Toll-like receptor-2 gene knockout results in neurobehavioral dysfunctions and multiple brain structural and functional abnormalities in mice

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Objective: Toll-like receptor-2 (TLR2), a member of TLR family, plays an important role in the induction and regulation of immune/inflammation. TLR2 gene knockout (TLR2KO) mice have been widely used for animal models of neurological diseases. Since there is close relationship between immune system and neurobehavioral functions, it is important to clarify the exact role of TLR2 defect itself in neurobehavioral functions. The present study aimed to investigate the effect of TLR2KO on neurobehavioral functions in mice and the mechanisms underlying the observed changes.

Methods: Male TLR2KO and wild type (WT) mice aged 3, 7, and 12 months were used for neurobehavioral testing and detection of protein expression by Western blot. Brain magnetic resonance imaging (MRI), electrophysiological recording, and Evans blue (EB) assay were applied to evaluate regional cerebral blood flow (rCBF), synaptic function, and blood–brain barrier (BBB) integrity in 12-month-old TLR2KO and age-matched WT mice.

Results: Compared to WT mice, TLR2KO mice showed decreased cognitive function and locomotor activity, as well as increased anxiety, which developed from middle age (before 7-month-old) to old age. In addition, significantly reduced regional cerebral blood flow (rCBF), inhibited long-term potentiation (LTP), and increased blood–brain barrier (BBB) permeability were observed in 12-month-old TLR2KO mice. Furthermore, compared with age-matched WT mice, significant reduction in protein levels of tight junction proteins (ZO-1, Occludin, and Claudin-5) and increased neurofilament protein (SMI32) were observed in 7 and 12-month-old TLR2KO mice, and that myelin basic protein (MBP) decreased in 12-month-old TLR2KO mice.

Conclusion: Our data demonstrated that TLR2 defect resulted in significantly observable neurobehavioral dysfunctions in mice starting from middle age, as well as multiple abnormalities in brain structure, function, and molecular metabolism.

1. Introduction

Toll-like receptors (TLRs) are a family of type-1 transmembrane receptors composed of an N-terminal ectodomain with leucine-rich repeats (LRRs), a transmembrane domain, and a C-terminal cytoplasmic signaling domain (Shi et al., 2019). TLRs interact with endogenous and exogenous molecules, activate different downstream inflammatory signaling pathways, and serve as important mediators for the activation of the innate immune system and of inflammatory responses (Kawai and Akira, 2010; Ye et al., 2016; Hua et al., 2011). Currently, a total of 13 TLRs (TLR1-TLR13) have been identified, 10 TLRs (TLR1-10) in humans and 12 (TLR1-9 and TLR11-13) in mice (Kawai and Akira, 2010; Shi et al., 2019). TLRs are found to present on various innate immune cells, such as polymorphonuclear neutrophils, monocyte/macrophage, dendritic cells, and NK cells, in which they trigger an immediate response against pathogens (Wang et al., 2013). Within
brain, TLRs are located on neuron, endothelial cell, and glial cells including microglia, astrocytes, and oligodendrocytes (Wang et al., 2013). Numerous experimental and clinical studies have confirmed that TLRs contribute to the onset and development of various neuroimmune diseases (Wasko et al., 2020) and neurological diseases, such as ischemia injury (Hua et al., 2015; Qin et al., 2013), traumatic brain injury (TBI) (Mao et al., 2012; Krieg et al., 2017), Alzheimers disease (AD) (Pourbadie et al., 2018). Emerging evidence indicates that TLRs also involve psychological and behavioral function. For example, expression of TLRs in the brain can be induced by stress, alcohol, and certain drugs (Crews et al., 2017). The mRNA levels of TLR3, TLR4, TLR5, TLR7, TLR8, and TLR9 were up-regulated in blood samples from the patients with major depressive disorder. Treatment with anti-depressants significantly decreased mRNA levels of TLR1 in those blood samples (Hung et al., 2016). Protein levels of TLR2, TLR3, TLR4, TLR6, and TLR10, and the mRNA of TLR2 and TLR3 were significantly increased in the prefrontal cortex of the patients with depressed suicide vs. non-psychiatric control brains (Pandey et al., 2019). TLR2 and TLR4 double gene knockout (KO) leads to impairments in behavior and cognitive functions in mice (Tuo et al., 2016).

