BioMed Research International

Molecular and Cellular Mechanisms of Neuroinflammation

Lead Guest Editor: Anna Di Vito
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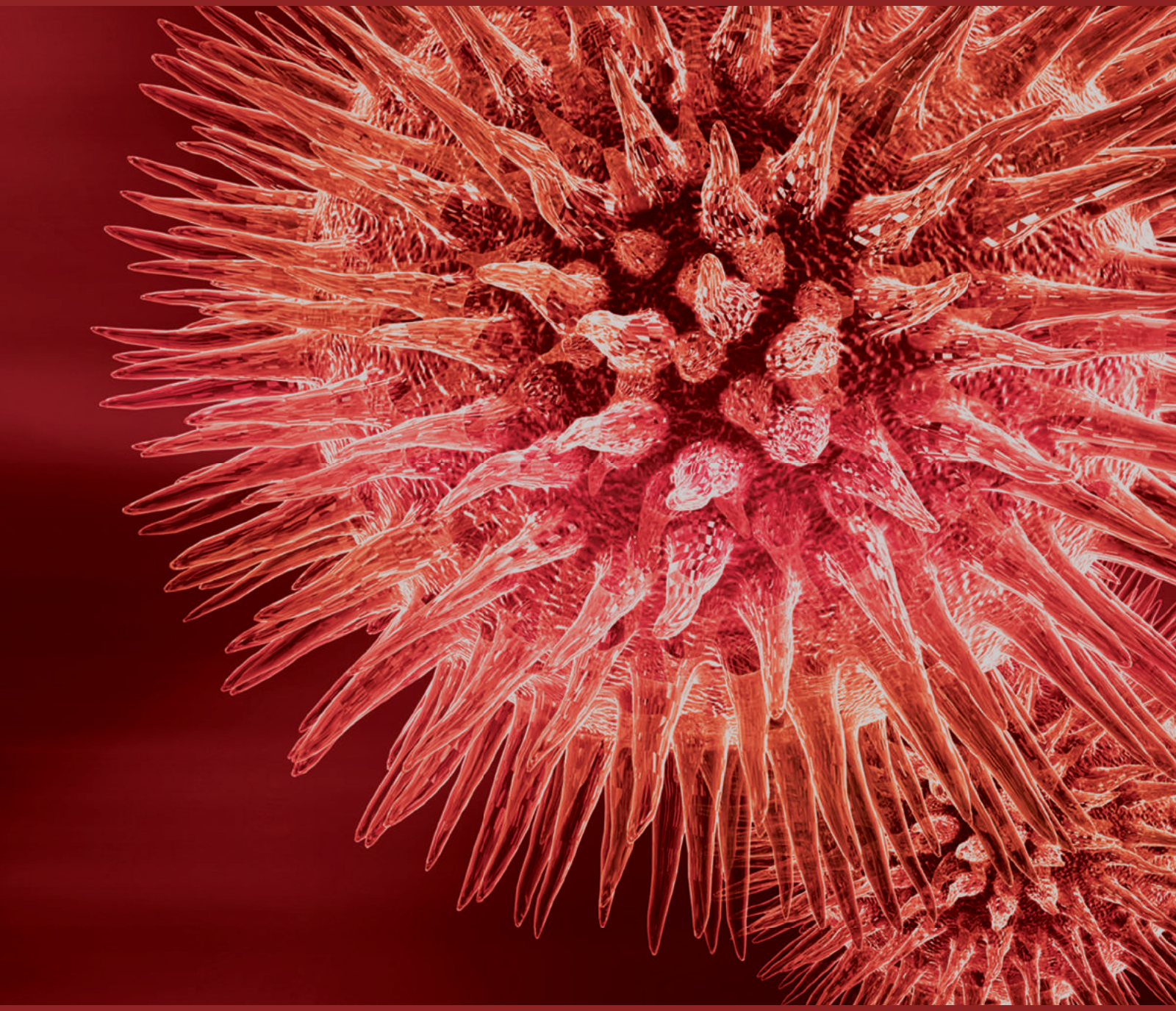

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Hindawi
BioMed Research International
Volume 2017, Article ID 8417183, 2 pages
https://doi.org/10.1155/2017/8417183

Editorial

Molecular and Cellular Mechanisms of Neuroinflammation

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Received 7 November 2017; Accepted 8 November 2017; Published 3 December 2017

In the central nervous system (CNS), the innate immune response plays a significant role in both physiological and pathological conditions. CNS diseases including traumatic brain injury, ischemic stroke, brain tumor, and cerebrovascular and neurodegenerative diseases trigger a cascade of events broadly defined as neuroinflammation, which is characterized by the activation of the microglia and astrocyte population. On the other hand, microglial and astrocyte activation, T lymphocyte infiltration, and overproduction of inflammatory cytokines have been demonstrated in association with neuronal alteration in both animal and human tissues. Neuroinflammation is thus a hot topic in contemporary neuroscience. Further complicating the neuroinflammatory landscape is the fact that the immune-privileged status of the CNS is on trial, given the numerous studies showing that CNS is an actively regulated site of immune surveillance, as recently reviewed by Negi and Das [1]. A key role in the control of immune-surveillance is played by microglia, which, in addition to keeping the brain free from damaging insults, also contribute to neuronal functions with providing neurotrophic substances to regulate neurotransmitters and hormones and mediating responses to pain and stress challenges [2]. In this special issue, we have invited a few papers that address such important issues.

Recently, great attention has been directed to the mechanisms by which innate immune system might activate the endothelial cells of the blood-brain barrier (BBB) to arise neuroinflammation that eventually becomes unregulated. The paper of B. W. Festoff et al. provides interesting details about the role of BBB as well as damaged associated-molecular pattern (DAMPs) and coagulation factors in the onset and progression of neurodegenerative disorders.

The paper of L. F. Hernández-Zimbrón et al. focused the attention on the activation of immunological responses in aged brain, which contribute to the development of neurodegenerative disease. The paper showed the accumulation of the amyloid-β peptide 1–42 (Aβ42), glial fibrillary acidic protein (GFAP), and presenilin 2, hallmarks of Alzheimer's disease, in endothelial cells, blood vessels, and neurons of the visual cortex in aged mice.

In the preclinical study, A. P. Herman et al. highlighted the importance of the interaction between the immunity and the neuroendocrine system. Acute and prolonged inflammation account for the release of proinflammatory cytokines, which ultimately interfere with the secretion of gonadotropin-releasing hormone (GnRH) and luteinising hormone (LH) altering the normal estrus. One possible strategy to block the inflammatory status is the stimulation of acetylcholine (ACh) secretion or the inhibition of acetylcholinesterase (AChE) activity. A. P. Herman et al. showed the possibility of blocking inflammatory-dependent changes in the GnRH/LH secretion via the administration of AChE inhibitors which did not pass the BBB, without interfering in the CNS.

Neuroinflammation is recognized as one of the potential mechanisms mediating the onset of a broad range of psychiatric disorders. The review of F. A. Radtke et al. provided a clear overview of the molecular mechanism underlying neuroinflammation in mental illness, such as schizophrenia, bipolar disorder, depression, anxiety, obsessive-compulsive disorder, and autism. An interesting interaction between mental disorders-associated copy number variants and inflammation was proposed.

G. Zhang and P. Yang explained the molecular mechanisms involved in the establishment of chronic neuropathic

pain associated with spinal cord injury and suggested the chemokine Ccl3 and the MAP kinase signaling pathway as potential therapeutic targets to alleviate neuropathic pain.

The paper of R. Maldonado-Ruiz et al. focused the attention on signaling pathways involved in the process of metabolic inflammation in obesity and its modulation by neuropeptides such as POMC-derived peptides, ghrelin, and leptin. In the CNS, neuropeptides modulate inflammation and migration of peripheral cells into the CNS via the BBB and may represent a molecular node during positive energy balance as is the obesity and maternal overnutrition.

The papers of this special issue provided important insights into CNS-peripheral immune system dialogue, highlighting the role of molecular factors and signaling pathways in the onset and progression of different neurological diseases.

Anna Di Vito
Giuseppe Donato
Daniele Tomassoni

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Hindawi
BioMed Research International
Volume 2017, Article ID 5071786, 21 pages
https://doi.org/10.1155/2017/5071786

Review Article

Modulating Neuroinflammation to Treat Neuropsychiatric Disorders

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Received 16 June 2017; Accepted 13 September 2017; Published 18 October 2017

Academic Editor: Daniele Tomassoni

Neuroinflammation is recognised as one of the potential mechanisms mediating the onset of a broad range of psychiatric disorders and may contribute to nonresponsiveness to current therapies. Both preclinical and clinical studies have indicated that aberrant inflammatory responses can result in altered behavioral responses and cognitive deficits. In this review, we discuss the role of inflammation in the pathogenesis of neuropsychiatric disorders and ask the question if certain genetic copy-number variants (CNVs) associated with psychiatric disorders might play a role in modulating inflammation. Furthermore, we detail some of the potential treatment strategies for psychiatric disorders that may operate by altering inflammatory responses.

1. Introduction

Neuropsychiatric disorders including devastating diseases such as schizophrenia, major depressive disorder, and bipolar disorder are generally considered to have a multifactorial pathophysiology including both genetic and environmental factors [1]. Neuroinflammation could be one of the potential mechanisms contributing to pathogenesis, anchored in the interplay of environmental factors such as hypoxia or infections and genetic susceptibility of the immune system.

In fact, increasing amount of evidence suggests that inflammatory processes have an important role in the pathophysiology of psychiatric disorders. The significantly higher level of different inflammatory markers such as cytokines, chemokines, and chemokine receptors in patients suffering from various forms of psychiatric disorder has laid molecular foundation for the important role of inflammation in the pathogenesis of neuropsychiatric disorder [2–6]. Furthermore, there is increasing evidence from genetic studies that these altered immune processes may play a primary role in the development of neuropsychiatric disorders, rather than simply being a consequence of associated brain pathology. Assuming inflammation plays a key role in the pathogenesis of psychiatric disorders, anti-inflammatory treatments may play a critical role in the treatment of these disorders.

To what extent and in which case has this anti-inflammatory therapy already been applied successfully is far from clear. The efficacy of prescribing anti-inflammatory drugs to treat depression and other psychiatric diseases, either alone or in conjunction with traditional medication, remains to be elucidated. In this review, we will try to summarize the answers to these questions and sum up treatment recommendations where available.

2. Relationship between Inflammation and Mental Illness

Recent studies on preclinical, genetics, and bioinformatics data have shown the activation of immune system molecules and pathways that can contribute to pathogenesis of psychiatric disorders [7]. Several lines of the evidence that support a role for inflammation as a contributing factor in psychiatric disorders include the following.

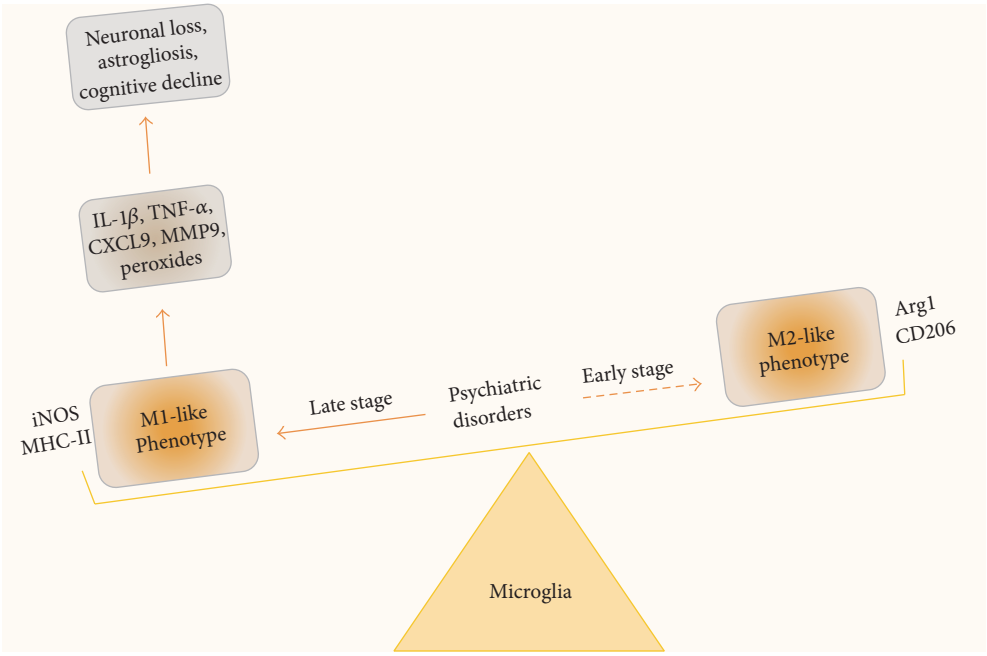

Figure 1: *Potential mechanism of microglia activation in psychiatric disorders.* Neuroinflammation is one of the key components of the pathogenic mechanisms underlying several psychiatric disorders and is often associated with microglial activation/dysfunction. Accumulating evidence indicate that M1-like microglia (proinflammatory) are significantly increased in comparison to ramified microglia (resting) and elongated M2-like microglia (anti-inflammatory) phenotypes in disease states. The levels of M1-like microglia in brain predominate and potentially can be associated with the severity of the disease, suggesting an imbalance in M1/M2 phenotype. M1-like microglia are characterized by the expression of MHC class II antigens and by the production of proinflammatory cytokines and nitric oxide synthase (iNOS). Continued production of proinflammatory cytokines can lead to neuronal damage, astrogliosis, plasticity, and cognitive decline. Peripherally derived macrophages and monocytes also participate in the inflammatory response. It is likely that during early stage of disease onset microglia can have phenotypic switch to an alternative state knows as M2-like phenotype, which are characterized by presence of surface markers like Arginase 1 and mannose receptor CD206, leading to resolution of inflammatory response by secretion of anti-inflammatory cytokines. The efficacy of the anti-inflammatory drug targeting M1/M2 balance will significantly depend on therapeutic time window and severity of symptoms associated with the diseases.

(I) It has been established that cytokines that are found typically during an ongoing inflammatory process are found to be elevated in blood samples of patients with various types of psychiatric disorders. This, depending on the study, includes both generally considered proinflammatory (i.e., interleukin- (IL-) 1-3, IL-5-9, IL-11-18, interferons (IFN), tumor necrosis factor (TNF), and chemokines) as well as anti-inflammatory (i.e., IL-4, IL-10, IL-11, and IL-13) cytokines and complement factors. Though the activation cascade of this elevated cytokine production is not yet understood, the findings may point to a significant role of peripheral inflammatory processes in psychiatric conditions. Following examination of blood samples from patients with schizophrenia [8, 9], depression [10, 11], anxiety [12], bipolar disorder [13–15], obsessive-compulsive disorder (OCD) [16, 17], posttraumatic stress disorder (PTSD) [18, 19], and autism spectrum disorder [20, 21] significantly elevated levels of all major kinds of cytokines were detected. This also included soluble interleukin receptors, interleukin antagonists, TNF, soluble TNF receptor IFN-γ, chemokines, and matrix metalloproteinases (MMP) (see Figure 1 and Table 1). The levels of some of these cytokines have also been correlated in some studies to the severity of disease symptoms [12, 17]. Work on the Whitehall II cohort shows that individuals with increased IL-6 levels over a prolonged period of time (one, two, or three measurements over a 5-year period) have an elevated subsequent 10-year risk for development of cognitive symptoms of depression [22]. There are multiple studies showing elevated high sensitivity- (hs-) C-reactive protein (CRP) levels in blood samples from patients with psychiatric disorders (schizophrenia, depression, PTSD, anxiety, and autism). Elevated levels of proinflammatory cytokines like IL-6 and IL-1β can lead to the production of this acute phase protein in the liver [14, 23–28]. Importantly, a meta-analysis of 8 longitudinal studies showed that increased CRP levels are significantly associated with the risk for later development of depressive symptoms. This result was independent of age and a range of other risk factors of depression [24]. Interestingly, higher levels of CRP and IL-6 during childhood (age of nine years) were shown to be a predictor of higher risk of depression and psychosis in later life [29]. Increased CRP was even suggested as a diagnostic tool of autism spectrum disorder due to a positive correlation with symptom severity [27].

It is difficult to examine cytokine and acute phase protein levels in the brain directly, hence findings from the peripheral

Table 1: Circulating cytokines and acute phase proteins that are found elevated in different psychiatric disorders.

Psychiatric disorder	Cytokines/acute phase proteins elevated
Schizophrenia	IL-1β, IL-6, IL12-p70, sIL-2R, TNF-α, IFN-γ [8, 9]
Depression	IL-1, IL-6, sIL-1R, sIL-2R, sIL-6, TNF-α, CRP [10, 11]
Bipolar disorder	IL-4, IL-6, IL-10, sIL-2R, sIL-6R, TNF-α, sTNFR1, IL-1 receptor antagonist, INF-γ, hs-CRP [13–15]
Anxiety disorder	IL-6, IL-8, IL-10, IL-18, MCP-1, MMP2, TNF-β [12]*
OCD	IL-2, IL-4, IL-6, IL-10, TNF-α, sTNFR1, sTNFR2 [16, 17]
PTSD	IL-1α, IL-1β, IL-2, IL-4, IL-6, IL7, IL-8, IL-10 and, IL12p40, IL12p70, IL-13, IL-15, TNF-α, MIP-1α, GM-CSF, IP-10, eotaxin [18, 19]**
Autism	IL-1β, IL-1RA, IL-5, IL-8, IL12p40, IL-12(p70), IL-13, IL-17, GRO-α [20, 21]

*After stimulation with LPS; **in PTSD and panic disorder patients.

blood therefore have to be interpreted with caution. This is why it is worth looking, among other aspects, into gene expression when reviewing results from postmortem samples of the brain.

