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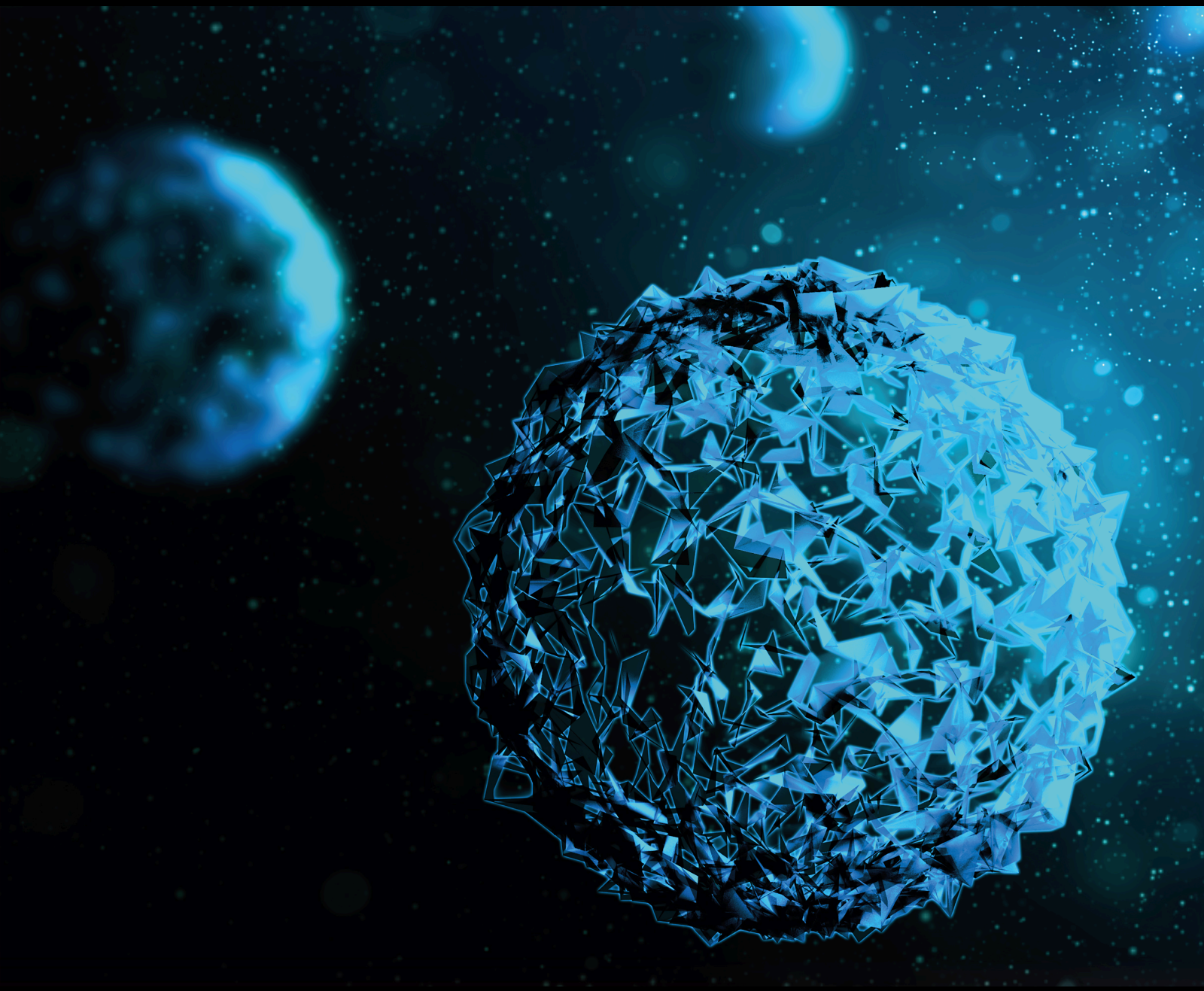
The Role of Neuroinflammation in Cellular Damage in Neurodegenerative Diseases

Guest Editors: Sangu Muthuraju, Rahimah Zakaria, Mohan K. M. Karuppan, and Badriya S. A-rahbi



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Editorial Board

Contents

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Editorial

The Role of Neuroinflammation in Cellular Damage in Neurodegenerative Diseases

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The role of inflammatory mediators in the central nervous system (CNS) has been investigated in different types of neurodegenerative diseases, such as Alzheimer's disease (AD), Parkinson's disease (PD), and Huntington's disease (HD). Interestingly, these inflammatory mediators have a dual role in both proinflammatory and anti-inflammatory processes, upregulating and suppressing cellular damage in injury sites, respectively. Immediately upon injury or stress, the first and foremost responses are initiated by immune cells in the brain called microglia. Inflammation causes positive and negative symptoms in the periphery and CNS. In the CNS, inflammation is called neuroinflammation. Periphery inflammation could affect the CNS and induce neurological problems. For example, gut microbiota (GM) are present in the human intestine. The connection between GM and inflammation was found in AD. Recently, H. Shen et al. (2020) investigated inflammatory response activated by NLRP3 inflammasome which reaches the brain through circulation. This NLRP3 inflammasome could trigger microglial activation and form the pathological progression of AD. This article suggested that neuroinflammation could be triggered through periphery infections. Y. Wang et al. (2019) reported that neuroinflammation has the main role in the pathogenesis of AD, cerebral ischemia, and PD by CysLT1 and CysLT2 modulate inflammation during brain injury conditions. These are the possible targets for reducing inflammation.

Neuroinflammation is not only causing damage in the brain but is also driving or modulating the behavior of the human/animals. There are plentiful factors triggering neuroinflammation, especially stress, it is the major problem among adults promoting inflammation in the brain especially in the prefrontal cortex and motivate the animal to drink alcohol (HG Chuang et al. 2020). HG Chuang et al. reported that toll-like receptor 4 antagonist diminished alcohol seeking and drinking behavior of mice following restraint and social isolation stress. Besides, R Wang et al. (2020) demonstrated that maternal separation could enhance neuroinflammation in the hippocampus and prefrontal cortex due to depressive behavior. Stress at the early stage of life could induce short- and long-term depression through the production of cytokine and microglial activation.

In the line of research, the same group also studied that neuroinflammation in the striatum and cerebellum disturbed motor behavior following lipopolysaccharide

(LPS) administration (data not published). This study directly helps to make new insight for approaching therapeutic for PD. I Parra et al. (2019) suggested that LPS administration is considered to be a better model for inflammatory and PD. ED Hamlett et al. (2020) reported that neuroinflammation induced memory loss in a down syndrome mouse model of Ts65Dn. Ts65Dn mice used for studying AD mouse model particularly age-related learning and memory loss associated with cholinergic neurons in the hippocampus and basal forebrain. ED Hamlett concluded that RvE1 could be potential therapy to reduce neuroinflammation and improve memory functions of persons with AD. This kind of preliminary findings lead us to establish animal studies to extend future research on AD and PD and further understanding of possible mechanisms to achieve effective therapy.

Recently, the literature has been reported that inflammation/neuroinflammation plays a key role in neurological diseases. Researchers have been involved to find out the crucial biomarkers of neuroinflammation in neurological diseases. Nevertheless, to understand neuroinflammation during disease condition is a major challenge for researchers. To find out, researchers and scientists, those who are working in the field of neuroinflammation and neurological diseases, making group, discuss the issues, these are the main idea for this special issue on "The role of neuroinflammation in cellular damage in neurodegenerative diseases". Our special issue was appreciated and received many research and review articles and based on the reviewer's comments, some of the articles were accepted. The purpose of the special issue is almost achieved with the quality of the research articles published now. Last but not least, again in the near future, neuroinflammation-related neurological disease special issues would be posted soon.

Conflicts of Interest

No authors declare that they have no conflict of interest.

Acknowledgments

We would like to express our gratitude to all the authors who made this special issue possible. We hope this collection of articles will be useful to the scientific community, those who are working in neuroinflammation and neurological disease.

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Review Article

Review on Cross Talk between Neurotransmitters and Neuroinflammation in Striatum and Cerebellum in the Mediation of Motor Behaviour

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Neurological diseases particularly Alzheimer's disease (AD), Parkinson's disease (PD), stroke, and epilepsy are on the rise all around the world causing morbidity and mortality globally with a common symptom of gradual loss or impairment of motor behaviour. Striatum, which is a component of the basal ganglia, is involved in facilitating voluntary movement while the cerebellum is involved in the maintenance of balance and coordination of voluntary movements. Dopamine, serotonin, gamma-aminobutyric acid (GABA), and glutamate, to name a few, interact in regulating the excitation and inhibition of motor neurons. In another hand, interestingly, the motor loss associated with neurological diseases is possibly resulted from neuroinflammation induced by the neuroimmune system. Toll-like receptors (TLRs) are present in the central nervous system (CNS), specifically and primarily expressed in microglia and are also found on neurons and astrocytes, functioning mainly in the regulation of proinflammatory cytokine production. TLRs are always found to be associated or involved in the induction of neuroinflammation in neurodegenerative diseases. Activation of toll-like receptor 4 (TLR4) through TLR4 agonist, lipopolysaccharide (LPS), stimulation initiate a signaling cascade whereby the TLR4-LPS interaction has been found to result in physiological and behavioural changes including retardation of motor activity in the mouse model. TLR4 inhibitor TAK-242 was reflected in the reduction of the spinal cord pathology along with the motor improvement in ALS mouse. There is cross talk with neuroinflammation and neurochemicals. For example, TLR4 activation by LPS is noted to release proinflammatory cytokines, IL-1β, from microglia that subsequently suppresses GABA receptor activities at the postsynaptic site and reduces GABA synthesis at the presynaptic site. Glial glutamate transporter activities are also found to be suppressed, showing the association between TLR4 activation and the related neurotransmitters and corresponding receptors and transporters in the event of neuroinflammation. This review is helpful to understand the connection between neurotransmitter and neuroinflammation in striatum- and cerebellum-mediated motor behaviour.

1. Introduction

Neurological diseases particularly Alzheimer's disease (AD), Parkinson's disease (PD), stroke, and epilepsy are on the rise all around the world. As the second leading cause of morbidity and mortality globally, it has become one of the greatest threats to public health [1]. All the aforementioned diseases share a common symptom of gradual loss or impairment of motor behaviour. Based on the Global Burden of Disease Study 2015 [1], neurological diseases were listed and emerged as the top disease to cause 250.692 million disability-adjusted life years (DALYs), comprising 10.2% of global DALYs, and 9.399 million deaths, comprising 16.8% of global deaths, the second highest in terms of global deaths. Therefore, from the statistics, it is evident how critical it is to research on ways to alleviate the distress, the physical constraint that is affecting the people [1]. It has been noted that the motor loss associated with neurological diseases are possibly resulted from neuroinflammation induced by the neuroimmune system [2]. The immune system is one of the major functional components of the body that is responsible for the occurrence of neuroinflammation. One of the major, active components of the neuroimmune systems is the toll-like receptors (TLRs).

Toll-like receptors (TLRs) are always found to be associated or involved in the induction of neuroinflammation in neurodegenerative diseases. For example, deficiency of toll-like receptor 2 (TLR2) and toll-like receptor 4 (TLR4) in mice exhibits reduced levels of proinflammatory cytokines, resulting in milder clinical disease following traumatic brain injury. Subsequently, increased expression of TLR4 is also found in PD, AD, and amyotrophic lateral sclerosis (ALS) patients as well as in animal models [3].

TLR4 particularly has been demonstrated in various studies to have a significant causal relationship with motor dysfunction in neurodegenerative conditions. Activation of TLR4 through TLR4 agonist, lipopolysaccharide (LPS), stimulation initiate a signaling cascade whereby the TLR4-LPS interaction has been found to result in physiological and behavioural changes including retardation of motor activity in the mouse model [4]. LPS is a component of Gram-negative bacteria known to trigger inflammation, specifically known to activate TLR4. A number of studies reported the suppression of neuroinflammation through TLR4 inhibition which consequently minimized motor deficit in animal models involving neurodegenerative diseases as well as traumatic brain injury [5]. Numerous studies had demonstrated a significant relationship between TLR4 and motor impairments in neurodegenerative disorders. A recent study demonstrated an increase in motor impairments in a mice model as a result of TLR4 activation using monophosphoryl lipid A [6]. However, the involvement of the striatum and cerebellum has not been thoroughly reviewed yet.

Striatum, which is a component of the basal ganglia, is involved in facilitating voluntary movement while the cerebellum is involved in the maintenance of balance and coordination of voluntary movements. Both these structures work together with the cerebral cortex in mediating movements, and various neurotransmitters are involved in the circuitries involved in the process. Dopamine, serotonin, gamma-aminobutyric acid (GABA), and glutamate, to name a few, interact in regulating the excitation and inhibition of motor neurons. Studies had long demonstrated the involvement of such neurotransmitters in the proper functioning of motor neurons in the striatum and cerebellum [7]. In the motor system, serotonin (5-hydroxytryptamine, 5-HT) is found to either enhance or depress glutamate-mediated transmission as well as GABA-mediated transmission in structures controlling movement [8]. Additionally, TLR4 activation by LPS is noted to release proinflammatory cytokines, IL-1β, from microglia that subsequently suppresses GABA receptor activities at the postsynaptic site and reduces GABA synthesis at the presynaptic site. Glial glutamate transporter activities are also found to be suppressed, showing the association between TLR4 activation and the related neurotransmitters and corresponding receptors and transporters in the event of neuroinflammation [9]. The aim of this review is focusing on the role of microglia in the central nervous system, neuroinflammation in different kinds of neurological disease, how TLR involves in motor behaviour and mediating neuroinflammation signaling and dealing with what are important of striatum and cerebellum neurotransmitters in motor behaviour.

2. Neuroinflammation

Motor deficit emerged as a prominent feature or symptom in neurodegenerative diseases such as Parkinson's disease (PD), Alzheimer's disease (AD), and stroke. Motor deficits linked to the aforementioned diseases usually shows in the form of motor slowing (bradykinesia), gait and posture disturbances, rigidity, and resting tremor [10]. It has been noted that the motor loss associated with neurodegenerative diseases are possibly resulted from neuroinflammation induced by the neuroimmune system [2]. In research studies involving PD and AD, neuroinflammation has been reported to play the central role in the pathogenesis of these diseases. Neuroinflammation is regarded as an important feature of many neurodegenerative diseases such as multiple sclerosis (MS), narcolepsy, and autism [11].

Neuroinflammation stems from the immune system of the central nervous system (CNS) and comprises of a complex series of local immune processes constituting CNS cells such as neuron and glia, cytokines, pattern recognition receptors (PRRs), and peripheral immune cells in response to threats such as pathogens, tissue damage, abnormal stimulation, neurotoxins, infection, or injury. Neuroinflammation can assume a neuroprotective role or it can be counterproductive, causing damage to the nervous tissues. A persistent acute neuroinflammation can turn to a chronic neuroinflammation as it accumulates damage, bringing about neuronal degeneration. The effects or outcome of neuroinflammation has been indicated to be dependent on the time span of the inflammatory response and the activation state of microglia [12].

2.1. Role of Microglia in Neuroinflammation. Microglia are the innate immune cells in the CNS whereby it monitors and regulates the brain homeostasis, maintaining it under normal physiological conditions by purging pathogens as well as clearing dead cells through phagocytosis. Most notably, the microglia are critically involved in the neuroinflammatory response, serving as the initial indication of neuroinflammation when activated. The presence of pathogens, tissue damage, abnormal stimulation, neurotoxins, infection, injury, or any threats to the microenvironment activates microglia and thereafter the complex neuroinflammatory pathway [11] Macrophages can be activated into several distinct activation states, and the microglia functions differently according to the different activation states. The classical M1 type activation is the response to microorganism threats and is associated with cytotoxicity and inflammatory responses including the upregulation of proinflammatory cytokine expression. On the other hand, the M2 type activation is associated with immunoregulatory functions and tissue repair as well as wound healing and regeneration [13]. In response to an extensive and diverse array of microbial stimuli, the differential activation of microglia regulates neuroinflammation by inducing the release of proinflammatory mediators that favour the permeabilization of the blood brain barrier (BBB), which results in either neurotoxicity or neuroprotection [14]. Such stimuli are recognized by an array of receptors on microglia. The microglia activation states are named based on their effects on synaptic plasticity, neurogenesis, and learning and memory. Recently, data showed that microglial phenotypes switch from M2 to M1 depends upon the disease progression. M2 microglia is subdivided into three such as M2a, M2b, and M2c. M2a is involved to repair tissue-undergone damage by triggering anti-inflammatory and nerve growth factors. M2b regulates the deactivating phenotype and then produces anti-inflammatory mediators. M2c actively participates in phagocytosis and helps in cleaning process in the brain [15].

2.2. Role of Astrocytes in Neuroinflammation. Another important cell in the brain is astrocyte, which is considered to be a key regulator in the immunological system of both innate and adaptive immune responses at the time of stress or injury. The crucial role of astrocytes in inflammation is currently highlighted from both *in vivo* and *in vitro* findings [16]. Present literature has reported that intracellular signaling pathways are completely controlled by astrocytes during inflammation. Astrocyte responses might be beneficial for tissue repair process followed by injury. Besides, astrocytes play a role in the maintenance such as neurotransmitter uptake and gliotransmitter release [17]. Moreover, astrocytes are involved in cellular and molecular functions for degeneration, vascular signaling, and glial-neuronal interactions [18]. GFAP is the relevant marker for neuroinflammation when astrocytes are involved in AD [16]. Astrocytes also have interaction with cytokines, resulting in increased level of inflammatory markers. Proinflammatory signaling and reduced immune response due to high level of IL-10 induce deactivation of astrocytes [5].

3. Toll-Like Receptor on Motor Behaviour

PRRs are employed as sensors in the signal transduction of the innate immune system for the initial detection of microbial threats. Activated PRRs effectuate downstream signaling pathways which induce the innate immune responses by producing proinflammatory mediators, resulting in inflammation. One out of the several distinct classes of PRRs includes the TLRs family. TLRs are always found to be associated or involved in the induction of neuroinflammation in neurodegenerative diseases. TLRs are known to regulate the production of proinflammatory cytokines, which may contribute to further neuronal damage [19]. There are a total of 10 members of the TLRs family in humans; TLR1–TLR10 and 12 members in mice; TLR1–TLR9 and TLR11–TLR13. TLRs are expressed either on the exterior of microglia cells or to intracellular compartments such as the ER, endosome, lysosome, or endolysosome. Cell surface TLRs include TLR1, TLR2, TLR4, TLR5, TLR6, and TLR10, whereas intracellular TLRs include TLR3, TLR7, TLR8, TLR9, TLR11, TLR12, and TLR13 [20].

Toll-like receptor 4 (TLR4) particularly has been demonstrated in various studies to have a significant causal relationship with motor dysfunction in neurodegenerative conditions. TLR4 and other cell surface TLRs mainly detect and identify microbial membrane components, for example, lipids, lipoproteins, and proteins [20]. TLR4 activates upon stimulation of the Gram-negative lipopolysaccharide (LPS) which are known to trigger inflammation. The TLR4-LPS interaction has been found to result in physiological and behavioural changes including retardation of motor activity, loss of interest or pleasure, impaired cognitive function, and social withdrawal as well as reduced food and water intake [21]. TLR4 blockage with Tat-TLR4 interfering peptides injection was reported to suppress the event of sickness behaviour and exhibited absence of motoric and motivational effects of LPS-induced sickness [22]. Additionally, morphological changes in microglia and cytokine production that are typically induced by LPS were also blocked. Inhibition of TLR4 signaling prevents changes in behaviour and motivation caused by inflammatory stimulation, further suggesting the role and contribution of TLR4 in motor deficit.

Furthermore, suppression of TLR4 was also observed to reduce motor deficit conditions in neurodegenerative disorders and traumatic brain injury animal model. Feng et al., in 2016 [5], administered resatorvid, the TLR4 inhibitor TAK-242, in a rat subjected to controlled cortical impact injury. The result showed a neuroprotective effect through the inhibition of the TLR4-mediated pathway whereby the expression of TLR4 and its downstream signaling molecules, including MyD88, TRIF, NF-κB, TNF-α, and IL-1β, was found to be significantly downregulated. However, a study by Zhu and colleagues [23] revealed a morphological-based analysis that linked TLR4 deficiency with thinning of the molecular layer of the cerebellum. The loss of TLR4 reduced the number of Purkinje cells (PCs) which are the sole output neurons of the cerebellar cortex, thus impairing motor function as PCs are responsible in regulating the function of

the cerebellum which plays an essential role in balance and motor coordination [23].

4. Toll-Like Receptor 4 (TLR4) Neuroinflammatory Signaling Pathway

Activation of TLRs initiates two signal transduction pathways, namely, the MyD88-dependent pathway and the MyD88-independent pathway. TLRs except TLR3 initiate intracellular signaling through ligand-induced dimerization of intracellular Toll-IL-1 receptor (TIR) domain [3]. TIR domains of TLR4 recruit TIR domain-containing adaptor proteins MyD88 and MAL of the MyD88-dependent pathway or TRIF and TRAM of the MyD88-independent pathway. The MyD88-dependent pathway activates IRAKs (IRAK1, IRAK2, and IRAK4) and TRAF6 that in turn activates TAK1. Subsequently, this leads to the activation of MAPKs (p38, JNK, and ERK1/2) and IKK pathways, resulting in NF-κB activation which then induces the production of proinflammatory cytokines. The MyD88-independent pathway on the other hand activates TRIF and TRAM adaptor proteins which then recruit TBK1/IKKε through the activation of TRAF3 (Figure 1). This then follows the activation of the transcription factor IRF3 in the nucleus leading to the production of type I interferons [3, 23]. Once LPS binds to TLR4 on the microglia surface, the signal transduction pathway is activated which in the end leads to NF-κB activation. Activated NF-κB functions to control DNA transcription, mediating the production of proinflammatory cytokines, chemokines, and inducible enzymes, namely, inducible nitric oxide synthase (iNOS) and COX-2 which are released from the microglia whereby all result in neuroinflammation [11, 24].

Previous studies had demonstrated that microglia in the brain region comprise an expression of TLR4 [25] and that the TLR4 activation activates microglia which in turn produces more proinflammatory factors such as tumor necrosis factor α (TNFα), interleukin-1β (IL-1β), and IL6 [26], resulting in a self-propelling and vicious cycle of neuroinflammation and neurodegeneration of dopamine neurons [27]. The production of proinflammatory cytokines is shown to be associated with reduced muscle mass and strength as well as affecting brain areas involved in motor coordination and fatigue [28]. To counter such reactions, IL-10, an anti-inflammatory cytokine, is produced by macrophages to suppress excess production of inflammatory cytokines and excessive inflammation [29]. Both NF-κB and IL-10 play a functional role in the production and regulation of such proinflammatory cytokines, respectively. Proinflammatory cytokines produced as a result of TLR4 activation and NF-κB triggering could affect the expression and regulation of neurotransmitters and receptors in the striatum and cerebellum in a way that possibly results in impaired motor functions.

5. Striatum and Cerebellum

The striatum is one of the main components of the basal ganglia which is involved in processes related to voluntary motor control. The striatum can be further divided into the dorsal striatum which consists of the caudate nucleus and putamen, and the ventral striatum which comprises of the nucleus accumbens and the olfactory tubercle. The striatum acts as the central glutamatergic and dopaminergic input receiving station and subsequently transmits these inputs to the rest of the basal ganglia. Within the striatum, the received inputs are projected onto two distinct classes of medium spiny neurons (MSNs) specified as the direct (striatonigral) and indirect pathway (striatopallidal) MSNs [30]. These two pathways differ whereby the direct pathway MSNs directly transmit inputs from the cortex and thalamus to the internal globus pallidus (GPi) and substantia nigra pars reticulata (SNr) while the indirect pathway MSNs receive input from the cortex and thalamus and indirectly transmit the outputs to SNr through the external GPe and subthalamic nucleus (STN). Moreover, the direct pathway MSNs express high levels of D1 dopamine while the indirect pathway MSNs have a high expression of D2 dopamine. Additionally, projections from the direct pathway MSNs are reported to mediate motor output, whereas projections from the indirect pathway MSNs impede motor output. The opposing activity of the two pathways is what regulates motor control [30].

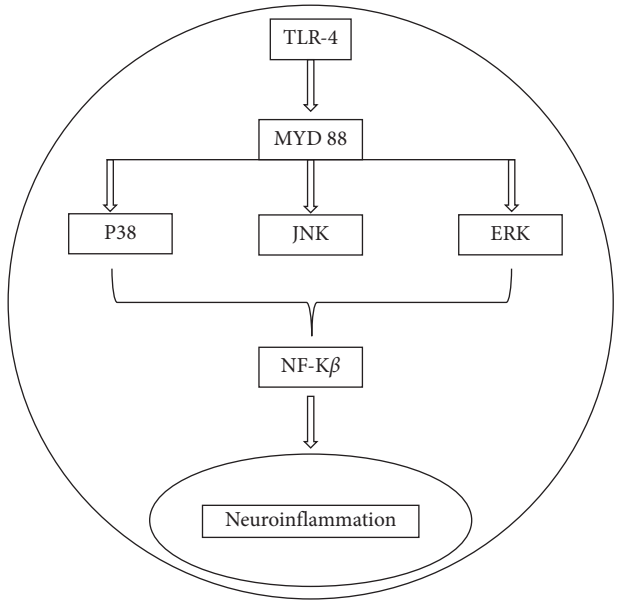

Figure 1: The schematic diagram shows the possible pathways to trigger neuroinflammation through toll-like receptor 4 in the brain.

Dysfunction of the connectivity or projections of the striatum is recognized as a notable cellular pathology in a number of motor and neurodegenerative diseases such as Parkinson's disease and Huntington's disease. Parkinson's disease (PD) is associated with a progressive decline in motor control. The causal circumstance of such decline is due to a dysfunction of the motor circuits within the striatum which is resulted from dopamine denervation in the dorsal striatum ascribable to the death of dopaminergic neurons in the SNr [30].

The cerebellum, also known as the "little brain," is the major folded structure of the hindbrain. It consists of two cerebellar hemispheres whereby the cerebellar cortex comprise of three layers, which are the internal granular layer with granule cells, the middle Purkinje cell layer consisting of single row of Purkinje cells, and the molecular layer of cerebellum which is mainly made up of basket cells and stellate cells, two types of GABAergic interneurons. These cells receive excitatory synaptic inputs from granular neurons, and their axons make an inhibitory synapse with Purkinje cells. Axons of granule cells and the dendrites of Purkinje cells stretch out all the way into the molecular layer. Inputs from the cerebral cortex are transmitted to the cerebellum by mossy fibres which then excite the granule cells of the granular layer. The granule cells then specialize into parallel fibres which synapse into Purkinje cell dendrites, transmitting excitatory signals. At the same time, Purkinje cells also receive regulatory input through their axons from climbing fibres that stem from the inferior olive. Purkinje cells then sends an inhibitory signal to the deep cerebellar nucleus neurons that proceed toward the motor cortex. Concurrently, both mossy fibres and climbing fibres excite the deep cerebellar nucleus neurons. The output from deep cerebellar nucleus neurons thus depends on the overall inhibitory and excitatory stimulation [31].

The cerebellum is critically involved in modulating various networks including voluntary motor control and cognition. Studies have showed a causal role of cerebellum dysfunction in motor impairment in a number of diseases such as PD and neurological movement disorders such as dystonia and multiple system atrophy (MSA). Mormina et al. [32] studied changes in the cerebellum in neurodegenerative diseases by using magnetic resonance imaging (MRI). All the aforementioned diseases are characterized with distinguished motor impairments and cerebellum dysfunction as their pathological hallmark. Loss in cerebellar volume was reported in PD patients with tremor due to cerebellar atrophy. Additionally, cerebellar hyperactivity was shown to be higher in PD patients. Similarly, atrophy of the middle cerebellar peduncles and volume loss of the middle and inferior cerebellar peduncles were also observed in MSA patients. Cerebellar atrophy and increased cerebellum activation together with the presence of cerebellar lesions and morphological cerebellar anomaly were observed in dystonia patients with hand stiffness. Dystonia is associated with continuous, unusual muscle contractions (Figure 2).

Another MRI study on the involvement of the cerebellum in the pathogenesis of ALS was conducted by [33]. ALS is a neurodegenerative disorder involving the motor neuron system in which it affects muscle contractions and progressively impact normal movement abilities. Motor impairments in ALS patients were linked with atrophy in the inferior cerebellum specifically the inferior lobules and vermis. Both the basal ganglia and the cerebellum interact with the cerebral cortex whereby the neuronal activity between the three structures is involved with parameters of movement [34]. In addition, past literatures reported that the primary brain regions most affected by inflammatory response include the basal ganglia, particularly the ventral striatum [35]. Both the striatum and cerebellum are selected as the areas of interest due to their involvement in motor control.

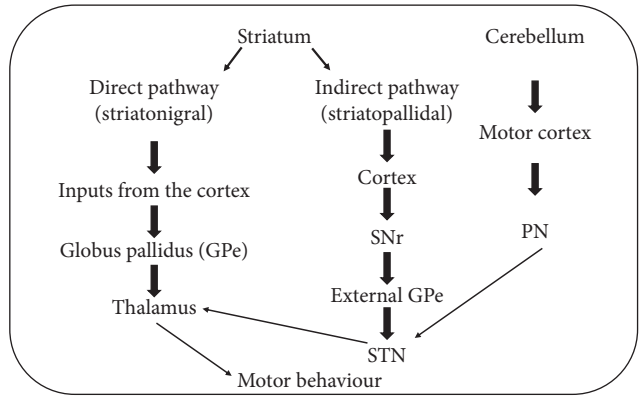

Figure 2: The schematic diagram shows the motor circuits from the striatum and cerebellum. The pathways connect the substantia nigra pars reticulata (SNr), subthalamic nucleus (STN), and cerebellum through the pontine nuclei (PN).

6. Neuroinflammation on Neurotransmitter's Receptors

Neurotransmitters are a diverse group of chemical compounds that are involved in the transmission of information in chemical synapses from the presynaptic site of one neuron to postsynaptic site of the adjacent neuron. Neurotransmitters from the presynaptic neuron diffuse into the synaptic cleft where they bind accordingly to their specific receptors to activate the respective signaling cascades. The neurotransmitters then either undergo the reuptake process by presynaptic transporter proteins and astrocytes or are degraded by specific enzymes that are present in the synaptic cleft. The resulting signaling cascade can elicit either an excitatory or inhibitory signal. Thus, neurotransmitters can be either excitatory or inhibitory in nature and are grouped accordingly based on structure and function [36]. Some of the neurotransmitter groups are as follows: acetylcholine, amino acids (glycine, glutamate and GABA (gamma aminobutyric acid)), amino acid derived amines (epinephrine, norepinephrine, dopamine, and serotonin), peptides (substance P and endorphins), purines (ATP), and gases (nitric oxide). Excitatory neurotransmitters include serotonin, acetylcholine, epinephrine, and norepinephrine, whereas inhibitory neurotransmitters include glycine and GABA. Studies had long demonstrated the involvement of various neurotransmitters in the proper functioning of motor neurons in the striatum and cerebellum [37, 38]. These studies involved the investigation of the functional relationship between neurotransmitters such as serotonin, GABA, dopamine, and glutamate with motor functioning.