TLR2, a key member of TLR family, is found to be widely expressed in glial cells and neurons in nervous system, and plays a pivotal role in neuroinflammation in neurological diseases, such as stroke, traumatic brain injury (TBI), and chronic neurodegenerative diseases (Hayward and Lee, 2014). Activation of TLR2 with its agonist, Pam3CSK4, protects against brain injury induced by ischemic stroke (Choi and Kim, 2017), and reduces axonal damage in spinal cord injury (Stivers et al., 2017). However, TLR2-deficient results in ameliorating brain injury in a mouse model of TBI (Yu and Zha, 2012). In addition, TLR2KO confers protection from impaired cerebral blood flow and hypoperfusion in diabetes in 16-week-old and 24-week-old mice, and improves diabetes-induced impairment of memory formation in mice (Haridigan et al., 2017), although there is no different in cortical cerebral perfusion and whole brain perfusion between normal TLR2KO and WT mice (Haridigan et al., 2017). Blocking TLR2 with anti-TLR2 antibody improves spatial learning in Alzheimers disease (AD) mouse model (McDonald et al., 2016). Our previous study demonstrated that TLR2KO exacerbated the impaired neurobehavioral functions and white matter damage (WMD) in a mouse model of AD (Zhou et al., 2019). In the study, we unexpectedly found that TLR2KO itself could induce learning disabilities, decreased locomotor activity, increased anxiety and depression in 12-month-old mice (Zhou et al., 2019). Since there is close relationship between neuroinflammation and neurobehavioral function (Di Benedetto et al., 2017; Pape et al., 2019), the role of TLR2 itself in neurobehavioral functions has attracted our attention. We looked at previous related studies, and found that the reports on the role of TLR2 in neurobehavioral function were inconsistent. For example, mice with constitutive loss of TLR2 (TLR2−/− mice) showed less day-to-day variability in feeding, drinking, and movement behaviors (DeKorver et al., 2017). Increased anxiety state and psychotic-like symptoms such as hyper-locomotion, anxiety-like behaviors, pre-pulse inhibition deficits, social withdrawal, and cognitive impairments were also observed in TLR2 deficiency mice (Ihara et al., 2019; Park et al., 2015; Haridigan et al., 2017). However, blocking TLR2 with anti-TLR2 antibody for 7 months from the age of 7 to 14 months did not induce cognitive dysfunction in wild type mice (McDonald et al., 2016). On the contrary, activation of TLR2 by its agonist (Zymosan) for 22 days resulted in cognitive deficit in mice (Ahuja et al., 2019). The inconsistent results can be explained as that TLR2 gene knockout is a condition of lifelong genotypic defect, which has developmental actions and is different from the temporary effect of acute administration of exogenous ligand or antibody. Nevertheless, these results strongly suggest that regulating TLR2 will affect neurobehavioral functions, and its exact role remains to be determined. Moreover, TLR2KO mice have been widely used for animal models of stroke (Lehardt et al., 2007; Gorup et al., 2019), TBI (Krieg et al., 2017; Yu and Zha, 2012), sepsis-induced cognitive dysfunction (Xing et al., 2018), and AD (Vollmar et al., 2010), in which, neurological and behavioral functions were often used as important evaluation indicators. Thence, the influence of TLR2KO itself on neurobehavioral function should be fully considered when evaluating its role in these diseases. However, the following issues are still unclear. First, the exact role of TLR2 defect in neurobehavioral functions and its relationship with age need to be clarified. Second, does TLR2 defect induce abnormalities in brain structure and cellular function? Third, what is the mechanism underlying the observed effect of TLR2 defect.

To answer these questions, in the present study, we evaluate neurobehavioral functions in TLR2KO and WT mice at different ages (3, 7, and 12 month old). Brain magnetic resonance imaging (MRI), electrophysiological recording, and Evans blue (EB) assay were applied to evaluate relative cerebral blood flow (rCBF), synaptic function, and blood–brain barrier (BBB) integrity in aged mice (12-month-old mice). In addition, tight junction (TJ) proteins and myelin associated proteins were also detected.

