaged on a confocal microscope (LSM 710, Carl Zeiss, Germany) to acquire a stack of images (z-spacing, 1 μm) of the apical dendrites from isolated DG granule neurons using a 63x oil objective (N.A., 1.4) with a DPS of 568 nm. Three to five secondary dendrites per neuron were imaged (1024 × 1024 pixels, x–y scaling = 0.0952 μm/pixel), and at least three neurons per rat were collected. All segments were imaged from secondary branches in the apical dendritic tree at a similar distance from the cell body across genotypes. Dendritic spine morphology was analysed using the Imaris software package (Version 7.0, Bitplane Inc., St Paul, MN). Spine density was determined by manually identifying spines. Spine area and length were automatically measured in the 3D reconstructive stacks, which can classify spines into stubby, mushroom, long thin, and filopodia on the basis of suitable morphological categories.

2.7. Western blot

Hippocampal tissue was harvested from the vaccinated and control pups at 2, 4, and 8 weeks (n = 4) and homogenised in ice-cold RIPA lysis buffer containing (in mM) 50.0 Tris–HCl, 150 NaCl, 5.0 $CaCl_2$, 0.02% NaN_3, and 1% Triton X-100 in the presence of a protease inhibitor mixture (1 mM PMSF, protease inhibitor cocktail; Sigma-Aldrich). The lysates were then centrifuged at 12,000 g for 20 min at 4 °C after incubation in ice for 30 min. The protein concentration was determined using a BCA Protein Assay Kit (Beyotime). The samples (20 μl per lane) were separated by 8% sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) and electro-transferred onto a polyvinylidenedifluoride (PVDF) membrane (Bio-Rad Lab, Hercules, CA, USA) at 60 V for 30 min and then 90 V for 1 h. Membranes were blocked with 5% non-fat milk in TBS for 2 h at room temperature. The following primary antibodies were used at the given concentrations: synaptophysin (1:500; Sigma-Aldrich), PSD-95 (1:2000; Sigma-Aldrich), NMDAR2A (1:1000; Millipore), NMDAR2B (1:1000; Millipore), NMDAR1 (1:1000; Cell Signalling Technology), and β-tubulin (1:1000; Beyotime). Membranes were then rinsed for 10 min three times in PBST (100 nM phosphate buffer, pH 7.5, containing 150 nM NaCl and 0.1% Tween-20) and incubated with a horseradish peroxidase (HRP)-conjugated secondary antibody (1:5000; Sigma-Aldrich) at room temperature for 2 h. Immunoblots were developed on films using the enhanced chemiluminescence technique (Pierce Chemical Co., Rockford, IL, USA). The intensities of protein bands were quantified and analysed using the NIH Image J software. All assays were performed at least three times.

2.8. Enzyme-linked immunosorbent assay (ELISA)

The levels of IFN-γ, IL-4, TNF-α, IL-6, and IL-1β in serum and in the hippocampus were assessed in duplicate via an ELISA kit (Neobioscience Technology Co., Ltd) as described previously (Xia et al., 2014a). The hippocampal samples were homogenised as described above by Western blotting. The levels of neurotrophic factors in the hippocampus were measured by an ELISA kit (Cusabio Life Science Co., Ltd) according to an optimised manufacturer's protocol at 2, 4, and 8 weeks. Samples were homogenised on ice in buffer (pH 7.6) containing (in mM) 50.0 Tris–HCl, 150 NaCl, 5.0 $CaCl_2$, 0.02% NaN_3, and 1% Triton X-100 and then centrifuged at 17,000 ×*g* for 30 min at 4 °C. The total hippocampal homogenate concentration was adjusted to 4.5 mg/ml by using an Enhanced BCA Protein Assay Kit (Beyotime) according to a previous report (Selenica et al., 2013). The prepared plates were analysed by the microplate reader (BIO-TEk ELx800, USA) at 450 nm.

2.9. Statistical analysis

Data are presented as the means ± SEM. Differences between groups were evaluated by two-way (vaccination × time) ANOVA followed by Bonferroni post-hoc test using SPSS 17.0. The analysis of the correlation between the IFN-γ to IL-4 ratio and BDNF/IGF-1 was performed using Pearson correlation analysis. Statistical significance was set to $p < 0.05$, and analyses were performed using Graph Pad Prism 5.0 (GraphPad Software).

3. Results

3.1. Neonatal vaccination and antibody levels

Each group of rats was administered BCG (BCG group), HBV (HBV group), or a combination of BCG and HBV (BH group) on P0. The serum anti-HBsAg antibody and anti-BCG titres of all control rats were kept at a baseline level. There was a significant increase in antibody titres in the HBV/BCG vaccinated rats compared with their control groups at all three time points (2, 4, and 8 weeks; Table S1 and Table S2). The increase was also observed in the BH groups. No significant differences were observed in physical conditions, such as weight, between the vaccinated rats and the controls (Table S1 and Table S2).

3.2. Neonatal vaccination affects hippocampal LTP in vivo

To investigate the effects of neonatal vaccination on synaptic plasticity, we examined LTP in the DG area in vivo at 2, 4, and 8 weeks postnatal. Two-way ANOVA revealed significant main effects of BCG ($F_{1,30}$ = 12.20, P = 0.002). Subsequent analysis revealed that BCG vaccination facilitated the induction of hippocampal LTP at 2 weeks (166.20 ± 9.11%, p = 0.01, Fig. 1A and E) and 4 weeks (173.22 ± 7.27%, p = 0.016, Fig. 1B and E) compared with the controls. There was no significant main effects of HBV, time or significant interactions of HBV × time. However, Subsequent analysis revealed that HBV vaccination inhibited hippocampal LTP at 8 weeks (126.12 ± 6.21%, p = 0.017, Fig. 1C and F). Interestingly, there was no significant difference in LTP between the BH group and the control group (Fig. 1D and G), which suggested that these two vaccines may counteract each other to a certain extent. Notwithstanding, these data indicated that neonatal BCG and HBV vaccination indeed influenced the synaptic activity in the hippocampus; moreover, the initial effect of BCG vaccination occurred at 2 weeks, whereas that of HBV was delayed to 8 weeks.