7. Role of Gamma-Aminobutyric Acid (GABA) on Motor Behaviour and Neuroinflammation

GABA is a major inhibitory neurotransmitter in the CNS. The inhibitory process is regulated by inonotropic and metabotropic receptors which are located in presynaptic and postsynaptic regions [17]. Besides, GABA is one of the predominant inotropic receptors in the basal ganglia. The chloride conductance increased due to actions of its inhibitory role. Alteration in the GABAa receptor could cause motor deficits. Over activity of the striatal pathway suppresses dopamine in pallidus neuorons which is responsible for motor behavior in parkinsonian symptoms [39]. Drugs, for example, flumazenil, could facilitate motor behaviour interact with the GABAergic system which indicates that GABA has specific role in the modulation of motor behaviour [18]. The increased level of GABA tone in the cerebellum causes motor impairment [40]. Another study suggested that the GABA level increased in extracellular may reduce motor coordination [41]. Recent literatures reported that peripheral and central nervous system inflammation in diabetes or surgeries alters the GABAergic system, resulting in altered motor behaviour [42, 43]. This study suggested that motor coordination regulated by reduced status of neuroinflammation is related with normalization of the GABA neurotransmitter in the cerebellum [43]. The molecular mechanism results suggested by neuroinflammation could alter the GABAergic system in the cerebellum [43]. These studies clearly connected with neuroinflammation with GABAergic neurotransmission.

8. Role of Dopamine on Motor Behaviour and Neuroinflammation

Dopamine, unlike other neurotransmitters, can act as both inhibitory and excitatory neurotransmitter depending upon its location in the brain and which receptor it binds to. In dopamine receptors (DRs), Dopamine Receptor D1 (DRD1) mediates excitatory signal while Dopamine Receptor D2 (DRD2) mediates inhibitory signals. DRD1 is highly distributed in the striatum, nucleus accumbens, olfactory tubercle, cerebral cortex, and amygdala. Additionally, DRD2 is also highly communicated in the striatum, olfactory tubercle, and nucleus accumbens as well as in the substantia nigra pars compacta (SNc) and ventral tegmental area. The striatum acts as one of the main target region for dopamine involving the regulation of motor functions. Dopamine is critically involved in numerous brain circuits in the nervous systems associated with mediating motor control, feeding behaviour, cognitive functions, emotion, motivation, and reward [44].

Dopamine is generally known to be involved in the modulation of motor functions, and this has been stated and reiterated in numerous studies and articles. Neurotransmission and projection of dopamine from the substantia nigra to the striatum and to the cerebellum from the ventral tegmental area have been noted to influence the fine tuning of movements [45]. Nuclei in both SNc and the ventral tegmental area are reported to make up the major dopaminergic tracts [44]. The corticostriatal circuit expresses high levels of both Drd1 and Drd2, demonstrating the involvement of such receptors in controlling movement, thus justifying the selection of Drd1 and Drd2 in this study. Additionally, varied connection strength between striatum-cortical, striatum-cerebellar, and cortico-cerebellar motor influenced by imbalanced neurotransmission of dopamine were observed in Parkinson's patients with akinesia [46]. Subsequently, the production of cytokine during neuroinflammation is found to be involved in the alterations in dopamine neurotransmission whereby cytokines ultimately lead to decreased dopamine synthesis, thus decreasing dopamine function which could lead to neurodegeneration.

9. Role of Serotonin on Motor Behaviour and Neuroinflammation

Serotonin (5-hydroxytryptamine, 5-HT) acts upon excitatory transmission and operates as a mediator in inflammatory processes. 5-HT neurons are widely dispersed in the raphe nuclei of the brain stem such as the pons and medulla oblongata and additionally other brain regions, for example, the striatum, hippocampus, amygdala, cerebral cortex, thalamus, hypothalamus, and spinal cord [47]. Besides governing the regulation of critical physiological processes such as motor activity, sleep, body temperature, and pain, 5-HT is also significant in mediating endocrine and autonomic systems as well as emotional behaviour and cognitive function [48]. 5-HT has been reported to enhance and/or depress glutamate-mediated transmission as well as GABA-mediated transmission in structures controlling the movement [8]. 5-HT receptors, sorted into 7 families consisting of 5-HT1, 5-HT2, 5-HT3, 5-HT4, 5-HT6, and 5-HT7, mediate the serotonergic signal transduction. These 7 families are further broken down into 14 subtypes which are 5-HT1A, 1B, 1D, 1E, 1F, 5-HT2A, 2B, 2C, 5-HT3, 5-HT4, 5-HT5A, 5B, 5-HT6, and 5-HT7. Found at both pre- and postsynaptic membrane, the 5-HT receptors with the exception of 5-HT3 receptors which are ligand-gated ion channels are G protein-coupled receptors [47, 48].

Serotonin is generally involved in the mediation of motor behaviour through the cerebellum. The serotonin innervations from other motor structures also influence the cerebellum to modulate motor behaviour [15]. Hoxha et al. reported that parallel fibre-Purkinje cell (PF-PC) synapse is proposed as mechanisms for motor learning. The PF-PC synapse is finely modulated by several neurotransmitters including serotonin. In Rett syndrome, serotonin neurotransmission mainly participates in motor control through the help of the hippocampus and cerebellum [49]. Schizophrenia of the human cerebellum also shows serotonin 5-HT (2A) receptor expression in Purkinje cells along with motor behaviour [50]. In the cerebellar atrophy 5-HT increases in the cerebellum related with alteration in motor coordination [51]. Previous studies reported that 5-HT1A role in cerebellar ataxia as well [52].

Various 5-HT receptors mediating 5-HT neurotransmission were reported in the regulation of extrapyramidal motor functions which are implicated in the pathophysiology of various neurological disorders. This is supported by the findings of the ameliorating effect of 5-HT1A receptors activation on antipsychotic-induced extrapyramidal side effects (EPS) and motor disabilities in animal models of Parkinson's disease in a study by 39. Specifically, 5-HT1A and 5-HT2A/2C receptors are amongst the multiple receptors that are of great significance in the modulation of motor disabilities in animal models of Parkinson's disease [47]. Past study of such findings include an experiment that demonstrated the weakening of L-DOPA-induced dyskinesia in 6-hydroxydopamine-lesioned rats through administration of mixed 5-HT1A/1B receptor agonist, eltoprazine [53]. Another study showed a decrement in tacrine-induced tremulous jaw movements in rats which are considered a primary motor symptom of tremor through the administration of 5-HT2A receptor inverse agonist and antagonist, ACP-103 [54]. Additionally, both 5-HT1A receptor agonist and 5-HT2A receptor inverse agonist and antagonist were shown to reduce L-DOPA-induced dyskinesia in MPTP-treated macaques. Serotonin has role in innate and adaptive immunity. Serotonin could trigger lymphocytes and monocytes which have an impact on the secretion of cytokines [55]. Recent literature reported that proinflammatory functions mainly active 5-HT2A receptors subtypes which inhibit TNF-α mediated inflammation (Yu, B). The animal studies showed that 5-HT2A blocks inflammatory response and prevents TNF-α activity (Nau).

Brain microglia expressed the mRNA of serotonin receptors. 5-HT2B receptor is expressed in microglia and supported for brain maturation [56]. From the literature, it is understood that serotonin has a main role in the modulation of neuroinflammation and motor behaviour.

10. Role of Glutamate on Motor Behaviour and Neuroinflammation

Glutamate is the most prevalent excitatory neurotransmitter in the CNS, having an extensive functional contribution in both the CNS and peripheral nervous system (PNS) processes as it is involved in various metabolic pathways. Present on glutamatergic neurons, glutamate execute glutamatergic signal transduction by binding to and, hence, activating both ionotropic and metabotropic glutamate receptors located on postsynaptic neurons. Regulation of glutamate is critical as unsuppressed glutamate release will result in glutamate dysregulation which poses excitotoxicity within the CNS. Such occurrence leads to neuronal damage and even neuronal death. Glutamate dysregulation has been well characterized in certain psychiatric, neurodevelopmental, and neurodegenerative disorders. The excitatory amino acid transporters (EAATs) hold the responsibility in preventing glutamate dysregulation by governing the release and reuptake of glutamate. Furthermore, glutamate transporters also contribute to learning, memory, and motor behaviour regulation. There are a total of five EAAT subtypes which are EAAT1 or GLAST (glutamate/aspartate transporter), EAAT2 (glutamate transporter-1), EAAT3 or EAAC1 (excitatory amino acid carrier-1), EAAT4, and EAAT5 [57].

Recently, it has been well studied that astrocytes play key role in the regulation of synaptic communication through modulating neurotransmitters and neuromodulators. It is reported that astrocytes control specifically and rapidly glutamate transmission [58]. Glutamate is a major excitatory neurochemical in the brain which is critical for maintaining normal CNS function. Glutamate and glutamine cycle are well mediated by astrocytes where released glutamate is recycled to glutamine in glial cells [59]. EAATs located in the neuron and astrocytes maintain glutamate release [60]. The excess of GLU is mainly regulated by GLU transporters on astrocytes [59]. Astrocytes regulate synaptic glutamate level by GLAST. The past and present animal studies reported knockdown or inhibition of GLAST results in increased level of GLU [61]. This caused motor impairment in rotarod. Astrocytes mainly involved in the mediation of pathological condition. The GLU and GABA are mostly regulated in the cerebellum by astrocytes. Neuroinflammation and glutamate toxicity play an important role in neurodegenerative process, resulting in elevated levels of inflammatory markers in ischemia and Parkinson's disease [62]. Besides, abnormal inflammatory mediates oxidative stress then may lead to glutamate excitotoxicity which plays an important role in pathogenesis [63]. Glial cells especially astrocytes are potentially involved in both glutamatergic and inflammatory process. Cytokine is well linked with neuroinflammatory process and glutamate-mediated toxicity. Glutamate released by neuronal vicious circle, microglia vicious circle, and astroglial vicious circle are regulated by glutamate transporters (Serafini).

Neurological disorders such as stroke, epilepsy, amyotrophic lateral sclerosis (ALS), Alzheimer's disease (AD), and Parkinson's disease (PD) exhibit alterations in the function or expression of glutamate transporters (EAATs) in their pathogenesis [64]. Zhang et al. [57] in their study showed that PD animal models exhibit a decreased expression and function of EAATs. EAATs especially EAAT1 are important in the maintenance of extracellular glutamate concentrations below glutamate excitotoxic levels, because high glutamate concentration results in glutamate neurotoxicity and subsequent dopamine neuronal death, movement disorder, and cognitive impairment [65]. Concentration of extracellular glutamate increases in the early stages of neuroinflammation due to microglia activation. A study by [66] demonstrated that fluctuation in neuronal glutamate transporter EAAT4 expression levels can alter the extrasynaptic glutamate signaling. Furthermore, both direct and indirect pathways of the corticostriatal circuit receive glutamatergic inputs that alter glutamate transmission in the dorsal striatum through NMDARs blockade which may contribute hyperactivity of motor function.

11. Future Perceptive

While the above studies provide valuable information regarding the potential associative mechanism between TLR, neuroinflammation and connection with neurotransmitters in the striatum- and cerebellum-mediated motor behaviour, there are still gaps in understanding the involvement and potential changes in functional neurotransmitter receptors and transporters that need to be investigated in order to know the complete mechanism of TLR4 activation in affecting motor behaviour. The known underlying pathway can provide alternative therapeutic treatment for existing neurological and motor neuron diseases. Therefore, this review was placed on the implication of the TLR toward the motor behaviour and associated neurotransmitter receptors and transporters using the animal model.

Conflicts of Interest

The authors declare there are no conflicts of interest.

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Research Article

Late Brain Involvement after Neonatal Immune Activation

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The neonatal immune system is still immature, which makes it more susceptible to the infectious agents. Neonatal immune activation is associated with increased permeability of the blood-brain barrier, causing an inflammatory cascade in the CNS and altering behavioral and neurochemical parameters. One of the hypotheses that has been studied is that neuroinflammation may be involved in neurodegenerative processes, such as Alzheimer's disease (AD). We evaluate visuospatial memory, cytokines levels, and the expression of tau and GSK-3β proteins in hippocampus and cortex of animals exposed to neonatal endotoxemia. C57BL/6 mice aging two days received a single injection of subcutaneous lipopolysaccharide (LPS). At 60,120, and 180 days of age, visual-spatial memory was evaluated and the hippocampus and cortex were dissected to evaluate the cytokines levels and expression of tau and GSK-3β proteins. The animals exposed to LPS in the neonatal period present with visuospatial memory impairment at 120 and 180 days of age. Here there was an increase of TNF-α and IL-1β levels in the hippocampus and cortex only at 60 days of age. Here there was an increase in the expression of GSK-3β in hippocampus of the animals at 60, 120, and 180 days of age. In the cortex, this increase occurred in the 120 and 180 days of age. Tau protein expression was high in hippocampus and cortex at 120 days of age and in hippocampus at 180 days of age. The data observed show that neonatal immune activation may be associated with visuospatial memory impairment, neuroinflammation, and increased expression of GSK-3β and Tau proteins in the long term.

1. Introduction

The neonatal period has the highest lifetime risk of serious infections. Neonatal sepsis is defined as a systemic inflammatory response, occurring in the first four weeks of life as a result of a suspected or proven infection. Sepsis is a leading cause of morbidity and mortality in newborn and preterm infants [1–3]. In this context, infections caused by Gram-negative bacteria are of high prevalence during prenatal and neonatal periods. The lipopolysaccharide (LPS) is the major virulent constituent of the outer membrane of Gram-negative bacteria and neonatal LPS exposition seemingly mimics physiological and behavioral alterations triggered by a Gram-negative bacterial infection [4].

The brain of neonates is more vulnerable to damage in response to systemic inflammation. The systemic proinflammatory cytokines is able to increase blood-brain barrier (BBB) permeability and lead to microglial activation. The immunological implications of brain immaturity, particularly with regard to the immaturity of central nervous system (CNS) immune cell regulation, might render brains especially vulnerable to damage by poorly controlled and pervasive inflammation [5, 6].

Studies have been demonstrated that neonatal immune activation by LPS in the early life periods can be associated with short- and long-term consequences. Animals that received a single injection of systemic LPS on the seventh postnatal day showed an increase in apoptotic cells within 24 hours and an increase in IL-6, IL-1β, and TNF-α levels between 6 and 48 h in brain tissue. Microglial activation was observed after 48 hours of exposure to LPS. These changes persisted up to 7 days after endotoxemia [7]. In the long term, it was reported that systemic immune activation by LPS on the third and fifth postnatal days increased levels of TNF-α and IL-1β in hippocampus of adult animals [8]. Cognitive impairment also was reported. Comim and collaborates (2016) [9] showed that animals exposed to LPS on the second postnatal days and evaluated when completed 60

days old presented with habituation and aversive and recognition memory impairment. A clinical study by Stoll and collaborates (2004) [10] observed the association between neonatal sepsis and cognitive deficits in children evaluated between 18 and 22 months old. Long-term alterations in brain development following being exposed to LPS result from the fact that neuronal migration, gliogenesis, and myelinogenesis occur at a late gestational age and predominate in the first two weeks of postnatal life [11].

This long-term impairment in brain function was suggested to result from neurodegenerative or ischemic mechanisms triggered by systemic inflammation [12, 13]. In the neurodegenerative processes the diagnosis is generally performed only in advanced or late stages, when brain function is impaired and the treatment is almost ineffective. Alzheimer's disease (AD), one of the most studied neurodegenerative diseases, is associated with an intense neuronal death occurrence, progressive formation of neurofibrillary tangles, and amyloid plaques. These neurofibrillary tangles are formed due to hyperphosphorylation of the protein tau, while amyloid plaques are synthesized by hydrophobic aggregates of misfolded amyloid-β peptide (Aβ) [14–16].

Studies showed that alterations in the GSK-3β protein expression are also associated with an increase of production and deposition of the Aβ protein in hippocampus [17] and frontal cortex [18] of AD patients. One of the functions of the GSK-3β protein is to regulate the phosphorylation of the tau protein. In this context, the increase of GSK-3β and tau expression may be involved in AD symptoms, including cognitive deficits [19]. Recently, it has been shown that mechanisms such as chronic neuroinflammation can occur before the classic pathological alterations mentioned above during AD [20]. The hypotheses of this study are that neuroinflammation in the early stages of life may be involved in neurodegenerative processes, such as AD. Thus, the objective of this study is to evaluate visuospatial memory, cytokines levels, and the expression of tau and GSK-3β proteins in hippocampus and cortex of animals exposed to neonatal endotoxemia.

2. Materials and Methods

2.1. Animals. Neonatal male C57BL/6 mice aged 2–3 postnatal days from our breeding colony were used for the experiments. All procedures were approved by the Animal Care and Experimentation Committee of UNISUL 17.003.4.01.IV, Brazil, and were in accordance with the National Institute of Health Guide for the Care and Use of Laboratory Animals (NIH publication no. 80-23), revised in 1996.

2.2. Animal Model of Endotoxemia. The technique consists of a single subcutaneous administration of 25 μg/kg of LPS (O26:B6 *E. coli* LPS, Sigma Chemical). The control group received a subcutaneous injection of PBS as a placebo in equivalent volume. The animals were monitored on the days following exposure to endotoxemia evaluated to mortality, resulting in survival of 25% after 80 h [9]. The literature reports that this experimental model is the one that most closely resembles neonatal sepsis, since there is a 60% mortality in the first days of life. Eighty percent of survivor animals showed a decrease of body weight and developmental delay until 60 days of age when compared to control animals that received PBS. After the inoculation, the animals returned to their cages and stayed with their mothers until 21 postnatal days. After that, they were separated into five animals per cage until 60, 120, and 180 days old. Each time, the animals were submitted to the behavioral tests and after that it was euthanized and the hippocampus and cortex were immediately isolated on dry ice and stored at −80°C for posterior analysis [9, 21].

2.3. Behavioral Tasks. To evaluate the behavioral response, the animals were separated into two groups: control and LPS (n=8 per group and 16 for each time task, n=32). Twenty-one days after inoculation, the animals were randomized and subjected to the Morris water maze test. Thus, using this design, we did not assess time-dependent memory, but memory over time (with new training at each test session). All behavioral procedures were conducted between 08:00 and 10:00 a.m. in a sound-isolated room, and a single animal performed only one behavior test session in only one time point. The behavioral test was recorded by the same person who was blind to the animal group.

2.4. Morris Water Maze. The Morris water maze aims to evaluate learning and the ability to acquire spatial memory using environmental tips. The animal should learn how to use the tips attached to the wall of the room to navigate to the submerged platform. During training, the latency is measured for seconds to find the submerged platform (10 cm2). The time the animal has to find the platform, during which each time it is put into the water, is 60 seconds. If the animal does not find the platform, it is gently led up to it; once it is on the platform, it stays on it for 10 seconds. The training takes place in the two days before the test, consisting of six batteries. At the time of testing, the platform is removed, and the animal is put into the water only once; during the three-minute period it will be free to swim. In the test, the time spent in the quadrant in which the platform was located is evaluated. To perform this test, a circular tank located in the center of a room of 12 m2 with an upper window at the south point was used, and the training and tests were performed from 9 o'clock in the morning. To carry out the training, water was added to the tank until the water level exceeded 2 cm of the platform height. Then, the water was left opaque with addition of corn starch, thus making the platform visibility difficult. The acquisition of visual-spatial memory was evaluated after the end of the test through filming and analyzed by the system Any-Maze ® [22, 23].

2.5. GSK-3β and Tau Protein Expression. Brain tissue samples were manually homogenized with micropistils in ice-cold RIPA buffer containing 1% protease inhibitor (Sigma-Aldrich, St Louis, MO) and then incubated on ice for 30 min. The tubes containing the lysates were centrifuged at 10,000 g for 20 min at 4°C, and the supernatants were collected. Protein concentration was determined using the Bradford method.

The electrophoretic separation was conducted using 30 μg of protein per well in 10% polyacrylamide gel electrophoresis (SDS-PAGE), running in a Mini-PROTEAN® Tetra cell apparatus under a PowerPAC™ HC power supply (both from Bio-Rad, CA, USA). The proteins were transferred onto a PVDF membrane (Bio-Rad Laboratories Inc., Hercules, CA, USA), blocked in 5% BSA (prepared in TBS-T buffer, pH 7.4; concentration in mmol/L: 20 Tris-HCl, 137 NaCl, 0.1% Tween 20), and incubated overnight at 4°C with primary antibodies to GSK-3β (1:1000, Cell Signaling Technology, Beverly, MA, EUA) and TAU (1:1000, Cell Signaling Technology, Beverly, MA, EUA). Peroxidase-conjugated monoclonal antibody against β-actin (dilution 1:45000) was used as a loading control for all samples tested. After incubation with primary antibodies, the membranes were washed three times (10 minutes each) with TBS-T solution and incubated with the specific secondary antibody conjugated to horseradish peroxidase (HRP) at room temperature for 1 h. The membranes were washed for another three times (10 minutes each) with TBS-T solution and exposed to HRP substrate (Pierce Biotechnology, Rockford, IL, USA), and immune complexes were visualized by chemiluminescence using ChemiDoc MP System (Bio-Rad Laboratories). Bands were quantified by densitometry using the software from the manufacturer (Image Lab, version 4.1, Bio-Rad Laboratories, Hercules, CA, USA). Values were normalized using the data obtained for β-actin and expressed as arbitrary units.

2.6. Cytokines Levels. The concentration of cytokines (IL-1β and TNF-α) was determined by ELISA (R&D Systems, Minneapolis, MN). All samples were assayed in duplicate. Briefly, the capture antibody (13 mL, contains 0.1% sodium azide) was diluted in phosphate-buffered saline (PBS), added to each well and left overnight at 4°C. The plate was washed four times with PBS and 0.05% Tween 20 (Sigma, St. Louis, MO, USA). The plate was blocked with 1% bovine serum albumin and incubated for 1h at room temperature before washing four times with PBS and 0.05% Tween 20. The samples and standards were added, and the plate was incubated overnight at 4°C. After washing the plate, detection antibody (concentration provided by the manufacturer) diluted in PBS was added. The plate was incubated for 2h at room temperature. After washing the plate, streptavidin (DuoSet R&D Systems, Minneapolis, MN, USA) was added and the plate was incubated for 30 min. At last, color reagent o-phenylenediamine (Sigma, St. Louis, MO, USA) was added to each well and the reaction was allowed to develop in the dark for 15 min. The reaction stopped with the addition of 1 M sulfuric acid to each well. The absorbance was read on a plate reader at 492 nm wavelength (EMax, Molecular Devices, Minneapolis, MN, USA). Total protein was measured using bovine serum albumin as a standard as described in the literature [24].

2.7. Statistical Analyses. Shapiro-Wilk normality test were utilized to determine the parametric and nonparametric data. Data from the behavioral test and biochemical analyses are parametric data and it were reported as mean±SEM and analyzed by Student's t test. A $^*p < 0.05$ was considered statistically significant.

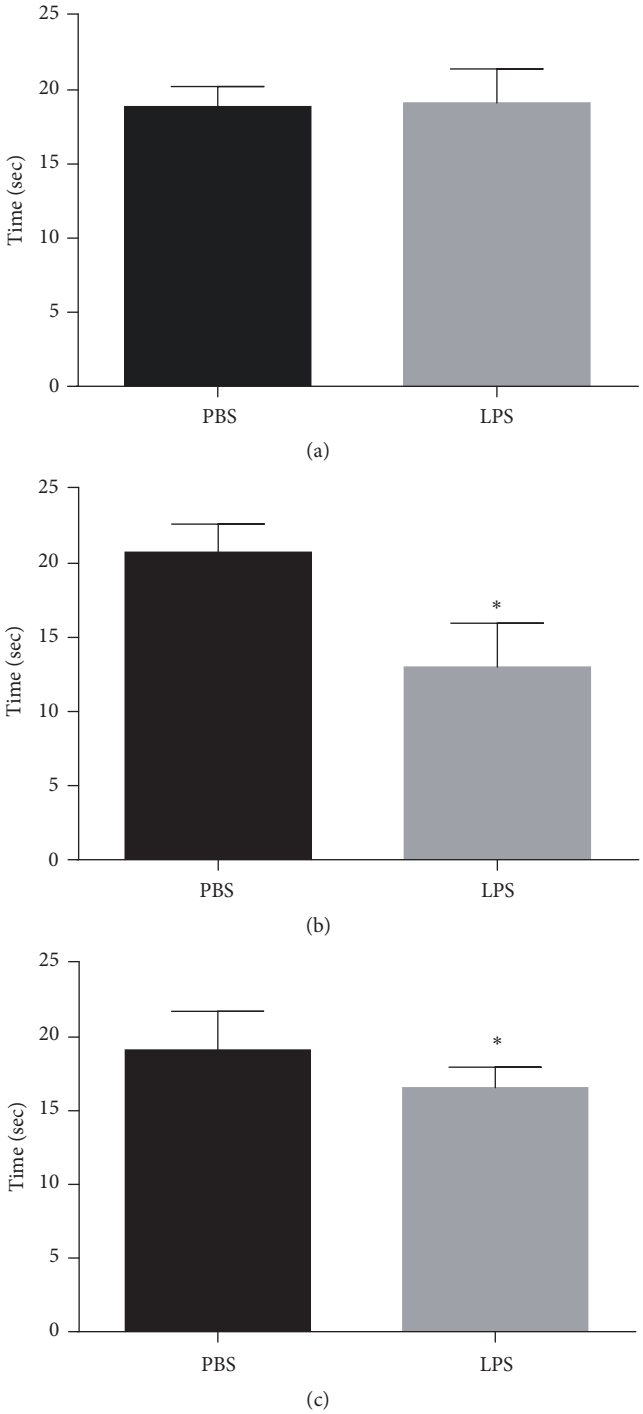

Figure 1: Morris water maze. (a, b, c) Demonstrating the results for 60, 120, and 180 days after neonatal immune activation, respectively. Data are expressed as mean and standard deviation. $^*p < 0.05$ versus PBS. n = 8.

3. Results

Figure 1 shows the results obtained in the Morris water maze test. The dwell time in the quadrant where the platform was

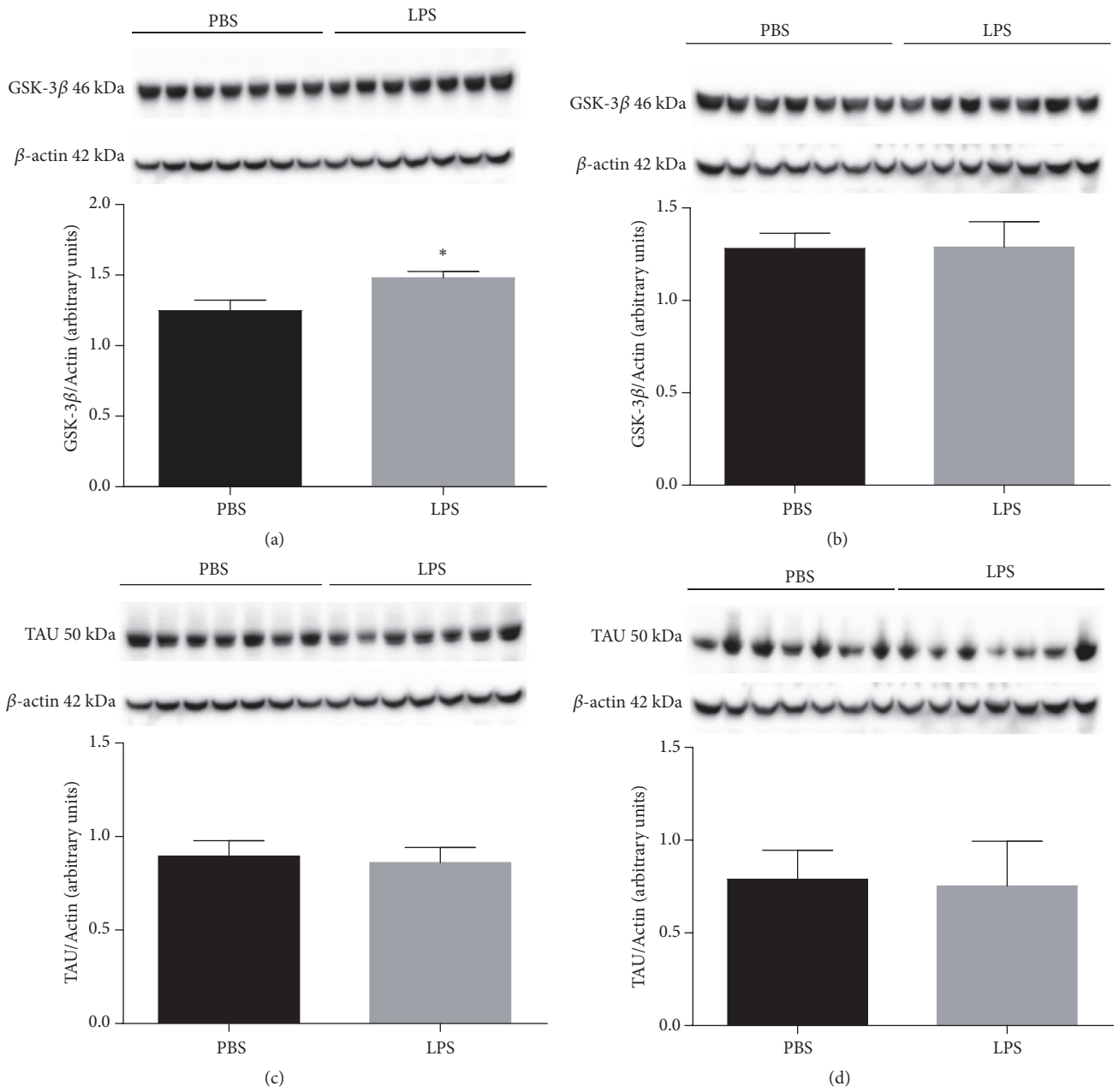

Figure 2: Expression of GSK-3β protein in hippocampus (a) and cortex (b) and Tau protein in hippocampus (c) and cortex (d) of animals 60 days after neonatal immune activation. Data are expressed as mean and standard deviation. $^{*}p < 0.05$ versus PBS. n = 8.

located was evaluated in the test session. It was observed that after 60 days (Figure 1(a)) of endotoxemia, there was no statistically significant difference between the groups evaluated ($p = 0.794$). However, after 120 (Figure 1(b)) and 180 (Figure 1(c)) days, a significant decrease in the residence time in the quadrant where the platform was located ($p < 0.0001$ and $p = 0.0403$, resp.) was observed.