2. Materials and methods

2.1. Animals

Toll-like receptor 2 knockout (TLR2KO) mice and wild type C57BL/6J background control (WT) mice were purchased from the Animal Model Research Center of Nanjing University, China. The mice were kept at the animal experimental center of Xuzhou Medical University under standard housing conditions and fed separately (temperature 22–23 °C; 12 h light-dark period; food and water available ad adimum). The animal feeding, administration, and experimental protocols of this study received approval from the Institutional Animal Care and Use Committee of Xuzhou Medical University. Both WT and TLR2KO male mice were randomly designed into groups of 3-month-old (n = 12), 7-month-old (n = 14), and 12-month-old (n = 15). The animals were subjected to behavioral tests at the age of 3, 7, and 12 months. For behavioral tests, all mice were adapted to the behavior laboratory for 1 week prior to administration of the tests, and stayed in the behavioral room until the end of behavioral test. The Morris water maze (MWM) test was conducted on the 1st to 8th day, the open field test was conducted on the 9th day, the elevated plus test was performed on the 10th day, and the tail-suspension test was performed on the 11th day. One day after the last behavioral test, 6 TLR2KO and WT mice aged 3, 7, and 12 months were sacrificed by decapitation after deep anesthesia with 5.0% isoflurane, and the brain tissue was harvested and stored at −80 °C for molecular biology testing. Four TLR2KO and WT mice aged 12 months were used for electrophysiological recording. Four TLR2KO and 5 WT mice aged 12 months were used for MRI scan, and then Evans blue (EB) assay. The remaining mice were sacrificed by transcardial perfusion with normal saline followed by 10% neutral formalin. The brains were removed and stored in the post-fixation solution for future use.

2.2. Morris water maze (MWM)

TLR2KO and WT mice aged 3 (n = 12/group), 7 (n = 14/group), and 12 (n = 15/group) months were used for MWM test to evaluate spatial learning and reference memory. MWM tests were performed using the process described previously (Dong et al., 2018) with a MWM system (Zhenghua biological instruments, China) containing a circular pool with an inner diameter of 122 cm and a water depth of 40 cm. The water temperature was maintained at 22 °C. A circular platform with a rough white surface (6 cm in diameter) was placed at the center of the first quadrant (target quadrant) and immersed 1.5 cm below the surface of the water. On days 1–7 (acquisition phase), each mouse was tested four times a day (60 s each), with a 10–15 min break between trials. During the acquisition phase, the platform was fixed in one place. Mice
were randomly placed into the water from four directions each day. When a mouse reached the platform successfully in 60 s, it was allowed to stay on the platform for 10 s. If the mouse failed to reach the platform within 60 s, it was guided to the platform and allowed to stay there for 10 s. After completing the 7-day learning phase, the probe test of platform location memory retention was conducted on the 8th day without the platform in the water. The mice were placed into the water from the opposite side of the original platform quadrant (the third quadrant) and were allowed to swim freely for 60 s to find the location of the original platform. The tracing was recorded with a monitor and stored in the computer system. The latency to arrive at the platform (latency to platform) and the distance traveled before finding the platform (path length) were calculated in the place navigation period. The time spent in the target quadrant, average swimming speed, and the numbers of crossings over the platform site were calculated using system software (Zhenghua animal behavior video analysis system, China).

2.3. Open field maze

In the present study, TLR2KO and WT mice aged 3, 7, and 12 months were used for open field maze test to evaluate spontaneous motor activity and exploratory behavior. Open field test was performed according to a previous report (Whyte et al., 2018). Briefly, the open field system (Zhenghua biological instrument, China) consists of four white Plexiglas (50*50*50 cm) open stages, a video capture, and an analysis system (Zhenghua animal behavior video analysis system, China). In the experiment, the mice were placed on one side of a stage and a video tracking system was used to record their movements for five minutes. Total traveled distance (mm) and the distance traveled in the central zone were recorded.

2.4. Tail-suspension test

TLR2KO and WT mice aged 3, 7, and 12 months were used for tail-suspension test to evaluate depressive behavior (Duarte et al., 2014). Briefly, the mice were suspended 40 cm above the ground by their tails using a nylon rope. A video analysis system (Zhenghua biological instrument, China) was used to record the behavior for 6 min. Lack of escape-related behavior was considered immobility and depression.