3.3. Neonatal vaccination influences dendritic spine density on hippocampal DG granule cells

Dendrites receive and process synaptic inputs from other neurons (Kampa et al., 2007). To elucidate whether the cellular mechanisms of LTP include the formation of new synapses or the remodelling of existing synapses, numerous studies have examined the number and structure of synapses following hippocampal LTP (Geinisman, 2000; Urbanska et al., 2012). To determine the morphological basis for the change in hippocampal LTP following neonatal vaccination, we investigated spine density, length, and area in hippocampal granule neurons at 2, 4, and 8 weeks. DiOlistic labelling was used to label spines. Although both neurons and neuroglia were labelled with DiI, they could be clearly distinguished on the basis of their morphological features (Cui et al., 2010). Granule cell bodies, apical dendrites, several lateral and basilar dendrites, and even dendritic spines could be recognised. There were significant main effects of BCG ($F_{1,\ 48}$ = 4.03, P = 0.049), time ($F_{2,\ 48}$ = 35.13, P = 0.000) and interaction of BCG × time ($F_{2,\ 48}$ = 3.49, P = 0.039) on the density of spines. Moreover, significant main effects of BCG ($F_{1,\ 48}$ = 10.89, P = 0.002) and time ($F_{2,\ 48}$ = 30.21, P = 0.000) on the area of spines were observed. Subsequent analysis revealed that BCG vaccination increased the spine density at 4 w (11.39 ±

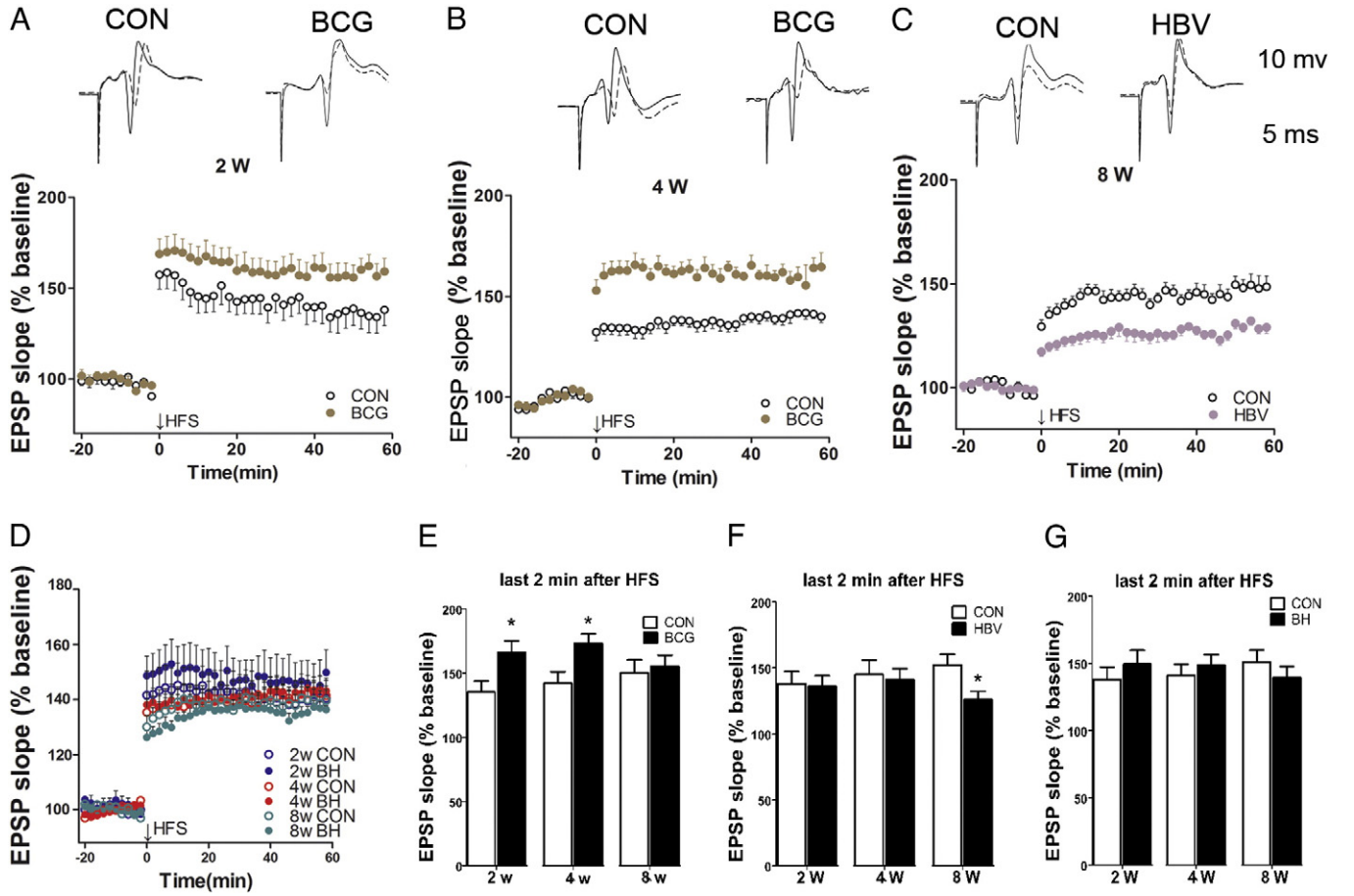

Fig. 1. Effects of neonatal vaccination with BCG, HBV, and BH on hippocampal LTP in vivo. (A) Representative traces (left) of PS before (basal) and after (60 min) HFS. BCG vaccination facilitated the induction of hippocampal LTP at 2 weeks (A) and 4 weeks (B) compared with controls. In contrast, HBV vaccination inhibited the induction of LTP at 8 weeks (C). BH vaccination caused no profound alterations at 2, 4, or 8 weeks compared with their controls (D). (E, F, and G) Summary histograms representing the effects of BCG (E), HBV (F), and BH (G) vaccination on LTP at 60 min post-HFS. Data are presented as the means ± SEM and were analysed with two-way ANOVA followed by Bonferroni post-hoc test. $n = 6–7$ for each group. $^*p < 0.05$.

1.33 10 μm^{-1}, $n = 9$ neurons, $p = 0.001$, Fig. 2B) and the spine area at 2 and 4 weeks (5.49 ± 0.26 μm^2, $p = 0.021$; 13.72 ± 0.17, $p = 0.032$, $n = 9$ neurons, Fig. 2C) compared with their controls. In contrast, HBV vaccination reduced the spine density (HBV: $F_{1, 48} = 4.86$, P = 0.033, time: $F_{2, 48} = 9.85$, P = 0.000) and area (time: $F_{2, 48} = 9.55$, P = 0.000, HBV × time: $F_{2, 48} = 3.69$, P = 0.033) at 8 weeks (HBV vs CON: density: 5.00 ± 0.52 10 μm^{-1}, $n = 10$ neurons, $p = 0.01$, Fig. 2B. Area: 2.45 ± 0.15 μm^2, $n = 10$ neurons, $p = 0.015$, Fig. 2C). Consistent with previous findings, the BH group showed no significant difference in spine density or area (Fig. 2B and C). No significant alternations were observed with respect to the spine length between any two groups (Fig. 2D). Altogether, there is a close correlation between hippocampal LTP and dendritic spine number and structure, suggesting that the functional and structural plasticity of synapses occurred simultaneously following neonatal vaccination. A total of three dendritic segments per neuron, three neurons per pup, and three to five pups were averaged to yield the mean spine density and area for each rat.