Figure 2 shows the results of GSK-3β protein expression in the hippocampus (Figure 2(a)) and cortex (Figure 2(b)) and TAU in the hippocampus (Figure 2(c)) and cortex (Figure 2(d)) in animals receiving LPS or PBS at 2 days of age and was evaluated when they completed 60 days old. It was observed that after 60 days of endotoxemia there was a significant increase in the expression of the GSK-3β protein in the hippocampus of animals receiving LPS when compared to animals receiving PBS ($p < 0.0001$, Figure 2(a)). However, there were no statistically significant alterations in the evaluations of GSK-3β protein expression in hippocampus (Figure 2(b)) and TAU protein in hippocampus (Figure 2(c)) and cortex (Figure 3(d)) ($p > 0.05$) after 60 days of endotoxemia.

Figure 3 expresses the results of GSK-3β protein expression in the hippocampus (Figure 3(a)) and the cortex (Figure 3(b)) and TAU in the hippocampus (Figure 3(c)) and

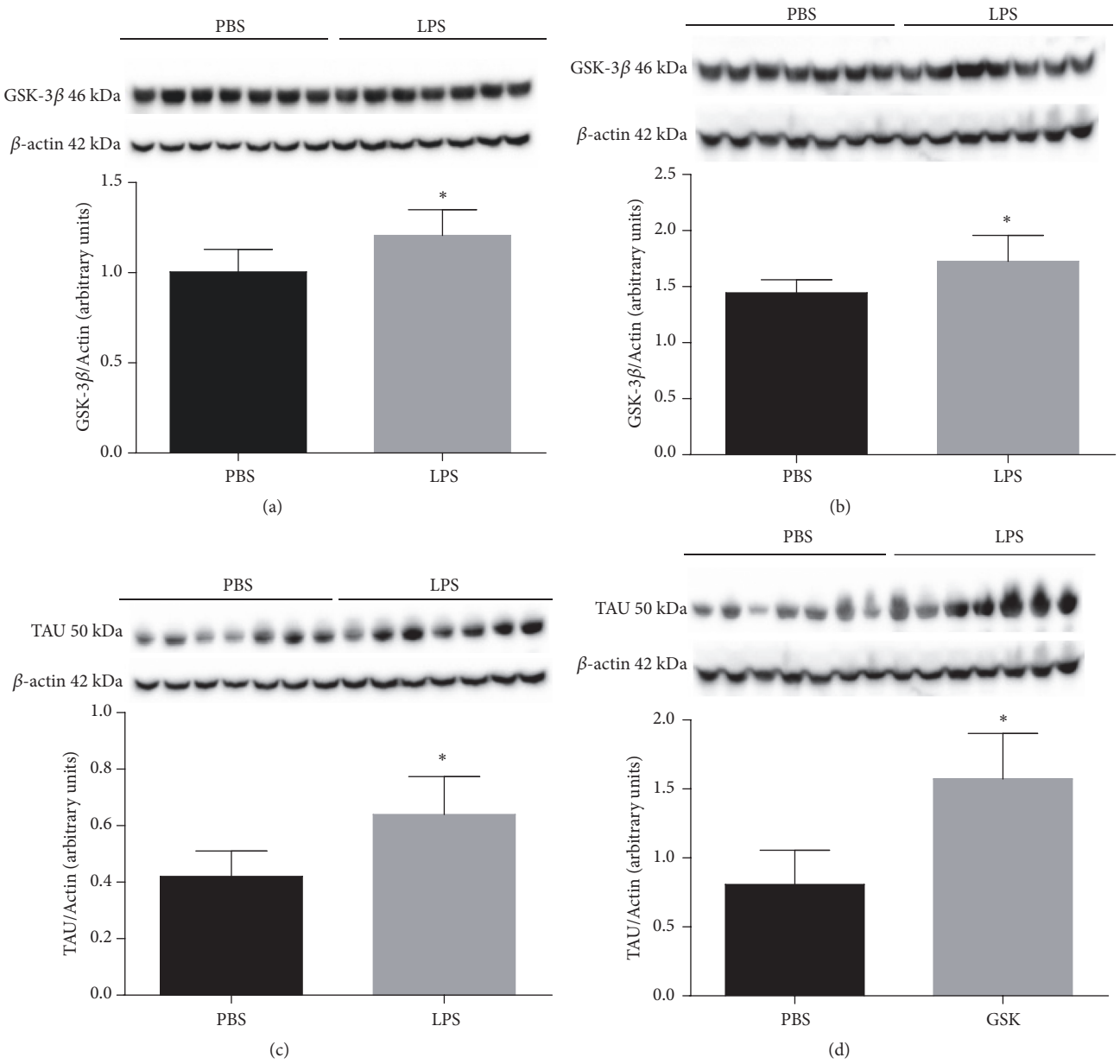

Figure 3: Expression of GSK-3β protein in the hippocampus (a) and cortex (b) and Tau protein in the hippocampus (c) and cortex (d) of animals 120 days after neonatal immune activation. Data are expressed as mean and standard deviation. $^{*}p < 0.05$ versus PBS. n = 8.

cortex (Figure 3(d)) in animals receiving LPS or PBS at 2 days of age and was evaluated when they completed 120 days old. It was observed that there was a statistically significant increase in GSK-3β protein expression in the hippocampus (Figure 3(a), p = 0.0150) and in cortex (Figure 3(b), p = 0.0396) when compared to the group of animals receiving PBS. There was also a significant increase in TAU protein expression in hippocampus (Figure 3(c), p = 0.0127) and in cortex (Figure 3(d), p = 0.0004) compared to the PBS group at 2 days old.

Figure 4 expresses the results of GSK-3β protein expression in the hippocampus (Figure 4(a)) and the cortex (Figure 4(b)) and TAU in the hippocampus (Figure 4(c)) and cortex (Figure 4(d)) in animals exposed to LPS or PBS at 2 days and was evaluated when they completed 180 days old. It was observed that there was a statistically significant increase in GSK-3β protein expression in hippocampus (Figure 4(a), p = 0.040) and in cortex (Figure 4(b), p = 0.0066) of the animals exposed to LPS when compared to the group of animals which received PBS. As for TAU protein, there was a significant increase in hippocampal expression (Figure 4(c), p = 0.0171). However, in the cortex (Figure 4(d)), there was no significant difference between the groups (p = 0.0680).

The cytokines levels were demonstrated in Figure 5. These levels were evaluated through the levels of TNF-α in hippocampus (Figure 5(a)) and cortex (Figure 5(b)) and IL-1β in hippocampus (Figure 5(c)) and cortex (Figure 5(d)) in animals exposed to LPS or PBS at 2 days and were

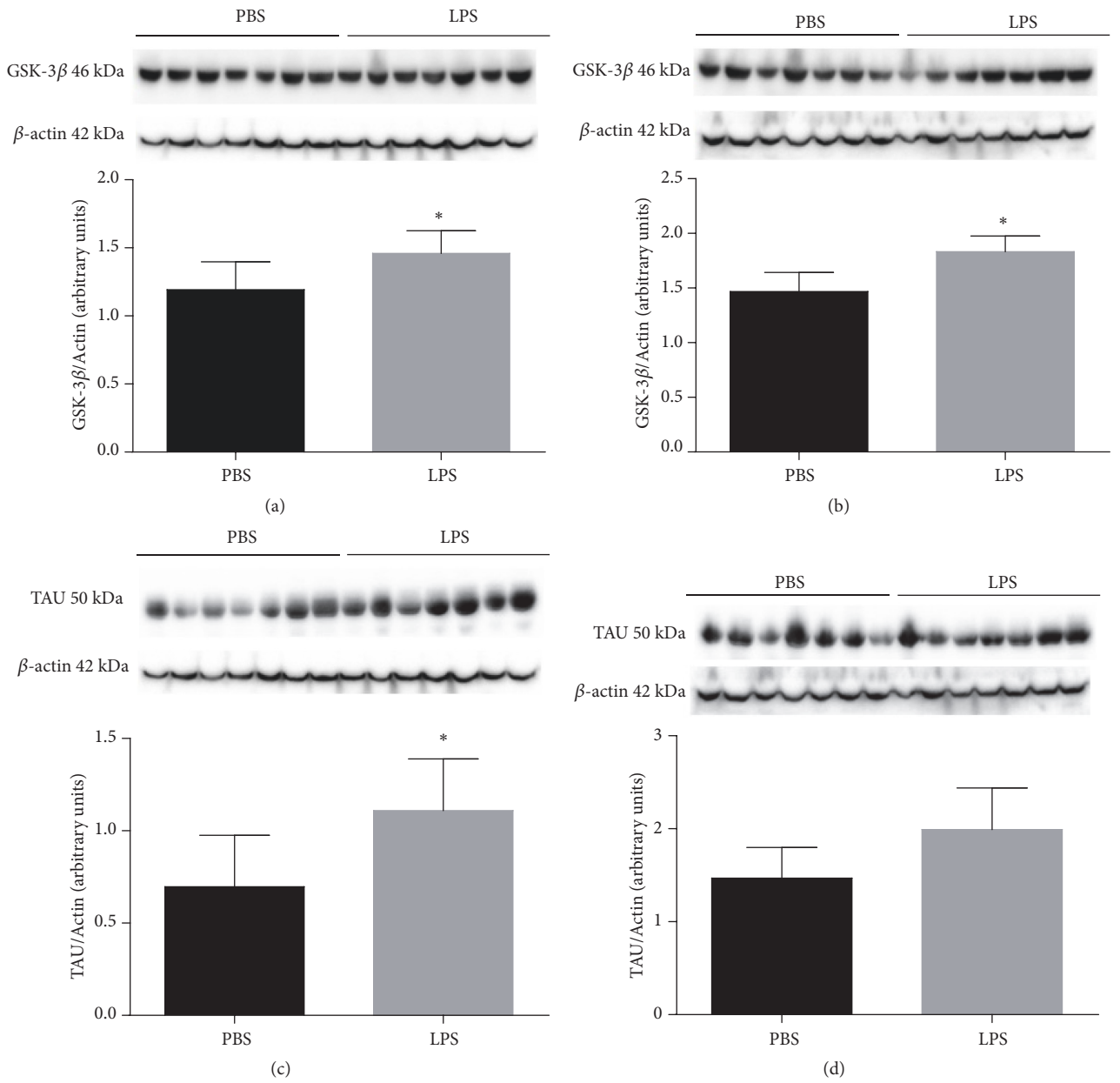

Figure 4: Expression of GSK-3β protein in hippocampus (a) and cortex (b) and Tau protein in hippocampus (c) and cortex (d) of animals 180 days after neonatal immune activation. Data are expressed as mean and standard deviation. $^{*}p < 0.05$ versus PBS. n = 8.

evaluated when they completed 60 days old. There was an increase of TNF-α in hippocampus (Figure 5(a), p = 0.0182) and (Figure 5(b), p = 0.0238) and IL-1β in hippocampus (Figure 5(c), p = 0.0376) and cortex (Figure 5(c), p = 0.0012) when compared with PBS group.

Figure 6 demonstrated the results of TNF-α in the hippocampus (Figure 6(a)) and the cortex (Figure 6(b)) and IL-1β in the hippocampus (Figure 6(c)) and cortex (Figure 6(d)) in animals exposed to LPS or PBS at 2 days and were evaluated when they completed 120 days old. There were no significant differences between groups in the TNF-α and IL-1β in the evaluated structures (p>0.05).

Finally, Figure 7 showed the results of TNF-α in the hippocampus (Figure 7(a)) and the cortex (Figure 7(b)) and IL-1β in the hippocampus (Figure 7(c)) and cortex (Figure 7(d)) in animals exposed to LPS or PBS at 2 days and were evaluated when they completed 180 days old. There were no significant differences between groups in the TNF-α and IL-1β in the evaluated structures (p>0.05).

4. Discussion

The results of this study demonstrate that the animals exposed to LPS in the neonatal period and evaluated at 120

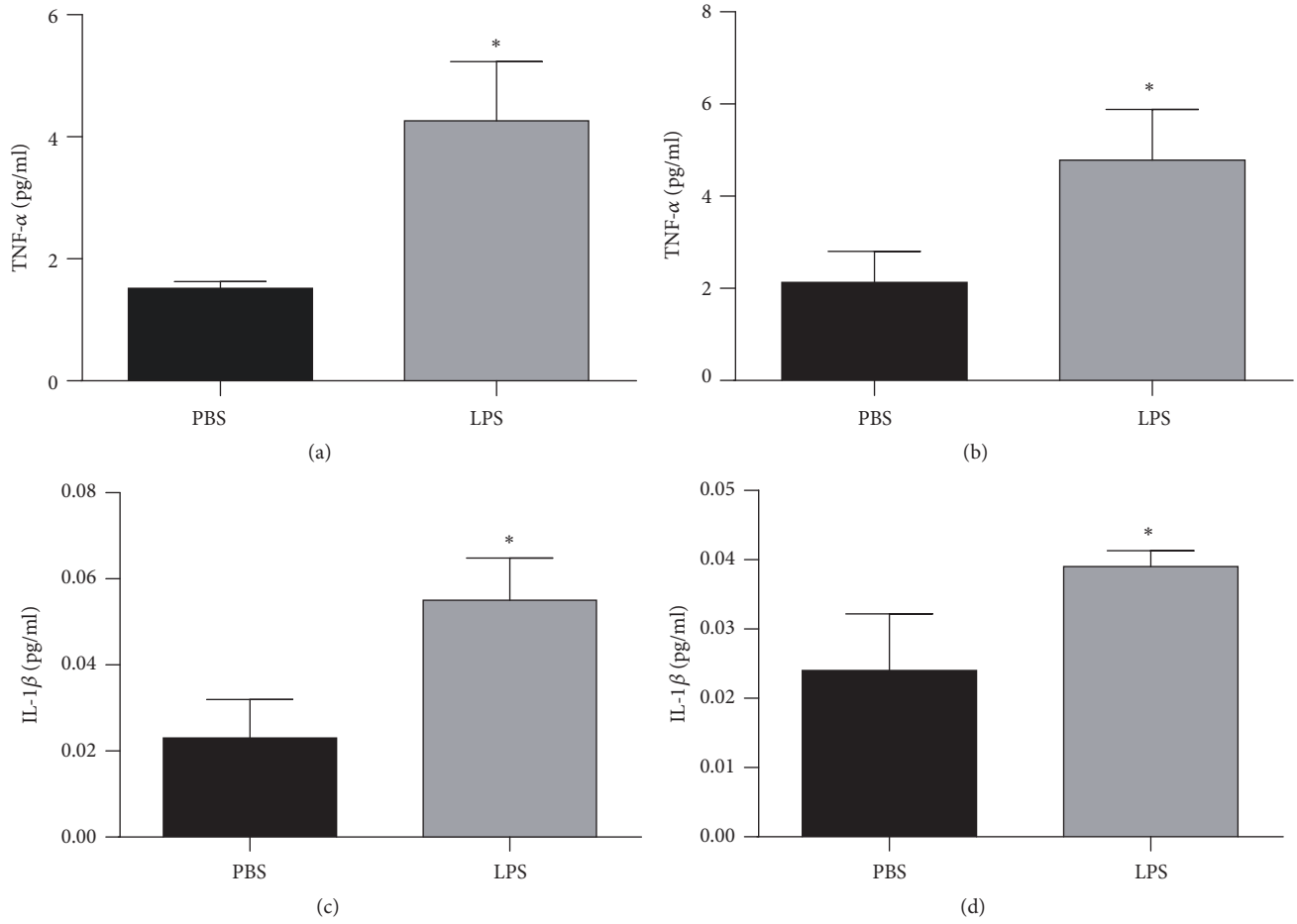

Figure 5: Levels of TNF-α hippocampus (a) and cortex (b) and IL-1β in hippocampus (c) and cortex (d) of animals 60 days after neonatal immune activation. Data are expressed as mean and standard deviation. $^{*}p < 0.05$ versus PBS. n = 8.

and 180 days old presented with visuospatial memory alteration. As regards protein expression, there was an increase in GSK-3β expression in hippocampus when the animals completed 60 days old. In animals with 120 days of age, there was an increase in GSK-3β and Tau expression in hippocampus and cortex. In animals with 180 days of age, there was a significant increase in GSK-3β protein expression in hippocampus and cortex and Tau protein only in hippocampus. There was also an observed increase of TNF-α and IL-1β levels in the hippocampus and cortex only at 60 days old.

The LPS entry into the brain after systemic administration is in small amounts; the increased expression of proinflammatory mediators is capable of increasing the permeability of the blood-brain barrier leading to a microglial activation. Once activated, microglia initiate a neuroinflammation process sustained by the release of proinflammatory cytokines directly into the brain tissue, intensifying damage to neuronal and glial cells [6, 25–27]. Microglia in the neonatal period are responsible for a process known as synaptic pruning, directly influencing synaptogenesis. Microglial dysfunctions in the early postnatal days are associated with cerebellar hypoplasia, neuronal loss and retraction, delay in the myelination process associated with changes in progenitor cell proliferation and differentiation, and increased expression of TLR-4 receptors and matrix myeloperoxidase 9 (MMP-9) and astrogliosis in brain tissue a few days after exposure to LPS in an animal model [28].

In a long term, the endotoxemia in the early life periods is associated with cognitive impairment in animal model [9] and patients [10]. The cognitive alterations are correlated to increase TNF-α and IL-1 in hippocampus [8]. In addition, cytokine overexpression has been associated with neuropsychiatric diseases such as depression [29–32] and neurodegenerative diseases such as AD and other dementias [33, 34]. Long-term alterations in brain development following LPS-induced neonatal immune activation result from the fact that neuronal migration, gliogenesis, and myelinogenesis occur at a late gestational age and predominate in the first two weeks of postnatal life [11]. In addition, it was demonstrated that a single systemic injection of LPS on the fourteenth postnatal day altered social behavior when the animals aged 21 days [35].

In addition to the behavioral consequences described above, pre- and postnatal exposure to LPS in rodents also include autism-like behaviors induced by prenatal exposure on the ninth embryonic day [36], schizophrenia-like behaviors induced by LPS exposure on the 15th embryonic day

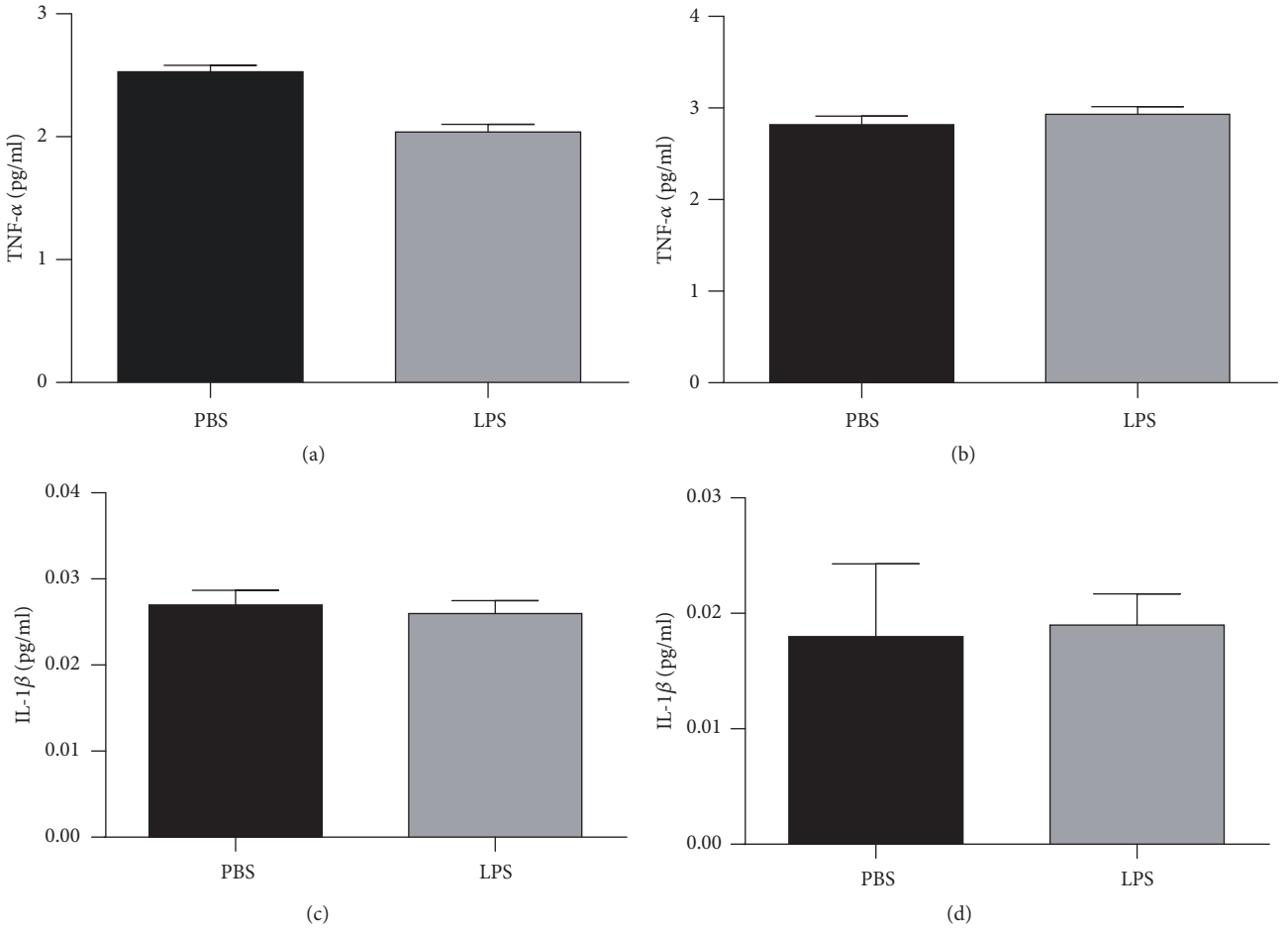

Figure 6: Levels of TNF-α hippocampus (a) and cortex (b) and IL-1β in hippocampus (c) and cortex (d) of animals 120 days after neonatal immune activation. Data are expressed as mean and standard deviation. n = 8.

[37], and anxiety-like behaviors induced by LPS exposure on the third and fifth postnatal days [8]. However, on the long-term consequences related to neurodegeneration, there are few reports in the literature.

Evidence suggests that the neuroinflammation processes are involved in the development of AD [38–40] and TNF-α plays an important role during the inflammatory response. The results of the present study demonstrated that animals exposed to endotoxemia in the neonatal period presented with increased levels of cytokines, TNF-α, and IL-1β in the hippocampus and cortex at 60 days old. In cross-sectional studies, these cytokines have been associated with cognitive deficits and dementia [41–43] and in clinical cohort studies in AD [39, 40]. As in studies with transgenic animals with AD, elevated TNF-α levels were observed in the brain tissues of these animals [44].

In the present study, it was possible to observe that endotoxemia in the neonatal period altered visuospatial memory only when the animals completed 120 and 180 days old. After 60 days, when the mice were considered young adults, there was no alteration of the visuospatial memory. This memory consists of the ability to encode, store, and retrieve information about spatial locations. The hippocampus and cortex are areas involved in the formation and storage of visual-spatial memory. Deficits in visuospatial memory are commonly observed in subjects diagnosed with neurodegenerative diseases, such as AD.

The AD is characterized by a progressive loss of visuospatial abilities associated with an accumulation of senile plaques, neurofibrillary tangles, and neuronal loss mainly in the hippocampus and temporal cortex [45, 46]. In addition to the changes described above, another important feature in the degenerative process of AD is the increased expression of GSK-3β and Tau proteins in brain tissue. Studies have shown that alterations in the GSK-3β protein are also associated with increased production and deposition of the Aβ protein. In this study, there was an increase in the expression of the GSK-3β protein in hippocampus of animals with 60, 120, and 180 days of age. In cortex, GSK-3β expression increased only in animals at 120 and 180 days of age. Visuospatial memory alteration was also observed only in animals with 120 and 180 days of age.

One of the functions of the GSK-3β is to regulate the phosphorylation of the Tau. Hyperphosphorylated Tau was found mainly in hippocampus and temporal lobe regions during the pathophysiological process of AD. Increased

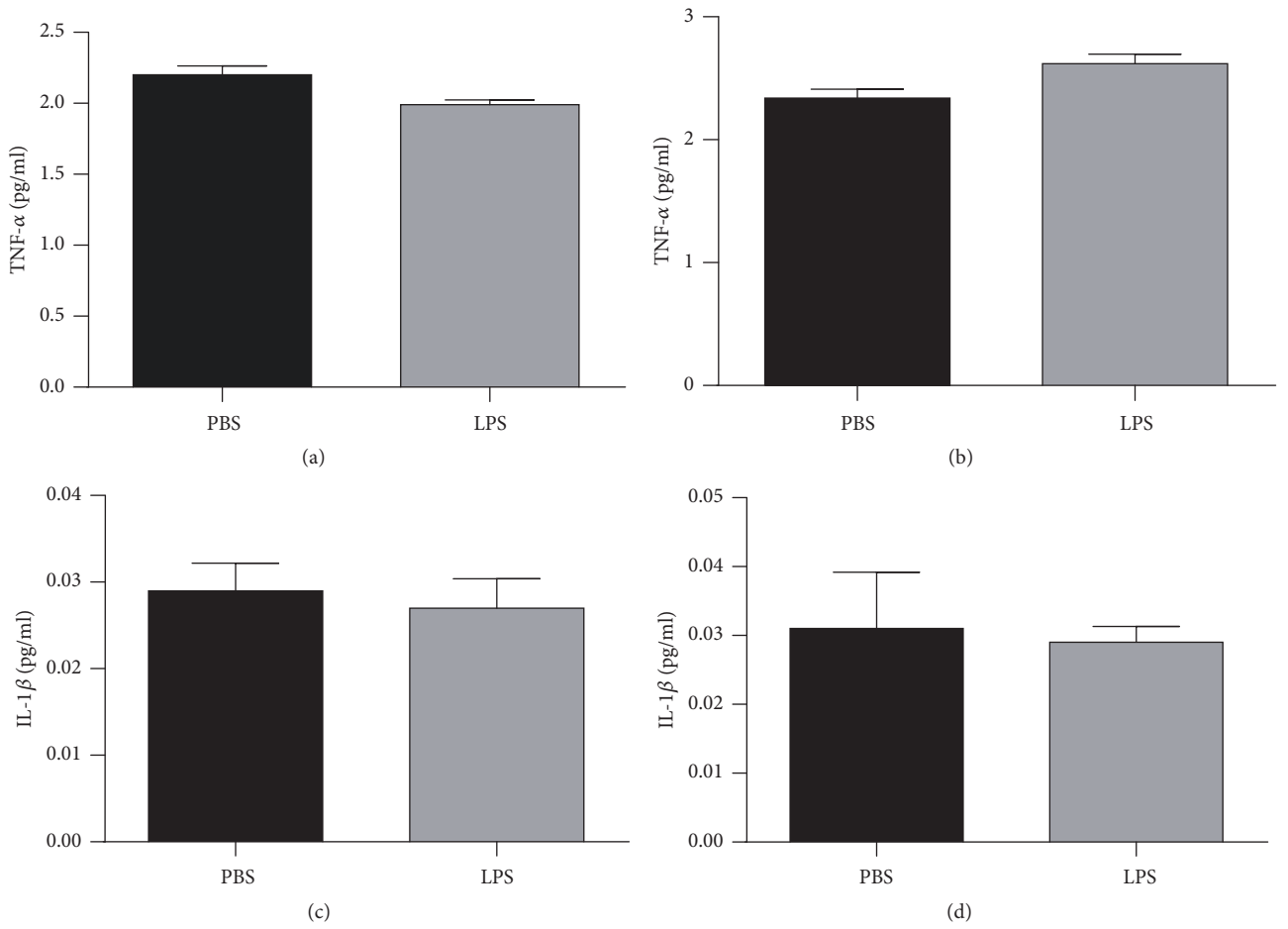

Figure 7: Levels of TNF-α hippocampus (a) and cortex (b) and IL-1β in hippocampus (c) and cortex (d) of animals 180 days after neonatal immune activation. Data are expressed as mean and standard deviation. n = 8.

expression of GSK-3β, Tau, and other substrates may be involved in AD symptoms, including cognitive deficits [18]. Changes in GSK-3β and Tau proteins are related to axonal transport, leading to impaired memory and learning as a result of synaptic dysfunction [18, 47, 48].

After the above explanation, increased expression of GSK-3β and Tau proteins in regions highly involved in learning and memory could be associated with impairment of visuospatial abilities. A consistent finding observed in this study was that, in animals at 60 days of age, increased GSK-3β expression was observed in the hippocampus associated with increased cytokines levels in hippocampus and cortex. However, these animals did not show visual-spatial memory alteration. Alteration in visuospatial memory was only demonstrated in animals at 120 days of age, that is, when there was an increase in the expression of GSK-3β and Tau proteins, both in the hippocampus and in the cortex. At 180 days old, the animals exposed to LPS in the neonatal period still presented with alterations in visuospatial memory associated with an increase in the expression of GSK-3β and Tau in hippocampus and only GSK-3β in cortex. In this time, we did not observe increase of cytokines levels.

5. Conclusion

In this context, it is believed that the results of this study may contribute to strengthening the evidence that a process of systemic neonatal immune activation can cause a change in visual-spatial memory and increase the expression of GSK-3β and Tau proteins in hippocampus and cortex in later periods.

Data Availability

The data used to support the findings of this study are available from the corresponding author upon request.

Conflicts of Interest

None of the authors or funding sources has conflicts of interest.

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Review Article

Cell Type Specific Expression of Toll-Like Receptors in Human Brains and Implications in Alzheimer's Disease

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Toll-like receptors mediate important cellular immune responses upon activation *via* various pathogenic stimuli such as bacterial or viral components. The activation and subsequent secretion of cytokines and proinflammatory factors occurs in the whole body including the brain. The subsequent inflammatory response is crucial for the immune system to clear the pathogen(s) from the body *via* the innate and adaptive immune response. Within the brain, astrocytes, neurons, microglia, and oligodendrocytes all bear unique compositions of Toll-like receptors. Besides pathogens, cellular damage and abnormally folded protein aggregates, such as tau and Amyloid beta peptides, have been shown to activate Toll-like receptors in neurodegenerative diseases such as Alzheimer's disease. This review provides an overview of the different cell type-specific Toll-like receptors of the human brain, their activation mode, and subsequent cellular response, as well as their activation in Alzheimer's disease. Finally, we critically evaluate the therapeutic potential of targeting Toll-like receptors for treatment of Alzheimer's disease as well as discussing the limitation of mouse models in understanding Toll-like receptor function in general and in Alzheimer's disease.