2.5. Elevated plus maze

TLR2KO and WT mice aged 3, 7, and 12 months were used for elevated plus maze to evaluate anxiety-like behavior based on the previously published method (Zhou et al., 2019; Sorregotti et al., 2018). Briefly, the maze system consists of two open arms and two closed arms with an open roof. The maze is 40 cm above the ground. The light intensity was

Fig. 1. TLR2 defect increases the latency and path length to find the hidden platform in place navigation. TLR2KO and WT mice at the ages of 3, 7, and 12 months were subjected to Morris water maze test (MWM) for place navigation for 7 consecutive days. The escape latency and the path length were analyzed using repeated measures two-way ANOVA. (A) Representative track plots of MWM for place navigation. (B) The latency (time taken to find the hidden platform) in 3-month-old mice was not significantly different between TLR2KO and WT mice. (C and D) The latency significantly increased in TLR2KO mice as compared to WT mice at the age of 7 months (P < 0.05, C) and 12 months (P < 0.05, D). (E) The path length (traveled distance before finding the hidden platform) in 3-month-old mice was not significantly different between TLR2KO and WT mice. (F and G) The path length significantly increased in TLR2KO mice as compared to WT mice at the age of 7 months (P < 0.05, F) and 12 months (P < 0.05, G). (Note: *P < 0.05, n = 12 ~ 15/group. Fig. 1B: F (1, 22) = 2.649; Fig. 1E: F (1, 54) = 0.0002227; Fig. 1C: F (1, 203) = 54.85; Fig. 1D, F (1, 203) = 69.02; Fig. 1F: F (1, 203) = 48.25; Fig. 1G: F (1, 203) = 30.56).
was a 4*4 cm² field-of-view (FOV) of 2 mm in thickness, with a single axial slice of the brain located on the point of the caudal axis that had the maximum cross-section of the hippocampus. The imaging geometry was designed at 45 lx inside the maze. During the experiment, the mice were placed in the middle of the maze in the beginning of test, and tested for 5 min each time. The video analysis system (Zenghuabao biological instrument, China) was used to record the total distance and time staying on the open arms of the maze.

2.6. Magnetic resonance imaging (MRI)

TLR2KO (n = 4) and WT (n = 5) mice aged 12 months were used for brain MRI scan 3 to 7 days after the last behavioral test according to the process previously reported (Zhou et al., 2019). Briefly, 12-month-old mice received brain MRI scans with a 7.0-T BioSpec 70/20 MRI scanner (Bruker BioSpin, Germany) equipped with a 500 mT/m (ascent time 80–120 s) gradient device (for performing high-resolution small animal imaging), a small diameter linear RF (RF) coil (ID119mm) as RF transmitter, and a four-channel surface array coil as RF receiver. During the MRI scan, all mice were anesthetized with 1.5% isoflurane (R510, RWD Life Science Co., China) in 70% nitrogen and 30% oxygen. The body temperature was maintained at 37 °C during the scan. Physiological parameters (heart rate, respiratory rate, and body temperature) were monitored to maintain normal physiological parameters. The body temperature was maintained at 37 °C during the scan. Arterial spin-labeling (ASL) perfusion maps were obtained on a single shot echo-planar encoding over a 64*64 matrix. Echo time (TE) of 13.5 ms, repetition time (TR) of 18 s, and inversion time (TIR) of 13.5 ms, repetition time (TR) of 18 s, and inversion time (TIR) of 26.0 ms were used. The flow-sensitive alternating inversion recovery scheme was used to obtain perfusion contrast. Regional cerebral blood flow (rCBF) values were obtained in the cortex and hippocampus areas (Towner et al., 2018).

2.7. Electrophysiological recording

TLR2KO and WT mice aged 12 months (n = 4/group) were used for measurement of field excitatory postsynaptic potentials (fEPSPs) and long-term potentiation (LTP) using electrophysiological recording method according to the process previously reported (Wang et al., 2011, 2020). Briefly, the mice were anesthetized with a mixture of ketamine and xylazine (100 mg/kg, 10 mg/kg, intraperitoneal injection), and perfused transcardially with ice-cold sucrose-rich slicing solution (SRSS) (75 mM sucrose, 85 mM NaCl, 2.5 mM KCl, 1.25 mM NaH2PO4, 25 mM NaHCO3, 4 mM MgCl2, 0.5 mM CaCl2, and 25 mM glucose) in order to prevent brain edema. The brains were quickly removed and chilled in SRSS. Horizontal hippocampal slices (300 μm) were cut in SRSS using a Leica VT1000S vibratome and transferred to a storage chamber containing ACSF (119 mM NaCl, 2.5 mM KCl, 1.3 mM MgSO4, 2.5 mM CaCl2, 1.25 mM NaH2PO4, 26 mM NaHCO3, and 10 mM glucose) at 37 °C for 30 min and maintained at 25 °C for an additional 1 h before recording. All solutions were saturated with 95% O2 and 5% CO2. The slices were transferred to the recording chamber and superfused (2 ml/min) with oxygenated ACSF at room temperature. A concentric circular stimulating electrode (FHC Inc. Bowdoin, USA) was placed in the Schaffer collateral (SC) to deliver test and conditioning stimuli. A glass microelectrode filled with ACSF (3–5 MΩ) was positioned in the CA1 stratum radiatum to record field excitatory postsynaptic potentials (fEPSPs). The input–output (I/O) curves of fEPSPs were recorded by stimulating the SC-CA1 pathway with gradually increasing intensities (0.05, 0.1, 0.15, 0.2, 0.25, 0.3, and 0.35 mA). For LTP recording, test fEPSPs were stimulated with an intensity that elicited ~ 50% of the maximum amplitude at 0.05 Hz. The fEPSPs were acquired with a MultiClamp 700B amplifier (Molecular Devices), filtered at 2 kHz and sampled at 10 kHz with a Digidata 1440A interface (Molecular Devices). Signals were obtained with Clampex 10.2 software and analyzed with Clampfit 10.2 (Molecular Devices).