3.4. Neonatal vaccination changes the dendritic spine morphology on hippocampal DG granule cells

The number of spines and their morphology have been demonstrated to be important for information processing and are associated with hippocampal LTP (Geinisman, 2000; Urbanska et al., 2012). The plastic changes in spine morphology reflecting the dynamic state of the correlative synapse are responsible to some extent for neuronal circuitry remodelling (Alvarez & Sabatini, 2007). Therefore, we assessed the density of the dendritic spines according to their specific morphology (filopodia, long thin, stubby, and mushroom) by DiOlistic labelling. Our findings revealed that BCG caused a selective increase in mushroom spines (BCG: $F_{1, 48} = 5.21$, P = 0.029, time: $F_{2, 48} = 32.19$, P = 0.000, BCG × time: $F_{2, 48} = 6.11$, P = 0.022) on hippocampal DG granule cells at 4 weeks (BCG vs CON: P = 0.017, Fig. 3B), whereas there was a significant reduction in the number of stubby spines (HBV: $F_{1, 48} = 7.98$, P = 0.009, time: $F_{2, 48} = 22.59$, P = 0.000, BCG × time: $F_{2, 48} = 17.11$, P = 0.002) in the HBV group at 8 weeks (HBV vs CON: $p = 0.037$, Fig. 3C). In line with the previous results, no significant differences were observed between the BH and CON groups (Fig. 3D). Therefore, it was inferred that the selective increase or reduction in mushroom and stubby spines, which have bigger heads, may contribute to the alterations in overall spine density and area.

3.5. Neonatal vaccination affects the expression levels of synaptic proteins in the hippocampus

Synaptophysin is the major integral membrane protein of synaptic vesicles (Thiel, 1993). The main functions of synaptophysin are docking, fusion, and endocytosis, otherwise known as membrane trafficking (Evans & Cousin, 2005). PSD-95, the major scaffolding protein contributing to the excitatory postsynaptic density (PSD) and a potent regulator of synaptic strength, has been considered a key synaptic protein that promotes synapse stability (Chen et al., 2011; Taft & Turrigiano, 2014). It is known that the induction of hippocampal LTP requires synaptic activation of postsynaptic NMDA receptors (Citri & Malenka, 2008).

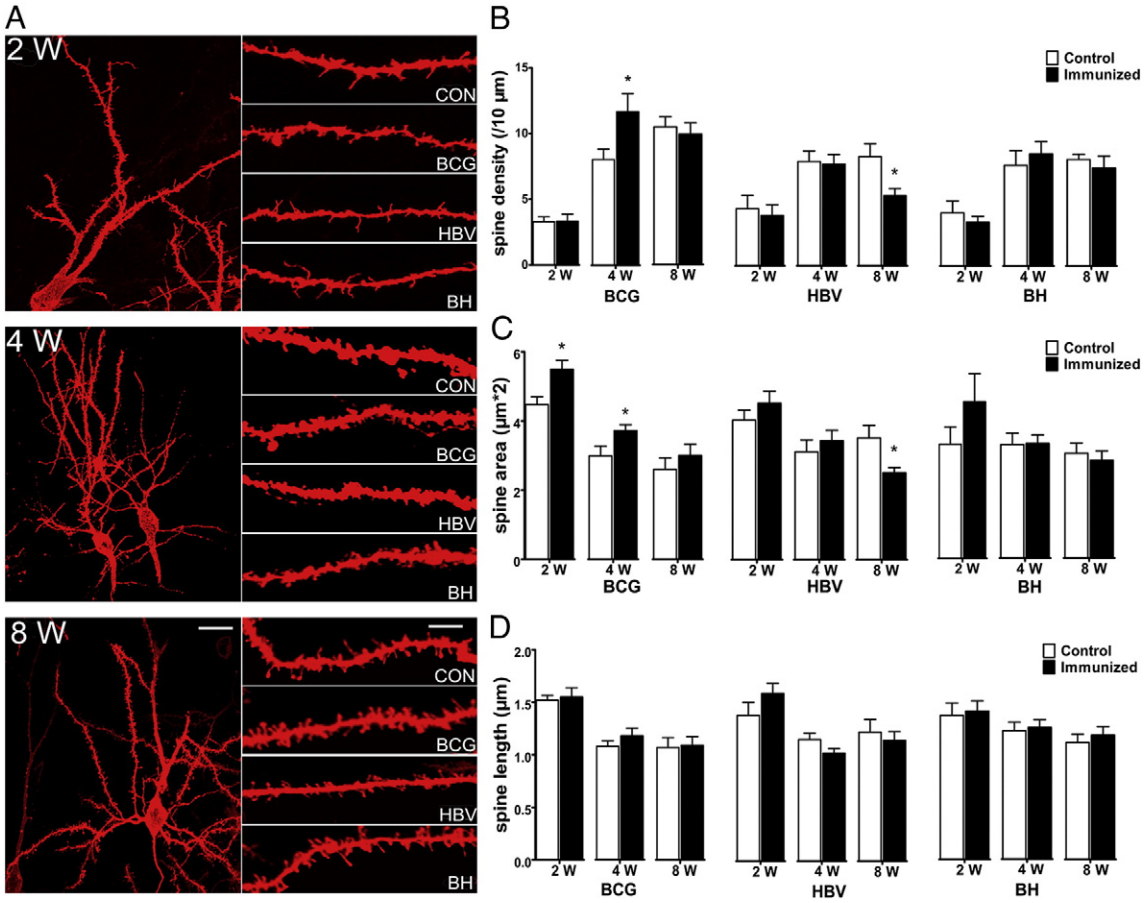

Fig. 2. Alterations in the dendritic spine length, area, and density of granule cells in the DG of the rats vaccinated with BCG, HBV, and BH. (A) A DiOlistic assay was used to visualise dendritic spines in granule cells. Individual granule cell at 2, 4, and 8 weeks, respectively (left), representative sections of lateral dendrites in the CON, BCG, HBV, and BH groups at 2, 4, and 8 weeks. The magnified images are on the right. BCG vaccination increased the spine density at 4 weeks (B) and the spine area at 2 and 4 weeks (C) compared with the controls. Conversely, HBV vaccination reduced the spine area and density at 8 weeks (B and C). BH-vaccinated rats showed no significant difference in spine density or area (B and C). No profound alterations were observed in the spine length between any two groups at any of the three time points (D). Data are presented as the mean ± SEM and were analysed with two-way ANOVA followed by Bonferroni post-hoc test. $n = 9$ neurons (3 dendritic segments per neuron, 3 neurons per pup, and 3 pups). $^*p < 0.05$ versus vehicle control. Scale bar, 10 μm (left), 5 μm (right).