1. Introduction

For many years it was believed that the brain did not possess an immune system, due to its isolation *via* the blood-brain barrier (BBB). The BBB represents a physical and anatomical barrier regulating uptake and release of molecules into the nervous tissue. In this manner, the brain is protected from the rest of the body, ensuring homeostasis of the cellular environment, which is essential for proper neuronal function [1]. The barrier limits entry of undesirable and/or toxic molecules and provides a means of removal of toxic substances produced in the brain. On the other hand, the BBB hinders delivery to the brain of nutrients and growth factors required for proper metabolism and nervous function [2]. Over the past decades it has become clear that the BBB is not as restrictive a barrier as previously assumed and that the brain is equipped with an innate immune system including specialized cells mediating such immune responses [3]. Research within, for example, the arena of Alzheimer's disease (AD) has revealed that neuroinflammation plays an important role in the disease mechanism and the brain's efforts to relieve the burden of amyloid plaques. Other well-documented examples of brain innate immune system activation involve traumatic brain injuries [4]. The core cells of the brain's innate immune system comprise microglia and astrocytes, which are gaining increasing attention as regards their involvement in disease development and progression. Amongst the neurodegenerative disorders, AD is considered to be the most common, affecting millions of people worldwide, with no curative treatment currently available. Historically, most researchers have focused their efforts on Amyloid beta (Aβ) plaques and neurofibrillary tangles (NFT), which together form the major histological hallmarks identified in postmortem AD patient brains [5]. Surprisingly and unfortunately, all therapeutic efforts to lower Aβ production and plaque load, manifesting in reversal of pathological hallmarks in AD mouse models, have to date shown negligible effects in human clinical trials. Consequently, the focus of research efforts that aimed at understanding AD pathology for therapeutic purposes has shifted and the inflammatory component of AD has taken a central stage, with immunotherapy being a potentially promising approach. Although a number of

antibodies targeting Aβ have to some degree been shown effective in reducing the Aβ burden in animal models, the overall clinical trials have not shown the same results [6]. The lack of positive results could however be due to the fact that treatment was started too late, and inflammation remains as a promising target in AD therapeutics. Neuroinflammation can be considered as a third hallmark in AD, highlighting the role of nonneuronal cell types and showing AD to be a multicellular pathogenesis. Increased activation of microglia and astrocytes has been identified in AD, manifesting in release of proinflammatory cytokines and neurotoxic mediators such as tumor necrosis factor-alpha (TNF-α), interleukin-1β (IL-1β), IL-6, IL-12, and IL-18, together with upregulated production of neurotoxic mediators including proteolytic enzymes, complement factors, nitric oxide (NO), and reactive oxygen species (ROS) [7]. These cell populations detect and respond to various stimuli such as pathogens and protein aggregates, as seen in AD, through the activation of various cell surface receptors. An important group of such receptors comprises the Toll-like receptors (TLRs). An increasing body of evidence supports an association between these receptors and various neurodegenerative disorders. TLRs not only are expressed by microglia and astrocytes, but have also been identified on neurons and oligodendrocytes in the brain [8]. However, the main focus of this review will be the immune cells of the brain, namely, microglia and astrocytes. We will provide a general introduction to AD and neuroinflammation, followed by a review and discussion of the involvement of TLRs in disease pathology and the inflammatory response.

2. Alzheimer's Disease

AD is a neurodegenerative disorder characterized by a progressive decline in memory and cognitive abilities. AD is the most common form of dementia, accounting for around 60-80% of all dementia cases, and is the leading cause of disability in the elderly population, affecting approximately 50 million people worldwide [9]. With age being the most recognized risk factor for developing AD, the disease is rapidly becoming an increasing health challenge with a continuously aging population and no curative treatments currently available. AD can be characterized as either familial (fAD) in which mutations in amyloid precursor protein (*APP*), presenilin 1 (*PSEN1*), or presenilin 2 (*PSEN2*) are causative of the disease, or sporadic (sAD) with no apparent heritability. The latter accounts for the majority of all cases and is associated with genetic risk factors that in combination with adverse environmental factors confer a certain risk of developing the disease [10]. fAD and sAD share the same pathophysiology. Primarily neurons degenerate and lose their function, eventually resulting in severe brain atrophy. The AD brain is characterized by two major neuropathological hallmarks, namely, extracellular deposits of Aβ in the form of senile plaques and intracellular formation of NFTs caused by tau hyperphosphorylation [5]. These hallmarks are mainly restricted to neuronal pathology. However, emerging evidence implies that immunological processes occur alongside the degenerating neurons, indicating potential roles of microglia and astrocytes, hence, neuroinflammation as a contributor to AD development and progression. Glial activation has been identified in patients with AD [11] and elevated levels of cytokines, chemokines, and complement factors in both the brain and cerebrospinal fluid (CSF) have been observed in these patients, indicating ongoing neuroinflammation. However, the results of studies investigating the levels of cytokines in the CSF of AD patients are controversial, and the time point of sampling and stage of disease have proved to be an important factor in such studies [12]. Distinct stages of microglial activation have been suggested to occur during the course of disease. In early phases of AD, activated microglia migrate towards Aβ deposits and clear them by phagocytosis, thus providing a protective effect of increased microglial activation. However, failure to adapt to chronic Aβ deposition results in incorrectly modulated activation levels and possibly leads to a shift towards a dysfunctional or neurotoxic microglial phenotype in later AD stages [13, 14]. However, whether neuroinflammation is in fact a cause or consequence of neurodegeneration remains to be elucidated: whether the inflammatory response precedes tau and Aβ aggregation is the focus of ongoing debate.

2.1. Neuroinflammation in AD. It has become clear that neuroinflammation is an important contributor to the complex pathology of AD. Genome-wide association studies have revealed a number of genes to be associated with increased risk of AD, with many of these being expressed by immune cells, indicating a multicellular pathogenesis and a primary role of neuroinflammation in AD aetiology [15]. A well-established example is the gene encoding the *triggering receptor expressed on myeloid cells 2* (*TREM2*). TREM2, which is highly expressed by microglia, acts as a regulator of phagocytosis and cytokine production; variants within this gene have been observed in AD patients [16]. Other identified genetic risk factors include CLU, CR1, and CD33, all associated with the innate immune system [17], thus further strengthening a link between inflammation and AD. Aggregation of proteins has been observed to activate microglia and astrocytes, and Aβ deposition in AD can trigger an innate immune response. Microglia respond to Aβ which initiates migration to the plaques and phagocytosis of Aβ, alongside release of proinflammatory cytokines. Accumulation of activated microglia has been observed around Aβ plaques in both mouse models and postmortem AD brains [18, 19]. However, several animal AD models have shown that prolonged activation decreases microglial efficiency in terms of Aβ clearance, while the production of neurotoxic cytokines sustains. The compromised Aβ clearance and persistent release of proinflammatory mediators in turn damage nearby neurons further promoting neurodegeneration, accelerating disease progression [20]. Aβ can react with microglial surface receptors and stimulate either the NF-κB-dependent pathway or activation of mitogen-activated protein kinase (MAPK) pathways, inducing proinflammatory gene expression. Aβ has also been documented to induce NADPH-oxidase-mediated ROS production in microglia, resulting in increased neurotoxicity and neurodegeneration [21]. Although some of the inflammatory mechanisms involved in AD are understood,

there is still much debate as to whether neuroinflammation is causative for AD, as the identification of genetic risk factors associated with the innate immune system might imply, or if it is a consequence of other AD pathologies such as Aβ accumulation. Precise pathways and other mechanisms of microglial response in disease thus remain to be elucidated.

As microglia are the resident immune cells of the central nervous system (CNS), dysfunction in this cell population is gaining increased attention in terms of the neuroinflammatory response in AD. Normally microglia exist in a "resting" state, fulfilling such duties as synaptic pruning, to ensure proper neuronal connectivity. In addition, they play a role in modulating cognitive functions, such as learning and memory, and maintain brain homeostasis by secreting neurotrophic factors that promote differentiation and survival of neurons and by scavenging and removing defective neurons by inducing neuronal death [22]. As such, microglia perform "immune surveillance" in the brain: they become activated in the presence of various stimuli, such as pathogens or tissue damage, to eliminate the potential threat. Traditionally, activation of microglia has been categorized as having either a proinflammatory, toxic state, or an alternative, protective state. In response to stimuli, these cells have been suggested to change their phenotype either into the classical "M1" state, with secretion of proinflammatory cytokines such as TNF-α, IL-6, and IL-1β and cytotoxic factors such as NO and ROS, or to the alternative "M2" state, with secretion of anti-inflammatory cytokines such as TGF-β and IL-10 and neurotrophic factors such as BDNF and GDNF. The latter is thus integral to the downregulation of inflammation to restore CNS homeostasis [23]. This classification can be limiting as it only represents two opposite sides of the activation continuum. Accumulating evidence suggests that the activation profile of microglia is multidimensional, indicating the need of new terminology based on emerging data within transcriptomics, gene expression, and proteomic analyses [24]. However, the traditional terms will be further used in this review, to clearly distinguish between a neurotoxic and neuroprotective microglial state and their potential beneficial and detrimental effects in neurodegenerative disorders. Many of the studies involving microglial activation have been performed in animal models and have yet to be confirmed in humans. Such studies give an important insight into the possible mechanisms of neuroinflammation but need to be proven in human brain studies to fully understand the human disease pathophysiology.

In addition to the release of inflammatory mediators, activated microglia facilitate the crucial process of phagocytosis, to clear pathogens, debris, or protein aggregates, maintaining the brain homeostasis.

Accumulation of activated microglia has been detected in tissue from AD brains, with this activation being particularly evident around Aβ plaques, indicating that microglia can be activated by Aβ. These findings go hand-in-hand with increased proinflammatory factors in these patients, which might exert detrimental effects on surrounding neurons, exacerbating disease progression [18]. Conversely, activated microglia can, as shown in transgenic mouse models, to some extent, clear the accumulating Aβ oligomers through phagocytosis, providing beneficial effects in AD pathogenesis [25]. The role of microglia in AD is thus very complex, with a potential beneficial activation in early disease stages and detrimental activation in late disease stages. It has been suggested that dysfunction in these cells promotes the neurotoxic effects and diminishes the neuroprotective effects of microglia. Targeting the regulation of microglial activation might thus serve as a potential avenue to pursue in the development of AD therapeutics. However, strategies for targeting microglia and neuroinflammation would have to be intricately tailored to the stage of the disease, promoting the beneficial neuroprotective activation in early stages and suppressing the neurotoxic effects in later stages of the disease course [26].

Alongside microglia, astrocytes are also currently attracting increased attention for their potential role in AD progression and likewise converge around Aβ plaques in the brains of AD patients [7]. Astrocytes are the resident cells of the CNS that play key roles in maintaining brain homeostasis, in processes such as uptake and recycling of neurotransmitters, release of gliotransmitters and nutrients, and regulation of synaptic activity and inflammation [27]. Astrocytes can release transmitters such as glutamate through calcium-dependent exocytosis. However, astrocytes can also take up glutamate *via* plasma membrane transporters, thereby serving important functions in both neuronal and glial communication and in glutamate balance, with potential impacts on excitotoxicity [28]. Astrocytes also closely interact with synapses and play a role in synapse formation, function, and elimination [29]. Astrocytes have also been suggested to contribute to degeneration in AD and potentially play an important role in the inflammatory profile observed in AD pathology [27]. Upon exposure to toxic materials or injury, astrocytes become activated, transforming both their morphology and function to become so-called "reactive" astrocytes. Much like microglia, two different states of reactive astrocytes have been proposed, namely, "A1" and "A2," depending on the stimuli. The A1 phenotype has been observed to be neurotoxic, whereas A2 astrocytes possess neuroprotective properties. The former predominates in AD conditions. Astrogliosis, with an increase in reactive astrocytes, has been observed in AD, and this reactivity is especially prevalent around Aβ plaques. Astrocytes have thus been suggested to be activated by Aβ, leading to overexpression of proinflammatory cytokines such as IL-1β, TNF-α, and IL-6, in addition to increased formation of ROS and NO. Resulting elevated oxidative stress levels might then initiate neuronal degeneration. It has also been proposed that reactive microglia can induce this A1 state by secreting cytokines, further promoting formation of reactive astrocytes and neuroinflammation [27, 30, 31]. Microglia and astrocytes can thus both play beneficial or detrimental roles in the CNS, whereby Aβ accumulation and inability to resolve plaque formation can lead to a chronic neuroinflammatory state as AD progresses, further exacerbating neurodegeneration (Figure 1).

Initiation of the immune response is triggered by recognition of various pathogens and stimuli, and immune cells are able to respond to different infections, trauma, brain

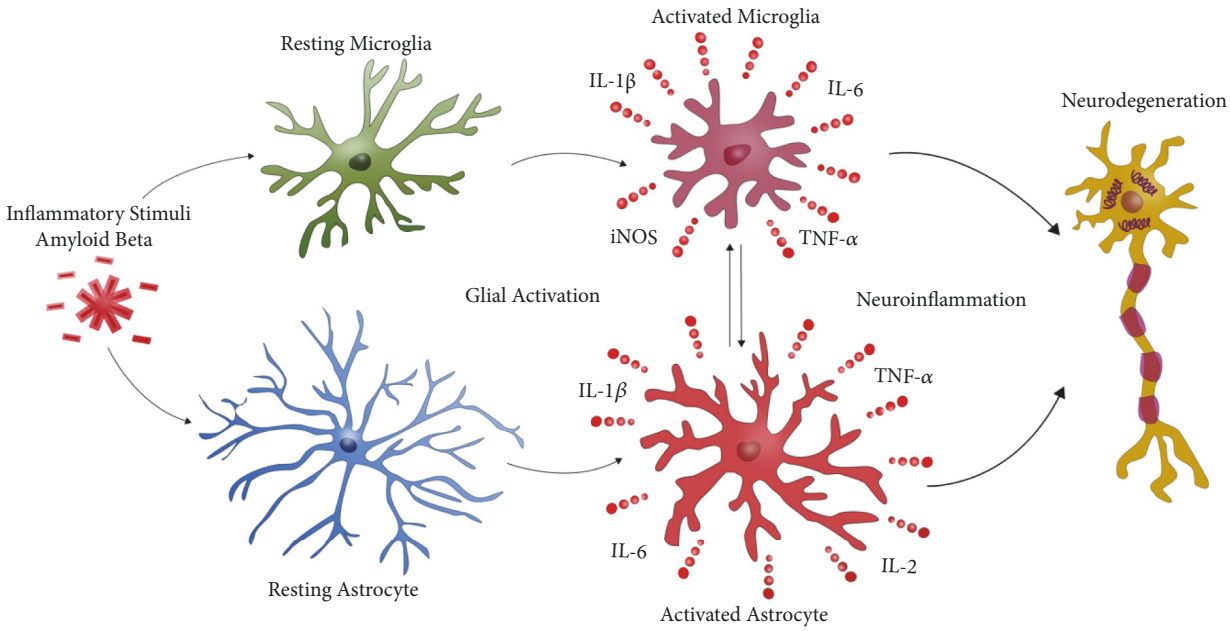

Figure 1: The potential role of neuroinflammation in Alzheimer's disease. Chronic exposure to inflammatory stimuli such as amyloid beta (Aβ) stimulates neurotoxic activation of microglia and astrocytes, triggering the release of proinflammatory cytokines and reactive oxygen species, promoting degeneration of neurons.

injury, protein aggregation, and neuronal death. Upon damage, immune cells migrate to the injury site and initiate an immune response. Microglia and astrocytes are able to recognize such stimuli owing to their expression of specific receptors, called pattern recognition receptors (PRR). These receptors can bind and respond to pathogen-associated molecular patterns (PAMPs) or danger-associated molecular patterns (DAMPs) such as Aβ and in this manner mediate the inflammatory response. It is through this receptor-complex that they can interact and react to the Aβ accumulation that occurs in AD [7]. Several types of PRRs are present on microglia and astrocytes including scavenger receptors, receptors for advanced glycation end products (RAGE), and toll-like receptors (TLRs), with the latter being implicated in AD pathogenesis. TLRs comprise an important group of PRRs, and various types of these receptors are expressed by microglia and astrocytes. TLRs are also found on neurons and oligodendrocytes, and emerging evidence has suggested involvement of these receptors in AD pathology [7, 32].

3. Toll-Like Receptors

Toll-like receptors are membrane receptors that can detect and be activated by the presence of pathogens *via* an extracellular domain, thereby generating an inflammatory response. These crucial components of the innate immune system were initially discovered on cells such as macrophages and dendritic cells [58, 59]. Subsequently, TLRs have been identified in a plethora of tissue and cell types including fibroblasts [60], eye tissue [61], blood cells [62] and, of specific interest for this review, brain tissue [63].

So far, TLR 1-13 have been identified in mice with the exception of TLR 10 [64] whereas 10 types of TLRs have been identified in humans (TLR 1-10) [65–68]. While not immediately appreciable, this difference is in fact notable and will be elaborated upon later in this review.

Common to all types of TLRs is their activation by the presence of a microorganism. Since types of microorganisms far exceed that of TLRs, the TLRs do not recognize a specific microorganism but instead recognize common pathogens expressed by different classes of microorganism. These are referred to as pathogen-associated molecular patterns (PAMPs). In addition to recognition of PAMPs, TLRs can interact with endogenous molecules such as proteins, polysaccharides, proteoglycans, nucleic acids, and other cellular components that are released from dead cells or damaged tissues [69]. These components are commonly known as damage-associated molecule patterns (DAMPs) and can also be released upon injury or during stress as an indicator of damage [70].

This section will give a general overview of TLRs with regard to their activation and signaling pathways. This overview will be based upon activation by pathogens, whereas more comprehensive details of activation by specific DAMPs associated with AD will be provided by other sections of this review.

TLRs can be segregated into two groups known as the cell surface TLRs and intracellular TLRs. Cell surface TLRs include TLR 1, 2, 4, 5, 6, and 10 and they can recognize various membrane components from bacteria such as proteins, lipids, and lipoproteins. TLR 3, 7, 8, and 9 are intracellular TLRs that are primarily located in the endosome and lysosome, where

Table 1: Overview of Toll-like receptors and their binding ligands.

Receptor	Ligand *(origin)*
TLR 2	Lipopeptides (*surface of gram positive bacteria*)[33], peptidoglycan (*surface of gram positive bacteria*)[34], Zymosan (*surface ligand on Fungi*)[35], Neisserial porins (*gram negative bacteria*)[36]
TLR 1/TLR 2	Triacylated lipopeptide (*surface of gram positive bacteria*)[37]
TLR 2/TLR 6	Diacylated lipopeptide (*surface of gram positive bacteria*)[38], FSL-1 (*synthetic lipopeptide derived from Mycoplasma salivarium*) [39], High mobility group box 1 protein (HMGB1) (endogenous *DNA-binding protein*) [40]
TLR 3	Polyinosinic:polycytidylic acid (Poly(I:C)) (*synthetic ligand with similar structure to dsRNA*)[41], genomic RNA and dsRNA (*Viral RNA*)[42], Stathmin (*endogenous human protein*) [43]
TLR 4	Lipopolysaccharide (LPS) (*molecule isolated from cell membrane of gram negative bacteria*)[44, 45], Glycosylphosphatidylinositol (GPI) (*membrane anchors in Protists*)[46], High mobility group box 1 protein (HMGB1) (endogenous *DNA-binding protein*) [40]
TLR 4/TLR 6	Amyloid beta (*peptides derived from the amyloid precursor gene*)[47]
TLR 5	Flagellin (*structural part of the flagella found on various bacteria*)[48]
TLR 7	ssRNA (*virus*)[49], Imidazoquinoline derivatives (*anti-viral organic compound*)[50]
TLR 8	ssRNA (*virus*)[51]
TLR 9	DNA (*virus* [52], *fungi* [53], *protists* [54] *and gram positive* [55] *and negative bacteria* [56]), CpG oligodeoxynucleotides (synthetic single stranded DNA molecules) [57]
TLR 10	Unknown

they can recognize various forms of RNA and DNA from viruses [71].

TLRs are activated by their respective ligand(s) (Table 1) binding to a leucine-rich repeat motif located on the outside of the membrane. The leucine repeats form a horseshoe structure which helps the ligand to attach to the TLR [72]. After attachment, the TLR will recruit specific adaptor molecules *via* its cytoplasmic Toll/IL-1 receptor (TIR) domain. Adaptor molecules that associate with the TIR-domain include MyD88, MAL, TRAM, and TRIF [73]. Depending on which adaptor molecule is recruited to the TIR-domain, various signaling pathways will be initiated (Figure 2). As an example, if TLR4 is stimulated by the presence of lipopolysaccharide (LPS), it will recruit the MyD88 adaptor molecule to its TIR-domain. MyD88 then associates with interleukin-1 receptor-associated kinase 4 (IRAK4) and IRAK1, forming an active complex that can add a phosphate group to the TNF receptor-associated factor 6 (TRAF6), allowing TRAF6 to form a complex that can phosphorylate the IKK-complex. The IKK-complex is responsible for recruitment of the transcription factor NF-κB to the nucleus where it increases expression of cytokines to mediate an inflammatory response [71, 74]. Studies have shown that, in order for TLR 4 to produce an inflammatory response to LPS, the cofactor CD14 is needed, as no production is seen in its absence [75]. The release of cytokines and other inflammatory factors, caused by TLR stimulation, can initiate a response in surrounding cells, thereby amplifying immune response. The activation of TLRs often results in an upregulation of TLR expression, allowing the cells to detect pathogens more efficiently, producing a stronger inflammatory response due to this positive feedback loop [76].

TLRs function as dimers with different types of TLR receptors forming heterodimers, so increasing ligand diversity. A high diversity of receptors and pathways allows for a highly tailored biological response according to the specific stimulus.

3.1. Toll-Like Receptors in the Human Brain. As elaborated upon in the previous section, TLRs respond not only to pathogens but also to the presence of DAMPs. Two main routes of TLR activation occur in neurodegenerative diseases: (1) cells undergoing apoptosis and necrosis release their cellular contents including DAMPs, triggering the immune response interacting with TLRs [77] and (2) other types of inflammation factors and protein aggregates directly activate TLRs [78]. Responses to DAMPs are of specific interest when studying neuroinflammation in the brain, since these are triggers from dying neurons and astrocytes and not caused by bacterial infections. Currently, the stimulation of TLRs *via* DAMPs is poorly studied: most investigations of TLR responses are still performed by presenting pathogen components to elicit an immune response. This following section will summarize the various types of TLRs identified in cells of the human brain, their ligands, and the downstream activating response.

All ten types of human TLRs have been found to be expressed in cells of the human brain [79] (Figure 3). It should be noted that many studies have investigated the expression of TLRs in mouse-derived tissue and cells. However, since TLRs are incompletely conserved between mouse and human, only mRNA and protein encoding TLRs, found in human brain tissue and cells, will be presented in this section.

3.1.1. Microglia. Microglia cells have been shown to express mRNA and protein for nine of the 10 TLRs identified in cells of the human brain (TLR 1-9) [80, 81]. This broad

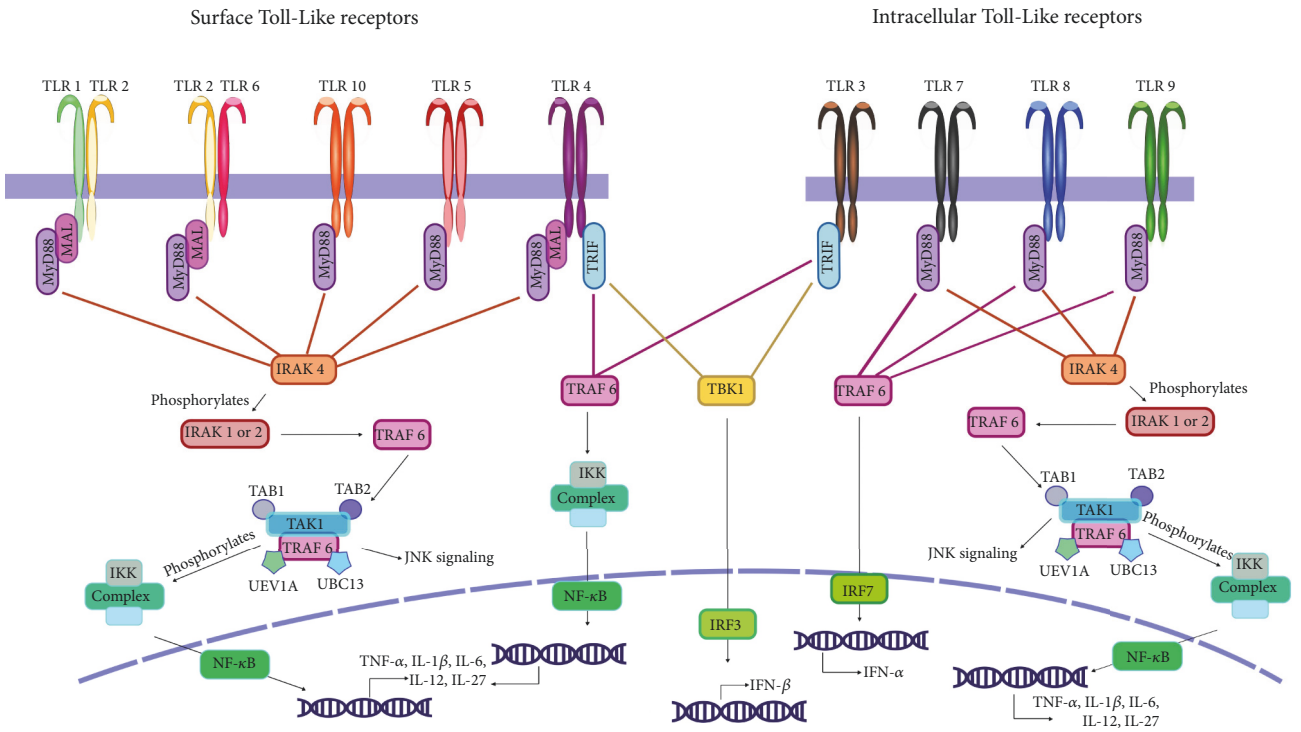

FIGURE 2: TLR signaling pathways. Depending on which TLR is stimulated, adaptor proteins MyD88, TRAM, TRIF, or MAL will associate with the TIR-site of the receptor. For the MyD88-dependent pathway, MyD88 recruits phosphorylated IRAK1 or 2 and associates with TRAF6. TRAF6 forms a complex with TAB1, TAB2, TAK1, UEV1A, and UBC13. The complex formation activates TAK1 which then phosphorylates the IKK complex. Once phosphorylated it can activate transcription factors involved in JNK signaling and NF-κB which results in production of various proinflammatory cytokines. The MyD88-independent pathway is initiated by TLR3 or 4 where TRIF associates and recruits TRAF6 of TBK1. TRAF6 results in NF-κB activation and TB1 in activation of the transcription factor IRK3, producing IFNβ. TRAF6 activation can also lead to IFNα production if activated by TLR 7, 8, or 9.

expression profile is not surprising given that microglia comprise the brain's innate immune system and that some of the inflammatory mediators that microglia produce are known to be regulated by TLRs. TLR 1 does not appear to be present in microglia as a homodimer but has been shown to form a heterodimer with TLR 2, responding to the spirochete *Borrelia burgdorferi*, and increases TLR protein and mRNA expression in astrocytes and glial progenitors [6]. This finding is informative in dissecting the pathways underlying neurodegeneration as the *Borrelia burgdorferi* infection in some cases affects the nervous system, leading to dementia [82]. Pathways identified in studies using *Borrelia burgdorferi* might therefore overlap with those involved in DAMP-initiated neurodegeneration.

In other pathological conditions, such as malignant tumors of the glial tissue of the nervous system (glioma), TLR 1/2 heterodimers, together with TLR 2/6 heterodimers and TLR 2 in microglia, facilitate infiltration of gliomas into the brain parenchyma of mice. Interventions into the activation of these TLRs might prevent tumor infiltration, increasing the likelihood of surgical resection [83].

Viral infections such as hepatitis C activate TLR 2 and TLR 6 in human microglia culture. These have been shown to respond to the presence of the hepatitis C virus antigen (virus NS3 protein), releasing the cytokines IL-8, IL-6, TNF-α, and IL-1β [84].

Another extensive study has systematically investigated the innate immune response mediated by TLRs in human microglia cells [81]. The major findings of this research were that human microglia express mRNA for TLR 1-9. Moreover, microglia could be activated through ligation of TLR 2 with synthetic lipopeptide, TLR 3 with synthetic dsRNA, and TLR 4 with lipopolysaccharide. All of these modes of activation triggered secretion of proinflammatory cytokines such as IL-6, IL-10, IL-12, and TNF-α.

All of these studies support the involvement of TLRs in the innate immune response mediated by microglia, as they produce proinflammatory cytokines. This immune response and inflammatory response is intensified by upregulated mRNA and protein expression of TLR 2 and TLR 3 and downregulated mRNA expression of TLR 4 [81]. For this reaction, microglia interact with astrocytes and mediate these responses. These findings underscore that glial activation results in an increased inflammatory response. Persistent activation of inflammatory responses in the glial compartment of the brain is characteristic for neurodegenerative diseases and if homeostasis cannot be restored after the pathogenic components have been removed, these

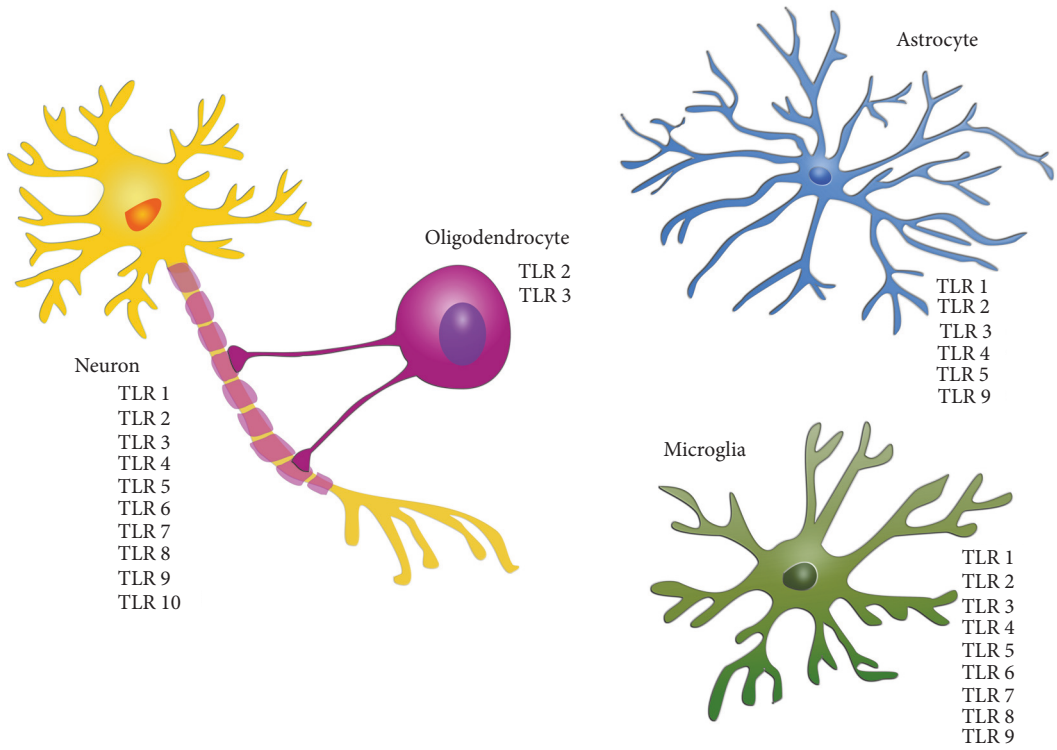

Figure 3: Expression of TLRs in human brain cells. Neurons express all ten human TLRs identified to date while microglia express nine of them. Astrocytes express fewer varieties of TLR and oligodendrocytes only express TLR 2 and TLR 3.

can be considered as potential triggers for disease pathology.