2.8. Western blot

TLR2KO and WT mice aged 3, 7, and 12 months (n = 6/group) were used for Western blot according to the process previously described (Dong et al., 2018). Briefly, the mice were killed by decapitation after deep anesthesia with 5.0% isoflurane. Cerebral cortical tissues were harvested (n = 6/group) and stored at ~ 80 °C. Proteins were...
extracted and isolated with SDS-PAGE system and transferred to 0.45 μm polyvinylidene difluoride (PVDF) membrane (Millipore IPVH00010, MA, USA). The PVDF membranes were then incubated with primary antibody at 4 °C overnight. After washing with buffer, the PVDF membrane was incubated with peroxidase-conjugated secondary antibody. The signal was detected using high-sig ECL Western Blotting Substrate (Tanon, Shanghai, China) and scanned using Bio-Rad Chemi Doc imaging system (Bio-Rad Inc. USA). The primary antibodies used in this study were anti-ZO-1 (Dilution: 1:500, 40–2200, Invitrogen, USA), anti-Occludin (Dilution: 1:1000, CN: 33–1500, Invitrogen, USA), anti-Claudin-5 (Dilution: 1:1000, CN: 35–2500, Invitrogen, USA), anti-MBP101 (Dilution: 1:1000, CN: ab62631, abcam), anti-SMI32 (Dilution: 1:500, CN: 801701, Biolegend, USA), and anti-GAPDH (Dilution: 1:5000, CN: 60004–1-Ig, Proteintech).

2.9. Evans blue assay

TLR2KO and WT mice aged 12 months (n = 4/group) were used for Evans blue (EB) assay to detect the permeability of the BBB according to the process described previously (Wang et al., 2019). Briefly, Evans blue (EB) solution (Sigma Inc, USA) at a concentration of 2% (4 ml/kg body weight) was injected into the tail vein. One hour after injection, under deep anesthesia, the mice were perfused with cooled normal saline to remove the EB dye in the blood vessels. Tissues from cortex,
3. Results

3.1. Deletion of TLR2 gene induced cognitive impairment in adult mice

Morris water maze (MWM) was performed at the ages of 3, 7, and 12 months. The water maze escape latency and the path length were analyzed using repeated measures two-way ANOVA. The number of platform crossings and platform quadrant activity time were analyzed using one-way analysis of variance. As shown in Fig. 1A, 1B, and 1E, in the 3-month-old mice, there was no significant difference between the TLR2KO mice and WT controls in the latency (Fig. 1B, (F (1, 22) = 2.649)) and path length (Fig. 1E, (F (1, 54) = 0.0002227)) during the acquisition/learning period. In the probe test, there was no significant difference in the time spent in the target quadrant (first quadrant) and the frequency crossing through the platform between TLR2KO and WT mice at the age of 3 months (Fig. 2A, 2B, and 2D). However, at the age of 7 and 12 months, TLR2KO mice spent significantly more time and longer path length in the acquisition/learning phase looking for a platform, compared to the WT controls from the 3rd day to the 7th day (P < 0.05, Fig. 1A, 1C (F (1, 203) = 54.85), 1D (F (1, 203) = 69.02), 1F (F (1, 203) = 48.25), and 1G (F (1, 203) = 30.56)). TLR2KO mice spent significantly less time in the target quadrant (first quadrant) than WT mice (P < 0.05, Fig. 2B, (F (5, 80) = 62.92)). In addition, the frequency of crossing over the platform was significantly lower in TLR2KO mice than that of WT mice (P < 0.05, Fig. 2D (F (5, 80) = 23.44)).