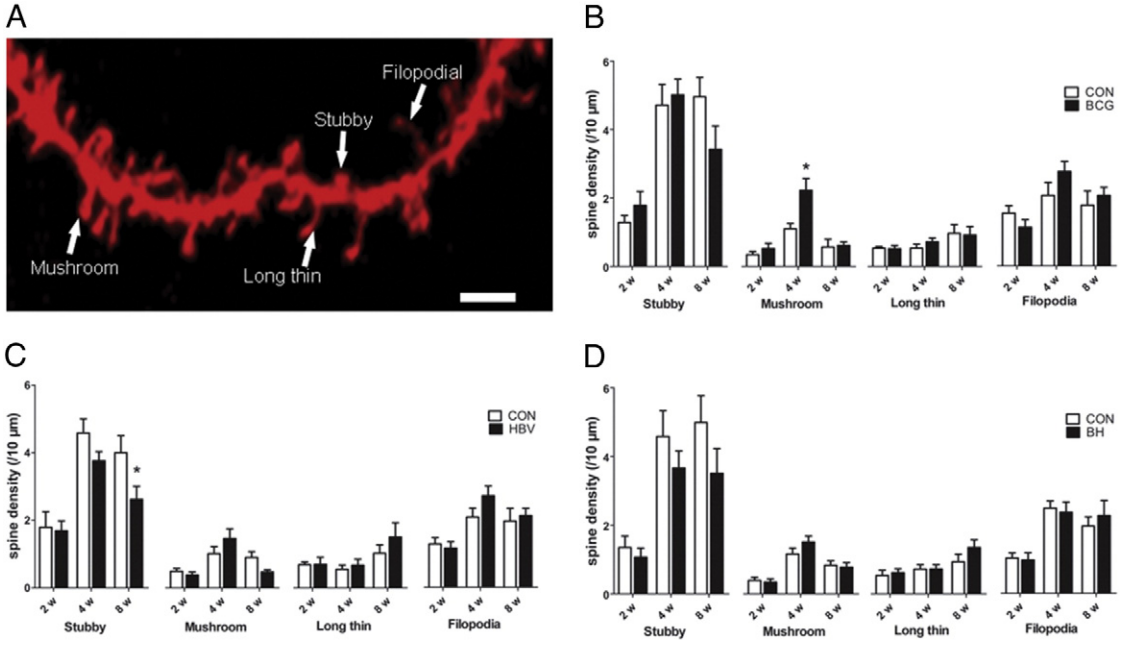

Fig. 3. Alterations in the morphology of the dendritic spines in the hippocampus of vaccinated rats. (A) Representative photomicrograph depicts different morphological subtypes of dendritic spines in relation to the dendritic shaft. BCG vaccination increased the density of mushroom spines at 4 weeks (B). HBV vaccination decreased the density of stubby spines at 8 weeks (C). Data are presented as the means ± SEM and were analysed with two-way ANOVA followed by Bonferroni post-hoc test. $n = 9$ neurons (3 dendritic segments per neuron, 3 neurons per pup, and 3 pups). For all graphs, $^*p < 0.05$ versus vehicle control and scale bar = 5 μm.

Therefore, we applied a Western blotting technique to assess whether the synaptic proteins were subject to modulation by neonatal vaccination. It was demonstrated that BCG vaccination promoted the expression of hippocampal synaptophysin (BCG: $F_{1,\ 30} = 12.29$, $P = 0.001$, time: $F_{2,\ 30} = 13.22$, $P = 0.000$. BCG vs CON: $p = 0.002$, Fig. 4B), PSD-95 (BCG: $F_{1,\ 30} = 5.90$, $P = 0.028$, time: $F_{2,\ 30} = 12.56$, $P = 0.000$) and NMDAR2A (BCG: $F_{1,\ 30} = 4.47$, $P = 0.043$, time: $F_{2,\ 30} = 7.91$, $P = 0.002$. BCG vs CON: $p = 0.01$, Fig. 4F) at 4 weeks.