3.1.2. Astrocytes. TLR 2 and TLR 3 are the prevalent TLRs in astrocytes and are both highly expressed on RNA and protein level [80, 85]. For the other TLRs such as TLR 1, 4, 5, and 9, astrocytes have lower expression levels of mRNA [81, 86, 87] and protein [86] while TLR 6, 7, and 8 mRNA and protein are either expressed at very low levels [81, 87] or wholly absent [86]. TLR 2 mRNA has however also been reported to be expressed at negligible levels or not at all in astrocytes [81]. The controversy surrounding levels of TLR astrocyte expression likely reflected differences in detection of TLR between studies. These might stem from astrocytes not being in the same activation status or stimulated in differing manners between studies. This hypothesis is supported by previous work in mice, showing that activation of TLR 2 heterodimers TLR 1/2 and TLR 2/6 in microglia is highly dependent upon the type of stimuli astrocytes have previously been exposed to [39]. Furthermore some use only fetal samples [86, 87], others adult [80], and others again both adult and fetal samples [81]. The culture time for the astrocytes varies from 2 passages [81] to 10 passages [86] which most likely affect the expression level of TLRs. This is supported by a study showing a 212-fold difference in TLR 4 gene expression between astrocytes extracted from human fetal brains and from human adult brains [88].

In regard to activation and response of TLRs in astrocytes, TLR 3 and TLR 4 have received the most attention so far. TLR 3 on human astrocytes has been shown to be activated by exposure to the synthetic compound poly (I:C) resulting in increased production of IL-6, IL-8, and TNF-α [85, 86, 89]. The protein expression of TLR 2, TLR 3, and TLR 4 in astrocytes is enhanced if the astrocyte has been activated by proinflammatory cytokines such as IFN-γ [80, 83]. This augmented activation by proinflammatory cytokines, mediated through activation of neighboring astrocytes or microglia, has been shown to lead to expression of anti-inflammatory cytokines rendering a neuroprotective effect [83]. Furthermore TLR 3 activation by poly (I:C) has been shown to increase ATP release from lysosomes, stimulating lysosomal clearance of pathogenic substances [90].

TLR 4 can be stimulated by lipopolysaccharides (LPS) from gram-negative bacteria [81] in the presence of CD14 protein [6]. Astrocytes stimulated with LPS increase their expression of TNF-α, IL-6, and IL-8 and activate NF-κB [6, 81], all of which are associated with proinflammatory signaling. All of these studies underline the importance of astrocytes within the innate immune response of the brain, closely collaborating with microglia.

3.1.3. Oligodendrocytes. Work on CNS TLRs has mainly focused upon microglia and astrocytes. However, such receptors have also been identified on oligodendrocytes and

neurons. Oligodendrocytes are the myelinating cells of the CNS, providing a supporting role for neurons *via* axonal insulation and release of neurotrophic factors. Although little is known in terms of TLR expression and function in oligodendrocytes, mRNA expression of TLR 2 and TLR 3 has been identified in these cells, and activation of these receptors has been suggested to play a role in CNS repair [80]. Besides these findings, an indirect effect of TLR activation *via* activated microglia and astrocytes has been proposed to cause demyelination of oligodendrocytes and their subsequent loss [91]. Therefore, the direct and indirect effects of TLR activation can contribute to degeneration of oligodendrocytes in the brain affecting neurons and their survival.

3.1.4. Neurons. Similar to glial cells, mRNA and protein expression of TLR have been identified in neurons in both the peripheral nervous system and CNS. There has been some controversy in regard to which TLRs are expressed in human neurons. Whilst some studies have identified only some of these, another study has detected all 10 TLRs in human neuronal populations, although the detectable mRNA expression level varied between different neuronal cell types [79]. The neuronal expression of such TLRs allows them to trigger an immune response, indicating the presence of specific neuronal innate immune machinery. The neuronal TLR signaling pathways have been suggested to involve glycogen synthase kinase 3β (GSK3β), jun-N-terminal kinase (JNK), and phosphatidylinositol 3-kinase/protein kinase B (PI3K/AKT). These factors and pathways have been implicated to play a role in the immune response of the brain as well as being important for brain development and maintenance of brain homeostasis [92].

4. Toll-Like Receptors in Alzheimer's Disease

Various cell types and pathways in the human brain display a connection with neurodegeneration. The precise mechanisms causing the neuronal death associated with AD are, however, still unknown. Several studies have implied a role of TLRs in AD pathology, and in this section the potential role of such receptors in AD pathogenesis will be discussed. Our aim is to provide a better understanding of disease mechanisms and the potential of TLRs as druggable targets in future therapeutics. Neuroinflammation and the activation of immune cells are considered a hallmark of AD, and TLRs have been suggested to play a significant role in this activation. Stimulation of TLRs and their response is dependent on the type of stimuli, receptor, and cell population expressing them and, in this section, the AD-specific TLR response will be reviewed.

In comparison to healthy brains, brain samples of AD patients display increased TLR mRNA expression. This tendency has been observed for all TLR groups, with the exception of TLR 2 mRNA [93]. The inflammatory response seen with TLR activation differs depending upon the type of receptor being stimulated and in what combination they are activated. For instance, simultaneous stimulation of TLR 4 and TLR 2, TLR 4 and TLR 9 or TLR 2 in combination with TLR 9 causes a significant increase in inflammation in mouse models [94]. In microglia from mice it has also been observed that inflammation is upregulated if both TLR 1 and TLR 2 are stimulated, compared to a solely TLR 2-mediated response [95]. Silencing of TLRs has been shown to decrease the inflammatory response, further indicating an important role for them in inflammation. This is, however, not seen for TLR 7 in human AD brains, although an upregulation of expression in AD mouse models has been reported [96]. These results indicate TLRs to be associated with noninflammatory processes, and TLR 7 has been suggested to be associated with autophagy in mice [97]. The role of TLRs in AD pathology is therefore very diverse, depending upon the exact receptors involved. However, there are clear indications that these are in fact involved in the neuroinflammation accompanying neurodegeneration. Further studies must be conducted in order to confirm the involvement of TLRs in disease conditions to fully understand the complex signaling mechanisms at play, as a study made on post-mortem brain samples from AD-patients and healthy controls showed a great variation in TLR expression from patient to patient [80]. In addition, some of these findings are based on mouse models and should be confirmed in human models. Animal models do not necessarily recapitulate the precise human disease pathology, also implicit in the divergence in CNS TLR expression between rodents and humans.

An article from 2018 has analyzed the expression profiles from 25 different genetic studies including AD studies [98]. This work has resulted in a public database that includes the changes in expression profile for a gene of interest. For an overview, Table 2 has gathered the results from human TLR 1-10 in AD studies in relation to a healthy control.

As evident from the genetic studies, there are differences between studies of whole tissue and studies of cells, but also differences between human and mouse studies (Table 2). In the following section, all results should be considered carefully, as small differences between studies can cause very different outcomes.

4.1. Aβ and Tau in relation to TLRs. The formation of insoluble Aβ plaques and NFTs, the main pathological hallmarks of AD, is suggested to initiate a cascade of pathological events that have been previously reviewed to cause neuronal dysfunction [99]. The involvement of TLRs has been implicated in this cascade: Aβ peptides have been suggested to stimulate TLRs in mice [100] leading to increased mRNA expression of these receptors [93, 101, 102]. Studies in APP mouse models have indicated upregulated levels of mRNAs for TLR 2, TLR 4, TLR 5, TLR 7, and TLR 9, compared to TLR expression in plaque-free tissue. In contrast, TLR 3 mRNA expression was shown not to be significantly altered in AD mouse models, indicating that both activation and response in AD conditions are specific for different types of TLRs [103]. These findings also emphasize the potential differences between TLRs in rodents compared to humans. In contrast to mice, TLR3 mRNA and protein are upregulated in human AD brains and TLR 2 expression is not significantly increased, as previously mentioned. Differences between model organisms

Table 2: Overview of TLR expression in various AD or LPS studies compared to a healthy control. Data from database (http://research-pub.gene.com/BrainMyeloidLandscape).

Receptor	Mouse cell studies	Mouse Whole tissue	Human Whole tissue	LPS treatment [1]*Microglia* [2]*Cortical*	
TLR 1	Up	Up	Not significant	[1]Up	[2]Up
TLR 2	Up	Up	Up	[1]Up	[2]Up
TLR 3	Down	Up	Up	[1]Down	[2]Up
TLR 4	Down	Up	Up	[1]Down	[2]Down
TLR 5	Down	Up	Up	[1]Down	[2]Down
TLR 6	Down	Up	Up	[1]Up	[2]Up
TLR 7	Not significant	Up	Up	[1]Down	[2]Up
TLR 8	Not significant	Not significant	Up	[1]Up	[2]Up
TLR 9	Down	Up	Up	[1]Up	[2]Same
TLR 10	Not tested	Not tested	Up	Not tested	

should thus be considered in future research, to fully understand the human disease aspect. The increase in TLR expression resulting from Aβ stimulation correlates with increased inflammatory response. For instance, addition of Aβ to mouse hippocampal neurons upregulates TLR 4 protein, which then shows a stronger response to lipopolysaccharide (LPS) treatment, and increased neuronal death [104].

Based on various mouse models, TLRs have also been suggested to play a role in Aβ clearance by microglia, and such phagocytosis is likely dependent upon TLR 2, TLR 4, and TLR 9 [105–107]. TLR 2 mediates interaction between microglia and Aβ and has been suggested to serve as an important trigger for neuroinflammation in AD. Deficiency of TLR 2 in mice has been suggested to reduce inflammation and increase clearance of Aβ, favoring the microglial M2 phenotype and neuroprotection, improving neuronal function. Such deficiency could thus be beneficial by inhibiting Aβ-induced neuroinflammation [95]. In addition, TLR 2 deficiency has been observed to relieve tauopathies in mice, indicating further beneficial effects. These studies imply that TLR 2 activation contributes to inflammation and neurodegeneration, and inhibiting TLR 2 function might potentially slow disease progression. However, there is some controversy regarding TLR 2 and its involvement in AD. Although deficiency of the receptor has been implicated as beneficial, conflicting results have demonstrated TLR 2-mediated Aβ uptake, and activation of TLR 2 with, for instance, peptidoglycan (PGN) has been reported to promote microglial phagocytosis of Aβ in mice. It has been suggested that this promotes M1 microglial activation and a proinflammatory state [108]. The hypothesis that TLR 2 is involved in the proinflammatory microglia response has also been supported by a study showing that the coreceptor CD14 must act together with TLR 2 and TLR 4 in order for fibrillary Aβ to bind and trigger a microglial response in mice [109]. Despite the controversy, these findings clearly indicate a role for TLR 2 in the inflammatory profile associated with AD.

TLR 4 is the other major receptor involved in Aβ activation of microglia. Upregulation of TLR 4 mRNA has been observed in AD transgenic mice, and TLR 4 expression is increased in brain tissue surrounding Aβ plaques [101]. Deficiency of TLR 4 in microglia from such mice has also been demonstrated to increase Aβ deposits [110], indicating that TLR 4 is also required for microglial activation [106].

Besides TLR 2 and TLR 4, the role of TLR 9 in AD pathology and inflammation has been probed by a number of studies. Stimulation of TLR 9 has been demonstrated to increase microglial recognition of Aβ42 [107] and Aβ uptake [110] in mice. TLR 9 can bind DNA containing unmethylated cytosine-guanosine (CpG) sequences, commonly found in bacteria and viruses, and such stimulation has been shown to reduce Aβ in the cortical regions of AD mouse models [111] and restore cognitive function in AD mice as a result of the TLR9 stimulation [112]. This has also been observed in cocultures of neurons and microglia in which stimulation of TLR 9 led to reduced toxicity of oligomeric Aβ, with increased microglial clearance without production of neurotoxic factors [113]. The use of TLR9 agonists in mouse studies has not raised any safety concerns [114], but tests need to be made in humans, as it is likely that the increased inflammatory response, caused by stimulation of TLR9, can have a negative effect even though a study has shown that activation of TLR9 in mice does not worsen Aβ-induced microglial activation [115]. Taken together, these findings render TLR 9 an attractive candidate to investigate further regarding the development of future AD therapies.

Although most studies involving TLRs in AD pathology have focused upon TLR 2, TLR 4, and TLR 9, other TLRs might also potentially play a role in AD development. Interestingly, some genetic variants of TLR 5 in mice have been suggested to be preventive for AD [116]. Expression of the ectodomain of TLR 5, mediated by Adenoviral vectors, has been shown to result in decreased Aβ accumulation. This ectodomain can form a complex with Aβ, thus preventing aggregation and toxicity, making it more susceptible for removal [116]. Studies involving both TLR 9 and TLR 5 thus point towards promising therapeutic potential of TLRs in AD.

Compared to the many studies on Aβ and its interaction with TLRs, limited data is available regarding tau tangles and TLR response. TLR 3 protein expression has been shown to increase correspondingly with the level of tau tangles in human cell culture and brain samples [93]. However,

stimulation of TLR 3 did not seem to impact microglial activity in these cases. Conversely, mild stimulation of TLR 4 with LPS in transgenic mice overexpressing human mutant tau in neurons resulted in enhanced autophagy and reduction in phosphorylated tau, indicating that neuroinflammation promotes autophagy. Chronic mild stimulation of TLR 4 might thus possibly attenuate AD-related tauopathy, by providing beneficial neuroinflammation, which might be exploited in AD treatment [117]. These studies hence indicate that TLR signaling might also be linked with tau pathology.

4.2. Activation of Microglia. As previously described, Aβ plays a major role in microglial activation in AD. Studies have shown that Aβ42 protofibrils, an intermediate preceding amyloid fibril formation, can trigger the MyD88-dependent pathway in microglia. Such activation favors the M1 microglial phenotype and causes secretion of proinflammatory mediators [118]. Microglial activation can be mediated by TLRs, and expression of TLR 1-9 mRNA is seen in microglial cells. Stimulation of TLR 2, TLR 4, and TLR 9 leads to activation of this cell population, characterized by release of cytokines such as TNF-α, IL-1β, and IL-12, in addition to nitric oxide. This has been confirmed by *in vitro* studies using agonists for TLR stimulation [119–121], and simultaneous stimulation of microglia with even low concentrations of TLR ligands has been shown to result in an additive effect, indicating that low amounts of pathogens can manifest in TLR activation, if multiple TLRs are targeted [121]. The exact mechanism(s) through which TLR activation can influence AD pathology and if this is the case is not fully understood, as well as the mechanisms causing AD. However, a constituent of the signaling pathways has been identified and is likely mediated by the MyD88/TRAF6//MAPK/IKKs/NF-κB pathway or the MyD88/PI3K/NF-κB pathway, both of which promote M1 activation [122].

Depletion of both TLR 2 and TLR 4 has been observed to decrease microglial activation. However, while deficiency for TLR 2 has been linked with reduced Aβ plaque burden [100], TLR 4 deficiency has been observed to increase Aβ deposition [123]. In contrast, TLR 4 inhibition has been observed to result in reduced secretion of proinflammatory cytokines [101]. The fact that deficiency of these receptors can reduce the inflammatory response in AD suggests an important role of these TLRs in microglial activation. However, activation is not solely dependent upon TLRs, and activation can occur in TLR 2- and TLR 4-deficient conditions *via* other factors such as ROS-mediated activation, but under such circumstances, very high levels of LPS are required to induce an inflammatory response [124].

Suppression of TLR 4 appears to improve cognitive deficits and decreases inflammatory injury in mice with AD mutations [125]. By targeting TLR 4 signaling pathways, inflammation can thus potentially be decreased. Chronic activation of TLR 2 and TLR 4 has been suggested to contribute to neuroinflammation. However, further studies are required as conflicting results remain in terms of the effects of activation and silencing of the same receptors. Conversely, all studies regarding TLR 9 activation of murine microglia have shown consistent results, leading to an ultimate decrease in Aβ [107, 111, 113] as described in the previous section. Aβ has also been observed to induce dimerization of TLR 4 and TLR 6 in mice, and inhibiting this process led to decreased release of proinflammatory cytokines from microglia, providing a neuroprotective effect [126].

Activation of microglia can result in different outcomes, either leading to the proinflammatory M1 state, or the anti-inflammatory M2 state, promoting neurotoxicity and neuroprotection, respectively. The balance between these two phenotypes is essential in terms of neuroinflammation and maintaining brain homeostasis [127]. In terms of neurodegeneration, this balance is shifted towards the M1 microglial phenotype, promoting the release of proinflammatory mediators and neuroinflammation [127]. Whether the observed inflammatory profile in AD is a direct cause of the disease or if it is in fact a secondary reaction to other AD pathologies is, however, still hotly contested.

Some studies have suggested that neuroinflammation in AD occurs due to the fact that microglia become senescent, and thus less responsive to stimuli [128]. Concordantly, repeated treatment of murine microglia with LPS has been demonstrated to drive them towards a senescent state [129]. Mouse studies showing no difference in the prevalence of active microglia between postmortem AD and control brains have supported this theory. The fact that LPS stimulation, which acts on TLR 4, can induce this microglial state invoked the hypothesis that chronic exposure to stimuli such as Aβ leads to less responsive microglia, decreased Aβ clearance, and thus accelerated AD progression [130]. Studies in mice have also indicated that age plays a role in microglial activation. Microglia from older mice have been observed to secrete higher amounts of proinflammatory cytokines compared to those from younger mice [131], and these microglia are less responsive to other stimuli. Chronic activation of microglia can thus lead to a state in which these cells are no longer able to respond to additional stimuli [132].

A recent study has shown that stimulation of microglia can lead to epigenetic reprogramming, traceable for up to 6 months [133]. In this study, two types of immunological imprinting were distinguished from one another, namely, training and tolerance, which can respectively enhance or suppress the inflammation [133]. This finding emphasizes that the type of stimulus can influence the inflammatory signaling pathway and produce distinct outcomes despite targeting the same TLRs.

4.3. Reactive Astrocytes. Besides microglia, reactive astrocytes play a role in neuroinflammation and neuronal death in AD. Activation of TLRs in human and rat astrocytes leads to secretion of TNFα, IL-6, IL-8, IL-10, IL-1β, and inducible nitric oxide synthase [86, 87, 134, 135]. TLR stimulation in these cells likely involves the NF-κB signaling pathway, which has been shown to induce astrogliosis and neuroinflammation in mice [136]. Increased TLR 2 expression in astrocytes has also been demonstrated to increase the secretion of proinflammatory cytokines, further indicating that TLRs are implicated in the inflammatory response. In contrast, astrocytes from TLR 2-deficient mice have been found to show reduced production of inflammatory mediators [137].

Furthermore, activation of TLR 3 in rats has been seen to increase the proinflammatory phenotype of astrocytes, contributing to neurotoxicity [138], whilst TLR 9 stimulation in mice has resulted in reactive astrogliosis, further emphasizing the role of TLRs in neurodegeneration [139].

Astrocytes can thus be activated by TLR recognition of different stimuli. However, these cells can also respond to cytokines of the adaptive immune system such as IFN-γ and TNF-α. Innate signals such as LPS and TLR ligands have been shown to elicit a stronger upregulation of TLRs and increase in cytokine release compared to cytokine-stimulated astrocytes. These findings clearly indicate that different stages of neurodegeneration can generate altered responses in astrocytes and are important for understanding the role of astrocytes in inflammation and neurodegeneration [140].

4.4. Implications of TLR Activation in Neurons. Activation of TLRs can produce either direct or indirect effects on the neuronal population of the CNS. The direct effect of TLRs can be seen from studies of knock-out mice. In TLR 2-deficient mice, differentiation of neural progenitor cells into neurons is favored over astrocytes, resulting in reduced plasticity while *TLR* $4^{-/-}$ mice show increased proliferation and differentiation of neural progenitor cells [141]. Together, these findings show that TLRs are involved in neurogenesis and therefore most likely are involved in neurodegenerative mechanisms of AD. In accordance with this notion, mouse models have shown that neurons can respond directly to the presence of Aβ through TLR 4, and such stimulation can lead to apoptosis [142]. By downregulating TLR 4, neurons showed greater survival and less sensitivity to Aβ. The same study looked at the levels of TLR 4 in brains from AD patients and healthy controls and found lower TLR 4 levels in AD patients, indicating that neurons expressing TLR 4 died. TLRs thereby directly impact neuronal health in AD. Because TLR 4 is also expressed in healthy neurons, apoptosis cannot be explained by the presence of TLR 4 alone but it is clearly involved in the process.

Furthermore, neurons can be affected by the neuroinflammation initiated by microglial activation as this process initiates a cascade of proinflammatory events. Stimulation of TLR 2 and TLR 4 in mice by Aβ activates microglia and causes secretion of proinflammatory cytokines [105] which can have detrimental effects on the surrounding neurons, hence promoting neurodegeneration.

A connection between neurons, neurodegeneration, and TLRs has also been found in human brain samples of patients with Parkinson's disease [143], where expression of the TLR 2 protein was found to be increased in patients. The same study showed that activation of TLR 2 in human cells increased the production of α-synuclein, a well-known hallmark of Parkinson's disease, but also a protein that has been associated with AD [144].

The activation of TLRs can thus affect the neuronal population, directly or through microglia-mediated inflammation, both of which should be studied further to increase our understanding of how these pathways work together to exacerbate neurodegeneration.

4.5. Aging/Stress. The number of people affected by dementia is expected to reach 152 million by 2050, due predominantly to increased longevity [145].

Many studies have shown that chronic stress increases the risk of developing AD as the body cannot normalize its homeostasis which progressively affects the physiological balance [146], leading to neurodegeneration [147]. Stress in fAD mice has been shown to mainly affect the hippocampal region of female mice, indicating stress pathology to be region- and sex-specific [148].

Chronic stress can lead to induction of proinflammatory mechanisms, causing oxidative stress due to generation of oxidative species [146]. As humans are exposed to stress throughout their lives, it is not a direct cause of AD, but stress might increase the level of damage in brains susceptible to neurodegeneration. It is therefore of interest to study the effect of stress on aging cells, as these are more susceptible to damage [149]. This effect has been studied in neonatal mouse microglia cells cultured for 16 days *in vitro* and investigated on days 2, 10, and 16 [127]. On day 2, microglia showed adaptable morphology and expressed markers of reactive phenotype whereas microglia on day 16 showed branched morphology, increased NF-κB activation, and glutamate release. Thus, old microglia cells (day 16) behave in a similar fashion as irresponsive/senescent microglia. Microglia from old mice secrete greater amounts of IL-6 and TNF-α compared to those from young mice and are less responsive to stimulation [131]. These findings indicate a higher detrimental effect of stress in aging microglia, supporting the hypothesis that brains of elderly people are more vulnerable to neuroinflammation.

In relation to TLRs, expression of TLR 2 and TLR 4 in microglia has been shown to decrease with age [104] together with the capacity to migrate and phagocytose. In correlation with this, the general level of functional TLR 1, 6, and 10 in human DNA from healthy old people has been shown to decrease [150], indicating that a downregulation of these TLRs in general might provide a beneficial effect in aging.

Other genetic studies have highlighted the potential influence of TLRs in AD, in which TLR 2 emerges as a potential risk factor in late onset AD [151, 152]. In a genetic study of a Chinese population, TLR 2 was not identified as a significant genetic risk factor for AD [153]. This might be explained due to differences in populations and testing protocols. All these findings together with the observation that mice deficient for TLRs show less cellular damage after exposure to stress [154] confirm that a relation between age, stress, TLRs, and inflammation exists but that further studies are needed to elucidate their relationships to one another.

4.6. Components Known to Decrease Inflammation via TLR Pathways. Ever-increasing numbers of studies have investigated potential therapeutics targeting the TLR signaling pathway to decrease neuroinflammation.

Treatment with Picroliv in mouse brains has been demonstrated to reduce the effect of the TLR 4/NFκB pathway, resulting in decreased expression of TLR 4, BDNF, IL-1β protein, and Aβ levels [155].

Stachydrine also reduces the levels of IL-1β, TNFα, and INF-γ *via* the TLR 4/NFκB pathway upon brain injury [156]. Treatment with Betainine and various polyphenols also exhibits anti-inflammatory effects by decreasing production of proinflammatory cytokines and increasing the release of anti-inflammatory cytokines [157, 158]. This shows that Betainine treatment promotes conversion of microglia from the M1 stage to the M2 stage, which is achieved by suppression of the TLR 4/NFκB pathway.

Together, these studies show that targeting the TLR 4/NF-κB-pathway decreases inflammation, rendering this pathway of therapeutic potential. As members of the NFκB family in general regulate inflammation by mediating synthesis of proinflammatory proteins, they are potential druggable targets for decreasing inflammation [159].

As another study has shown that combinatorial TLR activation results in increased inflammatory response and that the response depends on which specific TLRs are activated [94], other pathways and TLRs should be studied further to dissect potential involvement in AD pathogenesis.

5. Discussion

5.1. Study of TLR in Human versus Mouse. The various studies presented above were conducted in different models: while some pertain to human cells/tissues, the vast majority was performed in rodents. All of the TLRs identified in humans are also expressed in mice. However, the mouse exhibits three additional TLR members not found in humans [71].

While numbers of TLR members expressed between mouse and human brain cells diverge, so too do the expression *levels* of each member. Mouse astrocytes express TLR 1-6 and very low levels of TLR 7-9 [140, 160] whereas human astrocytes only express TLR 1-6 and 9. In neurons, humans express all 10 TLRs, whereas studies in mice have shown their cortical neurons to only express TLR 2, 3, and 4 [161]. Other significant differences between the innate immune systems of mouse and human include the finding that RNA is sensed by TLR 3, 7, and 8 in humans but by TLR 13 in mice, a receptor that does not exist in human cells [71, 162]. Furthermore, human TLR 9 recognizes the GTCGTT DNA sequence from bacteria whereas mouse TLR 9 recognizes the GACGTT sequence [163]. These studies clearly reveal substantial differences in numbers, expression levels, and cell type-specific expression patterns between mouse and human which need to be taken into consideration if mouse models are employed to study the role of TLRs in neurodegenerative diseases and to identify potential drugable targets.

Despite these differences, mice remain the most common model to investigate AD and other human diseases. Mice are important *in vivo* models since they can easily be bred, and knock-out, transgenic, and knock-in lines have been generated for diverse studies. Furthermore, humans and mice share many genetic and physiological similarities, which have helped elucidate many pathways in mice, which have then subsequently been confirmed in humans. [164]. However, major disadvantages of mouse models are that mice do not naturally develop AD and their longevity is too brief to develop the hallmarks of sporadic AD [165]. Therefore, in order to investigate AD pathology in mice, either transgenic mouse models with several strong pathogenic mutations are employed [166], or some of the pathogenic hallmarks such as Aβ or tau are directly injected into the mouse brain [167–169].

Taken together, owing to the challenges of TLR divergence between mouse and man and the difficulty in recapitulating AD pathology in mouse models, alternative experimental models should be sought.

5.2. Use of iPSC Models and Future Studies. One potential model for studying the functional roles of TLR in AD is the use of induced pluripotent stem cells (iPSC). iPSC possess the advantage that they can easily be generated from human fibroblasts [170] collected from skin samples, blood, or even urinary epithelial cells. This allows for investigation in cell lines generated from different individuals and thereby cell lines with different genetic backgrounds. Comparative studies can be made as samples can be taken from both AD patients and healthy controls.

Furthermore, gene-editing technologies such as TALENS and CRISPR-Cas9 allow for insertion of pathogenic mutations into healthy control iPSC or for correction of pathogenic mutations in patient iPSC, allowing for the establishment of isogenic control lines with the same genetic background. Many protocols have been developed to differentiate iPSC into various cell types, such as astrocytes [171, 172], neurons [173], and microglia [174]. It will be very interesting to investigate the expression patterns of TLRs in iPSC-derived neurons, astrocytes, microglia, and oligodendrocytes and to compare these with human brain samples in order to validate these *in vitro* models.

If the same expression patterns in the diverse iPSC-derived models can be validated, these cells would represent valuable tools for the identification of compounds to develop drugs targeting TLR activity and innate immune responses as well as for understanding the human-specific function of TLRs. Another possibility in order to study the TLR responses of human-derived cells in a complex *in vivo* system would be the transplantation of such cells into humanized AD mouse models, even though the investigation of such transplants is hindered by the fact that these mice must remain in an immunocompromised state.

6. Conclusion

In this review we have presented the different TLR expression patterns in the main cell types of human brains, their responses to pathogenic triggers, and secretion of proinflammatory cytokines. These different cell types are closely dependent on the innate immune responses of each other and facilitate either increased immune responses or restoration of the homeostatic state depending on the environmental situation in the brain. Moreover, we have described and discussed that microglia and astrocytes specifically respond to Aβ and tau, underlining the importance of TLR-mediated innate immune response in AD. Since the responses to Aβ and tau are late pathological events, the responses to

DAMPs released by degenerating neurons are even more intriguing in order to understand early AD pathology linked to inappropriate innate immune responses and potential drug development targeting the mild cognitive impairment state of the disease. Moreover, we have discussed the divergence in numbers and expression patterns of human- and mouse-specific TLRs in the brain, emphasizing the importance of human *in vitro* models, such as iPSC, to investigate the human-specific innate immune response in the various brain cell types facilitated by TLRs.

In conclusion, more studies are needed to elucidate the impact of TLRs in the human-specific context and in relation to AD.

Conflicts of Interest

The authors declare that they have no conflicts of interest.