(II) In schizophrenia, transcriptome analysis of brain tissue from autopsy of schizophrenic patients showed an increased expression of IFITM2, IFITM3, SERPINA3, and GBP1 was found. These genes are directly regulated by TNF-α, IFN-α, and IFN-γ, suggesting a proinflammatory state [30]. A recent study examining gene expression of the dorsolateral prefrontal cortex from patients with mood disorders demonstrate an elevated expression of inflammatory agents (IL-1α, IL-2, IL-3, IL-5, IL-8, IL-9, IL-10, IL-12A, IL-13, IL-15, IL-18, IFN-γ, and lymphotoxin-α) compared to control subjects [31]. Therefore, these results suggest that the findings seen using blood samples may be recapitulated in gene expression of key inflammatory agents in the brain, albeit in postmortem samples. A systematic review of 119 publications on postmortem tissue investigating further evidence of inflammation in schizophrenia concluded that there is high variability between the results found in different studies. For astrocyte or microglia markers no consistent increase or decrease was found across different studies. These investigations varied in patient age, diagnosis, and brain region examined. Out of 33 studies, 6 showed an increase in the astrocyte marker, glia fibrillary acidic protein (GFAP), while 6 showed a decrease. An increase in microglial markers (CD68, human leukocyte antigens (HLA), MHC antigens, CD11b, calprotectin, and quinolinic acid) was found in 11 studies and a decrease in 3 studies. In studies on general glia cell density, 7 of 34 studies showed a decrease in glial cell density and 2 studies an increase. Furthermore, changes, mostly increases, in mRNA expression levels of chemokines and cytokines (interleukins, TNF-α, TNF-α receptor, and IFN-γ) were frequently found in postmortem studies of schizophrenic brains. Other proinflammatory markers such as substance P, NF-κB, SERPINA3, and interferon-induced transmembrane protein (IFITM) were found elevated in concentration and/or expression in certain brain regions in schizophrenia patients in some, though not all, studies [32]. A similar meta-analysis of the literature on postmortems in mood disorders was somewhat more conclusive: in addition to findings of changes in the cytokine profile in the brain, it is argued that microglia and perivascular macrophages are found at increased density in the postmortem tissue. Meanwhile, oligodendroglia and GABAergic interneurons are found at a diminished number. One hypothesis that could explain this observation is that activated microglia release glutamate, which might damage neurons. The loss of oligodendroglia might also be a direct consequence of inflammation. So, pathways of neuroexcitation may be highly changed through neuroinflammatory mechanisms [33].

(III) In multiple studies elevations of CRP and/or proinflammatory cytokine levels were shown to return to normal when the psychiatric disorder was treated. For example, the level of IL-1β and IL-2 in depression patients returned to a similar level to that seen in controls after treatment with serotonin reuptake inhibitors over a 20-week period [34]. Accordingly, bipolar disorder patients treated with mood-stabilizers and antipsychotics responded with a significant decrease in high sensitivity CRP in the aforementioned study [14]. IL-1RA and IL-10 have also been found to be increased in drug-naïve schizophrenia patients decreased after 6 weeks of treatment with atypical antipsychotics. A strong correlation between IL-10 level and a score of negative, general, and total symptoms was found. Plasma levels of IL-2 and IL-6 were found to be decreased after antipsychotic treatment of schizophrenic patients in a meta-analysis of 8 similar studies [35]. Though little is said in the above studies on the correlation of patient outcome and the effects of pharmaceutics on cytokine levels, psychotherapy alone showed a decrease of IL-1Ra, IL-5,-6,-8,-10, G-CSF, IFN-γ, and TNF-α levels parallel to improvement of symptoms in major depression patients [36].

(IV) There is increasing evidence that some primary genetic risk factors for psychiatric disorders impact on immune functions. Most common psychiatric disorders are highly polygenic. Genome-wide association analysis of 5 common psychiatric disorders including schizophrenia, bipolar disorder, depression, ADHD, and autism identified genes in immune pathways as significantly contributing to risk [37]. The strongest and consistent association of SNPs for immune response and synaptic plasticity is associated with HLA region [38]. Recently, the most significant GWAS hit for schizophrenia risk (rs13194053), a SNP in the major histocompatibility complex region [39], was shown to exert

risk in part through impacting on the gene encoding complement 4 (C4) [40]. Some rare but recurrent copy-number variants (CNVs) associated with mental illness also impact on immune functions, including the 22q11.2 deletion associated with velo-cardio facial syndrome, a topic we will return to below.

(V) Conversely, patients with CNS inflammation caused by infection or brain injury can develop symptoms similar to those seen in psychiatric patients. In a study of 83 patients with viral encephalitis 67% of them were presented with psychomotor agitation, 55% with drowsiness, 47% with disorientation, and 43% with visual hallucinations and 34% showed aggressiveness as a symptom. One year after hospital treatment in 70 of the same patients, 16% had memory disorders, 9% showed signs of aggressiveness, and 8% suffered from aphasia, 8% from visual hallucinations, and 7% from auditory hallucinations [41]. Brucellosis, typhoid fever, Lyme disease, Leptospirosis, and Whipple's disease are all examples of inflammatory diseases caused by infection that can be accompanied by psychiatric symptoms ranging from depression, mania, personality trait, and behavioral changes to manifest psychosis [42]. Regarding inflammation during healing after trauma, a survey of 722 outpatients after brain injury with an average of 10 days' unconsciousness after injury found a rate of DSM-IV valuable diagnosis of depression in 42% of patients [43]. While it is not possible to fully disentangle the causal direction in these associations, they are consistent with the view that infection and brain injury may lead to psychiatric symptoms via the triggering of inflammatory processes.

(VI) Increasing number of studies support the hypothesis that autoantibodies that bind mainly to neuronal membrane protein or synaptic proteins result in psychiatric disorders. To date, the clear causative role of autoantibodies on psychotic symptoms has been most clearly shown for the N-methyl-D-aspartate receptor (NMDA-R), although further studies are still needed [44–46]. Similarly autoantibodies against dopamine-2 receptor, which regulates movement and behavior, have been associated with paediatric autoimmune neuropsychiatric disorders associated with streptococcal infection and subjects with Tourette's syndrome [47]. Autoantibodies against antiopioid receptor, 5-hydroxytryptamine receptor 1A, and muscarinic cholinergic receptor 1 are thought to induce a depressive state in psychiatric illness [48, 49].

(VII) Activation of inflammation in animal models leads to behavioral abnormalities in a range of experiments. In rodent models, peripheral injection of inflammation-provoking lipopolysaccharide (LPS, 0.83 mg/kg) has been shown to generate sickness/depression-like symptoms, that is, prolonged period of immobility in forced swim test and tail suspension test. This effect was ascribed to an activation of the tryptophan-degrading enzyme indoleamine 2,3-dioxygenase [50]. Injecting animals with proinflammatory cytokines like IL-1a or TNF-α has also been shown to lead to sickness behavior in a dose- and time-related manner [51]. LPS injection to pregnant rats could be demonstrated to lead to pronounced changes of the offspring's brain development: cortical and hippocampus thickness were found increased and markers for neural and glia progenitors decreased and abnormally distributed over the brain regions. This was accompanied by an inflammation reaction within the brain, as indicated by the elevated presence of cytokines [52]. It has been suggested that microglia activation through LPS-stimulation may lead to a damage of oligodendrocyte progenitor cells in a way that ultimately leads to severe losses of oligodendrocytes and myelination during development [53]. It is remarkable in this context that immune system stimulating drugs like β-interferon have been found to induce psychiatric symptoms, for instance, in hepatitis C treatment [54, 55].

All the above points support the hypothesis that inflammation plays a significant role in the pathophysiology of major psychiatric disorders. However it remains unclear to what extent immune changes are primary in the causation of these conditions and to what extent they are a consequence of brain pathology.

Finding pathways that can initiate neuroinflammation may help us find mechanistic key links between prolonged stress exposure or traumatic life events in relation to the development of a psychiatric disease. For instance, overexcitation of neurons in epilepsy has been demonstrated to activate microglia and inflammation [56]. If the same observation holds true for situations of sensory overload, experienced as stress and emotionally overwhelming feelings, remains to be elucidated. Similarly, it is unclear if metabolic changes, like changes in circulation or hormone levels and receptors in straining situations, lead to oxidative stress in the brain, which would in turn provoke neuroinflammation.

The identification of the molecular mechanisms that connect inflammation and altered behavior will be important in the development of new treatments for neuropsychiatric disorders. Some emerging studies hypothesize that synaptic damage seen in psychiatric disorders may in part be a consequence of neuroinflammation, reflecting the situation seen in other conditions such as multiple sclerosis. Importantly, dysfunctional synaptic activity is associated with high activity of glutamatergic synapses and loss of GABAergic synapse function in schizophrenia and autism spectrum disorders. This imbalance seems to be provoked through cytokines that are upregulated during inflammation, very much like the ones we see in psychiatric disorders, supposedly released by activated microglia [57]. In most cases, inflammation within the CNS can activate an array of cellular and molecular changes including activation of astrocytes, oligodendrocyte dysfunction, and mitochondrial damage, changes in neurogenesis and neural circuits, and a general imbalance of neurotransmitters with a surplus of dopaminergic and a loss of glutamatergic neurotransmission, and it is also crucial for synaptic plasticity and pruning. Arguably, in concert these alterations can then lead to an impairment of behavioral functioning like cognition or emotion regulation [58].

3. Microglial Pathology in Psychiatric Disorders

At a cellular level, microglia and astrocytes regulate the central nervous immune response. During the early embryonic

brain development, microglia are the first glial cells to develop alongside neurons [59] and they constitute ≅10% of total glial cells in the adult brain [60]. Besides contributing to neuronal function [61, 62] microglia regulate the CNS response to antigens and inflammation [63], accompanied by cytokine and chemokine expression [64–67]. Following damage or stress, microglia located in intact areas elicit an immune response on the affected area by extending their processes. Microglial processes are highly dynamic and can sense the released ATP secreted from astrocytes and neurons in the damaged area [68]. Following activation microglia secrete two proinflammatory cytokines (IL-1β and TNFα), chemokines (CCL3, CCL5), and reactive oxygen/nitrogen species (nitric oxide, peroxides, superoxide, and peroxynitrite) (Figure 1) [69–71]. In turn, this neuroinflammation may cause damage to neurons and other glial cells. Aging is another key factor that influences the status of microglial state and the associated inflammatory condition. The gene expression profile in the aged brain indicates a clear upregulation of immune-related genes, including enhanced expression of IL-1β, TNFα, and IL-6 [72], suggesting that age related changes also lead to enhanced activation of microglial cells and supposedly inflammation, in the absence of external damage.

Besides being the first line of defense in the mammalian brain, microglia are important for synaptic formation and synaptic pruning [62, 73]. Microglia derived IL-10 has been shown to promote synapse formation. Reduction in the level of microglia during the postnatal stage or young adult stage of development results in reduced spine formation in the motor cortex. This reduction in synapse formation is associated with reduced motor performance and reduced levels of synaptic proteins like GluN2B and vGlut1 [74]. These studies suggest that microglia are important for learning and memory by promoting learning associated synapse formation [75]. A recent study has demonstrated early life infection can lead to an increase in microglial derived IL-1β level, which in turn modulates hippocampus-dependent learning and memory [76]. This observation is further supported by animal studies, where neonatal bacterial infection of rats induces hippocampus-dependent memory deficits in adulthood [77]. Microglia also eliminate nonfunctional synapses through the fractalkine receptor (CX3CR1) in the hippocampus [78, 79]. In retinal ganglion cells they regulate pruning through the C3 complement pathway [80]. In a recent study it was noted that increased expression of the C4 gene, encoding complement 4, was associated with high risk of schizophrenia. In C4-deficient mice, C4 promotes C3 activation which targets subsets of synapse elimination by microglia, suggesting its involvement in synaptic pruning [40].

Furthermore, TGF-beta signaling has been demonstrated to regulate microglial mediated pruning [81]. Microglia also modulate the sleep-wake cycle through extraction and retraction of the process [82]. It has been hypothesized that the microglial circadian clock might play a causative role in cognitive deficits and depression [83]. Exposure to environmental factors, especially during development, has been demonstrated to have profound effects on microglial state, inflammatory status of the brain, and cognition [84–86]. Although the underlying mechanism behind this in the context of neuropsychiatric disorders is poorly understood, fractalkine signaling at least in part seems to mediate microglial response to environmental risk factors [87].

Given the important role that microglia play in normal brain development and neuronal homeostasis, it is not surprising their dysfunction leads to neuropsychiatric disorders. Recent postmortem studies and PET imaging of peripheral benzodiazepine receptors provide evidence for microglial activation in the brains of patients with neuropsychiatric disorders such as schizophrenia, depression, and autism [88–95]. In schizophrenia, a significant increase in microglial density along with signs of activation in prefrontal and visual cortex has been reported [96, 97]. These results are highly promising, even though more specific markers of microglial activation are still needed to confirm these findings. One possible candidate for that might be translocator protein 18 kDa (TSPO), as decreased radiotracing was associated with schizophrenia-like behavior and inflammatory cytokine elevation in a mouse model [98]. Furthermore, in animal models of Rett syndrome (RTT), a devastating neurodevelopmental disorder, which is caused by a mutation in the X-linked *MECP2* gene encoding methyl-CpG-binding protein 2, microglia release an abnormally high level of glutamate, causing excitotoxicity that may contribute to dendritic and synaptic abnormalities in RTT [99]. Furthermore, in *Mecp2*-null mice a reduced number of microglia, which fail to phagocytose debris as effectively as those in wild-type mice, were found. These finding suggest that microglia may also be responsible for the disorder [100]. Stress-induced depressive-like condition in rodents has shown to be caused by reduced levels of microglia within the hippocampus and reduced expression of activation markers, which in turn might contribute to reduced hippocampal neurogenesis and increased depressive-like behavior [101]. Microglia expressing glucocorticoid receptors have also been shown to undergo extreme alterations when exposed to prolonged stress, like cell body shrinkage and debris accumulation in lysosomes [102].

Sustained microglial activation has been shown to contribute to the alleviation of symptoms associated with neurological disorders including psychiatric disorders (Figure 1) [103]. Enhanced proinflammatory cytokine response in concert with microglia activation occurs in response to external immune challenges and has been associated with cognitive and behavioral deficits in rodents [104–106]. As described earlier, such associations between proinflammatory cytokine levels in blood and psychiatric disorders are numerous in humans. It is feasible that microglia, the most important proinflammatory secreting cells in the brain, might be responsible for or at least involved in this increase in blood cytokine levels. It is interesting in this context that microglia also are very important postnatal development defining cells [107]. It is plausible to speculate that microglial state and functionality in the context of inflammation may be altered in patients with a genetic susceptibility to psychiatric disorder tighter with environmental factors? With induced pluripotent stem cell based disease modeling, one way to further investigate this question would be to derive microglia with a known genetic high-risk profile for psychiatric disorders, which will help to understand the mechanisms and triggers

involved behind pathological inflammation. The potential mechanism of microglial activation that occurs during the course of psychiatric disorders is illustrated in Figure 1.

4. High-Risk Copy-Number Variants (CNVs): A Link between Genetic Alteration, Psychiatric Disorders, and Inflammation?

A strong association has been found between specific rare but recurrent chromosomal CNVs and psychiatric disorders, including schizophrenia, autism spectrum disorder, and mood and anxiety disorders [108]. Microarray experiments have now revealed abundant copy-number variations, a type of genetic variations in which stretches of DNA are duplicated or deleted [109, 110]. These structural alterations can range from 1 kilobase to several megabases and include either a single gene or contiguous sets of genes. CNVs can control the underlying psychiatric phenotype in multiple ways. They can affect the gene expression level by gene dosage effects or act as a faulty regulatory element to gene transcription cascades affecting other gene loci. They can also lead to frame shifts resulting in an abnormal genetic fusion product [111]. The mechanisms by which CNVs can lead to neuropsychiatric disorders and underlying complex behavioral traits are perhaps most clearly shown by rare and highly penetrant CNVs, involving the loss, gain, or disruption of a dosage-sensitive gene(s).