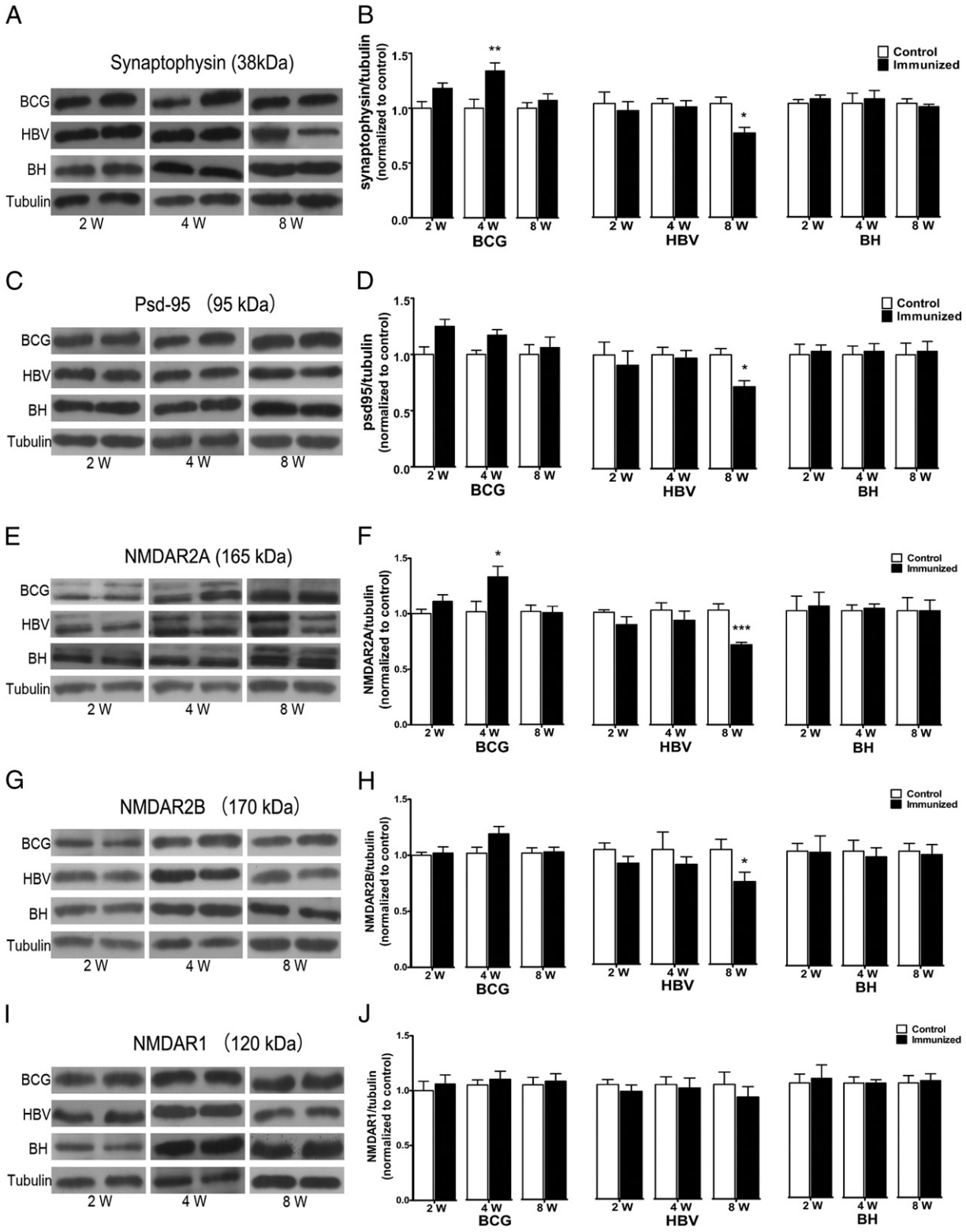

Fig. 4. Neonatal BCG, HBV, and BH vaccinations affect the expression of synaptophysin, PSD-95, NMDAR2A, NMDAR2B, and NMDAR1 in the hippocampus. (A, C, E, G and I) Representative immunoblots of synaptic proteins at 3 time points. BCG increased the synaptophysin (B), PSD-95 (D) and NMDAR2A (F) protein levels at 4 weeks compared with the controls. Conversely, HBV vaccination reduced the synaptophysin, PSD-95, NMDAR2A, and NMDAR2B levels at 8 weeks (B, D, F and H). BH rats showed no significant changes in synaptic proteins. Data are presented as the mean ± SEM and were normalised to the matched control. Two-way ANOVA followed by Bonferroni post-hoc test. $n = 4$ per group. $^{*}p < 0.05$ and $^{**}p < 0.01$ versus the control group.

Conversely, HBV vaccination decreased their expressions at 8 weeks (synaptophysin: HBV: $F_{1, 30} = 5.15$, $P = 0.031$, time: $F_{2, 30} = 16.74$, $P = 0.000$. HBV vs CON: $p = 0.012$, Fig. 4F; PSD-95: NMDAR2A: HBV: $F_{1, 30} = 13.70$, $P = 0.001$, time: $F_{2, 30} = 12.64$, $P = 0.002$. HBV vs CON: $p = 0.000$; NMDAR2B: HBV: $F_{1, 30} = 5.61$, $P = 0.025$, time: $F_{2, 30} = 14.02$, $P = 0.001$. HBV vs CON: $p = 0.042$. Fig. 4B, D, F, and H). No significant changes were observed in these proteins in the hippocampus between the BH and control groups. The results indicated that the alterations in the synaptic proteins may be associated with spine density and morphology because PSD-95, which is one of the most abundant PSD proteins, is involved in synapse maturation (El-Husseini et al., 2000; Prange & Murphy, 2001).

3.6. Neonatal vaccination alters the levels of cytokines and neurotrophins in serum and the hippocampus

Cytokines and neurotrophins play a critical role in the process of brain development under physiological/pathological conditions such as synaptic plasticity (Erion et al., 2014). Therefore, we considered the potential implications of the immune-related diffusible mediator in modulating synaptic plasticity and neural network functioning. Research in this field has focused on several pro-inflammatory cytokines, such as IL-1β, IL-6, and TNF-α, as having a detrimental effect on neuronal function and synaptic plasticity (Spedding & Gressens, 2008). However, IFN-γ, IL-4, BDNF, and IGF-1 are regarded as neurotrophic factors (Yirmiya & Goshen, 2011). Therefore, we investigated potential changes in the mediators related to immune activation and synaptic plasticity. The levels of cytokines and neurotrophins are shown in Table S3 (serum) and Table S4 (hippocampus). Our data revealed that the BCG group displayed a neurotrophic expression profile of increased IFN-γ, IL-4, BDNF, and IGF-1 and decreased TNF-α, IL-1β, and IL-6 at 2 and 4 w, whereas the HBV group exhibited a neurotoxic expression profile of increased TNF-α and IL-6 and decreased IFN-γ, BDNF, and IGF-1 at 8 w (see Fig. 5). Interestingly, the BH group exhibited no significant changes in serum molecules, but it exhibited slight alterations in the hippocampus compared with the controls. According to these data, the expression of cytokines and neurotrophins was altered in both serum and the hippocampus after neonatal vaccination. Moreover, the altered trend was almost the same in serum and the hippocampus, which suggested that there was an internal link between them.

3.7. Neonatal vaccination switches the bias of Th1 or Th2 in serum

Recent animal studies on ageing have indicated that hippocampal neurogenesis is associated with a decrease in the Th1/Th2 bias (Baruch et al., 2013). Therefore, to evaluate whether neonatal vaccination regulates the Th1/Th2 bias, we assessed the ratio of classical serum cytokine IFN-γ (Th1) to IL-4 (Th2). Consistent with the previous reports (Libraty et al., 2014; Marchant et al., 1999; Ota et al., 2002, 2004), our findings confirmed that BCG induced a Th1-like response at 2 weeks (BCG: $F_{1, 30} = 8.28$, $P = 0.007$, time: $F_{2, 30} = 3.8$, $P = 0.034$, BCG vs CON: $p = 0.043$, Fig. 6A). In contrast, HBV induced a Th2-like response at 8 weeks (HBV: $F_{1, 30} = 7.48$, $P = 0.01$, time: $F_{2, 30} = 29.65$, $P = 0.000$, HBV vs CON:$p = 0.012$, Fig. 6A). Moreover, no significant difference was observed in the BH group. The association between the Th1/Th2 bias and the central cytokine and neurotrophin milieu and, thus, the impact on synaptic plasticity were further demonstrated by the positive correlation between the IFN-γ:IL-4 ratio and BDNF (2 w: $r^2 = 0.572$, $p = 0.000$; 4 weeks: $r^2 = 0.507$, $p = 0.002$; 8 weeks: $r^2 = 0.386$, $p = 0.02$; $n = 36$; Pearson correlation analysis; Fig. 6B) and IGF-1 (2 weeks: $r^2 = 0.518$, $p = 0.001$; 4 weeks: $r^2 = 0.472$, $p = 0.004$; 8 weeks: $r^2 = 0.131$, $p = 0.446$; $n = 36$; Pearson correlation analysis; Fig. 6B) levels. Interestingly, the results showed decreased correlation coefficients between the IFN-γ:IL-4 ratio and BDNF and IGF-1 levels as time progressed.

4. Discussion

Our findings support the hypothesis that neonatal vaccination with BCG or HBV modulates hippocampal synaptic plasticity probably via the neuro-beneficial or neuro-detrimental expression profiles of hippocampal cytokines and neurotrophins. BCG vaccination facilitated the induction of hippocampal LTP, increased the spine density and area, elicited a selective increase in mushroom spines in the hippocampal DG area, and elevated hippocampal synaptophysin, PSD-95, and NMDAR2A protein levels. Conversely, HBV vaccination showed almost inverse alterations of all these aspects. In this study, BCG induced a Th1-like response followed by increased neurotrophins in the brain, whereas HBV induced a Th2-like response followed by decreased neurotrophins. In addition, positive correlations between the IFN-γ:IL-4 ratio and BDNF and IGF-1 were observed. Interestingly, BH showed no obvious shift to either a Th1 or Th2 response and no significant influence on synaptic plasticity.