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Review Article

Toll-Like Receptors as Therapeutic Targets in Central Nervous System Tumors

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In recent years, progress has been made in understanding the pathological, genetic, and molecular heterogeneity of central nervous system (CNS) tumors. However, improvements in risk classification, prognosis, and treatment have not been sufficient. Currently, great importance has been placed to the tumor microenvironment and the immune system, which are very important components that influence the establishment and development of tumors. Toll-like receptors (TLRs) are innate immunite system sensors of a wide variety of molecules, such as those associated with microorganisms and danger signals. TLRs are expressed on many cells, including immune cells and nonimmune cells such as neurons and cancer cells. In the tumor microenvironment, activation of TLRs plays dual antitumoral (dendritic cells, cytotoxic T cells, and natural killer cells activation) and protumoral effects (tumor cell proliferation, survival, and resistance to chemotherapy) and constitutes an area of opportunities and challenges in the development of new therapeutic strategies. Several clinical trials have been carried out, and others are currently in process; however, the results obtained to date have been contradictory and have not led to a definitive position about the use of TLR agonists in adjuvant therapy during the treatment of central nervous system (CNS) tumors. In this review, we focus on recent advances in TLR agonists as immunotherapies for treatment of CNS tumors.

1. Introduction

Central nervous system (CNS) tumors constitute a heterogeneous group of neoplasms that originate from many pluripotent and differentiated cell types, whose incidence and mortality are increasing worldwide. The GLOBOCAN 2018 database indicates that there were 296,851 new cases and 241,037 deaths from brain cancer compared with 257,000 and 189,000, respectively, in 2012 [1]. Factors that have been associated with increased risk of developing primary CNS tumors include hereditary syndromes and exposure to X-rays and gamma rays; however, in most cases, the etiology is unknown.

Several different types of tumors, both nonmalignant and malignant, have been identified in the CNS. The highest incidence of primary CNS tumors in adults is from meningiomas and neuroepithelial tumors (glioblastoma and pituitary tumors). Children and adolescents present with a higher incidence of embryonal tumors, mainly medulloblastoma, pilocytic astrocytoma, and ependymal tumors [1, 2].

Currently, conventional treatments, including surgical resection of tumors, craniospinal radiotherapy, and chemotherapy, are often successful. However, some CNS tumors are not amenable to surgical resection due to the depth of tumor infiltration or anatomical location of it. The side effects from treatments in patients significantly affect their neurological and psychological function and quality of life.

Advances have been made in understanding CNS tumor biology, but improvements in risk classification, prognosis,

and treatment have not been sufficient. Recently, great importance has been placed on the tumor microenvironment, which is composed mainly of cancer cells, stromal cells, and immune cells [3]. The activation of the immune response is an important factor in the onset, development, and metastasis of cancer, and therfore, the immune system is a potential therapeutic target.

2. Toll-Like Receptors

The initiation of the innate immune response begins with the recognition of exogenous molecules from microorganisms, called microbial-associated molecular patterns (MAMPs), as well as the recognition of endogenous molecules considered to be danger signals called danger-associated molecular patterns (DAMPs). MAMPs and DAMPs are recognized by germline-coded receptors, called pattern recognition receptors (PRRs). This family of receptors consists of mannose binding lectin (MBL), Toll-like receptors (TLRs), nucleotide-binding oligomerization domains (NOD) receptors, dectin-1, NOD-like receptors (NLRs), mannose receptors, and pentraxins, among others. Principal effector functions of PRRs include the activation of the transcription of genes involved in the immune response (cytokines, chemokines, growth factors, adhesion molecules, and costimulation), opsonization, phagocytosis, activation of the complement system, proliferation, and cell death [4, 5].

TLRs are type I transmembrane proteins, characterized by an extracellular domain with leucine-rich repeats (LRR) motifs. Through this domain, TLRs recognize structures present in certain groups of pathogens and endogenous molecules released in situations that result from physiological stress. Additionally, TLRs possess an intracellular domain, called TIR (Toll/IL-1R) similar to the interleukin-1 receptor (IL-1R) family, which leads to the activation of a signaling pathway [6]. The Toll protein was identified for the first time in *Drosophila melanogaster* as a fundamental receptor for dorsoventral polarity during the early phases of embryonic development of the fly [7]. Subsequent studies have shown that the Toll protein has a very important function in the immune system of the adult insect, mainly during infections by bacteria and fungi. Currently, it is known that TLRs are evolutionarily conserved from invertebrate organisms such as *Caenorhabditis elegans* to mammals [8]. To date, 13 members of this family have been found in mammals, including 10 in humans (TLR1 to TLR10) and one pseudogene [9]. The expression of TLRs is not limited to the cell immune system as they are expressed in other cell types. In the central nervous system, TLRs are present in glial cells (microglia, astrocytes, and oligodendrocytes), neuronal progenitors, mature neurons, and cancer cells (Table 1).

TLR ligands include MAMPs, endogenous molecules, and synthetic agonists. For example, TLR2, in conjunction with TLR1 or TLR6, recognizes a wide variety of MAMPs and DAMPs, such as lipoproteins, peptidoglycans, lipoteichoic acid, and zymosan. TLR3 recognizes double-stranded RNA (dsRNA) and polyinosinic:polycytidylic acid [poly(I:C)]. TLR4 recognizes lipopolysaccharide (LPS) and TLR5 flagellin [10]. TLR7 recognizes single-stranded RNA (ssRNA), microRNAs, small interfering RNAs (siRNA), and imidazoquinoline derivatives such as imiquimod (IMQ) and resiquimod (R848) and guanine analogs such as loxoribine. TLR8 is phylogenetically similar to TLR7 and preferentially recognizes bacterial RNA, ssRNA from viruses, and synthetic agonists such as R848. Finally, TLR9 is known to recognize cytosine-guanine motifs bound by nonmethylated phosphodiester (CpG) bonds and synthetic CpG oligonucleotides (ODN) and immunoglobulin-DNA complexes [5, 9, 10]. Activation of TLRs induces the recruitment of adapter proteins (MyD88, TRIF, etc.) that bind the TIR domain triggering a signaling cascade and activating transcription factors such as nuclear factor kappa B (NF-κB), activator protein-1 (AP-1), and interferon regulatory factors (IRFs) [11, 12], which in turn trigger transcription of genes that participate in the immune response and cellular processes such as proliferation, migration, and cell death [11–17].

3. TLRs and Immune Responses to Tumors

In the tumor microenvironment, TLR signaling can induce anti- or protumor effects which depends on the cancer subtype and the cells of the immune system that infiltrate the tumor [18]. There is great controversy for some TLRs which are explained by the tumor models used in the experiments. It has been documented that stimulation of TLRs may have antitumor effects through an intermediary immune cells response or directly on tumor cells, which improves the antitumor immune response and leads to apoptosis of tumor cells [19, 20]. Apoptosis of tumor cells can be generated by different mechanisms downstream of TLR3 activation in different cancer cell lines, as indicated by lower survivin expression and negative regulation of XIAP, FLIP, Bcl-xL, and Bcl-2 have been observed [21–23] and by a large number of cells positive for proapoptotic caspase-8 and caspase-3 [21].

The immune system has been shown to be more efficient activating the response to MAMPs than recognizing and eliminating tumor cells. Pharmacological studies have shown that the activation of signaling pathways initiated by TLRs through recognition of MAMPs and DAMPs, but not tumors, induces the production of mediators such as type I interferons (IFN), which can be used therapeutically to modify immunotolerance and produce antitumor effects.

The antitumor immune response depends largely on the cells presenting professional antigen-presenting cells, such as dendritic cells (DCs) [24, 25]. DCs express all TLRs and exert effects on T and B lymphocytes; they are the bridge between innate and adaptive immune responses. IFNs are necessary for an efficient immune response to tumors [26]. Therefore, activation of the TLR-IFN type I signaling pathway is of therapeutic importance in that it eliminates DC-induce tolerance and generates an antitumor response. Additionally, DCs activated by TLRs can mediate antitumor responses, by the presenting antigens, thereby initiating a T cell response, and by inducing cytotoxicity in tumor cells [27]. It has been documented that DCs activated by TLR7 ligands can induce antitumor responses by cell lysis [28]. On the other hand, the activation of TLR5 with flagellin can increase DC antitumor activity [29]. The death of tumor cells mediated

Table 1: TLR expression in normal and neoplastic CNS cells.

Receptor	Cell/Tissue	mRNA/Protein	Specie	References
TLR1	Microglia, neurons	+/+	human, mouse	[66–68]
	Astrocytes	+/nd	human, mouse	[40]
	Glioma ∗(GL261, U251, U87, SF126)	+/-	human, mouse	[40, 53]
	Astrocytoma, Glioblastoma ∗(U87MG, A172)	+/+	human	[69]
TLR2	Oligodendrocytes	+/nd	human	[66]
	Microglia, astrocytes, neurons	+/+	human, mouse	[66–68, 70–74]
	Glioma ∗(GL261, U251, U87, SF126)	+/-	human, mouse	[40, 53]
	Medulloblastoma	nd/+	human	[75]
	Astrocytoma, Glioblastoma ∗(U87MG, A172)	+/+	human	[69]
TLR3	Astrocytes	+ /-	human, mouse	[66, 70]
	Oligodendrocytes, microglia, neurons	+/+	human	[66–68, 70, 72, 73, 76]
	Glioma ∗(GL261, SF126)	+/+	human, mouse	[40, 53]
	Medulloblastoma	nd/+	human	[75]
TLR4	Microglia, neurons	+/+	human	[66–68, 72]
	Astrocytes	+/+	human, mouse	[40, 71, 72]
	Glioma ∗(GL261, U251, U87, SF126)	+/-	human, mouse	[40, 53]
	Astrocytoma, Glioblastoma ∗(U87MG, A172, U118, LN229)	+/+	human	[62, 69, 77]
TLR5	Microglia	nd/+	human	[66]
	Astrocytes	+/+	human, mouse	[40, 71, 72]
	Glioma ∗(GL261, U251, U87)	+/-	human, mouse	[40]
	Astrocytoma, Glioblastoma ∗(U87MG, A172)	+/+	human	[69]
TLR6	Microglia, microglia ∗(EOC13)	+/+	human, mouse	[66, 67, 78]
	Neurons	+/+	mouse	[72]
	Astrocytes	+/nd	human, mouse	[40]
	Glioma ∗(U251; SF126)	+/-	human	[40, 53]
	Astrocytoma, Glioblastoma ∗(U87MG, A172)	+/+	human	[69]
TLR7	Microglia	+/nd	human	[66, 67]
	Neurons	+/+	human	[76]
	Astrocytes	+/nd	human, mouse	[40, 79]
	Glioma ∗(GL261, U251, U87)	+/-	human, mouse	[40]
TLR8	Microglia	+/nd	human	[66, 67]
	Neurons	+/+	human, mouse	[66, 72, 76]
	Astrocytes	+/nd	human, mouse	[40, 67]
	Glioma ∗(GL261, U251, U87)	+/-	human, mouse	[40]
TLR9	Neurons (differentiated from ∗SH-SY5Y)	nd/+	human	[72, 80]
	Astrocytes	+/nd	human, mouse	[40, 71, 72]
	Glioma ∗(GL261, U251, U87, SF126)	+/+	human, mouse	[40, 53, 77]
TLR10	Microglia	nd/+	mouse	[66]
	Glioblastoma ∗(T387, T3832, T4121)	+/+	human	[81]
TLR11	Microglia	nd/+	mouse	[66]
	Brain	+/-	mouse	[82]
TLR12	Brain	+/-	mouse	[82]
TLR13	Brain	+/-	mouse	[82]

+, positive expression; -, negative expression; nd, not determined expression; ∗cell lines.

by DC generates a more efficient antigen presentation for cytotoxic T lymphocytes, amplifying the antitumor response. DCs stimulated with TLR9 also produce antitumor responses [30].

IFN has the ability to regulate the functions of natural killer cells (NK) and is very important for the modulation of tumor growth. A murine model of melanoma demonstrated that myeloid DCs can be activated via TLR3 with poly(I:C), inducing NK cell response and regression of tumors. It is now known that the NK-activating molecule dependens on IRF-3 to create a link between myeloid DCs and NK cells [31].

In addition, regulatory T cells (Tregs) (CD4+CD25+FoxP3+) have a very determinant role in the immune response, and to induce tolerance; it is known that the TLRs of DCs regulate the activation of Tregs through signals that block the immunosuppression by IL-6 [32]. Also, TLR8 agonists can inhibit the function of Tregs independently of DCs and promote an antitumor response [33].

The poly(I:C) agonist of TLR3 can cause tumor regression, such that tumor macrophages are transformed into tumor suppressor macrophages that produce inflammatory cytokines (M1 macrophages). This change is mediated by TNF-α through an independent pathway of MyD88 [34]. Furthermore, TLR9 agonists can also exert antitumor effects by suppressing angiogenesis [35]. TLR-induced interferon has an important role, because it reduces tumor growth blocking angiogenesis and metastasis [36, 37]. However, tumor cells may also activate TLRs and, coupled with a specific type of tumor, can cause death, survival, or proliferation of tumor cells, including resistance to chemotherapy [18].

4. Immunotherapy Targeted to the Activation of TLRs

TLR agonists have been considered therapeutic targets for treating different cancers [38]. Many synthetic ligands are being investigated for use in immunotherapy. ODNs are the most commonly used TLR agonists in therapy; they are potent activators of both innate and adaptive immununity and thus are capable of inducing cytokine production and activating NKs, dendritic cells, monocytes, and antitumor T cell responses [39]. Stimulation of TLR9 with ODN 1826 induces caspase-3-dependent apoptosis in gliomas and prolongs the survival of C57BL/6 mice with an intracranially implanted glioma cell line (GL261). Moreover, mice treated with ODN 2138 showed no evidence of enhanced survival [40]. In addition, ODNs enhance survival and prime long-term immunity in mice with two separate glioblastoma tumors in wich only one was treated. This may suggest that treatment with CpG-ODN could be effective in tumor cells located at some distance from the application site [41]. The antitumoral effect was not mediated by direct toxicity but instead involved cells of the immune system, including NK cells, macrophages, microglial cells, and CD8 T cells [41].

In another study, Grauer *et al*. showed that there are low to undetectable levels of TLR5, TLR7, and TLR9 in GL261 cells; surprisingly, in C57BL/6 mice, a single intratumoral injection of CpG ODN 1668 inhibited glioma growth and cell proliferation in a cell-type specific manner. CpG ODN 1668 was superior in the elimination of murine gliomas (median survival >90 days) when compared with PAM_3Cys-SK4 (median survival= 34.5 days), while LPS and poly(I:C) did not show a significant effect on tumor growth (median survival = 27 days). Similar to ODN 1668, R848 also extended the survival of glioma-bearing mice but not as effectively (median survival >36.5 days) [42].

Subcutaneous vaccination of CpG-ODN 2006 with glioma cell lysate (cell line GL261) in glioma-bearing mice had a potent antitumoral effect with a cure rate of 55%; the mice showed a significant increase in activated DCs and a considerable expansion of T lymphocytes, which produced IFN-γ and lysed glioma cells. These data support the idea that priming T cells extracranially with CpG-activated DCs with tumor antigens is better than administering intratumoral CpG ODN [41, 43]. According to the authors, this method is more effective and simple, and potentially safer for the administration of CpG ODN in glioma immunotherapy. However, it is known that cell line gliomas are more immunogenic than arising human gliomas; therefore, more studies are needed [44].

The CpG ODN effect is enhanced by using a vehicle that promotes internalization to target cells; carbon nanotubes (CNTs) have been tested as a thiolated CpG (sCpG) delivery vehicle into the tumor-associated inflammatory cells in a murine glioma model. CNT-sCpG delayed tumor growth, and 50-60% of mice with established gliomas were cured. This antitumoral effect was accompanied by a sustained elevation of NK cells in the circulation and macrophage infiltration into the brain. sCpG alone enhanced mouse survival, but the effect was less than when mice were treated with CNT-sCpG [45].

Another advantage of using of TLR agonists in immunotherapy is that TLR activation may also have a systemic effect. Xiong *et al*. demonstrated that topical administration of IMQ significantly increased the number of DCs and tumor-reactive T cells that reached the glioma site. Additionally, soluble IMQ inhibited the proliferation of GL261 cells in a TLR7-independent manner because TLR7 mRNA was not expressed in the tumor cells [42, 46]. The inhibitory effects of IMQ in glioma cells do not require TLR7 expression, and the mechanism by which IMQ inhibits tumor growth could be due to the adenosine receptor-mediated signaling pathway [46]. Similarly, another study found that TLR7/TLR8 is not expressed in the glioma rat model CNS-1; however, the activation of TLR7/8 by R848 alone was sufficient to cause rejection of the smaller established glioma in CNS-1 [47]. LPS injected intratumorally in a glioblastoma model induced near-complete subcutaneous tumor elimination in wild-type BALB/c mice and a 50% reduction in TLR4 knockout mice. However, it did not confer a substantial benefit in intracranial glioblastoma-bearing mice. Analysis showed no TLR4 expression in the tumors taken from wild-type mice. However, a neutrophilic and macrophage-rich infiltrates were found in both tumors. The evidence indicates that the immunity-related antitumoral effect of LPS is not completely mediated by TLR4 [48]. Together, these findings suggest the participation of the immunological and stromal components of the tumor microenvironment.

The use of LPS in therapy against CNS tumors requires careful study; several reports have found a neurodegenerative and inflammatory effect of this TLR4 agonist and hence have suggested less toxic alternatives [49, 50]. Kawanishi et al. reported that Spirulina complex polysaccharides (CPS) initiated an antitumoral response against glioma and induced a greater production of IL-17 than LPS in C3H/HeJ mice; however, this result is opposite that for C3H/HeN mice. The results confirm that these effects are dependent on TLR4 signaling. The anti-IL-17 antibodies inhibited the growth of glioma cells in both mouse strains (C3J/HeN and C3J/HeJ) but did not increase the growth suppression by Spirulina CPS in C3J/HeN mice. In addition, C3H/HeN mice treated with CPS had lower concentrations of IL-17, developed acquired immunity, and expressed low levels of CD31 (angiogenesis marker). Finally, T cells, macrophages, and NK cells were identified as being responsible for glioma growth suppression through Spirulina CPS-TLR4 signaling. The authors concluded that the antitumoral effect of CPS is due to angiogenesis suppression and in part to the ability to regulate IL-17. They also demonstrated that the antitumoral effect of *E. coli* LPS is induced by IL-17 and IFN-γ production, but LPS had no effect on glioma angiogenesis. In contrast, other studies showed that Spirulina CPS could cause NF-κB induction via TLR2 and TLR4. These findings may suggest that TLR4 is not the only path for Spirulina CPS to induce its effect [51]. Another study showed that the absence of TLR4 inhibited the growth of U-87 tumor xenografts. Furthermore, TLR4 gene deficiency induced apoptosis process (caspase-3-dependent), resulting in a decrease in tumor growth. This suggests that TLR4 is a biomarker of interest for tumor metastasis and prognosis [52].

Nevertheless, the antitumor effect of LPS has been shown. Hua et al. reported that LPS-TLR4 activation fosters glioma growth and decreases mouse survival; however, it did not promote proliferation in vitro. This activation also downregulated in a dose-dependent manner glial fibrillary acidic protein (GFAP). LPS treatment produced slight phosphorylation of MAPKs, ERK, JNK, and p38 but significantly increased phosphorylation of NF-κB and activation of the MyD88-dependent Notch pathway. Notch inhibition reversed the downregulation of GFAP, suggesting that LPS reverses glioma differentiation via the MyD88-dependent Notch pathway [53].

Despite these results, the participation of TLR2 in the antitumor responses in the CNS has been controversial. It was reported that TLR2 activation with a synthetic bacterial lipoprotein administered jointly with tumor antigen-specific CD8 T cells increased long-term survival and immune memory in a murine glioma model GL261 [54]. However, the protumorigenic function of TLR2 has also been demonstrated. In murine GL261 glioma cells implanted in TLR2 knockout mice, the lack of TLR2 resulted in significantly smaller tumors, reduced membrane type 1 matrix metalloprotease (MT1-MMP) expression, and enhanced survival rates compared with wild-type control mice. Agonists of TLR2 (Pam_3CSK_4 and MALP2) induce the upregulation of MT1-MMP expression, promoting glioma expansion and progression [55]. Nevertheless, a distinctive dysfunction in TLR2 ligand-induced tyrosine phosphorylation of STAT1 has been observed in malignant cells but not normal glia. Microglia, astrocytes, and neuroblastoma cells treated with LTA and Pam3CSK4, two TLR2 ligands, induced tyrosine phosphorylation of STAT1 in both astrocytes and microglia, but it was not detected in neuroblastoma or different glioma cell lines (GL26, U87, and U373) [56].

In addition, the possible therapeutic potential of some endogenous ligands (e.g., DAMPs) has been demonstrated. Curtin et al. developed immunotherapy using adenoviral vectors expressing Fms-like tyrosine kinase 3 ligand (Flt3L) and thymidine kinase (TK) administration into glioblastoma. While the Flt3L induces DC infiltration into the brain parenchyma, TK is a conditional cytotoxic gene. Later, researchers identified an endogenous TLR2 agonist called high-mobility-group box 1 (HMGB1), wich is released by dying tumor cells as a result of tumor cell killing. When HMGB1 was blocked, Flt3L/TK-induced glioma brain tumor regression was inhibited. Tumor-derived HMGB1 triggers a CD8+ T cell antiglioblastoma response and induces TLR2 signaling [57]. Nevertheless, HMGB1 is not a specific ligand for TLR2; it can also be recognized by TLR4, TLR9, and RAGE and activate multiple signaling pathways (NF-κB, ERK1/2, p38, and STAT3) and subsequently the regulation of cytokines, chemokines, adhesion molecules, cell proliferation, survival, differentiation, migration, phagocytosis, autophagy, and tumorigenesis [58–60]. These findings taken together demonstrate that TLR signaling in CNS tumors is highly heterogeneous as is the resulting response. Furthermore, there is evidence NF-kB can be activated independently of TLRs. Tumorigenesis has been associated with the activation of NF-kB in glioblastoma multiforme [61]. However, in glioma cell lines (A172 and LN229), TNFα-induced NF-κB activation is partially dependent on TLR4 and involves both MyD88 and TRIF [62].

Several clinical-phase studies have been carried out, and others are currently in process; however, the results obtained so far are controversial and have not led to a definitive position about the use of TLR agonists as adjuvant therapy to treating tumors of the CNS.

Phase I clinical studies have been conducted to establish the safety profile of CpG-28 in patients with recurrent glioblastoma. Patients were treated with increasing doses of CpG-28 and evaluated for at least four months. Two patients showed tumor reduction of 29% and 20% in the largest perpendicular diameters associated with reduced mass effect and decreased surrounding edema. Two other patients had a stable disease for more than four months. At the time of the antitumor response analysis, 20% of patients had died (n=24), and 28% experienced one-year survival; the median survival was 7.2 months. In conclusion, phase I trials and preclinical models demonstrated that local administration of CpG ODN in glioblastoma-bearing patients and those with recurrent glioblastoma is possible and tolerated at doses up to 20 mg [43]. Therefore, Ursu *et al.* conducted a phase I trial with patients with different types of cancer, including ependymoma (n=1), glioma (n=1), oligodendroglioma (n=1), oligoastrocytoma (n=1), and glioblastoma (n=15), and CpG-28 was administered to each patient. In some cases, patients

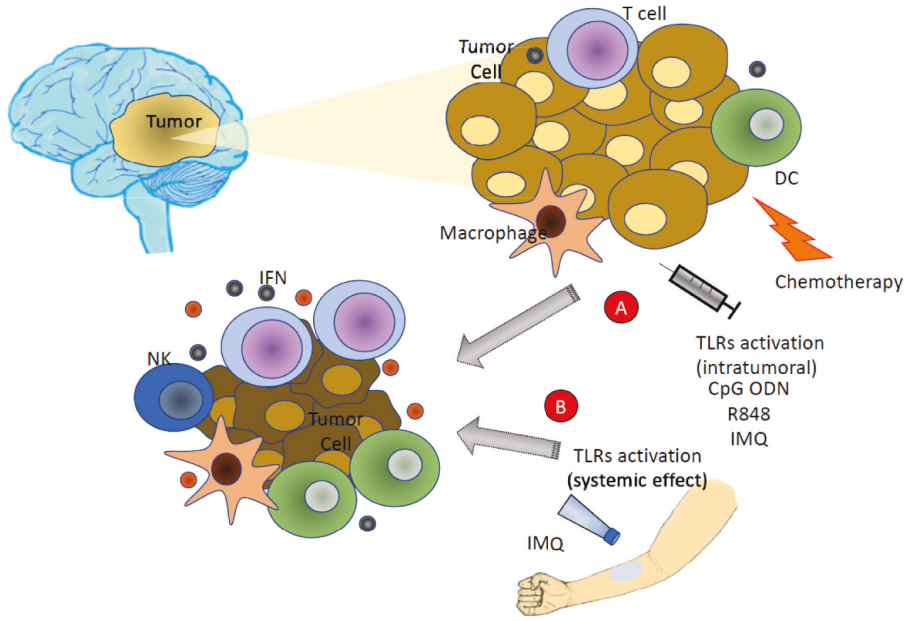

Figure 1: Summary mechanisms of TLR agonists as immunotherapy for CNS tumors. Activation of TLRs induces cytokine production, active NKs, dendritic cells, macrophage, T cell, and tumor cell apoptosis. TLR agonists can have local effect (A) and can also have a systemic effect (B).

received CpG treatment alone or in concomitantly with oncological treatment. The results showed heterogeneity among patients (n=29). Apparently, there was no significant survival between the groups treated with CpG-28 alone or CpG/oncological therapy. However, three patients showed remarkable changes. The patient with grade III ependymoma was stable during the protocol and remained alive 6 years after the study. The patients with grade III anaplastic oligoastrocytoma and glioblastoma showed clinical improvement after treatment with CpG28/bevacizumab, remained stable, and died at 12.5 months and 8.8 months, respectively [63].

In another phase I study, vaccination with autologous DC pulsed with glioma tumor lysate used as an adjuvant following surgical resection with standard chemoradiotherapy was determined to be safe, as it did not induce dose-limiting toxicities. In addition, the study authors used "boost" vaccinations with innate immune response modifiers (TLR agonists), 5% imiquimod, or poly-ICLC because these agonists may promote DC activation and priming of T cells; these vaccinations did not have any additional toxicity or adverse events. Interestingly, the median survival of patients treated with the vaccine was 31.4 months, compared to glioblastoma patients who had resection and were treated with concomitant chemoradiotherapy, where the median survival was 18.6 months [64] (Figure 1).

A phase II trial was performed to evaluate the efficacy and tolerance of a CpG ODN (10 mg/mL) treatment in recurrent-glioblastoma subjects. The authors did not find any progression-free survival in any of the patients evaluated, but the study had some long-term survivors, suggesting that some individuals might benefit from this treatment. Other studies need to be done with more patients to confirm whether side effects were caused by the CpG ODN treatment and to clarify which subgroup of patients benefit from the treatment [65]. Additional trials should be carried out with a greater number of patients to clarify the effect of immunotherapy targeting the activation of TLRs in tumors of the CNS.

5. Conclusion

The response induced by the activation of the TLRs leads to protumor or antitumor effects. Factors that determine the type of response include the agonists employed, type of cancer, the expression levels of TLRs, and the tumor microenvironment.

The molecular mechanisms through which TLRs modulate initiation, development, and tumor progression are not fully understood, but evidence shows their participation in processes such as apoptosis, angiogenesis, and proliferation of tumor cells. Clinical studies have shown the relevance and therapeutic potential of using TLR agonists in the treatment of tumors of the CNS. Future studies should be aimed at understanding the immunobiology of different malignancies originating in the CNS and establishing the efficacious and safety of immunotherapy based on the activation of TLRs that leads to establishing therapeutic alternatives for the treatment of cancer.

Disclosure

This work is part of the thesis of D. M. Abarca-Merlin, Facultad de Química, UNAM.

Conflicts of Interest

The authors declare that there are no conflicts of interest.

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Research Article

Assessment of the Levels of Level of Biomarkers of Bone Matrix Glycoproteins and Inflammatory Cytokines from Saudi Parkinson Patients

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Background. Parkinson's disease (PD) is the second most commonly neurodegenerative disease after Alzheimer's disease which occurs to nearly 1% of the population > 50 years old. Inflammatory and bone biomarkers have both become valuable tools for PD diagnosis and prognosis. However, no studies have examined these markers in Saudi patients diagnosed with PD. *Objectives.* To assess the biomarkers and proinflammatory cytokines from blood with PD in serum. *Methods.* In our study, we included 26 patients with PD and 24 controls. Blood samples were withdrawn from subjects with PD and their matched controls. Biomarkers multiplex assay from Milliplex was used to assess the levels of IL-1B, IL-6, TNF-α, osteoprotegerin (OPG), osteopontin (OPN), and PTH (parathyroid hormone). Data was analyzed using the Statistical Package, GraphPad Prism. *Results.* We found that IL-1ß cytokine is significantly higher in patients with PD (p value = 0.0014). However, there are no statistically significant variances found among the two studied groups with regard to the IL-6 and TNF-α cytokines levels. We also found that levels of PTH are decreased in the PD subjects than the age-matched controls (p value= 0.003). Also, the bone matrix glycoproteins, including osteoprotegerin (OPG) and osteopontin (OPN), are significantly upregulated (p value= 0.04 for OPG and p value= 0.003 for OPN), as compared to the controls. *Conclusions.* Our findings are reliable with the possibility that inflammatory and bone markers can be used as biomarkers in PD prognosis. However, to clarify the natural role and consequence of these markers in PD pathology, further larger cohort studies are needed.

1. Introduction

Parkinson's disease (PD) is a chronic, multifaceted disorder known as the second most commonly neurodegenerative disease, after Alzheimer's disease. PD is uncommon before the age of 50; however, the prevalence rises with age, affecting about 1% of the population > 60 years old. A few research studies have found that PD most commonly impacts men rather than women; however, other studies record no variations between the sexes [1].

Parkinson's disease is neuropathologically recognized via the inclusions of Lewy bodies and Lewy neurites containing α-synuclein (α-syn) [2]. While the etiology of PD is multifactorial, the protein α-syn is a critical component to the disease's pathogenesis. However, the mechanism that causes toxicity leading to neuronal death through α-syn malfunction remains unknown.

Parkinson disease is mostly characterized through the gradually death of dopaminergic nerves within the substantia nigra pars compacta (SNpc), subsequently principal of dopamine deficit on the striatum [3]. Clinically, patients with PD show a variety of symptoms, but the clinical hallmarks are resting tremor, postural imbalance, akinesia, bradykinesia, and rigidity, while nonmotor symptoms consist of cognitive impairment, depression, autonomic disorder, dementia, and visual hallucination [4].

Neuroinflammation, known in the pathogenesis of PD, has been suggested to play a crucial role in loosing neuronal in dopaminergic cells within the substantia nigra and then influencing the development of PD symptoms [1, 2].