Some of the most common CNVs associated with neuropsychiatric disorder include chromosomal regions 1q21.1, 3q29, 15q11.2, 15q13.3, 16p11.2, 16p13.1, and 22q11 [112]. The locus 1q21.1 is associated with duplications in autism and with deletions or duplications in mental retardation [113–115]. The 15q13.3 locus is associated with deletions in mental retardation with seizures and deletions or duplications in autism and other neuropsychiatric disorders [116–118]. The most common microdeletion syndrome in humans, 22q11.2, results in an increased rate of a range of psychiatric disorders in children with other developmental and intellectual disabilities [119–122]. A 15q13.3 microdeletion in humans results in a range of neurodevelopmental/psychiatric disorders, including autism spectrum disorder, schizophrenia, epilepsy, and intellectual disability [116–118, 123–131]. Microduplication or deletion at the 16p11.2 loci is associated with schizophrenia, bipolar disorder, mental retardation, autism, seizure disorders, and psychosis in Alzheimer's disease [132–135]. De novo CNV analysis has resulted in enrichment of genes that are associated with the NMDAR network, GABAA receptor, abnormal CNS synaptic transmission, abnormal learning/memory/conditioning, and abnormal cued conditioning behavior [112, 136–139]. However, these studies have not picked up genes that can influence inflammatory condition underlying psychiatric symptoms.

There is strong evidence that deletions at 22q11.2 directly impact immune function. Thymic hypoplasia is a common feature associated with 22q11.2 deletions resulting in $CD4^{+}CD45RA^{+}$ T cell counts, which can result in immunodeficiency [140]. A recent study looking at a wide range of cytokines in patients with 22q11.2 deletions demonstrates a significant increase in the serum levels of proinflammatory and angiostatic chemokine IP-10 in patients with 22q11.2 deletions compared to healthy individuals. The increased IP-10 profile was associated with conotruncal congenital heart defects [141]. It is less certain how these immune changes might relate to the psychiatric symptoms seen in the disorder, but a positive association between severity of autism-related behaviors and level of serum concentrations of inflammatory cytokines IL-1β, interferon gamma, and IL-12p70 in individuals with 22q11.2 deletions has been reported [142]. Furthermore, toddlers with 22q11.2 deletion have an elevated level of CD3+, CD4+, and IL-4-IFN-γ+ lymphocytes as compared to healthy control, suggesting that 22q11.2 deletion is associated with dysregulated Th1 cytokine production such as IL-4 and IFN-γ [143]. However, if the dysregulated Th1 cytokine production is also present in adulthood needs to be investigated. A recent study looking at cellular modeling of neurodevelopment by differentiating of hiPSCs carrying 22q11.2 deletions into neuronal lineage reports a reduction in the level of neural differentiation propensity and neurite outgrowth and migration as compared to controls [144]. This phenotype was further associated with reduced expression level of miR-17/92 cluster and miR-106a/b cluster. A number of studies have established direct linkage of these clusters with dysregulated inflammation [145–147], suggesting that the underlying psychiatric symptoms associated with 22q11.2 deletions is at least in part associated with altered inflammatory state and the subjects are prone to an increased risk for a variety of autoimmune diseases [148].

TNF-α was found to be downregulated in expression in cells carrying the 15q13.3 microdeletion. TNF-α is known as a potent activator of inflammation [149]. It is plausible to speculate that genes that are involved in the 15q13.3 duplication are capable of altering TNF-α expression leading to an altered inflammatory state of the central nervous system. The CHRNA7 gene is one of the genes in 15q13.3 deletions. It encodes the nicotinic acetylcholine receptor alpha 7 subunit (α7nAChR), which is associated with schizophrenia in clinical studies and rodent models. A recent study using immortalized lymphoblastoid cell lines with 15q13.3 homozygous deletion has shown the CHRNA7 gene to modulate the production of TNFα. Furthermore, they identified STAT3 as one of the four genes that are differentially expressed following 15q13.3 deletions [149]. It has been demonstrated that STAT4 null mice showed impaired IL12-mediated functions [150]. IL-2 is an activator of the nitric oxide synthase [89], which has been demonstrated to be important for synaptic plasticity [151] suggesting that 15q13.3 deletion might modulate the synaptic plasticity through the STAT4/IL12/NOS pathway.

5. Modulating Neuroinflammation to Treat Psychiatric Disease

5.1. Theories on Pharmacological Effects of Anti-Inflammatory Drugs in Psychiatric Disorder. A number of drugs with anti-inflammatory features have been investigated either retrospectively or prospectively in terms of potential impacts on psychiatric symptoms including nonsteroidal

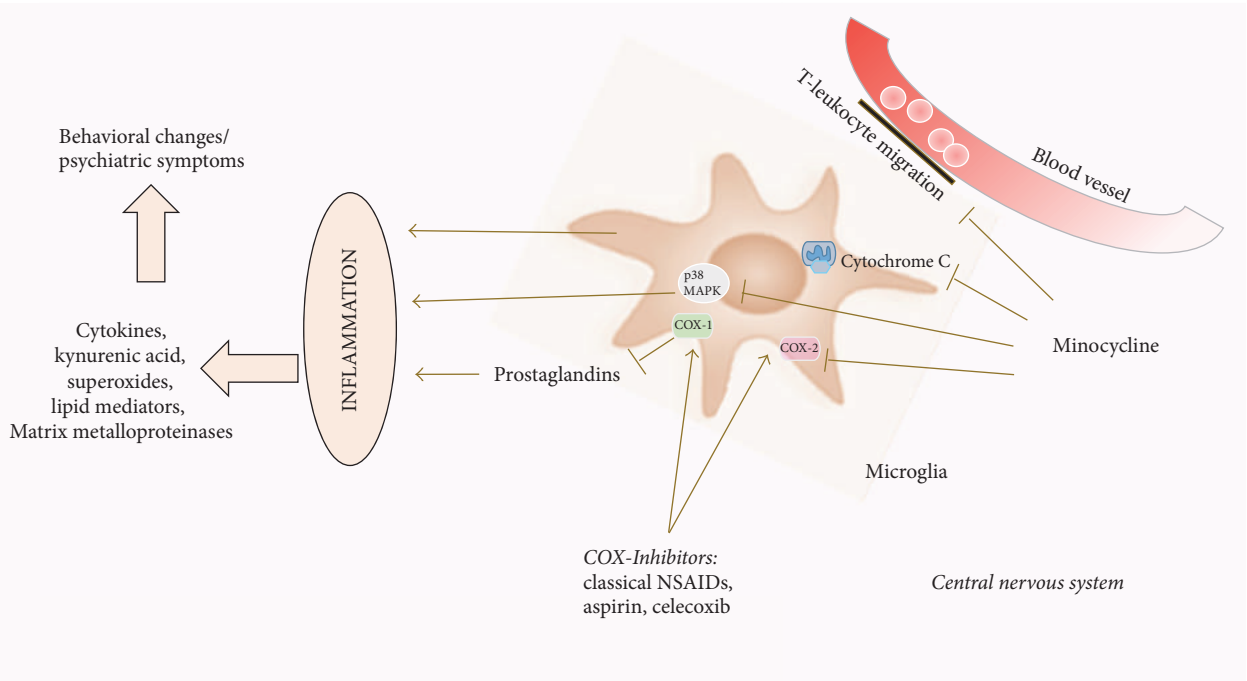

Figure 2: *Pictorial diagram summarizing the effects of anti-inflammatory medication on neuroinflammation mediated by microglia.* Inflammation, amplified through the activation of microglia, leads to the release of cytokines, kynurenic acid, superoxides, lipid mediators, and matrix metalloproteinases. These molecules may contribute to the pathological changes seen in neuropsychiatric conditions. Anti-inflammatory drugs like cyclooxygenase inhibitors block the prostaglandin-mediated cascade of this inflammation. Minocycline acts via (i) inhibition of T-leucocyte migration over the blood-brain barrier, (ii) cytochrome c inhibition in mitochondria, (iii) antagonism of MAPK p38 and subsequent products, and (iv) inhibition of COX-2 (COX: cyclooxygenase, MAPK: mitogen activated protein kinase).

anti-inflammatory drugs (NSAIDs) and the tetracycline related antibiotic minocycline. Aspirin, celecoxib, and the NSAID group of anti-inflammatory drugs, encompassing commonly prescribed drugs such as diclofenac, ibuprofen, or indomethacin, work by blocking the immune cascade enzyme cyclooxygenase (COX) [118], which result in reduced inflammation. COX is responsible for the translation of arachidonic acid into prostaglandins. COX-1, which is antagonized by classical NSAIDs, including diclofenac, ibuprofen and aspirin, is expressed ubiquitously in the body in addition to its role in inflammation. Celecoxib is a selective COX-2 inhibitor. COX-2 is mainly expressed during inflammation, but also in the brain and kidney [152]. In a rat model of neuroinflammation, created by injection of LPS, treatment with celecoxib significantly decreased the rate of microglia and astrocyte coactivation together with levels of IL-1β. Dopaminergic neurons and astrocytes with an upregulation of COX-2 were found in diminished numbers in treated individuals as compared to controls [153]. Inhibition of COX-2 has also been shown to lead to decreased production of kynurenic acid, which is a glycine antagonist at the NMDA-receptor and a nicotinic receptor antagonist. It has been found in elevated concentrations in schizophrenia patients' cerebrospinal fluid [154–156]. For minocycline, multiple effects seem to make up its anti-inflammatory character. In models of neuroinflammation, minocycline has been shown to inhibit activation and proliferation of microglia, as well as antagonizing important proinflammatory enzymes like COX-2, iNOS, NAPDH-oxidase, and P38 MAPK. It also seems to reduce T-leukocyte migration to the CNS by inactivation of MMP-9 (the enzyme rendering the brain-blood barrier permissive during inflammation).

In models of neurodegenerative disease, survival of neurons and glia cells can be increased through minocycline, through caspase dependent and independent mechanisms [157] (Figure 2). Little is known on why these drugs also work in psychiatric disorder, but one might speculate that the same cascades and factors of inflammation, notably microglia, are affected. We have summarized the clinical trials of compounds that aim to reduce the proinflammatory mediators in psychiatric disorders (Table 2).

5.2. Schizophrenia. In schizophrenic patients, several clinical studies exist on adjunctive treatment with drugs with anti-inflammatory features.

When 70 patients with at least a moderate symptom burden and a DSM-IV diagnosis for schizophrenia for under 10 years were treated with aspirin or placebo in addition to antipsychotic treatment, measurements by Positive and Negative Symptom Score (PANSS) showed an improvement in overall psychopathology and in positive symptoms. However, no differences in negative symptom or general scores were found [158]. In a similar study add-on aspirin given to

Table 2: Clinical studies on use of anti-inflammatory drugs in treatment of psychiatric disorder. N: proband number, T: time frame, PANSS: Positive and Negative Symptom Score, SANS: Schedule for the Assessment of Negative Symptoms, and CGI score: clinical global impression score.

Treatment	Psychiatric disorder	Probands	Trial design	Outcome	Authors
Aspirin 1000 mg/day plus 40 mg/day pantoprazole, in addition to antipsychotic treatment	Schizophrenia	Patients with at least moderate symptom burden and a DSM-IV diagnosis for schizophrenia spectrum disorder for under 10 years $N = 70$	Randomized controlled trial $T = 3$ months	Mixed model improvement in overall psychopathology and in positive symptoms as measured by PANSS No differences in negative symptom or general scores	Laan et al., 2010 [158]
Aspirin 1000 mg/day plus 40 mg/day pantoprazole, in addition to antipsychotic treatment	Schizophrenia	Patients with moderate or above on CGI score, moderate score on two of PANSS items: delusions, hallucinatory behaviors, conceptual disorganization or suspiciousness/persecution, and/or a total PANSS negative symptoms score above 18 $N = 400$	Post hoc analysis of randomized controlled trial $T = 16$ weeks	Improvements in patients with high CRP levels	Weiser et al., 2014 [159]
Celecoxib 200 mg bid in addition to 2–6 mg/day risperidone	Schizophrenia	Patients with acute exacerbation of schizophrenia $N = 50$	Double-blind, Randomized Placebo-controlled $T = 5$ weeks	Significant improvement of positive symptoms	Müller et al., 2002 [160]
Celecoxib in addition to olanzapine	Schizophrenia	Patients with acute exacerbation of schizophrenia $N = 94$	Open-label, prospective, controlled $T = 6$ weeks	Improvement of positive, negative, and general psychopathology and total scores as measured by PANSS	Baheti et al., 2013 [161]
Celecoxib (200 mg bid) in addition to risperidone (200 mg/day)	Schizophrenia	inpatients diagnosed with active phase schizophrenia $N = 60$	Double-blind randomized placebo-controlled $T = 8$ weeks	Significant improvement regarding positive symptoms as measured by PANSS	Akhondzadeh et al., 2007 [162]
Celecoxib	Schizophrenia	Patients with a first manifestation of schizophrenia $N = 49$	Double-blind randomized placebo-controlled $T = 6$ weeks	Significant improvement regarding positive and negative symptoms as measured by PANSS	Müller et al., 2010 [163]
Celecoxib (400 mg/day) in addition to antipsychotic treatment	Schizophrenia	Outpatients with a DSM-IV diagnosis of schizophrenia, experiencing persistent symptoms despite treatment for 3 months $N = 38$	Double-blind, randomized, placebo-controlled, $T = 8$ weeks	No difference in outcome, as measured by PANSS	Rapaport et al. 2005 [164]
Concomitant intake of NSAID or paracetamol	Schizophrenia	Schizophrenia patients $N = 2000$	Retrospective investigation	Higher risk for relapse to active psychosis	Köhler et al., 2016 [165]
Previous NSAID use	Schizophrenia	Patients prescribed antipsychotics 82 cases, 359 controls	Case-control trial of antipsychotics prescription	Significant reduction of risk to develop psychosis when NSAID had been taken, but only in male individuals	Laan et al., 2007 [166]
Use of COX-2 inhibitors	Schizophrenia	Case events indicating schizophrenia exacerbation in patients using antipsychotics $N = 1443$	Nested case-control study T = previous 93 days	No increase of risk for schizophrenia exacerbation	Stolk et al., 2007 [167]

Table 2: Continued.

Treatment	Psychiatric disorder	Probands	Trial design	Outcome	Authors
Minocycline in addition to usual treatment	Schizophrenia	Patients with early-stage schizophrenia $N = 94$	Double-blind, randomized, placebo-controlled $T = 1$ year	Significant improvement of negative symptoms as measured by PANSS	Chaudhry et al., 2012 [168]
Minocycline (200 mg/day) in addition to risperidone	Schizophrenia	Patients with DSM-IV diagnosis of early-phase (less than 5 years) schizophrenia who had been on a steady dosage of risperidone $N = 93$	Double-blind, randomized, placebo-controlled $T = 16$ weeks	Significant improvement in treatment response in the Scale for the Assessment of Negative Symptoms (SANS) total scores and PANSS for negative symptoms	Liu et al., 2014 [169]
Minocycline (200 mg/day)	Schizophrenia	Patients with DSM-IV diagnosis of schizophrenia, no treatment with therapeutics for one week before start of trial $N = 43$	Randomized, placebo-controlled $T = 8$ weeks	Significant decrease of SANS score after 8 weeks (though not 4 weeks) of treatment	Ghanizadeh et al., 2014 [170]
Minocycline (up to 200 mg/day) in addition to risperidone (up to 6 mg/day)	Schizophrenia	Chronic schizophrenia patients $N = 40$	Double-blind, randomized, placebo-controlled $T = 8$ weeks	Significant improvement of negative symptom as measured by PANSS	Khodaie-Ardakani et al., 2014 [171]
Minocycline	Schizophrenia	Early-phase schizophrenia patients $N = 54$	Double-blind, randomized, placebo-controlled $T = 6$ months	Significant improvement of negative symptoms and general outcome measured by PANSS, Clinical Global Impression (CGI) and insight score, and improvements of cognitive executive functions	Levkovitz et al., 2009 [172]
NSAID and paracetamol	Bipolar disorder	DSM-IV-TR diagnosis of bipolar disorder I or II and at least mild symptoms $N = 482$	Secondary analysis from the Bipolar CHOICE study $T = 6$ months	No difference as measured by CGI-BP	Köhler-Forsberg et al., 2017 [173]
Aspirin	Bipolar disorder	Patients taking lithium with erectile dysfunction $N = 32$	Double-blind, randomized, placebo-controlled $T = 6$ weeks	No differences in mania or depressive symptoms, significant improvement of erectile dysfunction	Saroukhani et al., 2013 [174]
Aspirin, low-dose of 30 or 80 mg per day and for an unspecified time or for more than 1, 45, 90, or 180 days, in addition to lithium	Bipolar disorder	Patients with at least five previous prescriptions for lithium and at least 1-year drug history $N = 5145$	Pharmacoepidemiological study on the PHARMO Record Linkage System (RLS) in the Netherlands $T = 10$ year period	Significantly fewer events of mood-stabilizer treatment alteration	Stolk et al., 2010 [175]
Celecoxib (400 mg/day)	Bipolar disorder	DSM-IV diagnosis of bipolar disorder during a depressive or mixed episode of the disease $N = 28$	Double-blind, randomized, placebo-controlled $T = 6$ weeks	Improvement of depressive symptoms (as measured by Hamilton Depression Rating Scale) found in the first week	Nery et al., 2008 [176]
Celecoxib in addition to sodium valproate	Bipolar disorder	Patients with diagnosis of acute bipolar mania with manic episode without psychotic features $N = 46$	Double-blind, randomized, placebo-controlled $T = 6$ weeks	Significantly higher remission rates as measured by young mania rating scale	Arabzadeh et al., 2015 [177]

Table 2: Continued.