3.2. Deletion of TLR2 gene reduced locomotor activity and increased anxiety

The swimming speed in MWM test was analyzed using one-way analysis of variance. The data demonstrated that the average swimming speed of TLR2KO mice at 7 months and 12 months was significantly slower compared with WT mice (P < 0.05, Fig. 2C, (F (5, 80) = 19.55)). In addition, the open field test was also used to evaluate locomotor activity and anxiety. The data was analyzed using one-way analysis of variance. The results showed that, in the 3-month and 7-month-old mice, there was no significant difference in the total distance and the distance traveled in the central area between TLR2KO and WT mice (Fig. 3A, 3B, and 3C (F (5, 80) = 9.6244)). In the 12-month-old mice, the exploration distance in the central region was significantly shorter in TLR2KO as compared to WT mice (P < 0.05, Fig. 3B, (F (5, 80) = 146.7)). In the tail-suspension test, the resting immobility time, which is positively correlated with depressive state, did not show significant difference between TLR2KO and WT mice at the ages of 3 months to 12 months (Fig. 3D, (F (5, 80) = 5.833)). The elevated plus maze was conducted to evaluate anxiety-like behavior. The results demonstrated that, at the age of 3 months, TLR2KO mice did not show a significant difference in the distance and time to enter the open arm as compared to WT mice. However, in 7- and 12-month-old mice, TLR2KO mice traveled significantly less distance in the maze and took less time staying on the open arm (P < 0.05, Fig. 3E (F (5, 80) = 8.972) and 3F (F (5, 80) = 13.82)).

3.3. TLR2KO decreased regional cerebral blood flow (rCBF) in cerebral cortex in 12 month old mice

Arterial spin-labeling (ASL) perfusion is an MRI technique that offers the prospect of non-invasive quantitative measurement of cerebral perfusion (Jezzard et al., 2018). The data was analyzed using independent sample t-test. The result showed that rCBF in the cerebral cortex of 12 month old TLR2KO mice was significantly reduced as compared to the WT control group (P < 0.05). However, rCBF in the hippocampus did not show significant difference between TLR2KO and WT mice (Fig. 4A, 4B, and 4C).
3.4. TLR2KO decreased long-term potentiation (LTP)

LTP, considered to be the neural mechanism for memory formation and storage, is closely related to neural cognitive ability (Snider et al., 2018; Gruart et al., 2015). Previous study demonstrated that activation of TLR2 and TLR4 with low-dose agonists in 3-month-old rats improved the disturbance in spatial and working memory and restored the impaired long-term potentiation induced by Aβ (Pourbadie et al., 2018). To evaluate synaptic function, the responses of fEPSP on hippocampal neurons (CA1 region, Fig. 5B) of 12-month-old mice before and after high-frequency stimulation (HFS) were measured. The data was analyzed using repeated measures two-way ANOVA. The results showed that after HFS, the normalized fEPSP slope was significantly lower in TLR2KO mice than in WT mice (P < 0.05, Fig. 5C), which indicated that TLR2KO induced a significant decrease in LTP. However, the stimulus–response curve of average amplitude of fEPSP in response to stimulus intensities did not show a significant difference between TLR2KO and WT mice (Fig. 5D (F (1, 147) = 1.173)).

3.5. TLR2KO induced myelin sheath lesion in mouse brain

Brain white matter, mainly composed of myelinated axons, plays a key role in regulating neuronal communication and maintaining cognitive function (Harauz et al., 2009). In the present study, the protein levels of myelin basic protein (MBP) and dephosphorylated nerve filament (SMI32) were detected. The data was analyzed using one-way analysis of variance. The results showed that the MBP level in TLR2KO mice was significantly decreased at the age of 12 months, not in younger mice, as compared to the age-matched WT controls (P < 0.05, Fig. 6B, 6C (F (1, 18) = 29.25)). The level of SMI32 was significantly increased in 7- and 12-month-old TLR2KO mice, not in 3-month-old mice, compared with age-matched WT controls (P < 0.05, Fig. 6C (F (1, 18) = 72.20)).
3.6. TLR2KO decreased the expression of the tight junction proteins

The BBB properties notably depend on tight junctions (TJs) between adjacent cells (Greene et al., 2016). As shown in Fig. 7, the data from Western blot indicated that, the levels of TJ proteins, including ZO-1, Occludin, and Claudin-5, were significantly lower in 7- and 12-month-old TLR2KO mice, not at the age of 3 months, compared to age-matched WT mice (P < 0.05, Fig. 7B (F (5, 80) = 1142.2), Fig. 7C (F (5, 80) = 152.8), and Fig. 7D (F (5, 80) = 71.89)).