LTP is the most extensively studied model of the cellular mechanisms of synaptic plasticity (Bliss & Collingridge, 1993; Bliss & Lomo, 1973). In the present study, PP-DG LTP was determined based on the particular sensitivity of the DG structure to both endogenous and exogenous signals, such as immune activation and pro-inflammatory cytokines, particularly IL-1β and TNF-a (Beattie et al., 2002; Di Filippo et al., 2008). The present study demonstrated that neonatal BCG vaccination transiently facilitated the induction of hippocampal LTP in rats. However, HBV impaired hippocampal LTP. These data confirm our speculation that altered immune status induced by vaccination modulates hippocampal synaptic plasticity during early life, which is different from immune activation models induced by LPS, poly (I:C), and *Escherichia coli* (*E. coli*) under pathological conditions.

Dendritic spines are important and pleomorphic structures in the collection, integration, and transmission of neural signals. Thus, the alterations of spine density and morphology may contribute to hippocampal LTP. We found that the changes in spine area and density were consistent with the results of hippocampal LTP in the DG area. It should be noted that only two subtypes of dendritic spines, namely, stubby and mushroom (mature and stable, with bigger heads that allow the passage of more current (Zhao et al., 2006; Urbanska et al., 2012)), were altered in granule neurons in the DG area in BCG/HBV-vaccinated rats (BCG rats/HBV rats), and no difference was observed in immature dendritic subtypes (thin and filopodia) in either experimental group. Together with spine density and area, the subsequent potential alterations in mature and efficient spine subtypes may contribute to the performance of hippocampal LTP in BCG and HBV rats. Recent evidence suggests that activated immune cells secrete cytokines and growth factors, which can modulate synaptic transmission (Henneberger et al., 2005; Pickering et al., 2005) and alter dendritic spine morphology (Schratt et al., 2006; von Bohlen und Halbach et al., 2006). The opposite findings regarding spine density and morphology between the BCG and HBV rats may be due to the different cytokine and neurotrophin networks induced by neonatal vaccination with BCG/HBV.

In addition to the structural plasticity of dendritic spines, the functional plasticity and molecular mechanisms involved in regulating synaptic transmission also require exploration. Both of these mechanisms may potentially contribute to the alterations in LTP observed in vaccinated rats. The synapse proteins, including synaptophysin, PSD-95, and NMDA receptors, play an important role in synaptic plasticity (Kamphuis et al., 1992; Liu et al., 2004; Monyer et al., 1992). Therefore, the modulation of these proteins by immune activation may influence synaptic transmission. Our findings showed that changes in synaptic proteins were almost parallel to the changes in LTP, spine density, and morphology. Based on these results, altered synaptic proteins induced by vaccination may be another contributory factor to the induction of hippocampal LTP observed in vaccinated rats. The BCG vaccination-induced increase in spine density and synaptic proteins may represent