Treatment	Psychiatric disorder	Probands	Trial design	Outcome	Authors
Concomitant NSAID use in addition to escitalopram or nortriptyline	Depression	Patients with major depression disorder treated in the GENDEP study $N = 811$	Retrospective on the GENDEP study T = up to 12 weeks	No change of serotonin reuptake inhibitor treatment	Uher et al., 2012 [178]
Previous aspirin use as anti-platelet treatment	Depression	Men of older age (69–87 years) $N = 5273$	Retrospective analysis of aspirin use after assessment of mood disorder symptoms T = last 5 years	Significantly higher risk for depression in individuals who had used aspirin as antiplatelet agent drug in the past, but had stopped the treatment before the mood assessment (measured by Geriatric Depression scale)	Almeida et al., 2010 [179]
Regular aspirin or statin use in the past	Depression	Depression patients $N = 1631$	Population-based study T = last 6 months	No higher risk for development of depression	Glaus et al., 2015 [180]
Celecoxib in addition to antidepressants	Depression	Patients with depressive episodes $N = 150$	Meta-analysis of 4 randomized controlled trials	Improvements in Hamilton Rating Scale for Depression scores, remission and response rate	Na et al., 2014 [181]
Diclofenac (50 mg bid) versus celecoxib (200 mg bid)	Depression	Outpatients with breast cancer and concomitant depression $N = 52$	$T = 6$ weeks	Significant improvements by HDSR score in both, celecoxib significantly more successful than diclofenac, analgesic effect comparable	Mohammadinejad et al., 2015 [182]
Celecoxib (200 mg bid)	Depression	Patients with depression due to brucellosis $N = 40$	Double-blind, randomized, placebo-controlled $T = 8$ weeks	Significant improvements on HDRS	Jafari et al., 2015 [183]
Celecoxib (200 mg bid) or sodium naproxen (220 mg bid)	Depression	Individuals older than 70 years and with a family history of Alzheimer's disease (though without any cognitive dysfunction) $N = 2312$	Alzheimer's Disease Anti-Inflammatory Prevention Trial Yearly follow-up	No significant change in individual or overall depression score measured by 30-item Geriatric Depression Scale	Fields et al., 2012 [184]
Minocycline (150 mg/day) as adjunctive therapy to fluvoxamine, paroxetine, or sertraline	Depression	Inpatients with a DSM-IV diagnosis of major depression with psychotic features $N = 25$	Open-label study $T = 6$ weeks	Significant improvement of depressive and psychotic symptoms as measured by Hamilton Depression Rating Scale and Brief Psychiatric Rating Scale	Miyaoka et al., 2012 [185]
100 mg bid minocycline	Depression	HIV positive patients with mild-to-moderate scores of depression $N = 46$	Double-blind, randomized, placebo-controlled $T = 6$ weeks	Significant reduction of depression score (Hamilton Depression Rating Scale)	Emadi-Kouchak et al., 2016 [186]
Celecoxib 200 mg bid in addition to fluvoxamine	OCD	OCD outpatient $N = 50$	Double-blind, randomized, placebo-controlled $T = 10$ weeks	Significant improvement of symptoms	Shalbafan et al., 2015 [187]
N-Acetylcysteine (up to 2400 mg/day)	OCD	OCD patients, nonresponsive to serotonin reuptake inhibitors $N = 48$	Double-blind, randomized, placebo-controlled $T = 12$ weeks	15% higher response rate	Afshar et al., 2012 [188]
Minocycline (100 mg/day) in addition to serotonin reuptake inhibitor	OCD	Treatment-resistant outpatients with OCD $N = 9$	Open-label study $T = 12$ weeks	No significant improvements in outcome in cohort as a whole, more than 30% reduction of the Yale–Brown Obsessive Compulsive Scale	Rodriguez et al., 2010 [189]

Table 2: Continued.

Treatment	Psychiatric disorder	Probands	Trial design	Outcome	Authors
Minocycline (100 mg bid) as add-on to fluvoxamine (100 mg/day for the first two 200 mg/day for the remaining 6 weeks of the trial)	OCD	OCD patients $N = 102$	Double-blind, randomized, placebo-controlled $T = 10$ weeks	Significantly higher treatment response rate (either complete or partial) measured by the Yale-Brown Obsessive compulsive scale. Specifically patients scored significantly lower in total and on the obsessive subscale	Esalatmanesh et al., 2016 [190]
Celecoxib (up to 300 mg/day) in addition to risperidone	Autism	Children with autistic disorder $N = 40$	Double-blind, randomized, placebo-controlled $T = 10$ weeks	Significant improvements of scores in irritability, social withdrawal, and stereotypy	Asadabadi et al., 2013 [191]
Minocycline (1.4 mg/kg body weight)	Autism	Children with autism spectrum disorder $N = 11$	Open-label $T = 6$ months	No clinical improvements (Clinical Global Impression Severity Scale, Clinical Global Impression Severity Scale Improvement, and Vineland Adaptive Behavior Scales)	Pardo et al., 2013 [192]

patients improved symptoms, however only in patients with high CRP serum levels [159]. In patients with an acute exacerbation of schizophrenia treatment with cyclooxygenase-2 inhibitor, celecoxib in addition to risperidone led to a significant improvement of positive and negative symptoms in excess of the relief assignable to the antipsychotic [154, 161–163]. On the other hand, when patients who had suffered from symptoms for at least three months despite treatment, rather than patients with an acute exacerbation of psychosis, were treated with celecoxib in a similar study design, no difference in outcome was found [164]. So it might be that the effect of celecoxib add-on treatment in schizophrenia patients is dependent on the duration of the disorder. Looking at anti-inflammatory drugs as a possible risk or protective factor for psychiatric disorder, in a retrospective investigation looking at more than 2000 schizophrenic patients who had concomitantly taken nonsteroidal antirheumatic drugs or paracetamol, researchers found a higher risk for relapse to active psychosis. It is not clear whether this may be simply assignable to the parallel comorbidity treated with nonsteroidal anti-inflammatory drugs (NSAIDs) or the drug use per se [165]. Then, again, previous NSAID use was examined in a case-control trial (82 cases, 359 controls) of antipsychotics prescription. This showed a significant reduction of risk to develop psychosis when NSAID had been taken, but only in male individuals [166]. A nested case-control study of 1443 case events indicating schizophrenia exacerbation in patients using antipsychotics looked back at recent use of COX-2 inhibitors. The risk for schizophrenia exacerbation was not increased when the anti-inflammatory drug had been taken beforehand (for 0–93 days) [167].

Double-blind, randomized placebo-controlled studies showed that adjunctive treatment with the tetracycline minocycline to usual treatment could significantly improve negative symptoms [156, 168, 170]. Though unchanged by the treatment with minocycline, positive symptom score seems to be predictor for that improvement of negative symptoms [171]. Furthermore, minocycline seems to have positive effects on cognitive executive functions, that is, working memory, cognitive shifting, and planning [172]. A proportion of schizophrenic patient respond to clozapine. In a recent study positive outcome along with improved cognitive outcome has been reported when patient received minocycline together with clozapine in a 10-week double-blind, placebo-controlled trial involving 52 patients [193]. So minocycline add-on therapy seems especially interesting in negative and cognitive symptom treatment of schizophrenia.

Although the above data are interesting, it can only reflect implication of inflammation in pathogenesis of schizophrenia. Therefore, randomized prospective trials with a larger sample size and longer follow-up duration are crucial to establish anti-inflammatory therapy as an effective, safe, and tolerable add-on treatment in schizophrenia.

5.3. Bipolar Disorder. In bipolar disorder, investigations of proinflammatory cytokines, namely, TNF-α, INF-γ, IL-6, and high sensitivity CRP, found elevated levels in a cohort of 30 patients in acute mania as compared to healthy controls. Most significantly, only levels of CRP were decreased after treatment with antipsychotic drugs (± electroconvulsive therapy) while patient showed good outcomes [14]. In a study originally aiming at the examination of a potential drug blocking effect of NSAID on treatment with mood-stabilizers no difference in outcome for bipolar patients was found with and without NSAID [173].

A trial on the effect of aspirin on lithium-using patients with erectile dysfunction found no differences in depressive or mania symptoms before and with the add-on treatment [174]. Nevertheless, aspirin had beneficial effects in a pharmacoepidemiological studies looking at mood-stabilizer substance change or dosage increase in patients on lithium (interpreted as worsening of bipolar disorder) in correlation to the use of further drugs. Prescribed at a low-dose of 30 or 80 mg per day and for an unspecified time or for more than 1, 45, 90, or 180 days, the patients, picked from the nationwide Netherlands PHARMO Record Linkage System, had significantly fewer events of mood-stabilizer treatment alteration [175].

In 2008 Nery et al. investigated the effect of celecoxib treatment in 28 patients with a DSM-IV diagnosis of bipolar disorder during a depressive or mixed episode of the disease. The study was randomized, double-blind, and placebo-controlled and celecoxib was given out at a dosage of 400 mg per day over 6 weeks. This was in addition to a stable treatment with mood-stabilizers or atypical antipsychotics. Among the patients who finished the complete trial, a significant improvement of depressive symptoms (as measured by Hamilton Depression Rating Scale) could be found in the first week of celecoxib treatment [176]. Similarly, a study with celecoxib or placebo in 46 patients, in addition to sodium valproate treatment, showed that remission rates were significantly higher with the adjunctive treatment than without assessed by the young mania rating scale (YMRS) following treatment of 6 weeks. At day 42, YMRS scores were significantly lower in the celecoxib group [177]. Trials over multiple centres for treatment of psychiatric disease with add-on minocycline and/or celecoxib/aspirin are currently ongoing [194, 195].

The above adjunctive anti-inflammatory therapy has more efficacy and comparable tolerability compared with control and future prospective studies with a longer study duration which are based on larger sample sizes are needed to comprehensively evaluate the efficacy and tolerability of anti-inflammatory therapy in bipolar disorder.

5.4. Major Depression Disorder. Treatment of major depression with NSAID has been tried reluctantly so far because of reports in which parallel NSAID or COX-2 inhibitor treatment of comorbidity lead to supposed drug interaction and higher resistance level to antidepressant treatment [196]. However, there is a number of noteworthy, even if sometimes contradictory, clinical trials that frequently come from investigations of anti-inflammatory comorbidity treatment. In a study on the effect of NSAID use in addition to escitalopram or nortriptyline treatment in a cohort of 811 major depression patients no significant effect of NSAID use on the outcome of the antidepressant therapy was found [178]. An Australian study in over 5000 men of older age (69–87

years) investigated the correlation between aspirin use and development of depressive symptoms. Individuals who had used aspirin as antiplatelet agent drug in the past but had stopped the treatment before the mood assessment were at a significantly higher risk for higher scores of depression [179]. However, a population-based study of 1631 patients could not find any lower rates of depression in patients who had taken aspirin on a regular basis [180].

A meta-analysis of 4 randomized controlled trials with celecoxib adjunctive to treatment with antidepressants was able to show consistent improvements in depression scores and remission and response rate [181]. Similarly, in 52 outpatients with breast cancer and concomitant depression a trial compared outcome after treatment with diclofenac versus celecoxib. At the endpoint, both treatments showed significant improvement as indicated by HDSR score. The celecoxib treatment was significantly more successful than the diclofenac treatment, while the analgesic effect was comparable [182]. Patients with depression due to Brucellosis showed significant response to treatment with celecoxib (200 mg bid) as measured by Hamilton Depression Rating Scale in a study on 40 patients over 8 weeks [183].

Then, again, in the Alzheimer's Disease Anti-inflammatory Prevention Trial, a study in more than 2000 individuals older than 70 years and with a family history of Alzheimer's disease (though without any cognitive dysfunction), the application of celecoxib or sodium naproxen was tested against placebo. In the beginning of the trial, around 20% of the test persons had an increased score for depression as measured by 30-item Geriatric Depression Scale. However, there was no significant change in that percentage over the trial, or in scores of individual probands [184]. Thus, one could argue that the effect of celecoxib might be age-dependent.

A 6-week open-label study in 25 inpatients with a DSM-IV diagnosis of major depression with psychotic features showed that minocycline (150 mg/day) as adjunctive therapy to fluvoxamine, paroxetine, or sertraline was beneficial. Both depressive and psychotic symptoms were significantly improved [185]. Similarly, HIV positive patients ($n = 46$) with mild-to-moderate scores of depression (Hamilton Depression Rating Scale) were treated with 100 mg bid minocycline or placebo in a double-blind randomized trial. A significant reduction of depression score was observed by the end of six weeks [186].

5.5. Anxiety/Anxiety Disorder. Even though to our knowledge there are no clinical trials with celecoxib, other NSAIDs, or tetracyclines in anxiety disorder patients to date, there are some interesting observations on anti-inflammatory manipulation of the immune system in animal models of anxiety symptoms. One trial in experimental autoimmune encephalitis in a rat model of multiple sclerosis showed that gene treatment with IL-10 diminished anxiety and depression symptoms, indicated by voluntary wheel running frequency [197]. In mouse models of anxiety after traumatic brain injury [198] and of anxiety following neonatal immune activation by infection treatment with minocycline led to a significantly positive effect on psychopathologic outcome [199].

A recent study provides evidence of high levels of IFN-γ and TNF-α but low IL-10 serum levels in anxiety patients when compared to healthy control subjects [200]. Furthermore, a large cohort study looking at the association between anxiety disorders and inflammation identified an elevated level of CRP in male patients with anxiety disorders [201]. In another study which included patients with rheumatoid arthritis receiving anti-TNF-α therapy had less frequent prevalence of any mood or anxiety disorders [202] demonstrating a beneficial role of anti-inflammatory therapy in anxiety disorders. Coumarin-derivate esculetin [203] as well as phenolic-rich *Aronia melanocarpa* berry juice attenuated anxiety and depressive symptoms in mice and rats, as measured during LPS-inducement and stress test, respectively. Both substances are known for their antioxidative power and it is thus feasible that the effect is assignable to an anti-inflammatory impact [204].

A synthetic derivative (4-phenylselenyl-7-chloroquinoline) with anti-inflammatory and antioxidative potential was shown to have anxiolytic effects in mice, as measured by elevated-plus maze and light dark tests. The quinolone was applied 0.5 hours prior to onset and anxiety-related behavior seemed avoided for up to 72 h. However, the authors of this study assigned the effect primarily to a change in glutamate levels provoked by the drug rather than anti-inflammatory effect [205]. The polyphenol Honokiol is primarily known as a drug used in traditional Chinese medicine. In mice, application of Honokiol (for 2 days at either 2 or 5 mg/kg i.p.) prior to induction of inflammation by LPS injection led to a decrease of anxiety levels, as measured by behavioral testing using the elevated-plus maze and open field test. Furthermore, a significant decrease in plasma levels of IL-1β, IL-6, TNF-α, and BDNF was found in those mice pretreated with Honokiol as well as a liver-protective effect [206]. Of note, physical exertion is shown by several lines of evidence to have anti-inflammatory effects and at the same time is one of the most effective nonpharmaceutical remedies of anxiety [207].

5.6. Obsessive-Compulsive Disorder. In a double-blind randomized trial in 50 OCD outpatients, fluvoxamine in combination with anti-inflammatory COX-2 inhibitor celecoxib (200 mg bid) showed a significant improvement of symptoms in comparison with fluvoxamine alone and placebo control [187]. In a similarly designed study 48 OCD patients who were nonresponders to serotonin reuptake inhibitors were add-on treated with N-acetylcysteine (up to 2400 mg/d) or placebo over 12 weeks. At the end of this period 15% more than in the placebo group were responsive to therapy in the N-acetylcysteine group (52.6%) [188].

In an open-label study, 9 treatment-resistant outpatients with OCD were given 100 mg/day of minocycline adjunctive to their treatment with serotonin reuptake inhibitors. In diagnostic testing by Yale–Brown Obsessive-Compulsive Scale every 2 weeks over 12 weeks, no significant improvements in outcome could be found in the cohort as a whole. However, in 2 patients with early-onset OCD, more than 30% reduction of the Yale–Brown Obsessive-Compulsive Scale was reached, which was initially defined as a marker for treatment response

[189]. Indeed, in a double-blind randomized trial on 102 OCD patients, therapy with minocycline (100 mg bid) as add-on to fluvoxamine (100 mg/day for the first two days and then 200 mg/day for the remaining 6 weeks of the trial) was associated with a significantly higher rate of patients responding completely or partially to the treatment. Measured by the Yale–Brown Obsessive-Compulsive Scale, total score and obsession subscale score were significantly lower in the minocycline than in the placebo group [190].