Several studies have confirmed an increase in the peripheral cytokines, such as TNF and IL-6, in patients with PD when compared with control subjects [5, 6]. Moreover, a study found that fatigued PD patients showed elevated levels in IL-6 serum concentration when compared with nonfatigued patients. These outcomes suggest that IL-6 might also have a role that may cause fatigue in cases with PD [7].

Osteopontin (OPN) was revealed to be elaborate in inflammatory and degenerative mechanisms of the neurones (Carecchio et al., 2011). OPN plays a critical role in PD due to its anti-inflammatory and antiapoptotic properties and its role in regulating iNOS transcription, reactive oxygen species production, and cytokines levels [31–33]. In addition, it is been found that OPN sera and cerebrospinal fluid (CSF) amounts are greater in PD patients than controls, with CSF extent positively linked with concomitant dementia (Maetzler et al., 2007).

Parkinson disease is a complicated heterogeneous disease that requires multimodal biomarker technique to track and control the disease progression and analysis. Within the Saudi population, the association between these biomarkers and PD was not previously studied. Understanding the pathophysiological mechanisms of PD would lead to the development of effective therapies. Therefore, it is very crucial to study and discover a likely relationship between PD pathogenesis and serum concentrations of these biomarkers.

2. Methodology

This project was approved by the King Fahad Medical City (KFMC), IRB Committee. Seventeen PD patients were enrolled from the Outpatient Neurology Clinic at KFMC, Riyadh. Additionally, 9 cases were recruited from King Abdulaziz University Hospital (KAUH) in Jeddah. A formal written consent form was provided to all included subjects to read and sign prior to the study.

2.1. Subjects. Seventeen patients were included from KFMC in this study and 9 patients from KAUH Applicable demographic and clinical data were collected from each patient's medical record. Additionally, the control group included 24 Saudi individuals, which were matched with PD patients in regard to their age and sex (IRB number 16-450, IRB registration number with KACST, KSA H-01-R-012, IRB registration number with PHRP/NIH/USA = IRB00010471, Approval number federal wide assurance NIH, USA = FWA00018774).

2.2. Vitamin D, Calcium, and Phosphate Assay. Vitamin D was evaluated by determining 25-dihydroxyvitamin D (25-OH D) levels using ELISA with a commercially available radioimmunoassay (R&D systems, refRDKAP1971). 150 μL of Incubation Buffer was added to microplate. Then, the plate was incubated for 2 hours at room temperature (RT), on a plate shaker (400 rpm). After that, the plate was rinsed 3 times by washing solution. Then, 200 μL of the Working HRP conjugate solution was added to plate wells and then incubated for 30 minutes at RT on a plate shaker (400 rpm). Again, the plate was rinsed 3 times by washing solution. Later, 100 μL of the chromogenic solution was added to each well within 15 minutes following the washing step. Then, the plate was incubated for 15 minutes at RT, on a plate shaker (400 rpm). Finally, color development was stopped via addition of 100 μL of Stop media into separate well; then absorbance was calculated at 450 nm within 1 hour.

2.3. Cytokines Multiplex Assay. Human bone magnetic bead panel, cytokines multiplex assay from Milliplex (Cat No HBNMAG-51K), was used to assess the levels of IL-1β, IL-6, TNF-α, osteoprotegerin, osteopontin, and PTH. Frozen serum samples, from PD versus non-PD subjects, were assessed for all the above parameters in duplicate at one time by using a single plate. The procedure was done according to the assay protocols provided by the manufacturer. Luminex 200 machine and Milliplex Analyst software were used for data analysis, at the Neuroscience Unit in KAUH.

The kit uses a 96-well format, containing a lyophilized standard cocktail and 2 quality controls that can measure up to 38 serum samples in duplicate. Multidimensional fatigue inventory (MFI) measurements were obtained, and data was analyzed accordingly for high sensitivity, consistency, and reproducibility.

In summary, 25 μg of serum (1:2 diluted) was incubated with antibody-conjugated magnetic beads for overnight at four-degree temperature inside the fridge. Bead-complexes after rinsed were kept with 50 μL biotinylated detection antibody for half an hour on a plate shaker at room temperature. After that, they were incubated with 50 μL streptavidin-phycoerythrin for half an hour on a plate shaker at (20-25°C). After washing 3 times 100 μL of Sheath Fluid was added to all wells. Bead-complexes were then read on Run plate on Luminex® 200TM and analyzed by MAGPIX® with xPONENT® software.

2.4. Statistical Analysis. GraphPad Prism 7 software was used to perform an unpaired t-test to compare between the PD and control groups using all the investigated biomarkers.

3. Results

3.1. Patients' Characteristics. Information on the sociodemographic characteristics and initial laboratory parameters of vitamin D (25-Hydroxyvitamin D), calcium, and phosphate was attained via closed question form (Table 1).

3.2. Status of the Inflammation in the PD. In comparison to the controls, IL-1ß cytokines were significantly higher in patients with PD (p value = 0.0014), (Figure 1). However, the IL-6 and TNF-α cytokines did not significantly differ between the two studied groups.

3.3. Imbalance of Bone Matrix Glycoproteins and PTH in PD. Despite the lower levels of PTH produced in the PD subjects than the age-matched controls (p value= 0.003), the bone matrix glycoproteins, including osteoprotegerin (OPG) and osteopontin (OPN), were significantly upregulated (p value= 0.04 for OPG and p -value= 0.003 for OPN), as compared to the controls. The results are depicted in Figure 2.

Table 1: Sociodemographic characteristics of the subjects.

Variables	PD cases (n = 26)	Control Cases (n = 24)	*P-value*
Age (years)	60.35 ± 11.5	60.22 ± 9.2	*0.9769 NS*
Sex	20 M / 6 F	18 M / 6 F	
Duration of disease (years)	6.23 ± 4.5	NA	
Vitamin D Supplement use			
Yes	16	6	
No	10	18	
Sun exposure			
Yes	3	4	
No	23	20	
Family history of PD (first degree)			
Yes	9	0	
No	17	24	
Laboratory parameters			
Vitamin D (25-Hydroxyvitamin D)(nmol\l)	58.7 ± 12.8	156.9 ± 13.7	0.0001
Calcium levels (mg\dl)	8 ± 0.0	9.2 ± 0.22	0.0253
Phosphorus levels (mg\dl)	2.6 ± 0.1	3.4 ± 0.29	0.0561

M, male; F, female; NA, not applicable; NS, not significant. Data are expressed as mean ± standard deviation.

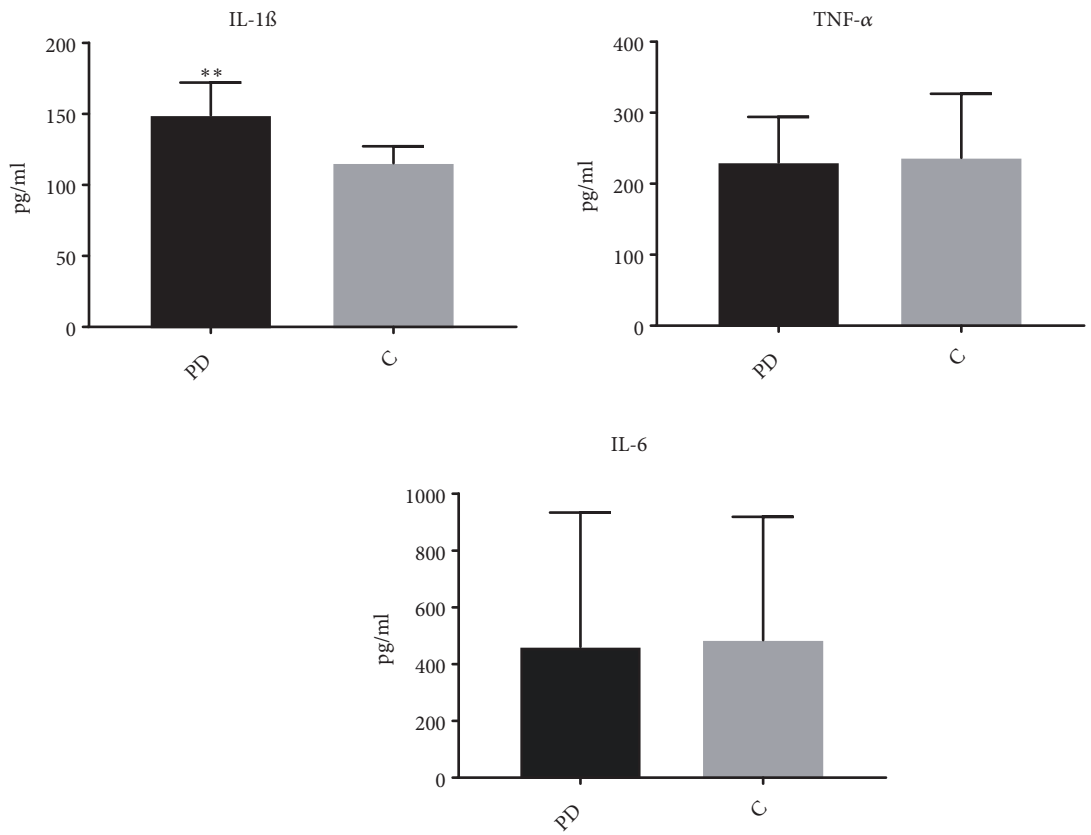

Figure 1: *IL-1ß cytokine is upregulated in the PD*. Results of inflammatory cytokines IL-1ß, IL-6, and TNF-α are differentiated between the PD and control (C) subjects. Data are expressed as mean ± standard error of the mean. An unpaired t-test was used for comparison (n = 50); $**p < 0.001$.

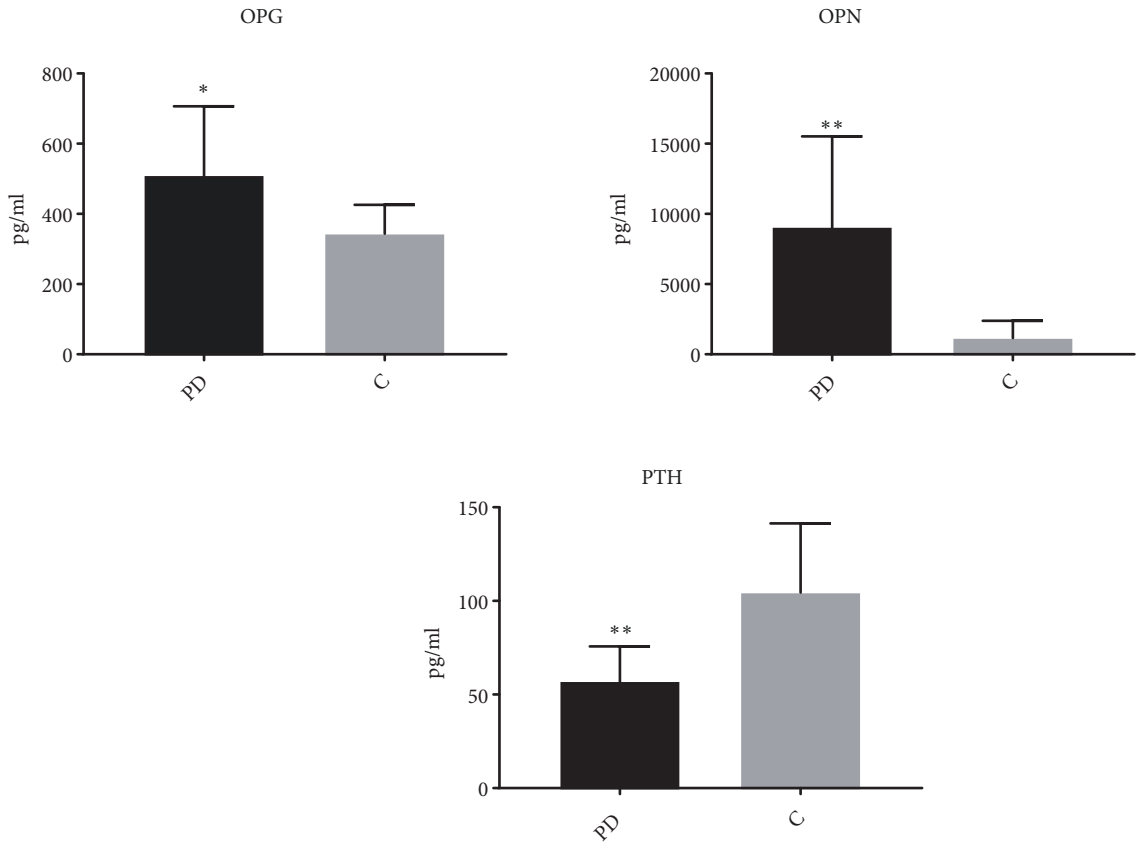

Figure 2: *Bone matrix glycoproteins are overshoot from PD.* Results of bone matrix glycoproteins: osteoprotegerin (OPG) and osteopontin (OPN) and parathyroid hormone (PTH) are differentiated between the PD and control (C) subjects. Data are expressed as mean ± standard error of the mean. An unpaired t-test was used for comparison (n =50); $* * p < 0.05$ and $* * p < 0.001$.

4. Discussion

Biomarkers are commonly used to serve as a predictor tool in pathogenic processes and normal biological function to expand our understanding and treatment of complex diseases. The function of microglia in the pathology of PD had been established twenty years ago from a postmortem study when scientists found T-lymphocytes activation and microglia dysfunction in the SNpc of PD subjects [8]. Thus far, various investigations have exhibited the connection of inflammatory microglia in the pathology of PD.

Cytokines display a functioning role in several diseases and conditions, which are inflammation host responses to infection, injury, sepsis, and malignant growth. Cytokines are nonstructural proteins that contain low molecular weights running from 8,000 to 40,000 Da [9]. Various immune and nonimmune cells (e.g., macrophages, T-lymphocytes, Schwann cells, and fibroblasts) are recognized to create cytokines that are essential in cell signalling. Cytokines, including interleukins, interferon, and chemokines, are in charge of inciting numerous biological impacts, for example, inflammatory responses, inhibition or stimulation of cell development and differentiation, cytotoxicity/apoptosis, and antiviral action [10]. Additionally, cytokines are imperative in the inflammatory or anti-inflammatory processes relying upon the need of the host's biological status [10].

There are two types of cytokines based on their actions: (1) proinflammatory cytokines (IL-1, TNF) that are engaged with starting inflammation and (2) anti-inflammatory cytokines (IL-4, IL-10, IL-13, and TGF) which control the proinflammatory cytokines' action. Moreover, cytokines have a vital role in both inflammatory and anti-inflammatory forms in numerous neurological diseases [10–12]. Various cytokines are secreted by neurons or glia, thereby changing cytokines concentration in the brain, blood, and cerebrospinal fluid (CSF) of patients with PD [11].

Our results as shown in Figure 1 confirmed previously published data that the Th1 cytokines (TNF-α,) and some proinflammatory cytokines (such as IL-6) are similar in the serum of PD patients. Moreover, we found a significant increase in IL-1β serum concentration in PD subjects.

Various investigations have reported that IL-1 has a primary function in the inflammation process, together with the dynamic role in the development of a multifaceted hormonal and cell inflammatory course [13]. Although abnormal increase in IL-1 level was linked with neuronal degeneration, elevated levels of IL-1 concentrations have been seen in CSF and brain parenchyma of humans and rodents after brain trauma [11, 13, 14].

Additionally, up expression of IL-1 is the fundamental element associated in the initiation of the inflammatory

course, which consequently initiates a vicious cycle of various reactions that subsequently lead to neuronal loss. In the present study, serum concentration of IL-1β was remarkably higher in patients with PD in comparison to controls as indicated in Figure 1. Our finding is in agreement with previous investigations that associated increase in IL-1β serum concentration with PD diagnosis.

Moreover, it has been reported that IL-6 may be correlated with inflammation process or neuronal survival in the brain which may affect the neurodegeneration process in PD [12]. Although we did not find a significant difference between IL-6 concentrations in our patients with PD and control cases Figure 1, it is important to know that IL-6 neuroimmune dysfunction was linked with central nervous system (CNS) inflammation. This is merely because it was documented in the CSF and postmortem brain of PD patients and, it is not likely to determine the origin of cytokines when serum cytokines levels are measured.

On the other hand, TNF-α is a member of the peptide ligands family which contributes the stimulation of a set of structurally interassociated receptors [15]. TNF-α is a protein of 17 kDa molecular weight, that consists of a nonglycosylated protein of 157 amino acids. The TNF-α is biological response to the stimulation via two structurally different receptors. Both types of receptors are transmembrane glycoproteins that have more than one cysteine-rich which repeats in the extracellular N-terminal domains [16]. In the peripheral system, the activated macrophages and T-lymphocytes are mainly accountable for the creation of pro-TNF (molecular weight 26 kDa) protein [15]. TNF is expressed on the plasma membrane and then processed via matrix metalloproteinase, leading to cleavage of the extracellular domain and finally yielding 17 kDa soluble structure of TNF [17]. Keep in mind that a range of pathological approaches including inflammation process, ischemia, and traumatic damage can lead to the mediation of microglia and astrocytes in order to introduce TNF in the CNS [18].

TNF is known as a circulating element leading to tumor necrosis. Although studies have validated that TNF has crucial and numerous function in the pathological development of several chronic diseases such as neurodegenerative disorders [18]; typically, in the brain TNF performs a critical position structurally and functionally such as normal behavior, sleep, and synaptic plasticity [19]. In contrast, activation of the microglia in the CNS causes elevated TNF-α expression which subsequently stimulates NFK-α function [20]. iNOS is needed in the production of NO as well as peroxynitrite, known as NO-derived reactive nitrogen species [14]. Therefore, upregulation of large reactive molecules results in the production of lipid peroxidation, tyrosine nitrosylation, and oxidative damage to DNA ensuing in neurodegeneration [14]. Subsequently, this neural damage may play a possible role in PD pathology [14, 20].

A study has found that TNF levels were elevated in a cohort of Japanese early-onset PD patients in comparison to late-onset of PD and control subjects [21].

A notable number of studies which have been done in peripheral inflammatory/immune markers suggested the hypothesis of inflammation involved in PD [22]. Furthermore, research of cytokines in plasma and serum was found to raise levels of proinflammatory cytokines such as TNF-α and its soluble receptors sTNFR1 and IL-1β in PD cases in comparison with matched controls [22, 23].

In this study, levels of TNF-α did not change between PD patients and healthy controls Figure 1. Therefore, our findings can be explained by the heterogeneity of PD pathophysiology. Although IL-6 plasma level was prospectively related to an elevated risk of developing PD [24]; several studies have failed to point out noteworthy changes in cytokines (IL1-α, IL-6, and TNF-α) concentration in PD.

5. Conclusions

The interpretation of the findings of this study should take into account many limitations, such as medications used by PD patients, disease severity, and small sample size. In addition, the cytokine methodology assessment may differ which directly affects result's sensitivity. In conclusion, our findings demonstrated that serum and IL-6, IL-IB, and TNF could be used as promising applicable biomarkers of PD inflammation. However, in this study, we did not find a significant impact of IL-6 and TNF-α concentrations on PD patients. Our results revealed that inflammatory mechanisms are an essential factor in PD pathophysiology.

Data Availability

The data used to support the findings of this study were included within the article.

Conflicts of Interest

The authors declare that they have no conflicts of interest.

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Research Article

Glutamine Improves Oxidative Stress through the Wnt3a/β-Catenin Signaling Pathway in Alzheimer's Disease In Vitro and In Vivo

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Background/Aims. Alzheimer's disease (AD) is the most common neurodegenerative disease, and all researchers working in this field agree that oxidative stress is intimately associated with Alzheimer disease. In this study, we hypothesized that glutamine (Gln) offers protection against oxidative stress injury in SAMP8 mice as well as the underlying mechanism. *Methods*. The SAMP8 mice received glutamine intragastrically for 8 consecutive weeks to evaluate the protective effect of glutamine on oxidative stress in AD mice involving Wnt3a/β-catenin signaling pathway. In addition, rat pheochromocytoma tumor cell line PC12 was pretreated with 32 μM glutamine for 2 h followed by 24 h incubation with 40 μM Aβ25-35 to obtain in vitro data. *Results*. In vivo the administration of glutamine was found to ameliorate behavioral deficits and neuron damage, increase superoxide dismutase (SOD) and glutathione peroxidase (GSH-XP) activity, reduce the malondialdehyde (MDA) content, and activate the Wnt3a/β-catenin signaling pathway in SAMP8 mice. In vitro glutamine treatment decreased the toxicity of Aβ25-35 on PC12 cells and prevented apoptosis. Additionally, glutamine treatment increased SOD and GSH-XP activity and decreased MDA content and increased Wnt3a and β-catenin protein levels. Interestingly, the DKK-1 (Wnt3a/β-catenin pathway inhibitor) decreased the antioxidant capacity of glutamine in Aβ25-35-treated PC12 cells. *Conclusion*. This study suggests that glutamine could protect against oxidative stress-induced injury in AD mice via the Wnt3a/β-catenin signaling pathway.

1. Introduction

Alzheimer's disease (AD), also known as senile dementia, is a chronic neurodegenerative disorder characterized by progressive cognitive impairment and behavioral damage [1]. It is predicted that the number of AD patients will rise to 90 million in 2050 worldwide [2]. More worrying is that China's aging population is increasing by more than 8 million every year, and the number of AD patients in China is expected to be the sum of all developed countries in 2040 [3]. AD has become the fourth leading cause of death in humans after cardiovascular disease, cancer, and stroke. Amyloid cascade is an important hypothesis in the pathogenesis of AD. Aggregation of Amyloid β-protein (Aβ) induces an oxidative stress response that damages mitochondria. Oxidative stress, in turn, further promotes the aggregation of Aβ and the phosphorylation of tau, a microtubule-related protein, aggravating the imbalance of REDOX reactions in the brain of AD patients. Oxidative stress is a common key point connected with various pathogenic mechanisms [4]. Therefore, finding suitable antioxidants for appropriate clinical intervention is an effective means to prevent AD.

Glutamine (Gln) is the most abundant-free amino acid in plasma, acting on oxygen-free radicals and playing an important role in vascular disease [5], diabetes [6], neurodegenerative diseases [7, 8], and various cancers. As the main energy supply substance for mitochondria to form ATP, the oxidation of Gln can eliminate some strong oxidizing substances to protect some important components of cells from oxidative damage [9]. The intracellular and extracellular glutamine is essential for neuronal health. A previous research pointed out that the dietary supplementation of

glutamine has significant neuroprotective effects and help restore homeostatic functions that are lost in AD [10]. However, whether the glutamine can affect the level of oxidative stress to play a neuroprotective effects in AD mice model is not clear.

As a key molecular pathway, the Wnt3a/β-catenin signaling pathway regulates neuronal survival, differentiation, axonal extension, neurogenesis, synapse formation and plasticity, and neuroprotection [11]. Previously, the pathway was related to research on the treatment of Parkinson's disease [12]. Increasing evidence showed that this pathway participated in the neuronal differentiation and apoptosis in Alzheimer's disease model [13, 14]. Wnt3a/β-catenin signaling is also critically associated with the occurrence and development of oxidative stress [15]. Thus, our study aimed to evaluate whether glutamine protects against oxidative stress-induced injury via activation of the Wnt3a/β-catenin signaling pathway in a mouse model of AD.

2. Materials and Methods

2.1. Reagents. Aβ25-35 [Amyloid beta-peptide (25-35)] was purchased from MCE (HY-P0128; NJ, USA) and the purity of Aβ25-35 was ≥ 98%. DKK-1 (Wnt3a/β-catenin pathway inhibitor; SRP3258) and glutamine (Gln; 1294808) were purchased from Sigma-Aldrich (Merck KGaA, Darmstadt, Germany). Additional reagents employed in the present study were commercially available and of analytical purity.

2.2. Animals and Grouping. Thirty male senescence accelerated mouse SAMP8 (20~30 g, three-month-old) and ten normal aging control mouse SAMR1 were purchased from the animal center of the West China Medical College of Sichuan University, Chengdu, China. All animals were housed in the animal laboratory under controlled, conventional conditions (temperatures of 24±1°C, relative humidity of 60±10%, and 12 h light-dark cycle) and were allowed free access to food and water during the experimental session. Following a week of adaptation, mice were randomly divided into four groups (n=10): the control (SAMR1), model (SAMP8), glutamine-low (250 mg/kg), and glutamine-high (500 mg/kg) groups. After grouping, the mice were immediately administered treatments. The mice in the control and sham groups received 1 mL saline by intragastric administration, and the mice in the glutamine groups received an equal volume of 250 mg/kg and 500 mg/kg glutamine intragastrically. The treatment was given once a day for 8 consecutive weeks.

2.3. Behavioral Assessment. A step-down passive avoidance test was performed to detect the learning and memory of mice [16]. The platform reaction box (10x10x5 cm) was divided into two sections by a copper gate with continuous electrical stimulation (36 V) at the bottom of the box. A 4.5 cm inner diameter and height rubber pad was placed at the right rear corner of each box to serve as a safe area for mice to avoid electric shock. In the training session the animals were placed on the platform for 3 min. The time taken to react to jump to the pad (reaction time) and the number of electric shocks they received within 5 min (error frequency) were recorded as learning achievements. After 24 h, the animals were again placed into the platform for 3 min and then set on the pad. The first time they jumped off the pad (latent period) and the number of electric shocks they received within 5 min (error frequency) were recorded as memory achievements.

2.4. HE Staining and TUNEL Staining. Five mice in each group were anesthetized by an intraperitoneal injection of 3% pentobarbital sodium (50 mg/kg); brain tissues were taken out immediately and fixed with 4% paraformaldehyde for 48 ~ 72 h. The hippocampus was embedded in paraffin, and 5-μm-thick paraffin sections were prepared. The pathological changes of tissues were observed under light microscope following hematoxylin and eosin (HE) staining. The apoptotic cells were detected using a TUNEL assay kit (T2190; Beijing Solarbio Science & Technology Co., Ltd., Beijing, China). The apoptotic cells exhibited brown staining within the nucleus. Images were captured with a fluorescence microscope (Olympus Corporation, Tokyo, Japan).

2.5. Detection of Superoxide Dismutase (SOD), Glutathione Peroxidase (GSH-Px), and Malondialdehyde (MDA) Contents. After the intraperitoneal injection of 3% pentobarbital sodium (50 mg/kg) for anesthesia, brain tissue was removed immediately, placed on an ice tray to isolate hippocampus tissues, and weighed on an accurate electronic scale. Then, the tissues were shredded using an ophthalmic scissor and normal saline was added (1:10) to produce 10% brain tissue homogenate. The homogenate was centrifuged at 4°C for 10 min, obtaining the supernatant for future use. SOD, GSH-Px activity, and MDA contents were detected by the colorimetry method using a microplate reader according to the kit instructions (SOD: A001-1-1; GSH-Px: A005; MDA: A003-1; Nanjing Jiancheng Bioengineering Institute, Jiangsu, China).

Cells from each group were collected and digested with trypsin. Following the cells were disrupted at 4°C by an ultrasonic cell disruptor and the lysate was centrifuged at 1000 r/min at 4°C for 10 min. A total of 100 μl supernatant was obtained to detect the OD values using a microplate reader according to the instructions of SOD, GSH-Px, and MDA kit, and the content was calculated.

2.6. Western Blot Assay. Protein samples were prepared from brain tissues and PC12 cells using RIPA lysis buffer (AR0105; Boster, Wuhan, China) and quantified with a Protein Assay kit (AR0146; Boster). In order to detect the levels of protein expression, protein samples were separated by 10% SDS-PAGE gel and then transferred onto a PVDF membrane (C3117; Millipore, MA, USA). Following sealed with 5% skimmed milk powder at room temperature for 1 h, the membranes were incubated with rabbit anti-Wnt3a (#2721), rabbit anti-β-catenin (#8480), and rabbit anti-β-actin (#4970; 1:1000; Cell Signaling Technology, MA, USA) at 4°C overnight and then incubated with goat anti-rabbit IgG at room temperature for 1 h. β-actin was used as inner

Table 1: Glutamine affects the learning and memory abilities of SAMP8 mice.

Group	Learning ability		Memory ability	
	Reaction time (s)	Error time (s)	Reaction time (s)	Error time (s)
Control (n=10)	16.58±1.62	3.84±1.08	197.40±24.05	3.54±0.97
Model (n=10)	64.69±5.55**	6.93±1.82**	71.32±8.12**	7.07±1.40**
Gln-low (n=10)	60.01±5.32**	6.82±2.14**	76.22±6.70**	6.52±1.71**
Gln-high (n=10)	18.91±2.19##	4.21±1.09#	182.26±15.79##	4.32±1.46##

**$p < 0.01$, compared with control group. #$p < 0.05$ and ##$p < 0.01$, compared with model group.

loading control. Protein bands were visualized using an ECL chemiluminescence kit (WBULS0500; EMD Millipore).

2.7. Cell Culture and Proliferation Assay. Cells of the rat pheochromocytoma tumor cell line PC12 were purchased from Procell Life Science & Technology Co., Ltd (CL-0412; Wuhan, China). PC12 cells were cultured in RPMI-1640 medium (PM150115; without glutamine; Procell Life Science & Technology Co., Ltd) supplemented with 10% Fetal Bovine Serum (10099-141; FBS; Gibco, CA, USA) and incubated at 37°C in 5% CO_2.

Cell proliferation was measured using a Cell Counting Kit-8 (CK04; CCK-8; Dojindo, Kumamoto, Japan) assay. Cells in log phase were collected and seeded into 96-well plates at a density of 6×10^3/well. Then cells were cultured in 10% CCK-8 for 1 h and the absorbance value was measured at 450 nm using a microplate reader (Thermo Fisher Scientific, MA, USA).

2.8. Annexin-V/Propidium Iodide (PI) Double-Staining and Flow Cytometry Assays. Following washing, trypsin digestion, and centrifugation, PC12 cells were resuspended in 100 μl binding buffer ($1x10^5$ cells) with 5 μl Annexin V-FITC and 5 μl PI (CA1020; Beijing Solarbio Science & Technology Co., Ltd.) for 15 min in the dark. Subsequently, cell apoptosis was detected using a FACSCaliburTM Flow Cytometer (BD Biosciences) within 1 h.

2.9. Statistical Analysis. Statistical analysis was performed using SPSS20.0 software (IBM Corp., Armonk, NY, USA). Values were presented as the means ± standard deviation (SD) from three separate experiments. Differences among multiple groups were compared by one-way analysis of variance (ANOVA) with Dunnett's post-tests or two-way ANOVA with Bonferroni's post-tests. The differences were considered statistically significant at $p < 0.05$ and $p < 0.01$.

3. Results

3.1. Glutamine Enhances Learning and Memory Abilities of SAMP8 Mice. A passive avoidance apparatus was performed to examine the learning and memory abilities of SAMP8 mice following glutamine treatment. As shown in Table 1, compared with the control group, with respect to learning ability, the reaction time and error time in the model and glutamine-low groups were significantly increased, respectively. Compared with model group, the Gln-high group displayed reduced reaction time and error time. With respect to memory ability, the reaction time in the model and glutamine-low groups was significantly decreased compared to control group; the reaction time in the Gln-high group was significantly increased compared to model group. These results indicated that the high concentration of glutamine could enhance the learning and memory ability of SAMP8 mice.