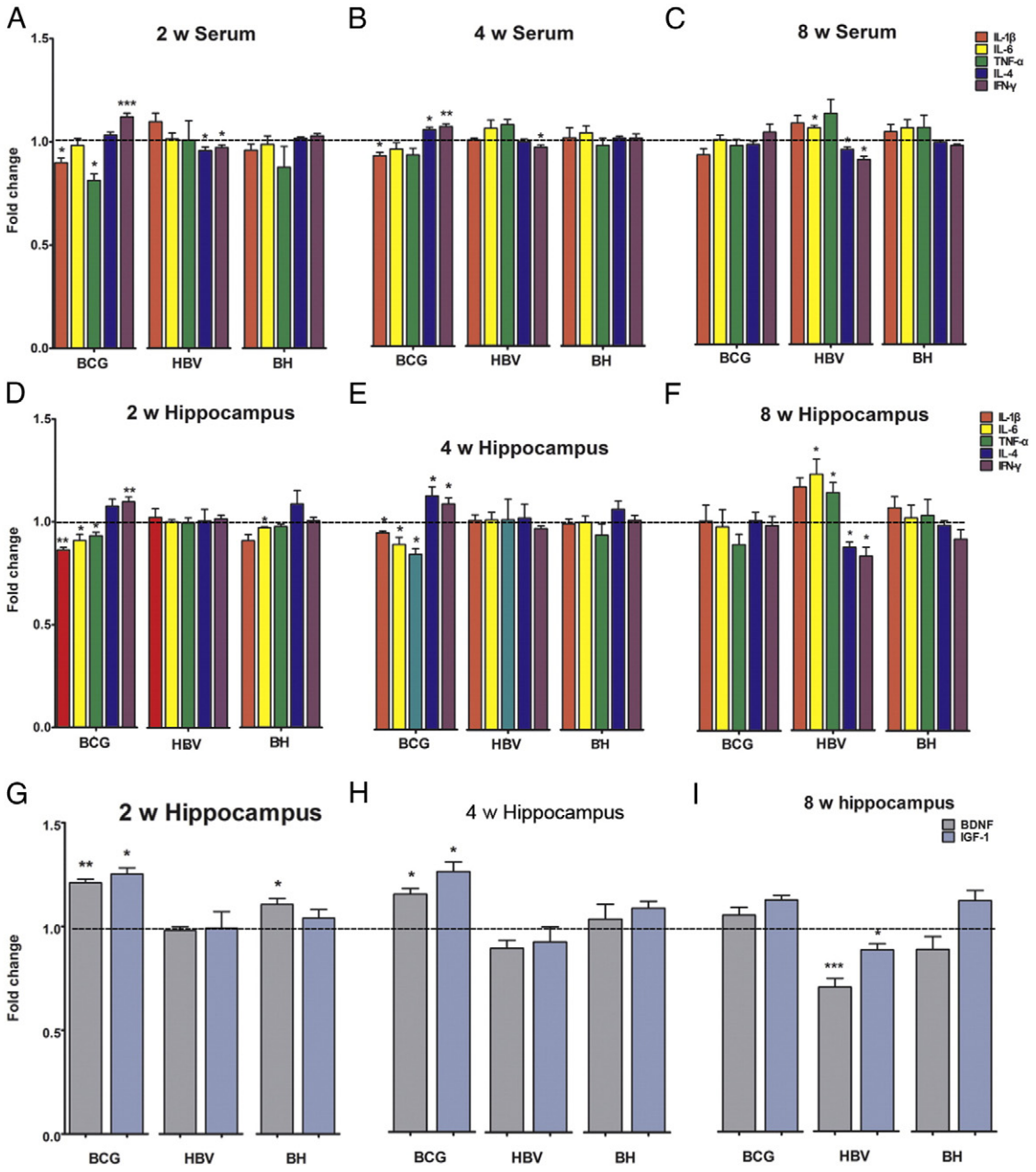

Fig. 5. Neonatal BCG, HBV, and BH vaccination alters the levels of cytokines and neurotrophins in the hippocampus and serum. The levels of IL-1β, IL-6, TNF-α, IL-4, IFN-γ, BDNF and IGF-1 in serum (A, B and C) and the hippocampus (D, E, F, G, H and I) were normalised and analysed at 2, 4, and 8 weeks. In serum, BCG vaccination up-regulated IL-4 at 4 weeks (B) and IFN-γ at 2 weeks (A) and 4 weeks (B), whereas it down-regulated IL-1β and TNF-α at 2 weeks (A) and 4 weeks (B) and down-regulated IL-6 at 4 weeks (B). HBV vaccination up-regulated the level of IL-6 at 8 weeks (C), which decreased the level of IFN-γ at 2 weeks (A), 4 weeks (B), and 8 weeks (C) and decreased the level of IL-4 at 2 weeks (A) and 8 weeks (C). Alterations in the hippocampus were almost consistent with those in serum (D, E and F). BCG vaccination increased the expression of BDNF and IGF-1 at 2 weeks (G) and 4 weeks (H), whereas HBV decrease them at 8 weeks (I). Data are presented as the means ± SEM normalised to the controls and were analysed with two-way ANOVA followed by Bonferroni post-hoc test. $n = 6$ for each group. $^{*}p < 0.05$, $^{**}p < 0.01$, and $^{***}p < 0.001$ versus the control group.

an enhanced excitatory synaptic connectivity in the early stage of synaptogenesis. Accordingly, it has been reported that there was a correlation between spine density, PSD-95, and the inflammatory environment (Chugh et al., 2013; Jakubs et al., 2006). Although recent studies have demonstrated that inflammatory cytokines participate in physiological and pathological events depending on PSD-95 protein level or NMDA receptor activation (Gardoni et al., 2011), how the vaccination-related cytokine network modulates the expression of synaptic proteins remains elusive.

The most important question for further study is the underlying mechanism mediating synaptic transmission and structure and the potential difference between the two vaccines. It has been reported that early life events altered this normal developmental trajectory of the brain, specifically synaptic plasticity, via their specific impact on cytokine and neurotrophin expressions (Goshen et al., 2007; Yirmiya & Goshen, 2011). Therefore, the hippocampal homogenate was collected to determine the profile of these mediators in relation to immune activation. IL-1β, IL-6, and TNF-a, which have been associated with cognitive decline, inhibited synaptic plasticity and caused hippocampal LTP impairment in previous studies (Balosso et al., 2008; Viviani et al., 2003; Viviani et al., 2006). It has also been demonstrated that both IL-4 and IFN-γ contribute to hippocampal LTP and neurogenesis (Nolan et al., 2005; Zhu et al., 2011). In line with previous reports, our results showed that the levels of IL-4 and IFN-γ were significantly

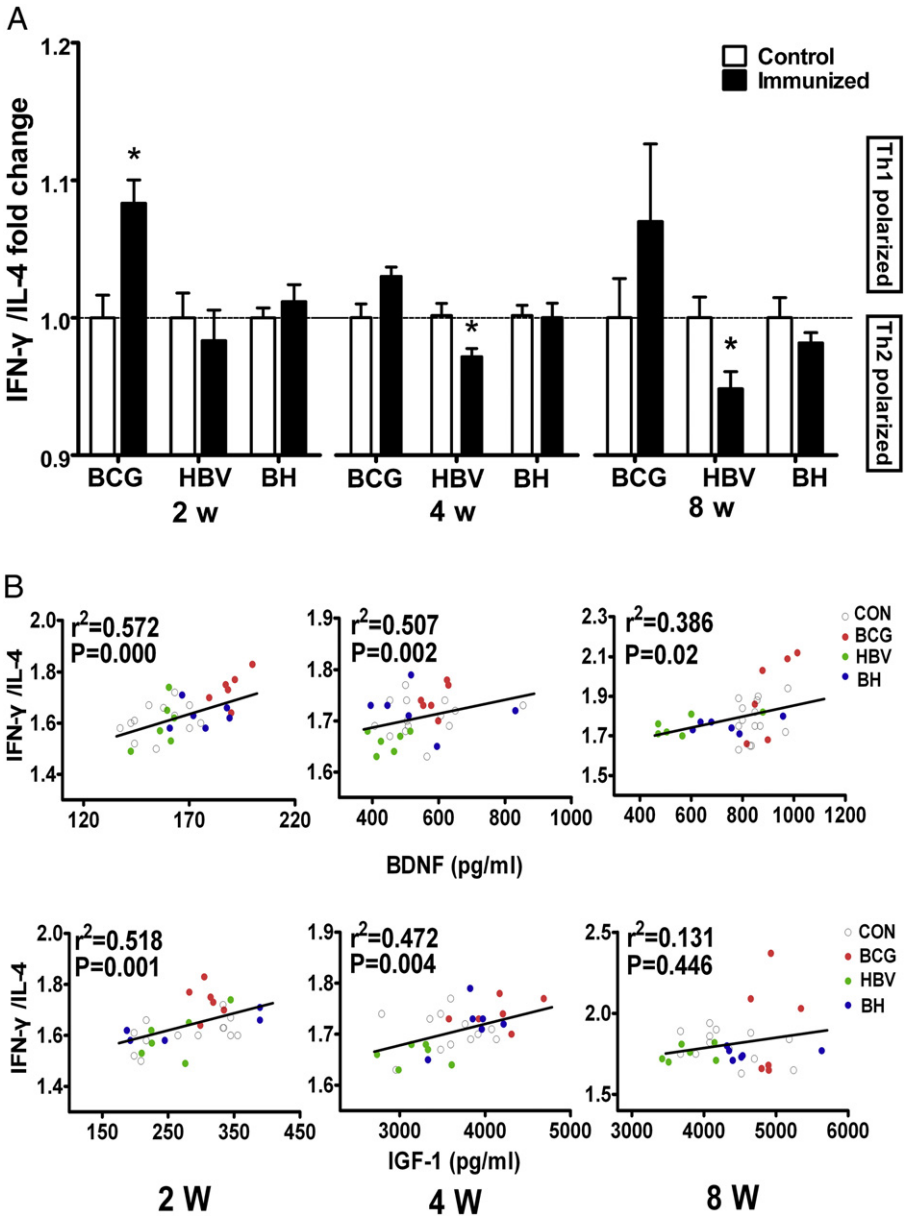

Fig. 6. Neonatal BCG, HBV, and BH vaccination alters the Th1/Th2 bias. BCG vaccination induced a Th1-like response, while HBV led to a Th2-like response in the periphery. Bars in (A) represent the fold-change of the average concentration of IFN-γ with respect to that of IL-4 in each group in serum. Data are presented as the means ± SEM normalised to the control and were analysed with two-way ANOVA followed by Bonferroni post-hoc test. $n = 6$ for each group (A). Correlation analysis was performed using the serum IFN-γ:IL-4 ratio and the hippocampal BDNF or IGF-1 level (B). Pearson correlation analysis (BDNF: 2 weeks, $r^2 = 0.572$, $p < 0.01$; 4 weeks, $r^2 = 0.507$, $p < 0.01$; 8 weeks, $r^2 = 0.386$, $p < 0.05$; IGF-1: 2 weeks, $r^2 = 0.518$, $p < 0.01$; 4 weeks, $r^2 = 0.472$, $p < 0.01$; $n = 36$).