5.7. Autism Spectrum Disorder. Little is found on anti-inflammatory treatment in autism spectrum disorder so far. Add-on of celecoxib (up to 300 mg/day) to risperidone treatment in a study on 40 children suffering from autism leads to significant improvements of scores in irritability, social withdrawal, and stereotypy [191]. However, an open-label study in 11 children on treatment with 1.4 mg/kg body weight of minocycline found no clinical improvements after 6 months (measured using Clinical Global Impression Severity Scale, Clinical Global Impression Severity Scale Improvement, and Vineland Adaptive Behavior Scales). IL-8 was found significantly decreased in serum and cerebrospinal fluid, while other cytokines, that is, TNF-α, CD40L, IL-6, IFN-γ, and IL-1β, were unchanged [192].

6. Conclusions

A close examination of the literature demonstrates that the increased understanding of the microglia driven inflammatory effect on psychiatric diseases has not yet been translated into the clinical treatment for these disorders. The results from experimental animal models as well as from patients cohort studies are however quite promising. Adjunctive therapy with anti-inflammatory medication, especially in cases where conventional therapy has failed to bring complete recovery, may provide a novel route to treatment of these conditions.

Conflicts of Interest

The authors declare that they have no conflicts of interest.

Acknowledgments

Franziska A. Radtke is supported by a CMU studentship. Gareth Chapman is supported by the Neuroscience and Mental Health Research Institute and Waterloo Foundation studentship. Jeremy Hall is supported by Wellcome (DEFINE Strategic Award), Hodge Foundation (Centre Grant), MRC Centre Grant, and TWF Changing Minds Programme. Yasir A. Syed is supported by CMU fellowship and Hodge Foundation project grant.

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Hindawi
BioMed Research International
Volume 2017, Article ID 6423021, 11 pages
https://doi.org/10.1155/2017/6423021

Review Article

Bioinformatics Genes and Pathway Analysis for Chronic Neuropathic Pain after Spinal Cord Injury

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Received 7 April 2017; Revised 9 August 2017; Accepted 7 September 2017; Published 15 October 2017

Academic Editor: Giuseppe Donato

It is well known spinal cord injury (SCI) can cause chronic neuropathic pain (NP); however its underlying molecular mechanisms remain elusive. This study aimed to disclose differentially expressed genes (DEGs) and activated signaling pathways in association with SCI induced chronic NP, in order to identify its diagnostic and therapeutic targets. Microarray dataset GSE5296 has been downloaded from Gene Expression Omnibus (GEO) database. Significant analysis of microarray (SAM), Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway enrichment analysis, and pathway network analysis have been used to compare changes of DEGs and signaling pathways between the SCI and sham-injury group. As a result, DEGs analysis showed there were 592 DEGs with significantly altered expression; among them Ccl3 expression showed the highest upregulation which implicated its association with SCI induced chronic NP. Moreover, KEGG analysis found 209 pathways changed significantly; among them the most significantly activated one is MAPK signaling pathway, which is in line with KEGG analysis results. Our results show Ccl3 is highly associated with SCI induced chronic NP; as the exosomes with Ccl3 can be easily and efficiently detected in peripheral blood, Ccl3 may serve as a potential prognostic target for the diagnosis and treatment of SCI induced chronic NP.

1. Introduction

Neuropathic pain (NP) is a common consequence following spinal cord injury (SCI), which compromises a person's life satisfaction and quality. It is estimated that the mean cost was $47,518 for each SCI patient with NP in the USA [1]. According to the statistics of the GoPubMed website (http://www.gopubmed.org/web/gopubmed/), the molecular mechanisms of NP following SCI remain elusive. NP in SCI can be classified as "at-level" pain [2–4], "below-level" pain [2, 3], and "above-level" pain [5–7]. According to the International Spinal Cord Injury Pain Classification system [8], pain experienced at or within three dermatomes below the neurologic level of injury is considered at-level neuropathic pain, while pain that is present more than three dermatomes below the level of injury is classified as below-level neuropathic pain. While at-level pain results from lesion of nerve roots and/or the spinal cord and is felt at the corresponding segment, below-level pain is a central pain caused by damage to spinal cord pathways, suggesting different pathological mechanisms of pain generation [9, 10]. However, there is not too much description for above-level neuropathic pain following SCI in previous study. Not rarely, patients suffer both at-level and below-level pain, but at-level pain seems to appear earlier than below-level pain clinically [11].

The prevalence of NP syndromes in the general population is as high as 7 to 8% [12, 13], and approximately 30–50% of patients with a SCI will develop chronic NP [14–16]. And, according to previous studies [17], a total of 140 participants were analyzed, 70 of them were SCI-NP subjects and the remaining 70 controls did not show neuropathic symptoms. SCI can result from trauma, tumor, infection, and degenerative condition; among them the traumatic SCI plays a pivotal role in inducing chronic NP [18]. There are annually 0.25–0.5 million SCI cases around the world

and more than 90% are due to traumatic injury [19]. SCI results in cell loss, disruption of neural circuitry, and chronic functional impairment [20]; thus patients with chronic NP after SCI may benefit from strategies aiming to promote neurogenesis, neural plasticity, and functional recovery such as human umbilical cord-derived mesenchymal stem cell transplantation [21], astrocyte transplantation [22–25], and neural stem cell transplantation [26, 27]. The mechanisms underlying SCI induced chronic NP remain elusive, and recent advance in neuroscience has implicated that SCI is a polygenic disease and its pathogenic mechanism is associated with changes of gene expression; therefore identification of related genes in chronic NP after SCI could provide new insights into gene function as well as potential diagnostic and therapeutic targets. In this study, we used microarray technology to identify differentially expressed genes (DEGs) and activated signaling pathways in association with SCI induced chronic NP in a mouse SCI model.

2. Material and Methods

2.1. Data Source. Microarray technology is a widely used high-throughput tool for measuring gene expression [28–30]. Moreover, previous studies have shown that data from DNA microarray analysis can be reliable and useful for identifying novel targets for clinical diagnostic and therapeutic approaches [31]. Thus, we used the microarray expression profiles (GSE5296), which were extracted from the GEO (https://www.ncbi.nlm.nih.gov/geo/) database, to identify DEGS associated with SCI induced chronic NP. A C57BL6 mouse SCI model was used (https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE5296). The experimental group ($n = 12$) was subjected to a moderate injury at the T8 spinal cord segment under isoflurane anesthesia. Total RNA was extracted from sections of rostral regions, caudal regions, and lesion centre from T8 spinal cord injury (0.4 cm in length each one). The expression of genes was detected at a series of time points: 0.5, 4, 24, 72 h, and 7 and 28 days after injury, respectively. According to clinical circumstance, patients commonly experience NP during the initial 3–6 months and 3–5 years after SCI [2]. Therefore, it must be noted that neuropathic pain did not appear during the time points we studied. The control group is sham-injured ($n = 8$), with laminectomy only. Global changes were evaluated using Affymetrix Mouse Genome 430 2.0 arrays. The experiments were performed three times.

Altered DEGs and signal pathways were compared between injury group and sham-injury group in different regions: rostral, lesion centre, and caudal regions and at serial time points. The lesion centre has the most significant alteration in DEGs and signal pathways, and thus the dates in different time point from lesion centre were chosen to perform the next analysis before normalization processing by RMA (robust multichip average).

2.2. Data Preprocessing. The RMA method [32] is for computing an expression measurement with three steps' process: background-correction, normalization, and summary. The method includes a probe-specific background-correction and a probe selection strategy in which a subset of probes with highly correlated intensities across multiple samples is chosen to summarize gene expression.

2.3. Analysis Methods. GCBI platform (https://www.gcbi.com.cn/gclib/html/index) was mainly used in the whole process. Initially, DEGs were significantly identified in the spinal cord total RNA samples from C57BL6 mouse model of contusion injury in comparison with samples from animals of laminectomy only. Significant analysis of microarray (SAM) is used to study DEGs. $P = 0.05$ was used as the significance threshold of screening DEGs, and fold change > 2 was used as the threshold to determine the significance of gene expression difference. Cluster analysis based on Pearson correlation calculation was used to ensure that the screened genes perfectly expressed the differences between SCI group and sham-injury group. Furthermore, GO functional [33] and KEGG analysis were performed to identify the altered pathway involved in the SCI. Significantly enriched GO terms and KEGG pathways with Fold Discovery Rate (FDR) < 0.05 and $P < 0.05$ were screened out. Finally, pathway net analysis was to explore the relationships among each pathway. What is more, it must be noted that SAM (significant analysis of microarray) is used to study DEGs (differentially expressed genes), while RMA (robust multichip average) is for normalization procession, and their function are totally different.

3. Results

3.1. Data Preprocessing from Lesion Centre. The original data were preprocessed by RMA function with the Affymetrix package of R language [34]. The original CEL files were switched into probe expression measures, and the probe-level data were converted into gene names by an annotation package supported by the GCBI platform. After excluding the influence of background, the signal values of the samples are still high (Figure 1(a)), assuring the reliability of the analysis results. It can be seen that the black lines were almost on the same line (Figure 1(b)), indicating an excellent degree of standardization, which ensure the accuracy of subsequent data processing. The correlations of all samples are basically very strong (Figure 1(c)), providing the basis of the subsequent cross analysis and system analysis. The data preprocessing results showed that the samples were sufficiently, precisely, and stably enough to support the following analysis.

3.2. Screening of Differentially Expressed Genes from T8 Lesion Centre (Ccl3 Was the Maximum Changed Gene Expression Profile among the 592 DEGs). DEGs that were significantly differentially expressed were screened out by use of significance analysis of microarrays (SAM) [35, 36] in the GCBI platform. $P < 0.05$ and fold change > 2 were used as the threshold of screening differentially expressed genes. When the number of samples becomes large, we implement a standard analysis method for screening difference genes. In fact, we use the two samples' Welch t-test (unequal variances) for two groups' difference analysis and use analysis of variance (ANOVA) for multiple groups (groups count no

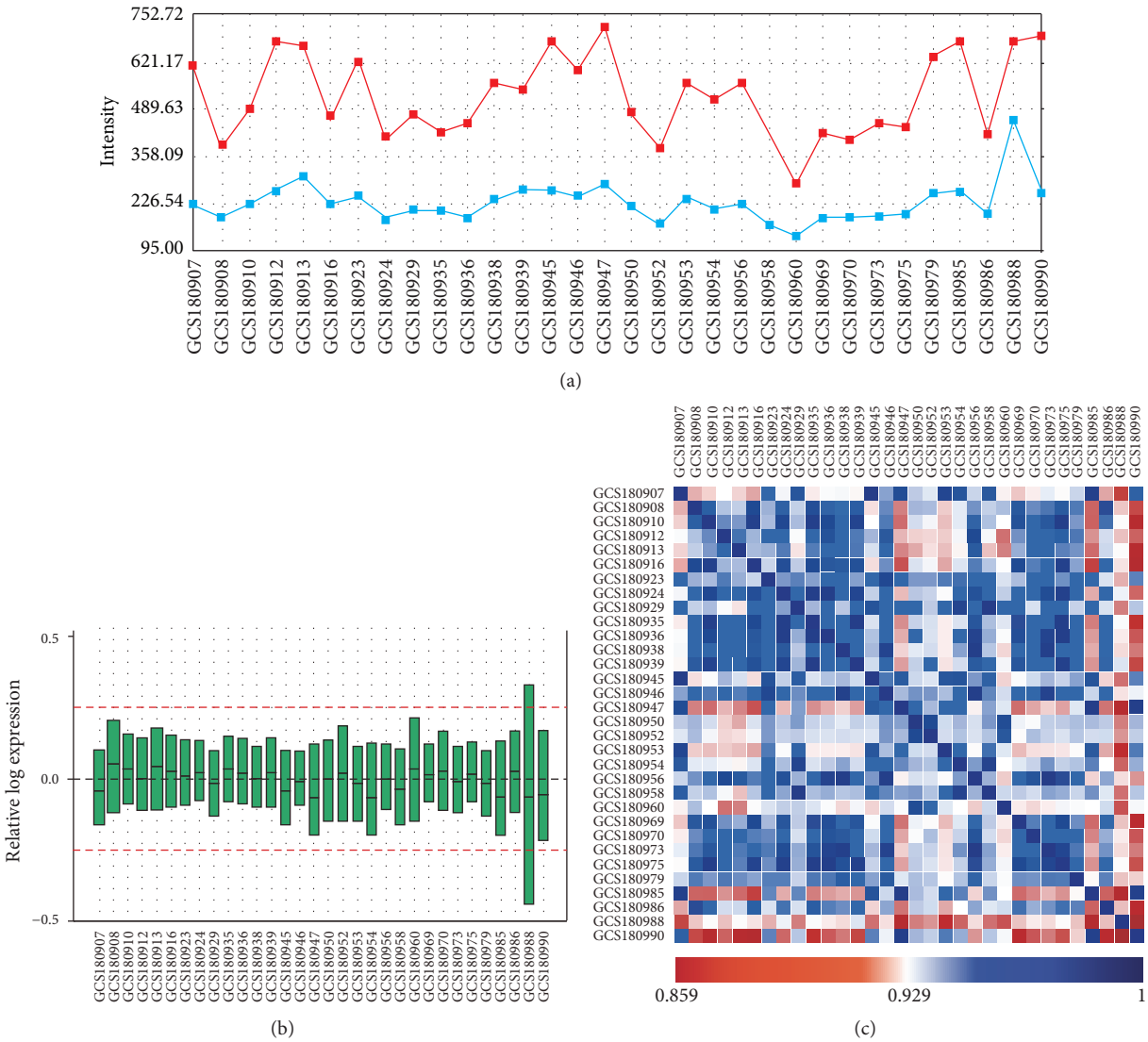

Figure 1: The background and signal value for each sample. Red represents the average of the values of the sample signals for each sample. Blue represents the average of the background values for each sample. It demonstrated that, after excluding the influence of background, the signal values of the samples are still high (a). The horizontal axis represents the name of samples, while the vertical axis represents the expression value after log conversion. The black lines stand for median, which can be used to identify the degree of standardization after normalization of all samples by the package of R/Bioconductor. It can be seen that the black lines were almost on the same line (b). Sample correlation calculated by injury associated genes expression. Both the horizontal axis and the vertical axis represent the name of samples. The gene expression level from different sample was calculated with Pearson correlation. The closer the point is to the blue color, the greater the correlation is between the two samples. It shows that the correlations of all samples are basically very strong (c).

less than 3). For multiple comparison analysis, we computed the q-value to control the false discovery rate [37]. The top ten largest differences in DEGs were screened with fold change > 2 and $P < 0.05$, and the maximum change of gene expression profile was upregulated Ccl3 (Table 1). After heatmap of gene expression differences by gene coexpression network analysis, we found 592 statistically significant DEGs (Figure 2(a)), which is consistent with the known results that SCI is a polygenic disease and its pathogenic mechanism is associated with changes of gene expression. The abscissa value of Ccl3 is 3.45 (Figure 2(a)), which shows Ccl3 is the maximum change among all DEGs.

We further calculated the Pearson correlation to construct the distance between the genes and samples and implemented the hierarchical clustering based on used average method for linkage [38]. The top 10 DEGs were listed according to the size of difference and Ccl3 is the top DEGs which are upregulated. From the horizontal axis at the top, it can be concluded that the samples can be divided into clusters generally: the control group of sham-injury and the experimental group of injury (Figure 2(b)). Moreover, the maximum change of gene expression profile was upregulation of Ccl3 (fold change = 10.91, $P = 2.20E - 05$).

Table 1: Top 10 significantly enriched up- and downregulated DEGs. The P values associated with each term are calculated by the Fisher Exact Test which represents the "degree of enrichment." Q-value is the correction for multiple comparison by Benjamini and Hochberg [33]. The rank was according to the difference determined by d Score, fold change, and P value. The top ten largest differences in DEGs were screen with fold change > 2 and $P < 0.05$, and the maximum change of gene expression profile was upregulation of Ccl3.