3.2. Glutamine Alleviates Neuron Damage in SAMP8 Mice. Morphological changes in the hippocampus were measured with H&E staining (Figure 1(a)). Compared with the control and Gln-high groups, the model and Gln-low groups had sparsely and disorderly arranged cells, substantial reduced pyramidal cells, nucleus pycnosis observed, and nucleus stained with deep blue. Apoptotic cells were detected by TUNEL staining (Figure 1(b)). The TUNEL-positive cells were observed in the model and Gln-low groups, and almost no TUNEL-positive cells were observed in the control and Gln-high groups. These results indicated that the high concentration of glutamine could improve the abnormal structure and apoptosis of the hippocampus cells in SAMP8 mice and could improve the damage of hippocampus neurons.

3.3. Glutamine Strengthens the Antioxidant Capacity in SAMP8 Mice. As shown in Figure 2, compared with control group, the activity of SOD and GSH-XP in hippocampus of the model and Gln-low groups was significantly decreased, where the content of MDA was markedly increased. Compared with model group, the Gln-high group displayed reduced MDA content, increased SOD, and GSH-XP activity. These results showed that the high concentration of glutamine could improve antioxidant capacity of SAMP8 mice.

3.4. Glutamine Treatment Activates the Wnt3a/β-Catenin Signaling Pathway in SAMP8 Mice. The western blot result indicated that, compared with control group, the expression levels of Wnt3a and β-catenin in hippocampus of SAMP8 mice were significantly decreased. Compared with model group, the Wnt3a and β-catenin protein levels were significantly increased in Gln-high group (Figure 3). These results indicated that the Wnt3a/β-catenin signaling pathway was inhibited in SAMP8 mice, and the high concentration of glutamine could reverse.

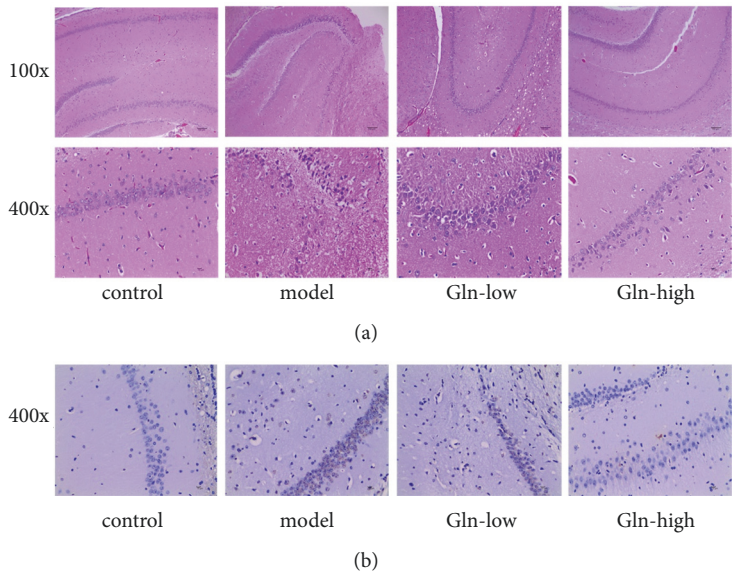

Figure 1: Effects of glutamine on the damage of hippocampus neurons in SAMP8 mice. (a) Images of brain tissue of mice following hematoxylin-eosin staining. (b) Images of brain tissue following TUNEL staining; nucleus of apoptotic cells were stained brown.

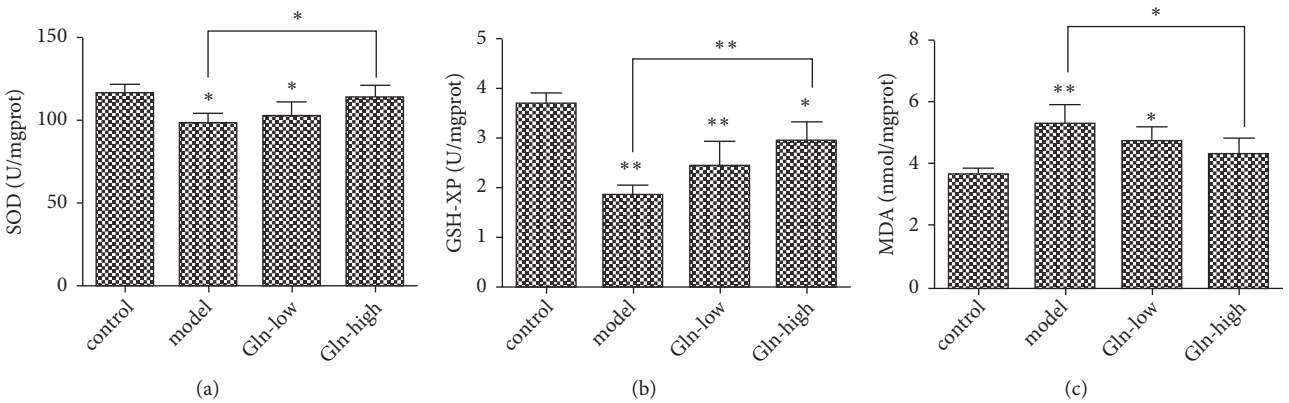

Figure 2: Effect of glutamine on activity of SOD and GSH-XP and content of MDA in hippocampus of SAMP8 mice. (a) SOD activity in each group. (b) GSH-XP activity in each group. (c) MDA content in each group. Data were obtained from three independent experiments. The results were presented as the mean ± standard deviation. $^{*}P < 0.05$ and $^{**}P < 0.01$.

3.5. The Cytotoxicity of Aβ25-35 on PC12 Cells. To simulate pathological damage of AD in vitro, PC12 cells were treated with different concentrations of Aβ25-35 for 24 h, and the inhibition of cell proliferation was detected via a CCK-8 assay. As shown in Figure 4(a), the cell proliferation was significantly decreased in a dose- dependent manner, with IC50 value of 41.601 μM. Thus, we chose 40 μM and 24 h as the concentration and time of the follow-up study. To determine whether the cytotoxicity of Aβ25-35 against PC12 cells induces apoptosis, the present study analyzed apoptotic rate by flow cytometry following 24 h of treatment with Aβ25-35. As shown in Figure 4(b), the apoptotic rate of PC12 cells was significantly increased in a dose-dependent manner. These results indicated that the PC12 cells damage can be induced by Aβ25-35 and can be used in the establishment of AD cell model.

3.6. Glutamine Suppressed Cytotoxicity Induced by Aβ25-35 in PC12 Cells. To investigate whether the glutamine plays a protective role on Aβ25-35 induced PC12 cell injury, cells were pretreated with different concentrations of glutamine for 2 h and then incubated with 40 μM Aβ25-35 for 24 h, followed by observation of PC12 cell proliferation and apoptosis. As shown in Figure 5(a), the cell proliferation was significantly increased following glutamine pretreatment, and the cell proliferation was the highest when glutamine concentration was 32 μM. The flow cytometry results showed that the apoptotic rate of PC12 cells in the G0+A0 group was

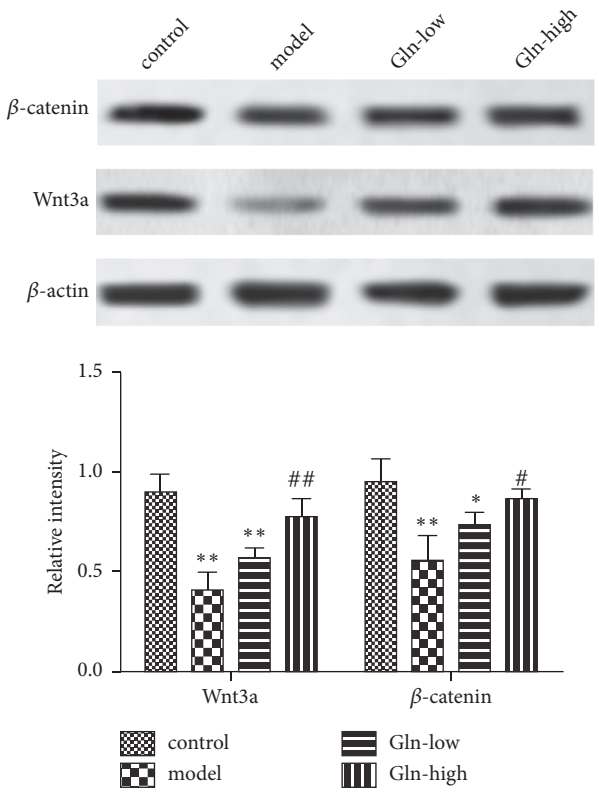

FIGURE 3: Wnt3a and β-catenin protein expression levels in hippocampus of SAMP8 mice following pretreatment with glutamine. The expression levels of Wnt3a and β-catenin protein were examined by western blotting. The experiments were repeated three times. The results were presented as the mean ± standard deviation. $^{*}P < 0.05$ and $^{**}P < 0.01$, compared with control group. $^{\#}P < 0.05$ and $^{\#\#}P < 0.01$, compared with model group.

significantly lower than that in the A0 group (Figure 5(b)). These results suggested that the glutamine protected PC12 cells from damage induced by Aβ25-35.

3.7. Glutamine Treatment Activates the Wnt3a/β-Catenin Signaling Pathway in AD Cell Model. To elucidate whether the protective effect of glutamine on PC12 cell damage induced by Aβ25-35 is through activation of the Wn3a/β-catenin signaling pathway, western blot assay was performed to detect the expression of Wn3a and β-catenin proteins. As shown in Figure 6, the expression of Wnt3a and β-catenin protein was decreased in different degrees in the remaining groups compared with the control group, indicating that the Wnt3a/β-catenin pathway was inhibited in AD cell model. Compared with the A0 group, the expression of β-catenin protein was significantly increased in the G0+A0 group, indicating that glutamine pretreatment of cells can alleviate the inhibition of Wnt3a/β-catenin pathway. Compared with the G0+A0 group, the Wnt3a and β-catenin proteins in the A_0+DKK-1 and G_0+A_0+DKK-1 groups were significantly decreased, indicating that the DKK-1 can inhibit the activation of Wnt3a/β-catenin pathway.

3.8. The Blocking of Wnt3a/β-Catenin Signaling Pathway Decreased the Antioxidant Capacity of Glutamine in PC12 Cells. To elucidate the role of Wn3a/β-catenin signaling pathway on glutamine protecting PC12 cell against oxidative stress, DKK-1, Wn3a/β-catenin signaling pathway inhibitor was used. As shown in Figure 7, the activity of SOD and GSH-XP in A0+DKK-1, A0, and G0+A0+DKK-1 groups was significantly decreased compared with the control group, where the content of MDA was markedly increased, indicating that the antioxidant capacity was inhibited in AD cell model. Compared with the A0 group, the activity of SOD and GSH-XP in G0+A0 group was significantly increased, indicating that glutamine could improve antioxidant capacity of AD cell model. Compared with the G0+A0 group, the activity of SOD and GSH-XP in A0+DKK-1 and G0+A0+DKK-1 groups was significantly decreased, where the content of MDA was markedly increased, indicating that the glutamine strengthens the antioxidant capacity through activating the Wnt3a/β-catenin signaling pathway in vitro.

4. Discussion

Alzheimer disease (AD) is a multifactorial and fatal neurodegenerative disorder which has an influence on a large number of senior citizens. The neuropathological hallmarks of AD are Aβ plaques, neurofibrillary tangles, synapse loss, and neuronal loss [17, 18]. The pathogenesis and progression of AD are related to many risk factors, and the oxidative damage was served as the earliest pathological events [19]. The present study aimed to investigate the effect of glutamine on oxidative stress-induced injury in AD mice. Our results indicated that glutamine could protect against oxidative stress-induced injury in AD mice through activating the Wnt3a/β-catenin signaling pathway.

Initially, we performed the rotation experiment and found that the SAMP8 mice had an obvious rotational behavior. Following the administration of glutamine, the learning and memory abilities of SAMP8 mice were significantly increased. Concerning H&E and TUNEL staining experiments, we found that the hippocampus neuronal cells were abundant, arranged in neat rows, with morphological integrity and zonal distribution in SAMP8 mice following glutamine administration. And the administration of glutamine also prevented hippocampus neuronal cells from apoptosis. These results indicated that the glutamine could alleviate the damage of hippocampus. Glutamine, a free amino acid, confers various biological effects. The value of glutamine is particularly apparent during stress, which is extensively applied for the treatment of diseases related to inflammation and oxidative stress [20, 21]. Previous studies have showed that the glutamine levels are lower in the brains of patients with Alzheimer's disease (AD), and the glutamine supplementation could reduce inflammation-induced neuronal cell cycle activation, tau phosphorylation, and ATM-activation in a mouse model of AD [10] . Therefore, we speculated that the glutamine could reduce oxidative stress levels in hippocampus of AD mice to control AD progression. Superoxide dismutase (SOD) and glutathione peroxidase

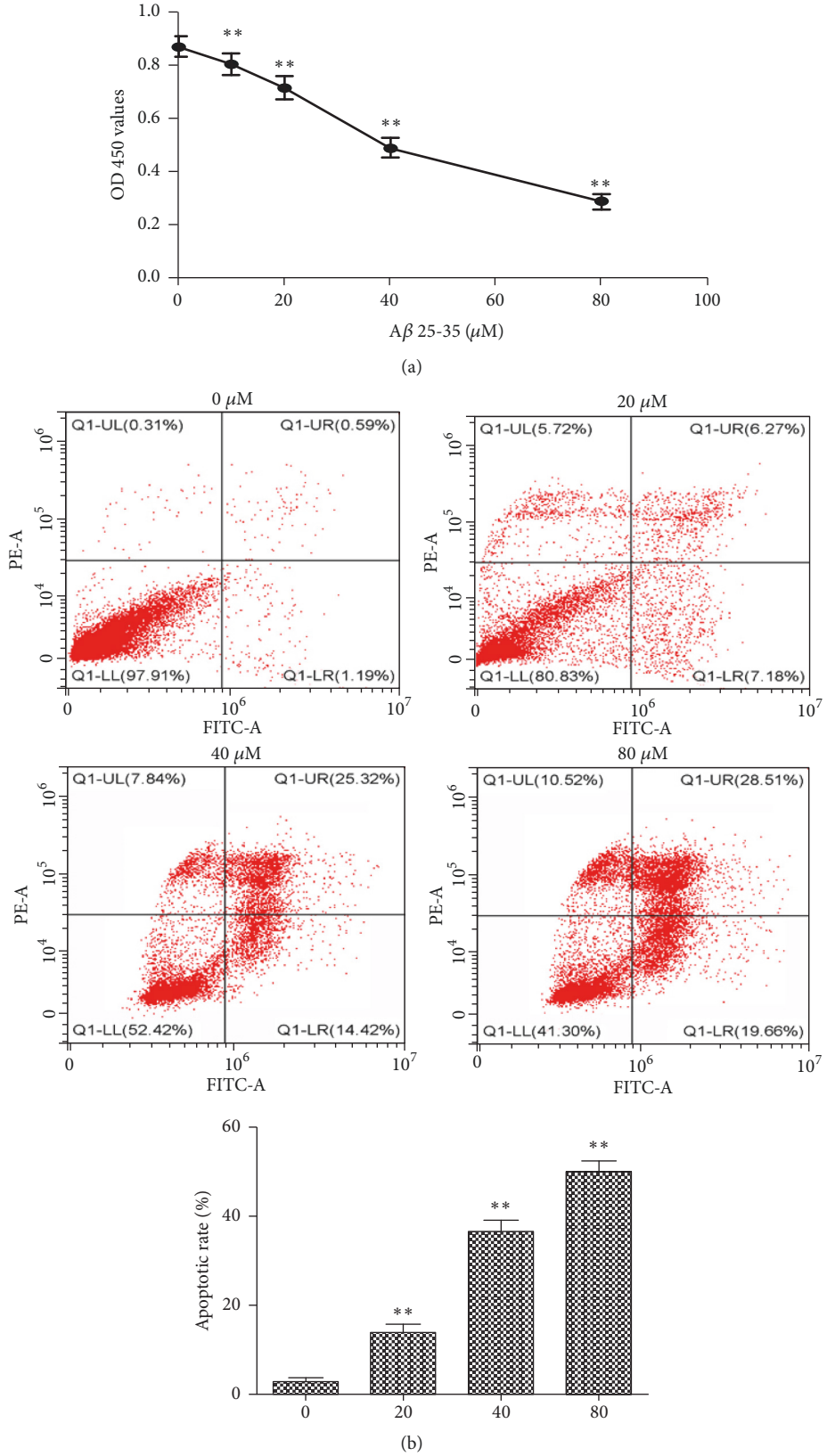

Figure 4: Effects of Aβ25-35 on the proliferation and apoptosis of PC12 cells. (a) PC12 cells were incubated with Aβ25-35 (0, 10, 20, 40, and 80 μM) for 24 h. Cell viability was determined by a Cell Counting Kit-8 assay. (b) Cell apoptosis was detected by flow cytometry following treatment with Aβ25-35 (20, 40, and 80 μM) for 24 h. Data was obtained from three independent experiments. The results were presented as the mean ± standard deviation. $^{**}P < 0.01$.

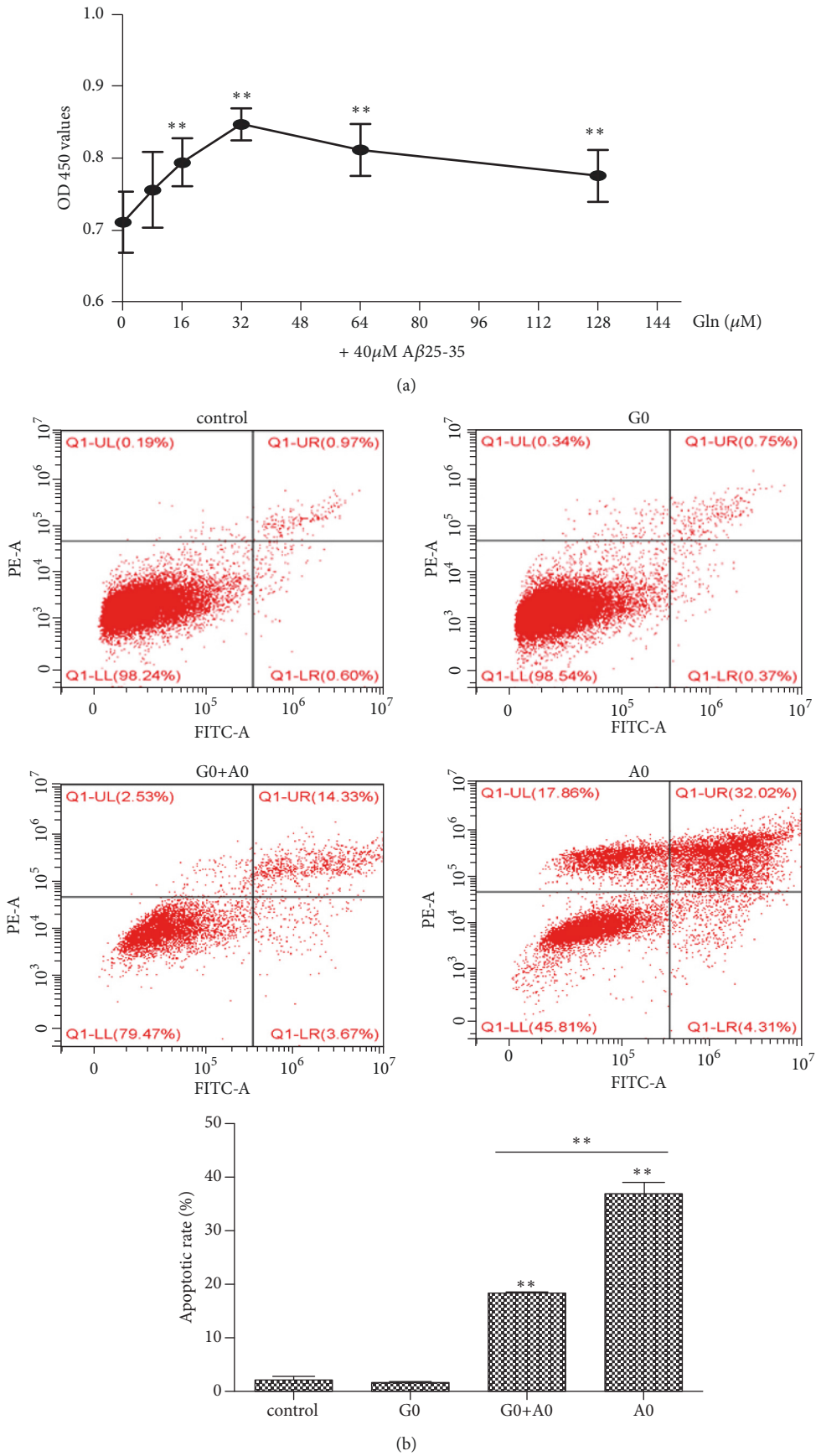

FIGURE 5: Protective effect of glutamine on PC12 cell injury induced by Aβ25-35. (a) PC12 cells were incubated with 40 μM Aβ25-35 for 24 h following pretreatment with glutamine (0, 8, 16, 32, 64, and 128 μM) for 2 h. Cell viability was determined by a Cell Counting Kit-8 assay. (b) PC12 cells were pretreated with glutamine (32 μM) for 2 h, followed by incubation with or without Aβ25-35 (40 μM) for 24 h. Cell apoptosis was detected by flow cytometry following. Data was obtained from three independent experiments. The results were presented as the mean ± standard deviation. $^{**}P < 0.01$. 32 μM of glutamine showed as G0, and 40 μM of Aβ25-35 showed as A0.

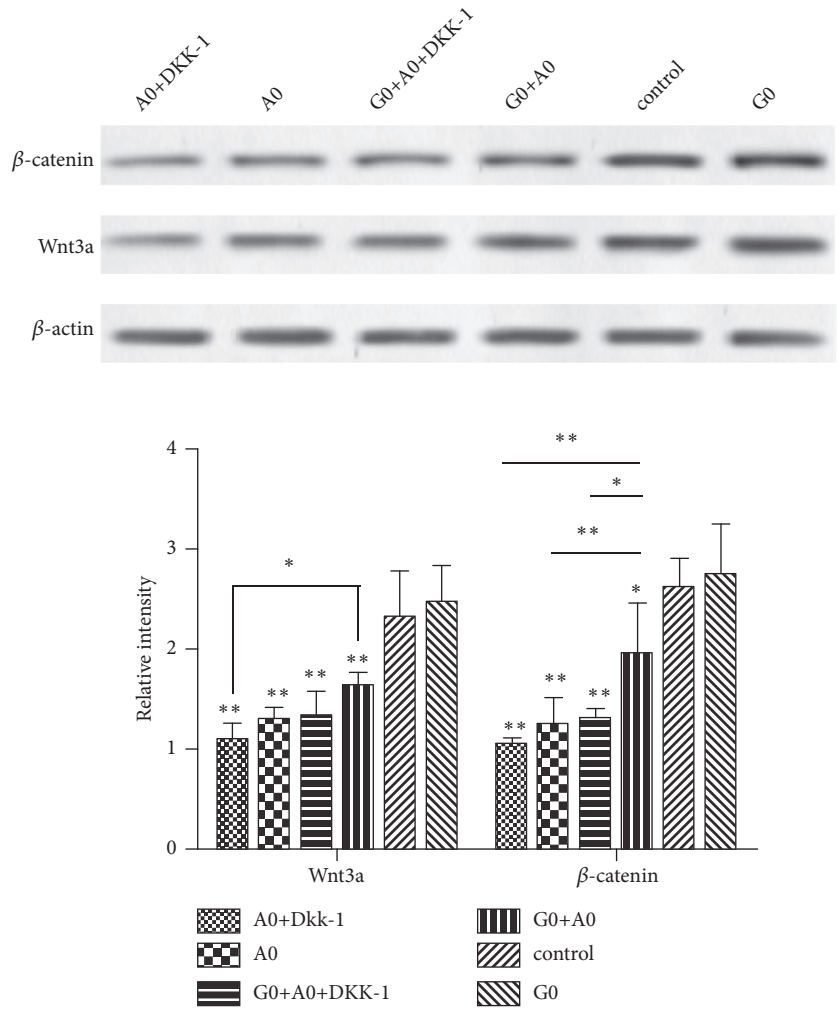

Figure 6: Wnt3a and β-catenin protein expression levels in AD cell model following pretreatment with glutamine and DKK-1. The expression levels of Wnt3a and β-catenin protein were examined by western blotting. The experiments were repeated three times. The results were presented as the mean ± standard deviation. $^{*}p < 0.05$ and $^{**}p < 0.01$.

(GSH-XP) are important antioxidant enzymes in vivo, which are related to free radical scavenging ability. MDA is a lipid peroxidation product, and its content can reflect the level of free radicals [22]. At present study, we found that the glutamine restored the decrease of SOD and GSH-XP activity and the increase of MDA content in SAMP8 mice, indicating that the glutamine could improve antioxidant capacity of SAMP8 mice. Wnt3a/β-catenin signaling cascade was the common final pathway for neuroprotection and self-repair through antioxidative stress [23]. Wang Y L et al reported that the curcumin reduced oxidative stress-induced injury through activating Wnt3a/β-catenin signaling pathway in PD rats [24]. At present study, the Gln-high group exhibited significantly higher protein expression of Wnt3a and β-catenin as well as enhanced activity of SOD and GSH-XP and reduced MDA content, indicating that the glutamine may improve antioxidant capacity through activating Wnt3a/β-catenin signaling pathway in SAMP8 mice.

Additionally, we found that Aβ25-35 could cause injury to PC12 cells, while glutamine could provide protective effects against damage to PC12 cells. As a widely used neurotoxin in the construction of AD models, Aβ25-35 increases neuronal loss, inflammation, oxidative stress, and cognitive and memory impairment [25]. Our study found that the glutamine treatment increased SOD and GSH-XP activity and decreased MDA content. The expression of Wnt3a and β-catenin protein was significantly increased following glutamine treatment. Interestingly, when the Wnt3a/β-catenin signaling cascade was inhibited by DKK-1, SOD, and GSH-Px activity was reduced, while MDA content was increased. These results indicated that the glutamine exerts its antioxidant capacity by activating the Wnt3a/β-catenin signaling pathway.

In summary, our results demonstrated that glutamine plays a protective role against oxidative stress-related injury in SAMP8 mice and PC12 cells through activating the Wnt3a/β-catenin signaling pathway to enhance viability and attenuate apoptosis. Our study provides potential therapeutic strategies in the future treatment of oxidative stress-related injury involved in AD as well as the underlying mechanism of

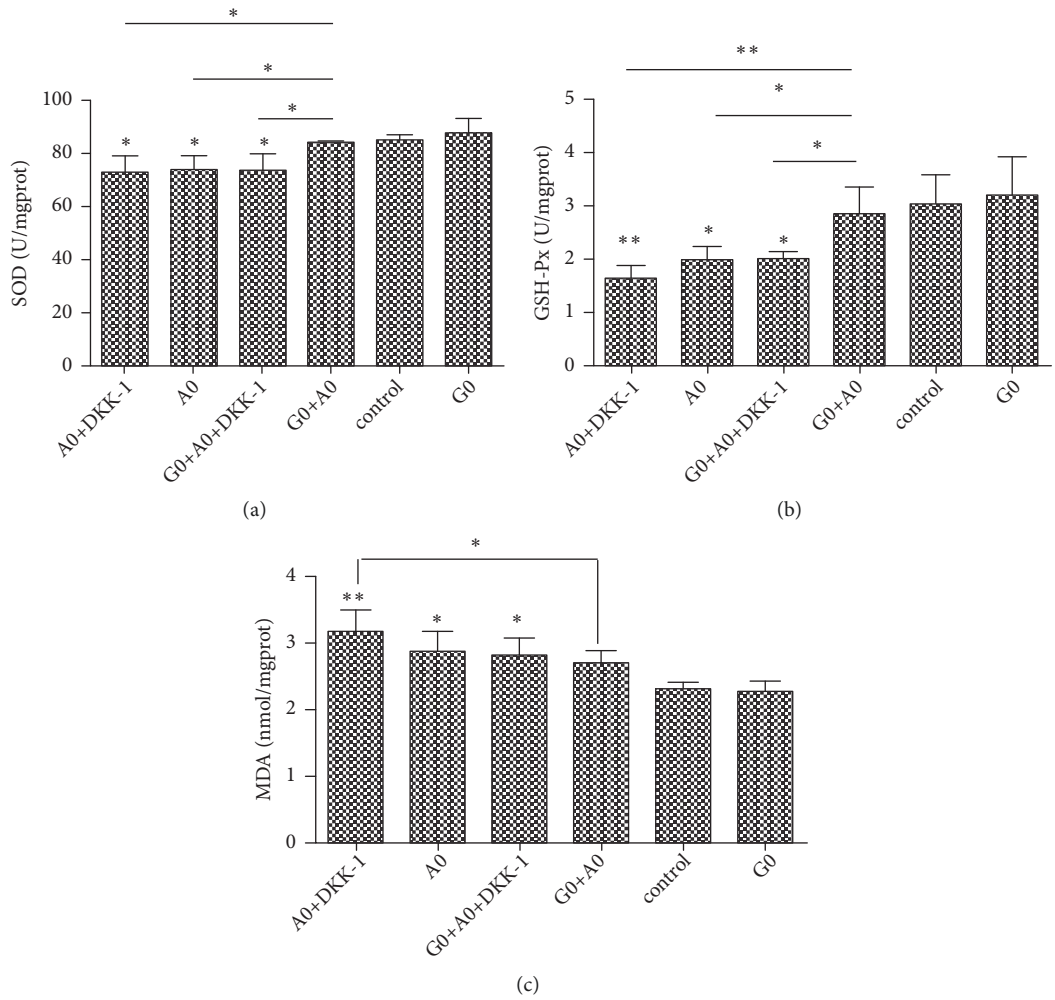

Figure 7: Glutamine strengthens the antioxidant capacity through activating the Wnt3a/β-catenin signaling pathway in vitro. (a) SOD activity in each group. (b) GSH-XP activity in each group. (c) MDA content in each group. Data were obtained from three independent experiments. The results were presented as the mean ± standard deviation. $^{*}p < 0.05$ and $^{**}p < 0.01$.

the neuroprotection function of glutamine. Considering the complexity of molecular and neurological systems, further efforts are needed to confirm our findings.

Data Availability

The data used to support the findings of this study are available from the corresponding author upon request.

Conflicts of Interest

The authors declare that they have no competing interests.

Acknowledgments

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