3.7. TLR2KO increased the permeability of blood–brain barrier (BBB)

The blood–brain barrier (BBB) selectively restricts the blood-to-brain paracellular diffusion of compounds (Butler et al., 2020). The integrity of BBB plays an important role in maintaining cerebral homeostasis and neuronal function (Luissint et al., 2012). In the present study, the EB assay method was used to study BBB permeability in mice aged 12 months. The data was analyzed using independent sample t-test. The results showed that the Evans blue contents were significantly higher in the cortex (A), hippocampus (B), and striatum (C) in TLR2KO as compared to WT mice (P < 0.05). Note: *P < 0.05, n = 4/group. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

4. Discussion

In the present study, TLR2KO and WT mice at ages of 3, 7, and 12 months were applied for neurobehavioral functions, respectively. In the MWM test, spatial learning and reference memory represented by the latency and the traveled distance to find the hidden platform during the acquisition/learning period, the frequency crossing the platform, the time spent in the target quadrant, and the average swimming speed in probe test did not change significantly at 3 months old, but began to show statistical differences at 7 months old and lasted until 12 months between TLR2KO mice and WT controls. In addition, the exploration distance in the central region in the open field test, distance traveled in the maze, and the time staying the open arm, demonstrated that the locomotor activities reduced and the anxiety increased in TLR2KO mice compared to WT controls. The resting immobility time in the tail-suspension test, which is positively correlated with the depressive state, was not significantly different between TLR2KO and WT mice at the ages of 3 months to 12 months. Our data demonstrated that TLR2 gene defect resulted in neurobehavioral dysfunction, including declined cognitive function, reduced locomotor activity, and increased anxiety, which began in middle age and aggravated with age. This novel finding not only confirmed that TLR2KO could cause a variety of neurological dysfunctions, but also clarified the
relationship between these changes and the age of onset. More importantly, these data provide an important reference for the application of TLR2KO mice as animal models of nervous system diseases, in which neurobehavioral functions are used as important evaluation indicators.

The biological basis of neurobehavioral functions includes the anatomy of the brain, structure and function of neurons and brain white matter, production and metabolism of neurotransmitters, and blood supply to the brain. Changes in these factors can directly or indirectly affect neurobehavioral functions. Our previous study demonstrated the reduced cortical thickness, impaired white matter, and neuron loss in 12-month-old TLR2KO mice (Zhou et al., 2019), which might be the structural basis of neurobehavioral dysfunction induced by TLR2 defect. However, more structural and functional changes associated with neurobehavioral function in TLR2KO mice brain are unknown and worthy of further study. Since our data revealed that behavioral dysfunction induced by TLR2 gene knockout appeared in middle-aged mice and worsen with age, which was most severe in 12-month-old mice, we assumed that the impaired brain structure and abnormal function might be most obvious in aged mice. Considering that the damage is a gradual change, in order to more clearly observe the difference between the TLR2KO and WT mice, in this study, we selected the mice with the most damage (12-month-old mice) to detect the cortex rCBF, synaptic long-term potentiation (LTP), and blood–brain barrier (BBB) permeability.

Reduce of rCBF or cerebral hypoperfusion is closely associated with cognitive dysfunction, brain atrophy, neuron loss, and white matter lesion (Kazumata et al., 2019; Ishikawa et al., 2016; Kim et al., 2009). Previous study demonstrated that cortical cerebral perfusion in 16-week-old mice and whole brain perfusion in 24-week-old mice were not difference between TLR2KO and WT mice (Hardigan et al., 2017). Our data demonstrated that, in 12-month-old TLR2KO mice, cortical rCBF decreased significantly as compared to age-matched WT controls. Long-term potentiation (LTP) is an activity-dependent process by which transmission is persistently enhanced at the chemical synapses in the brain. LTP is considered the neural mechanism for memory formation and storage, and is closely related to learning and memory ability (Guarri et al., 2015; Snider et al., 2018). To detect synaptic function, field excitatory postsynaptic potential (fEPSP) at CA1 synapse in hippocampal slices in 12-month-old mice before and after high-frequency stimulation (HFS) was measured using electrophysiological recording. The data, showing lower fEPSP slope in TLR2KO as compared to WT control, indicated that deletion of TLR2 resulted in a decreased LTP. However, the stimulus–response curve of average amplitude of fEPSP in response to HFS did not show a significant difference between TLR2KO and WT mice, which suggested that the basal synaptic strength was not affected by deletion of TLR2 gene.