DEGs	Accession number	Signal of control	Signal of experimental	d Score	Fold change	P value	q-value	Expression change	Rank
Ccl3	*NM_011337*	*5.37*	*8.81*	*10.04*	*10.91*	*2.20E − 05*	*0*	*Up*	*1*
Atf3	NM_007498	6.12	9.18	10.10	8.37	2.20E − 05	0	Up	2
Plek	NM_019549	6.67	9.20	8.10	5.77	2.20E − 05	0	Up	3
Ctla2b	NM_001145801	5.45	7.90	8.08	5.45	2.20E − 05	0	Up	4
Bcl2a1a	NM_007534	7.23	9.59	8.37	5.14	2.20E − 05	0	Up	5
Ch25h	NM_009890	6.82	9.13	8.27	4.95	2.20E − 05	0	Up	6
Plek	NM_019549	5.87	8.12	7.58	4.76	2.20E − 05	0	Up	7
Tnfaip3	NM_001166402	5.75	7.45	8.12	3.24	2.20E − 05	0	Up	8
Tgif1	NM_001164074	6.05	7.59	8.52	2.91	2.20E − 05	0	Up	9
Gpr84	NM_030720	6.31	7.83	7.92	2.86	2.20E − 05	0	Up	10

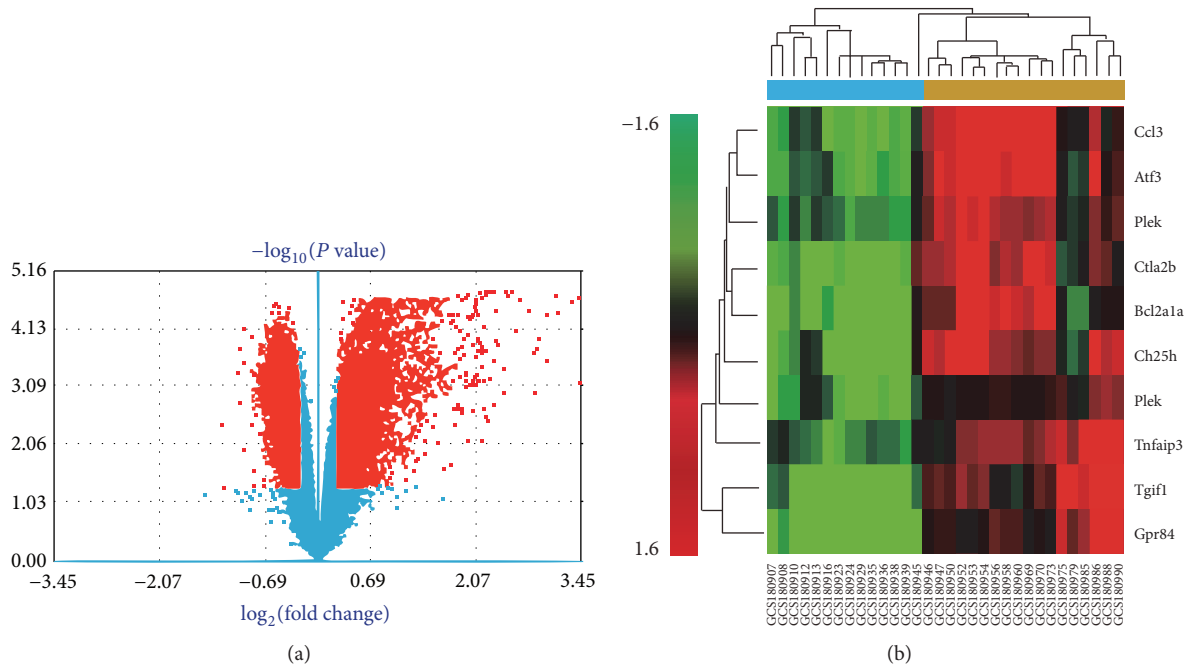

Figure 2: Heatmap of gene expression differences by gene coexpression network analysis. Red dot indicates a differentially expressed gene with statistical significance. Red dots on the right indicate upregulation of gene expression, whereas red dots on the left indicate downregulation of gene expression. Blue indicates that there is no statistically significant difference in gene expression. The greater the ordinate value corresponding to the point is, the greater the difference in gene expression corresponding to that point is. Similarly, the greater the absolute value of the abscissa corresponding to the point is, the greater the difference in gene expression corresponding to that point is. Note that there are 592 statistically significant DEGs. The abscissa value of Ccl3 is 3.45, which means Ccl3 is the maximum change among all DEGs (a). Hierarchical clustering dendrogram of gene expression: the horizontal axis at the bottom represents the name of samples and the vertical axis on the left side represents the degree of gene clustering. The vertical axis on the right side represents the name of genes and the horizontal axis at the top represents the degree of clustering of samples. The red color stands for upregulated while the green color stands for downregulated. The darker red indicates a stronger upregulation in expression and the darker green indicates a stronger downregulation in expression. It can be concluded that the samples can be divided into clusters generally: the control group of sham-injury and the experimental group of injury (b). Moreover, the maximum change of gene expression profile was upregulation of Ccl3 (fold change = 10.91, $P = 2.20E - 05$) (b).

3.3. KEGG Pathway Enrichment Analysis from T8 Lesion Centre (MAPK Signaling Pathway Was the Most Important among the 209 Pathways). Significantly enriched GO terms and KEGG pathways with FDR < 0.05 were screened out. The rank was according to the enrichment score, P value, and FDR. KEGG biological pathway enrichment analysis found that MAPK signaling pathway (enrichment score = 5.68, $P = 3.38E{-}74$, and FDR = $8.78E{-}72$) was the most important one among the 209 pathways according to the enrichment scores (Table 2).

3.4. Pathway Network Analyses from T8 Lesion Centre (MAPK Signaling Pathway Was Also the Most Important Pathway). The interaction in KEGG was used to construct the interaction network between pathways. The overall and systematical pathway analysis of the relationship between marked pathways can help to disclose the synergistic effect module of important pathways. In the top 10 altered pathway interaction nets with 111 nodes and 404 relationships between each other, MAPK signaling pathway was the most important one with the largest degree (outdegree = 5, indegree = 39, and degree = 44) (Table 3) and it was in the centre of the altered pathways interaction network (Figure 3).

3.5. Systematic Analysis of DEGs and Altered Pathway in Different Section and Time Points by SAM and KEGG. The DEG Ccl3 and MAPK signaling pathways are not necessarily in the top 10 list (Tables 4 and 5) because of the difference of data analysis. We only discussed, verified, and confirmed the correlation between DEGs Ccl3, MAPK signaling pathway, and SCI induced chronic NP because of the great diversity of genes and the huge complexity of the whole work. However, what is the most important is that systematic analysis of DEGs and altered pathway in different section and time point by SAM and KEGG suggests another method and strategy to study the target gene and pathway of nerve-related disease.

4. Discussion

SCI has been demonstrated to be a polygenic disease and its pathogenic mechanism is associated with changes of many genes. In this study, we have used microarray technology to identify differentially expressed genes (DEGs) and activated signaling pathways in association with SCI induced chronic NP in a mouse SCI model. We showed that Ccl3 and MAPK were the most upregulated DEG and the most activated signaling pathway, respectively. Our results are consistent

Table 2: Top 10 GO terms and KEGG pathways enrichment results of DEGs. Significantly enriched GO terms and KEGG pathways with FDR < 0.05 were screened out. KEGG biological pathway enrichment analysis found that MAPK signaling pathway (enrichment score = 5.68, $P = 3.38E-74$, and FDR = $8.78E-72$) was the most important one among the 209 pathways according to the enrichment score.

Pathway ID	Pathway name	Enrichment score	P value	FDR	Rank
4010	*MAPK signaling pathway*	*5.68*	*3.38E − 74*	*8.78E − 72*	*1*
1100	Metabolic pathways	2.73	3.22E − 65	4.19E − 63	2
4151	PI3K-Akt signaling pathway	4.31	8.74E − 57	7.57E − 55	3
5200	Pathways in cancer	4.36	2.57E − 53	1.67E − 51	4
4380	Osteoclast differentiation	6.77	2.20E − 52	1.14E − 50	5
5166	HTLV-I infection	4.54	6.12E − 52	2.65E − 50	6
4810	Regulation of actin cytoskeleton	5.06	3.43E − 49	1.28E − 47	7
4062	Chemokine signaling pathway	5.26	6.63E − 49	2.15E − 47	8
4510	Focal adhesion	5.11	2.31E − 47	6.68E − 46	9
5205	Proteoglycans in cancer	4.73	3.22E − 45	8.36E − 44	10

Table 3: The top 10 altered pathways of network analyses. The outdegree and indegree represent, respectively, the number of upstream and downstream signal pathways. The degree represents the sum of the outdegree and indegree. In the top 10 altered pathway interaction nets with 111 nodes and 404 relationships between each other, MAPK signaling pathway was the most important one with the largest degree (outdegree = 5, indegree = 39, and degree = 44).

Pathway ID	Pathway name	Outdegree	Indegree	Degree	Rank
4010	*MAPK signaling pathway*	*5*	*39*	*44*	*1*
4210	Apoptosis	3	29	32	2
5200	Pathways in cancer	28	0	28	3
4110	Cell cycle	3	20	23	4
10	Glycolysis/gluconeogenesis	5	15	20	5
4020	Calcium signaling pathway	5	14	19	6
4115	p53 signaling pathway	2	17	19	7
4310	Wnt signaling pathway	8	9	17	8
4060	Cytokine-cytokine receptor interaction	0	16	16	9
620	Pyruvate metabolism	7	8	15	10

with that of previous studies. It will be very interesting to further this study into SCI patients. Throughout the analysis, the factors affecting the results include sample attributes (sample source, sample size, and sample quality), treatment tools, treatment methods, and results screening. In addition, various analysis methods were used, including the screening of differentially expressed genes, KEGG pathway enrichment analyses, and pathway network analyses. All the analysis processes were performed on the GCBI platform in order to avoid the error difference resulting from running different analysis at the different platforms.

The activation of resident cells and the inflammatory cells (macrophages, neutrophils, and lymphocytes) in PNS was involved in peripheral sensitization. In the spinal dorsal horn, glial cells (microglia and astrocytes) are activated to account for central sensitization. Neuropathic pain induced by peripheral and central sensitization is mediated by some inflammatory mediators (IFMs) including chemokines and cytokines (e.g., Ccl3) [39]. After SCI, Ccl3 were induced significantly in the dorsal horns 2 days after lesion and remained at high levels with significantly higher intensities

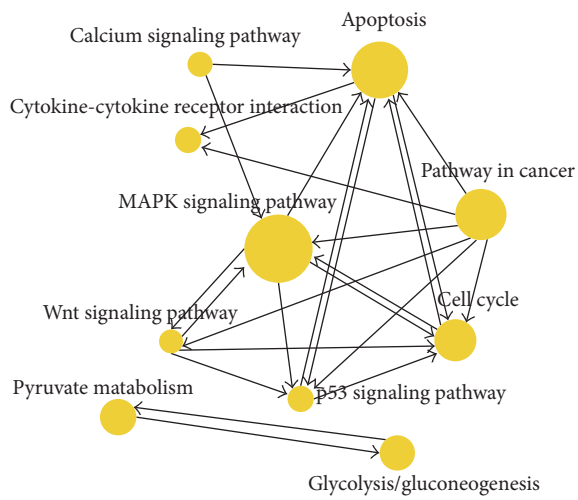

Figure 3: Pathway network after spinal cord injury. The more important the signaling pathway is, the larger the ball is. The importance was ranked according to the degree. MAPK signaling pathway was in the centre of the altered pathways interaction net.

Table 4: Top 10 DEGs and pathways between sham-injury and injury in lesion centre at different time points. At different time points, top 10 DEGs and pathways between sham-injury and injury in lesion centre are showed, respectively.

Time point	Top 10 DEGs between sham-injury and injury in lesion centre	Top 10 pathways between sham-injury and injury in lesion centre
0.5 h	Npas4, Gm2083, Socs3, Socs3, Fosb, Ccl3, Il6, Cyr61, Ptgs2, Myh1	Pathways in cancer, MAPK signaling pathway, Transcriptional misregulation in cancer, focal adhesion, proteoglycans in cancer, PI3K-Akt signaling pathway, hippo signaling pathway, HTLV-I infection, regulation of actin cytoskeleton, metabolic pathways
4 h	Ucn2, Gm2083, Atf3, Hspa1b, Hspa1b, Ccl3, C330006P03Rik, Hspa1a, Hspa1b, Egr3	Metabolic pathways, MAPK signaling pathway, pathways in cancer, PI3K-Akt signaling pathway, HTLV-I infection, focal adhesion, proteoglycans in cancer, osteoclast differentiation, transcriptional misregulation in cancer, olfactory transduction
24 h	Gm2083, Socs3, Chi3l3, Adam8, Gp49a, Hmox1, Serpine1, Tgm1, A130040M12Rik, Tnc	Metabolic pathways, MAPK signaling pathway, pathways in cancer, HTLV-I infection, PI3K-Akt signaling pathway, focal adhesion, protein processing in endoplasmic reticulum, regulation of actin cytoskeleton, Epstein-Barr virus infection, proteoglycans in cancer
3 d	Gpnmb, Cd36, Abca1, Cd5l, Cd36, Ccnb1, Thbs1, Rrm2, Rrm2, Sprr1a	Metabolic pathways, HTLV-I infection, pathways in cancer, focal adhesion, regulation of actin cytoskeleton, PI3K-Akt signaling pathway, proteoglycans in cancer, MAPK signaling pathway, lysosome, osteoclast differentiation
7 d	Gpnmb, Gp49a, Cd36, Cd36, Ms4a7, Cd5l, C3ar1, Clec7a, Cd68, Atp6v0d2	Focal adhesion, PI3K-Akt signaling pathway, metabolic pathways, pathways in cancer, proteoglycans in cancer, MAPK signaling pathway, regulation of actin cytoskeleton, osteoclast differentiation, HTLV-I infection, tuberculosis
28 d	Gpnmb, Clec7a, Cst7, Gp49a, Lgals3, Cd68, C3ar1, Ms4a7, Sprr1a, Cd48	Metabolic pathways, MAPK signaling pathway, pathways in cancer, HTLV-I infection, focal adhesion, proteoglycans in cancer, PI3K-Akt signaling pathway, regulation of actin cytoskeleton, chemokine signaling pathway, phagosome

Table 5: Top 10 DEGs and pathways between sham-injury and injury in different sections. By processing data from all time points in each section, top 10 DEGs and pathways between sham-injury and injury are showed, respectively.

Section	Top 10 DEGs between sham-injury and injury	Top 10 pathways between sham-injury and injury
rostral regions	Ccl3, Plek, Slc15a3, Bcl2a1a, Plek, Tlr2, Ccl4, Clec7a, Plek, Palld	Osteoclast differentiation, cytokine-cytokine receptor interaction, PI3K-Akt signaling pathway, phagosome, Chagas disease (American trypanosomiasis), leishmaniasis, toll-like receptor signaling pathway, chemokine signaling pathway, tuberculosis, transcriptional misregulation in cancer
lesion centre	Ccl3, Atf3, Plek, Ctla2b, Bcl2a1a, Ch25h, Plek, Tnfaip3, Tgif1, Gpr84	MAPK signaling pathway, metabolic pathways, PI3K-Akt signaling pathway, pathways in cancer, osteoclast differentiation, HTLV-I infection, regulation of actin cytoskeleton, chemokine signaling pathway, Focal adhesion, proteoglycans in cancer
caudal regions	Atf3, Tlr2, Irgm1, Trim30d, S1pr3, Bcl2a1a, Slc45a3, Plek, Trim30a, Zfp36l1	Tuberculosis, phagosome, *Staphylococcus aureus* infection, leishmaniasis, osteoclast differentiation, antigen processing and presentation, herpes simplex infection, Fc gamma R-mediated phagocytosis, viral myocarditis, Toll-like receptor signaling pathway

[40], while, after peripheral nerve injury, Ccl3 and their receptors (CCR2 and CCR1/CCR5, resp.) were increased [41]. In addition, there were differences in gene expression at the different stages of pain. For example, the expression of Ccl3 at the six time points was reflected in the ranking of top 10 DEGs (Table 5). Because of the existence of ongoing pain and evoked pain following SCI, here the neuropathic pain we discussed is defined as the evoked pain following SCI. Moreover, we did not perform animal experiments to confirm the relationship between the protein function and SCI-NP, and we did not exclude the false positive microarray results, which result from insufficient conditions.

Ccl3, the ligand of CCR1 [42] and CCR5 [43, 44], was upregulated after SCI and elicits chronic inflammation, resulting in NP [45, 46]. Peripheral Ccl3 [47, 48] and Ccl3 in the spinal cord [49, 50] can produce pain behaviors through the activation of chemokine receptors in the dorsal root ganglia (DRG). Ccl3 was found to be upregulated in activated Schwann cells and infiltrating macrophages close to the injured nerves and found to participate in the development of neuropathic pain through its dominant receptors CCR1 and CCR5, which are also located in Schwann cells and macrophages [39].

CCR1 were found to be induced in the early phase (first 7 days after SCI), while in the late time course (42 days after SCI) elevated chemokine levels were only found after severe SCI [42]. CCR5 was involved in the development of other inflammatory diseases through macrophage activation

[51–53], which was located in primary afferent neurons or secondary neurons of the spinal dorsal horn [47, 54]. Ccl3 and its receptor, CCR5, are upregulated in the spinal cord after injury by using qRT-PCR analysis [43, 44].

Microglia and astrocytes constitutively express CCR1 and CCR5 [55, 56]. It has been shown that microglia proliferate robustly after SCI and were essential to induce NP sensitization [57, 58]. Furthermore, minocycline, a microglial inhibitor, was reported to prevent, delay, or relieve NP [59, 60]. On the contrary, microglial activation is sufficient to induce pain sensitization [61]. Microglia are referred to as a main source of IFMs in the CNS [62, 63], which plays a crucial role in neuropathic pain development [64].