increased in the hippocampus of BCG rats, whereas the levels of IL-1β, IL-6, and TNF-a, known to be detrimental to LTP, were reduced. Importantly, the concentrations of BDNF and IGF-1, which are thought to enhance brain functional plasticity (Nolan et al., 2005), were up-regulated in the BCG rats. In contrast to BCG, those levels declined in the HBV rats. Interestingly, the BH rats showed no significant alterations in these cytokines and neurotrophins in the brain. These data indicated that the alterations in synaptic plasticity regulated by the cytokine network were accompanied by the alterations in neurotrophins, such as IL-4, BDNF, and IGF-1, which modulate synaptic efficacy and neurotransmission (Figurov et al., 1996; Levine et al., 1995; Neal-Perry et al., 2014).

Previous studies have demonstrated that manipulations of individual cytokines can modulate learning, memory, and synaptic plasticity. However, there are many conflicting findings that have prevented a clear understanding of the precise role of cytokines in synaptic plasticity. Given the complexity of inflammatory signalling, we speculated that it is primarily the cytokine network that contributes to the fine-tuning of neural transmission rather than an individual cytokine (Xia et al., 2014a). In our study, the levels of cytokines in the hippocampus displayed similar trend as those in the serum, which suggests a close coincidence between the brain and peripheral blood system. It has been reported that peripheric cytokines may permeate into the CNS and affect neuronal transmission directly (Banks, 2005). The interplay between cytokines and neurotrophins is complex. Neurotrophins can be secreted by several types of immune cells, including T cells, microglia, macrophages, and mast cells (Elkabes et al., 1996; Nakajima et al., 2001). Cytokines in the CNS have crosstalk with resident immune cells (e.g., microglia) and regulate their phenotypes and therefore alter their local molecule production, including cytokines and neurotrophins (Schwartz and Shechter, 2010).

BCG or HBV vaccination induced a shift toward a dominance of the Th1 or Th2 response, respectively. Given that mediator-related

immunity in the hippocampus resulted from immune activation in the periphery, the positive correlation between systemic Th1:Th2 ratios and hippocampal neurotrophins bridges the vaccination and neurogenic niche and explains the change in synaptic plasticity.

Furthermore, it has been confirmed that the Th1/Th2 cytokine balance can modulate neurotrophin expression and, thus, affect neuronal function (Besser & Wank, 1999). Thus, an integrated network is formed between the extrinsic Th1/Th2 serum cytokines followed by intrinsic CNS-derived cytokines and the neurotrophin network to build a beneficial/detrimental neurogenic niche. Therefore, we propose a hypothesis that a systemic Th1/Th2 bias modulates central cytokines and neurotrophins and thereby affects the neurogenic niche, which is tightly correlated with synaptic plasticity. Previous reports support this hypothesis. It was reported that cognitive deficit was related to decreased Th1/Th2 balance in periphery and could be recovered when the balance was restored (Jakobsson et al., 2014; Palumbo et al., 2012). In addition to this, influenza vaccines administered during pregnancy induced a systemic Th1 bias and increased neurotrophins in both dams and their offspring (Xia et al., 2014a; Xia et al., 2014b). In our study, the probable underlying mechanism of the Th1/Th2 bias modulating synaptic plasticity was demonstrated by the following results: 1) BCG vaccination induced a Th1 serum cytokine response and yielded beneficial effects on synaptic plasticity; conversely, HBV induced a Th2 bias and exerted detrimental effects; 2) the correlation analysis showed a positive correlation between systemic Th1:Th2 ratios and hippocampal BDNF and IGF-1 levels; 3) it has been demonstrated that BDNF and IGF-1 contribute to the enhancement of synaptic transmission (Figurov et al., 1996; Levine et al., 1995; Neal-Perry et al., 2014); and 4) BH vaccination showed no obvious shift in Th1 or Th2, and no significant effects were observed on synaptic plasticity. In summary, the possibility arises that altered synaptic plasticity during early life may be modulated by the balance of two forces, namely, intrinsic CNS-derived signals and extrinsic signals that permeate to the CNS. However, the underlying mechanism is complex and diverse. Other mechanisms may exist and require further study, such as the neuro-protective or neurotoxic microglial cells reactivity to TH1/TH2 response. Moreover, the implication of immune molecules, such as MHC of class I, toll like receptors and complement system, which have been recently related to neonatal synaptic plasticity, may contribute to the alteration of synaptic plasticity.

Interestingly, we found a decline in the correlation coefficients between the IFN-γ:IL-4 ratio and BDNF and IGF-1 levels as time progressed, which may explain why the effect on synaptic plasticity induced by neonatal vaccination disappeared with age. Although we speculated that the influence on synaptic plasticity induced by neonatal vaccination was associated with Th1/Th2 bias accompanied by changes in BDNF and IGF-1, other immune cells, such as regulatory T lymphocytes and local microglia affected by immune activation, also play critical roles in modulating synaptic plasticity (Lagranderie & Guyonvarc'h, 2014; Yong et al., 2011).

We found that the timing of the effect on synaptic plasticity was different between the BCG and HBV rats. This may be due to immune reactions, bacterial/virus antigens, or the humoral/cellular immune response that contribute to different latencies. It is well known that the cellular immune response is activated faster than the humoral immune response under normal physiological conditions, which may explain why BCG vaccination is more quickly effective in synaptic structures and transmission than HBV. However, the present analysis remains speculative, and the reason for this speculation is considerably complicated and requires further exploration.

In summary, we worked specifically with a model of neonatal vaccination in rats that modulates hippocampal synaptic plasticity. The present findings provide innovative information regarding the correlation between neonatal vaccination and synaptic transmission. Moreover, our data suggested that combinations of different vaccines can mutually interact (enhance or counteract). The mechanism of synaptic plasticity modulation through neonatal BCG/HBV vaccination may be via systemic Th1/Th2 bias accompanied by a specific profile of cytokines and neurotrophins in the brain. Our work highlights a critical role of neonatal vaccination in synaptic plasticity outside of infectious disease prevention, which suggests the necessity of further studies on the association of vaccination with brain development under normal physiological conditions.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.jneuroim.2015.08.019.

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