BBB is formed by the endothelial layer of cerebral vessels selectively and restricts the blood–brain paracellular diffusion of compounds (Butler et al., 2020). The integrity of BBB is important for maintaining cerebral homeostasis and neuronal function (Luisint et al., 2012). Our data demonstrated that the BBB permeability evaluated by EB assay increased in the cortex, hippocampus, and striatum in TLR2KO mice at the age of 12 months.

The data from the present experiment and our previous study (Zhou et al., 2019) have confirmed that TLR2 deficit not only leads to neurobehavioral dysfunction, but also results in various changes in brain structure and function, such as impaired white matter and BBB integrity. However, the molecular mechanism of action remains unclear.

Brain white matter, which plays a key role in neuronal communication and cognitive function, is mainly composed of myelinated axons. Oligodendrocytes enwrap neuronal axons with extensions of their plasma membrane to form the myelin sheath (Kim et al., 2009). In myelin, myelin basic protein (MBP) is abundantly found. In mature myelin, the main role of MBP is to keep the oligodendrocyte membranes together, thereby maintaining the structural integrity of the myelin sheath (Harauz et al., 2009) and playing an importance role in myelin synthesis (Miller et al., 2013). On the other hand, mammalian neurons express the neural intermediate filament protein neurofilament (NF), which is major constituent of axonal cylinder. NF consists of three subunits: NF-H (high medium molecular weight; heavy NF), NF-M (medium molecular weight), and NF-L (low molecular weight) (Kashiwagi et al., 2003). Any abnormality in synthesis, delivery, or processing of these critical proteins could lead to severe impairments in axon structure and function. Increased NF protein was considered as an indicator of brain aging, nerve fiber neurodegeneration, axonal transport damage, and Wallerian degeneration (Kashiwagi et al., 2003).

In the present study, the protein levels of MBP and non-phosphorylated NF-H (SMI32) were detected in 3, 7, and 12-month-old mice. We found that, in TLR2KO mice, MBP significantly decreased at the age of 12 months, and that SMI32 increased in 7- and 12-month-old mice, compared with age-matched WT mice. Our data indicated that defect in TLR2 lead to the damage in white matter integrity by reducing MBP and increasing non-phosphorylated NF-H.

The BBB properties notably depend on tight junctions (TJs) between adjacent cells (Greene and Campbell, 2016). TJs are dynamic structures consisting of a number of transmembrane and membrane-associated cytoplasmic proteins, which are assembled in a multicomponent complex and act as a platform for intracellular signaling (Winkler et al., 2020). In the present study, TJ proteins were detected in 3, 7, and 12-month-old mice. We found that the levels of TJ proteins, including ZO-1, Occludin, and Claudin-5, significantly decreased in TLR2KO mice at the ages of 7 and 12 months, but not 3 months. Our data indicated that TLR2 defect resulted in impaired BBB integrity by reducing TJ proteins.

In summary, the present study is the first to report the relationship between age and neurobehavioral dysfunction caused by TLR2 deficiency. The neurobehavioral abnormalities and the changes in MBP, SMI32, and TJ proteins in TLR2KO mice were observed at the age of 7 and/or 12 months, not 3 months. The possible reason for this phenomenon may be that TLR2 gene defect may lead to a chronic biochemical, structural, and functional changes, which appear in adulthood and become more obvious with age. Moreover, other novel findings include the impaired synaptic function, cortical hypoperfusion, lesions in the white matter, and damaged BBB integrity observed in 12-month-old TLR2KO mice, which may serve as the basis of the structure and function of neurobehavioral dysfunction. The underlying mechanism involves multiple pathways, such as abnormal synthesis of myelin and TJ proteins, and more molecular mechanisms are worthy of further investigation by using agonist or antagonist for TLR2.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.bbi.2020.10.004.

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