MAPK signaling pathway and chemokine signaling pathway are involved in SCI, which play a very important role in SCI induced NP [65]. MAPK family includes three major members: p38, extracellular signal regulated kinase (ERK), and c-Jun N-terminal kinase (JNK), regulating different signaling pathways. MAPKs are activated by phosphorylation and transduce a broad range of extracellular stimuli by both transcriptional and nontranscriptional regulation, leading to different intracellular responses. Asiaticoside attenuates SCI induced NP through anti-inflammatory effects and inhibition of the p38-MAPK mechanism [66]. Intrathecal injection of the anti-inflammatory cytokine alleviated SCI induced inflammation, suppressing the SCI induced activation of p38-MAPK [67]. Moreover, CCR5 is one receptor of Ccl3, knockout of CCR5 suppressed SCI induced neuropathic pain [67]. Inhibition of p38 MAPK signaling pathway can alleviate neuropathic pain [68].

p38 MAPK is activated by upstream kinase MKK3/MMK6, whose activation in spinal cord microglia was reported after SCI model [69]. p38α and p38β are two major p38 isoforms among the four isoforms: α, β, γ, and δ in the mature nervous system [70]. p38β appears to be expressed in spinal cord microglia, and the knockdown of p38β but not p38α prevents acute pain sensitization [71]. p38 is involved in the maintenance of neuropathic pain, and its inhibitor can attenuate and reverse NP symptoms [57].

Activation of cytokine receptors (CCR1 and CCR5) results in p38 MAPK activation in spinal cord microglia. p38 activation results in increased expression, through the transcription factor NF-κB or other transcription factors (e.g., ATF-2), of secreted inflammatory mediators/growth factors (e.g., cytokines and BDNF) or of genes encoding membrane receptors. In addition, p38 also induces release of PGE2 and IL-1β via rapid posttranslational regulation. Upon release, these mediators will sensitize nociceptive dorsal horn neurons via presynaptic and postsynaptic mechanisms, leading to persistent pain hypersensitivity [57].

Furthermore, exosomes and other extracellular vesicles are emerging as a novel form of information exchange within the nervous system, and exosomes can play both neuroprotective and neurotoxic roles [72]. Exosomes are released by neurons in a way depending on synaptic activity, and these exosomes can be retaken by other neurons, suggesting a novel way for interneuronal communication [73]. Exosomes derived from heat-stressed tumor cells (HS-TEX) which contain chemokines, such as CCL2, CCL3, CCL4, CCL5, and CCL20, could chemoattract and activate dendritic cells (DC) and T cells more potently [74]. Schwann cells-derived exosomes enhance axonal regeneration and increase neuronal survival after prodegenerative stimulation [75]. The cotransplantation of Schwann cells and OECs reduced number of astrocytes, microglia and macrophage infiltration, and the expression of chemokines (CCL2 and CCL3) at the injured site, which provide a better immune environment for SCI repair [76].

Ccl3 and its receptors, CCR5 and CCR1, are upregulated after SCI, and knockout of Ccl3 as well as inhibition of p38 MAPK signaling pathway can alleviate neuropathic pain [67]. Thus, Ccl3 antagonists may be potential new drugs for the treatment of neuropathic pain.

5. Conclusions

In this study, the maximum change of gene expression profile Ccl3 (fold change = 10.91, $P = 2.20E - 05$) was identified among the altered 529 DEGs after SCI with threshold of $P < 0.05$ and fold change > 2. Furthermore, KEGG analysis found that 209 pathways with significance were identified, among which the most important was the MAPK signaling pathway according to the enrichment score (enrichment score = 5.68, $P = 3.38E - 74$, and Fold Discovery Rate (FDR) = $8.78E - 72$). According to previous study, in SCI induced chronic NP, exosomes in the peripheral blood would contain Ccl3, which was derived from Schwann cells. The exosomes could cross blood-spinal cord barrier and combine with Ccl3's receptor, CCR5, which accounts for the chronic neuropathic pain syndromes. Ccl3 and its receptor, CCR5, are also upregulated after SCI, and knockout of Ccl3 as well as inhibition of p38 MAPK signaling pathway can alleviate neuropathic pain. Since the exosomes with Ccl3 can be easily and efficiently detected in peripheral blood, Ccl3 may serve as a potentially prognostic and predictive target for the diagnosis and treatment of SCI induced chronic NP in clinical applications. What is the most, the systematic analysis of DEGs and altered pathway in different section and time point by SAM and KEGG suggests another method and strategy to study the target gene and pathway of nerve-related disease.

Conflicts of Interest

All authors declare no conflicts of interest.

Acknowledgments

This work was supported by grants from the National Natural Science Foundation of China (NSFC no. 81171719) and from Chongqing Natural Science of Foundation of China (CSTC, 2008 BB5281). The authors thank Dr. Dongsheng Wu for reading and revising this manuscript.

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Hindawi
BioMed Research International
Volume 2017, Article ID 3706018, 10 pages
https://doi.org/10.1155/2017/3706018

Research Article

Markers of Alzheimer's Disease in Primary Visual Cortex in Normal Aging in Mice

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Received 4 June 2017; Accepted 9 August 2017; Published 12 September 2017

Academic Editor: Anna Di Vito

Aging is the principal risk factor for the development of Alzheimer's disease (AD). The hallmarks of AD are accumulation of the amyloid-β peptide 1–42 (Aβ42) and abnormal hyperphosphorylation of Tau (p-Tau) protein in different areas of the brain and, more recently reported, in the visual cortex. Recently, Aβ42 peptide overproduction has been involved in visual loss. Similar to AD, in normal aging, there is a significant amyloid deposition related to the overactivation of the aforementioned mechanisms. However, the mechanisms associated with visual loss secondary to age-induced visual cortex affectation are not completely understood. Young and aged mice were used as model to analyze the presence of Aβ42, p-Tau, glial-acidic fibrillary protein (GFAP), and presenilin-2, one of the main enzymes involved in Aβ42 production. Our results show a significant increase of Aβ42 deposition in aged mice in the following cells and/or tissues: endothelial cells and blood vessels and neurons of the visual cortex; they also show an increase of the expression of GFAP and presenilin-2 in this region. These results provide a comprehensive framework for the role of Aβ42 in visual loss due to inflammation present with aging and offer some clues for fruitful avenues for the study of healthy aging.

1. Introduction

Traditionally, the aging population has been associated with developed countries, but currently two-thirds of the world's oldest persons live in developing countries, where the elderly population is growing faster than in developed regions.

AD is the most common cause of dementia in older people, and it is estimated that 27 million people are affected worldwide [1, 2]. As the life expectancy of the population increases, the number of affected individuals is predicted to present a threefold increase by 2050 [2, 3]. Different risk factors have been associated with the development of AD, such as environmental, dietary, and pathological factors, altered glucose metabolism, chronic inflammation, gender, and oxidative stress. Nevertheless, age continues to be the main risk factor for AD, although early-onset disease can occur before the age of 60 years [4, 5].

AD is a paradigm of a neurodegenerative disorder that is caused by the detrimental progression of age-dependent loss of cognitive function. The hallmarks of this disease are accumulation of amyloid aggregates (also known as amyloid plaques), principally constituted by abnormal local deposits of Aβ42 in the extracellular brain parenchyma and the hippocampus, as well as the formation of neurofibrillary tangles within the neurons in the aforementioned regions [6]. These neurofibrillary tangles consist of cross-linked

protein strands that generate a double helix structure; the principal component of these tangles is the pathologically hyperphosphorylated Tau protein [7].

The production of Aβ, a critical event in AD, results from the cleavage of the amyloid precursor protein (APP), whose levels are high in AD. This peptide is toxic and induces several detrimental effects on cells, like cell membrane disruption, excessive production of reactive oxygen species, interactions with several proteins that affect their normal function, synaptic failure, chronic local inflammation, glial hyperactivity, and cell death [8]. These changes have been studied and identified mainly in brain areas such as entorhinal, prefrontal, and visual cortices, hippocampus, and olfactory bulb.

In addition, it has been reported that AD patients lose certain visual functions that are not correlated to structural damage of the eye but with loss of neurological function. Aβ42 peptide toxicity has been related to several disrupted molecular mechanisms in normal vision; for example, inhibition of long-term potentiation and cognitive processes [9, 10] increases the proinflammatory effects in the occipital visual cortex and induces gliosis and apoptosis among other toxic effects. These alterations have been correlated with ophthalmic disorders such as age-related macular degeneration (AMD) and glaucoma [11–13]. However, there is not enough information about these pathological changes in the visual cortex during normal aging.

Aging is accompanied by chronic inflammation, demonstrated by the increase of inflammatory mediators such as cytokines and oxidative stress markers and chronic antigenic stress and influenced by the genetic background [14–16]. Chronic inflammation appears to be involved in the pathogenesis of some age-related diseases such as AD, atherosclerosis, diabetes, age-related macular degeneration, and cancer [17, 18]. As in Alzheimer's disease, aging is also characterized by the accumulation of Aβ42 in "inflammaging," a term used to highlight the importance of inflammation in many age-associated diseases [19]; as previously mentioned, the presence of Aβ42 is associated with vision loss. The presence of multiple Aβ42 reservoirs in the eye (especially in the retina and the optic nerve) induces different pathologies that lead to potentially blinding disorders [20]. However, the presence of Aβ42 in the visual cortex and the role it plays in vision loss, related to normal aging, have not been described.

Brains of 4-month-old and 25-month-old C57BL/6J mice were used as an aging model. Visual cortices (VC) were analyzed to evaluate whether an AD-like pathology develops during normal aging in this area.

Our results demonstrated intracellular accumulation of Aβ42, Aβ42 deposition in blood vessels, and disturbances in the pattern of p-Tau protein distribution in the VC of 25-month-old mice. This murine model also showed overexpression of the enzymes involved in the production of Aβ42 and an increase in the number of astrocytes expressing GFAP protein in the aged mice compared to the young ones.

2. Materials and Methods

2.1. Animal and Animal Care. Young (4-month-old and 25-month-old) male C57BL/6J mice were maintained on a 12-hour light/dark cycle in a temperature-controlled room, within a clean air box, and food was provided ad libitum (NutriCubo, Purina, USA). The animals were maintained and treated in accordance with the NORMA Oficial Mexicana NOM-036-SSA2-2002, the National Institutes of Health Guidelines for Animal Treatment, and the Ethics Committee of the Asociación para Evitar la Ceguera en México, "Hospital Dr. Luis Sanchez Bulnes" IAP.

2.2. General Procedure. Mice were randomly separated into two experimental groups ($n = 6$ per group). Group 1 was composed of 4-month-old animals and group 2 was composed of 25-month-old mice.

The visual cortices of control and aged mice were obtained for IMHQ assays. Briefly, six animals from each group were transcardially perfused with 4% paraformaldehyde (Sigma-Aldrich Chemie, Germany) in 0.1 M phosphate buffer (J.T. Baker, NJ; PB, Tecsiquim; pH 7.4) for the immunohistochemistry assays. Male 4-month-old and 25-month-old C57BL/6J mice were evaluated in the study. Animals were perfused transcardially with phosphate-buffered saline (PBS) and 4% (w/v) paraformaldehyde under sedation. The brains were postfixed in 4% paraformaldehyde for 20 h and immersed in a 30% sucrose solution (w/v) in PBS for 24 h.

Coronal sections (20 μm) from visual cortex were cut on a freezing microtome (Leica CM3050s) and mounted serially. Slides were used for immunofluorescence detection.

2.3. Immunohistochemistry and Immunofluorescence. Rabbit monoclonal anti-Aβ42 antibody (obtained from Abcam, MA, USA) was used to detect the Aβ42 peptide. Goat polyclonal anti-GFAP and rabbit polyclonal anti-p-Tau and rabbit anti-presenilin-2 (PS2) antibodies were from Santa Cruz Biotechnology (CA, USA). Alexa Fluor 594 goat anti-rabbit IgG (H+L), Alexa Fluor 488 mouse anti-goat IgG (H+L), Alexa Fluor 488 goat anti-rabbit IgG (H+L), and Alexa Fluor 594 mouse anti-goat IgG (H+L) were from Molecular Probes, OR, USA.

For the double immunofluorescence (IF) assays, rabbit monoclonal anti-Aβ42/goat polyclonal anti-p-Tau (dilution 1 : 100) antibodies were used and visualized with Alexa Fluor 594 goat anti-rabbit IgG (H+L) and Alexa Fluor 488 mouse anti-goat IgG (H+L). Besides, rabbit monoclonal anti-PS2/goat polyclonal anti-p-Tau (dilution 1 : 100) antibodies were used and visualized with Alexa Fluor 488 goat anti-rabbit IgG (H+L) and Alexa Fluor 594 mouse anti-goat IgG (H+L). Samples were mounted onto glass slides in VECTASHIELD Medium (Vector Laboratories, Burlingame, CA, USA) containing DAPI. Representative brain sections from each group were processed in parallel next; these sections were examined with an Olympus BX41 Microscope (Japan) and photographed with an Evolution-QImaging Digital Camera Kit (Media Cybernetics, Rockville, MD, USA) for DAB reaction and the double IF assays were observed through a Leica DM-LS epifluorescence microscope at 40x and 100x (Leica Microsystems, Wetzlar, GmbH, Germany). The fluorochromes were visualized with their specific filters and analyzed in three channels.

2.3.1. Image Analysis. Fluorescence pixel intensities were measured in several regions of interest (ROIs) within each image using ImageJ. Average pixel intensities were calculated from five ROIs for measurements in different regions from each image. The measures were realized in six animals per time point. All signal intensities were background-subtracted from the average of three.

2.3.2. Statistical Analysis. All of the data are expressed as mean Chi-square for trends and Fisher's exact tests were used for multiple comparisons. Prism GraphPad software was used (Systat Software, Inc., Point Richmond, CA, USA).

3. Results

We studied the deposition of Aβ42, changes in Tau expression patterns, GFAP overexpression, and the enzymes involved in the production of the Aβ42 peptide in the visual cortex of 4-month-old (4 M) and 25-month-old (25 M) aged symptomatic mice (C57BL/6J WT). To evaluate the overproduction and accumulation of the Aβ42 peptide in the VC, we performed double immunofluorescence assays in brain sections derived from control (4 M) and aged mice (25 M). The double IF assays showed qualitative increases in the intracellular accumulation and blood vessel deposition of Aβ42 (Figures 1 and 2) and an increase in its expression, as well as a change in p-Tau's distribution in the VC (Figures 1, 2, and 3) of 25 M aged mice.

In order to confirm Aβ42 deposition in blood vessels, we repeated the IF to detect Aβ42 accumulation in 4 M and 25 M aged mice. Again, there is an increase of Aβ42 in blood vessels of 25 M old mice (Figure 2).

The Aβ42 peptide is a product of the proteolytic cleavage of amyloid precursor protein (APP). APP's cleavage is done first by beta-secretase, followed by a second cleavage by the gamma-secretase complex. This complex is a multisubunit protease comprised of four components: presenilin-1 and presenilin-2 (PS1 and PS2, resp.), nicastrin, anterior pharynx defective-1 (APH-1), and presenilin enhancer 2 (PEN-2). Among these proteins, PS2 is a transmembranal protein and it has been confirmed as the main enzyme involved in the production of Aβ42 in AD. To evaluate changes in PS2 expression which could be related to the increase in the production of Aβ42, we performed IF assays in the same aging model. In these assays, because PS2 is a membranal protein (as mentioned before), we decided to use p-Tau to mark microtubules to identify the localization of PS2.

As shown in Figure 3, there is an increase of PS2 expression and there are changes in the expression pattern of this enzyme in the VC of aged mice compared to the VC of young mice. Interestingly, we observed changes in the localization of p-Tau protein in aged mice (white arrows in Figure 3).

As shown in Figure 3, there is an increase of PS2 expression and there are changes in the expression pattern of this enzyme in the VC of aged mice compared to the VC of young mice. Interestingly, we observed changes in the localization of p-Tau protein in aged mice (white arrows in Figure 3).

To evaluate whether Aβ42 deposition induces an inflammatory response in aged mice, we performed IF assays to detect astrocytes using a GFAP antibody. GFAP is a commonly used marker for astrocytes, and it has been related to brain inflammation and to the proper functioning of the blood-brain barrier in health. In this aging model, we observed an increase in the number of astrocytes positive to GFAP in 25 M mice (Figure 4). Besides, there were large numbers of astrocytes associated with blood vessels in aged mice.

Finally, to demonstrate these results in a semiquantitative manner, we performed fluorescence intensity quantification on every protein or peptide studied in this report.

As shown in Figure 5, we observed a statistically significant increase in the intracellular Aβ42 peptide (a), p-Tau (b), PS2 (c), and GFAP (d) in the VC of aged mice.

4. Discussion

In the present study, we demonstrated that, during normal aging, in mice, there is an increase of AD-like pathological changes, like intracellular accumulation and blood vessel deposition of Aβ42 in the VC; these increases were correlated with significant overexpression of one member of the gamma-secretase complex (PS2). Besides, GFAP, a common marker for astrocytes, showed an increase in its expression, and the number of GFAP-positive astrocytes also increased, indicating the activation of immunological responses in aged brains.

AD is a neurodegenerative disease with a complex and progressive pathological phenotype that is initially characterized by hypometabolism and impaired synaptic function and, subsequently, by pathological burden [21]. The Aβ42 peptide is the pathological hallmark of AD produced by the sequential cleavage of APP by β-secretase and the gamma-secretase complex. In contrast, the activation of ∂-secretase leads to nonamyloidogenic processing of APP and the generation of truncated nontoxic sAPPa fragments [5, 22, 23]. Neurofibrillary tangles formed by the pathological hyperphosphorylation of Tau protein, an associated-microtubule protein that helps to stabilize microtubules, are the other AD hallmark.

The results presented herein show similar AD pathologic changes and, importantly, this model lacks other factors that could be inducing this pathology. It is important to mention that the amino acid sequence of the Aβ42 peptide in mice does not form amyloid plaques. However, our results indicate an increase of intracellular Aβ42 in the VC of aged mice which could be related to the increased expression of PS2, the main enzyme associated with Aβ42 production in AD [24, 25], and supported by previous studies that have shown that all the components of the gamma-secretase complex increase under stressful conditions, as in AD [26].

It has been reported that Aβ42 can bind to a great number of proteins and to extracellular and intracellular macromolecules that affect normal neuronal function due to increases in the production of hydrogen peroxide, induction of oxidative stress, disturbances in Ca^{2+} homeostasis, and mitochondrial dysfunction (promoting the opening of the

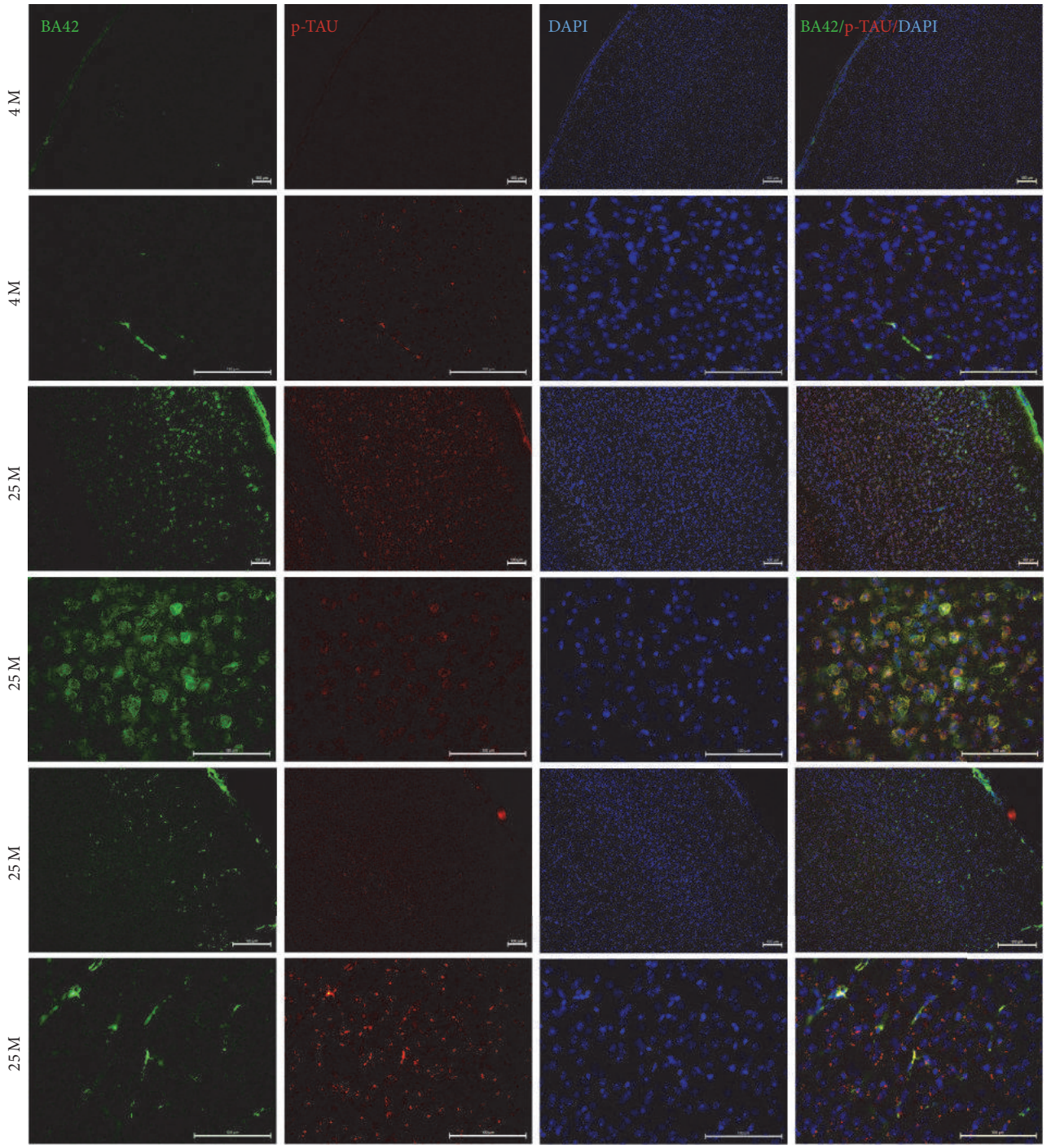

Figure 1: Double IF to detect Aβ42 (green channel) and p-Tau (red channel) in VC of 4 M and 25 M old mice. 15 um thick brain tissue sections of VC from mice were used. DAPI stain for nuclei (DAPI) and Merge are shown. Observe the intracellular accumulation, blood vessel deposition of Aβ42 peptide, and the increase of p-Tau signal in VC from 25 M old mice. Some tissues of mice show more Aβ42 deposition in blood vessels (two bottom last lines). Scale bar: 100 μm.

membrane permeability transition (MPT) pores or disruption of neuronal signal transduction pathways in AD [13, 19, 27–30]); however, it is not known whether Aβ42 accumulation in the VC activates these pathological processes and, as a consequence, they are affecting normal vision. Our results showed an increase of p-Tau, another hallmark of AD. It has been demonstrated that an abnormal increase in p-Tau affects the normal functioning of microtubules, impairs intracellular communication, and, finally, induces cell death [31, 32]. As we observed in Figures 1 and 2, p-Tau not only increases

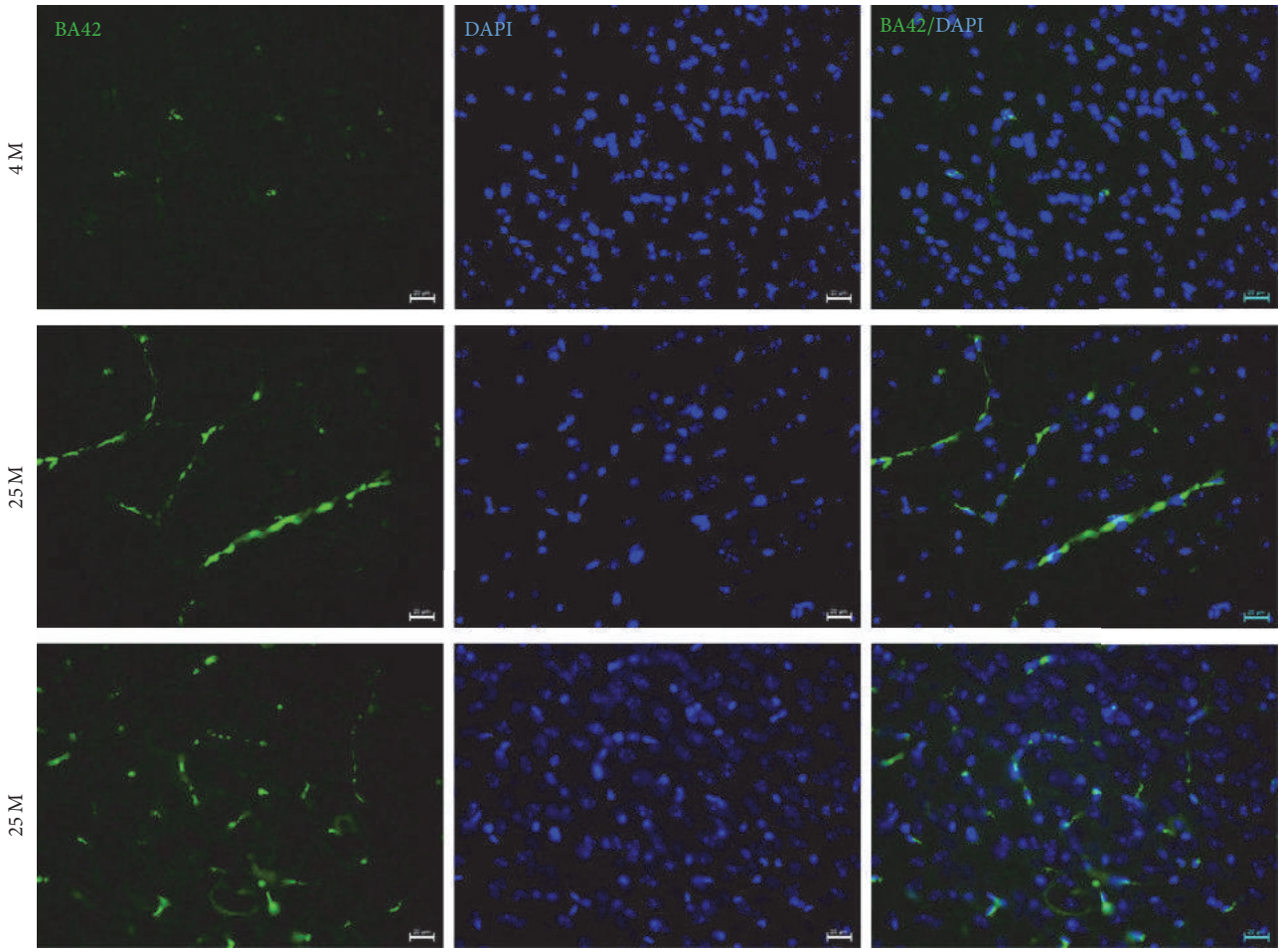

Figure 2: Double IF to detect Aβ42 in the VC of 4 M and 25 M old mice. 15 um thick brain tissue sections of VC from mice were used. Observe Aβ42 accumulation in blood vessels in the VC of 25 M old mice. Scale bar: 20 μm.

its expression but also shows a change of its distribution and localization, suggesting that there may be changes in its intracellular transport in aged mice. More experiments need to be performed to evaluate the impact of this differential expression and localization of p-Tau in the VC during aging.

AD represents a chronic inflammatory state caused mainly by the presence of the Aβ42 peptide. It has been broadly reported that there is an overreactivity of immune cells, such as astrocytes and microglia, during normal aging and AD [33]. Our results show that some GFAP-positive astrocytes are associated mainly with blood vessels in aged mice, and quantification of GFAP reveals that glial response is also prominent in the VC, as observed in AD. This could be relevant, because disruption of the blood-brain barrier in AD and activation of proinflammatory mechanisms (like oxidative stress) due to the presence of Aβ42 have been previously reported. Interestingly, the Aβ42 peptide accumulates in blood vessels in 25 M old mice. This could suggest that these factors are related, and the presence of growing numbers of astrocytes in blood vessels is a protective response to avoid or repair blood-brain barrier damage caused by the Aβ42 peptide's presence in the aged visual cortex.

There are several inflammatory factors in the aging brain which originate from microglia and astrocytes, as they adopt a senescence-associated secretory phenotype [19, 34]. Some aging astrocytes release more cytokines, which is consistent with the aforementioned phenotype [35]. However, more studies are needed to demonstrate the production levels of proinflammatory cytokines in the visual cortex of aged mice. Together, these events contribute to neuronal dysfunction in primary visual areas, supposedly protected from beta-amyloid deposition [36].

In another way, in order to discuss the translation of our findings and their application in human, it is necessary to mention some interesting points about the different animal models used in aging and AD.

The simplest and best model of aging is an old organism. Mouse is an attractive model for studying mammalian biology due to the genetic manageability of its genome, ease of breeding, and the large amount of available baseline phenotypes; they are relatively economical to maintain for long-term aging studies and, more importantly, they are similar to humans genetically and physiologically [37, 38]. These similarities and differences between mouse and man

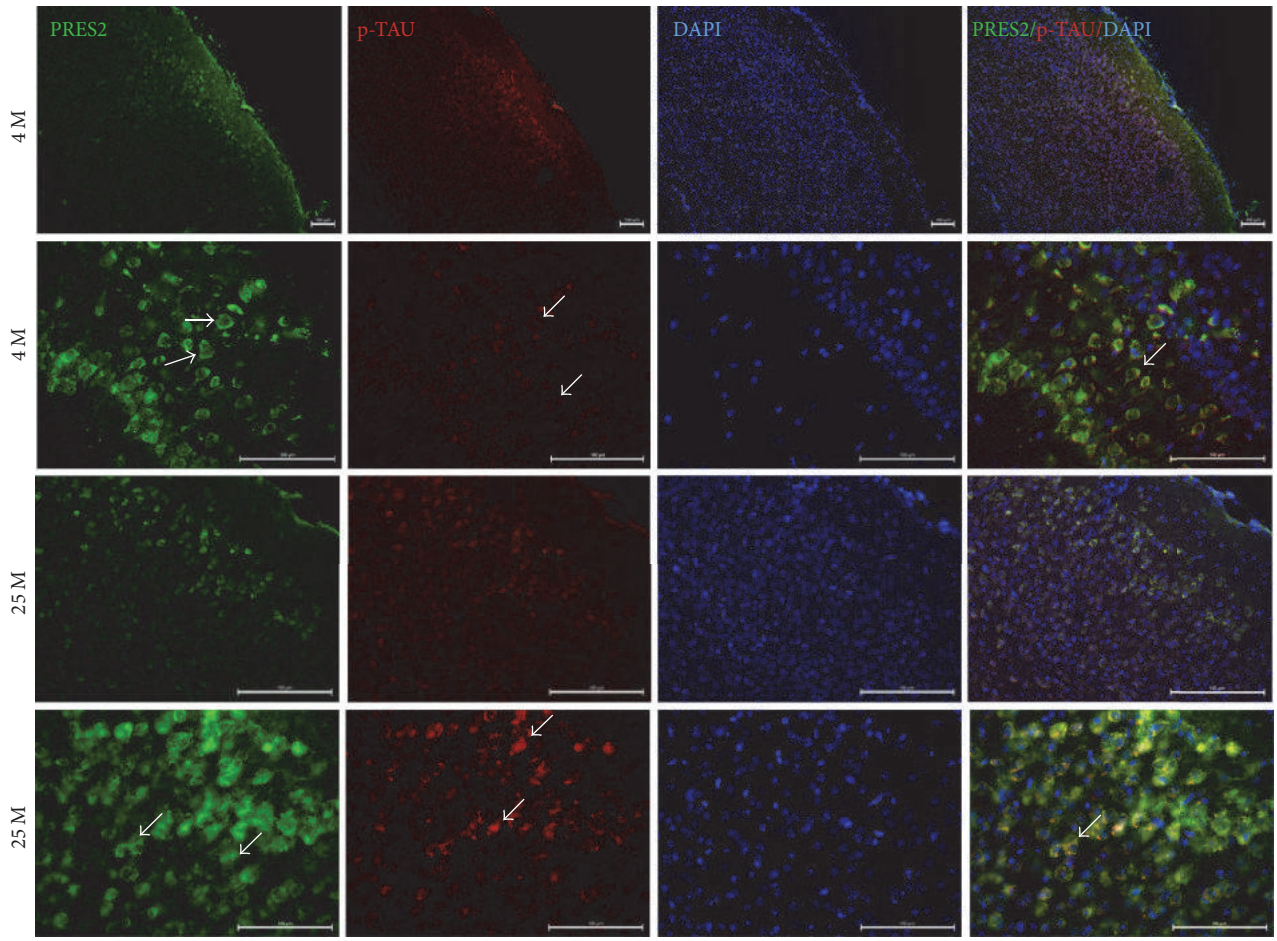

Figure 3: Double IF assays to evaluate the overexpression and changes in the localization of presenilin-2. Observe the increase of PS2 expression and the change in the localization of this enzyme in aged mice (25 M, white arrows). In young mice, PS2 presents a cytoplasmic expression pattern, but, in aged mice (25 M), it is apparently located in the cell membrane (white arrows). Scale bar: 100 μm.

in relation to studies on aging have been extensively reviewed [15, 39–42].

To replicate the pathology of AD in humans, several animal models of AD pathology have been developed. These mouse models have been useful to study the mechanisms involved in the progression of AD and to predict outcomes from pharmacological interventions. No animal model fully replicates the pathogenesis and the cognitive deficits observed in human AD and therefore it is important to understand both the utility and limitations of particular animal models.

According to the recent NIA-AA sponsored consensus reports on three defined stages in a clinical continuum for AD including preclinical and mild cognitive impairment and dementia, the latter is related to the presence and extent of neuropathological changes of AD patients observed at autopsy [43, 44]; we present here these neuropathological changes in healthy aging in mice. Our results suggest that these changes could be affecting the normal vision in AD patients as it has been previously reported.

Although the mouse might not be the perfect model for studying aging, its use as a model of mammalian biology will contribute to gaining important insights into the pathobiology of different diseases, fundamental processes involved in aging, and the relationship between aging and AD. Besides, the validation of these models is totally necessary for a better understanding of the effects of healthy aging and AD on vision loss to translate these advances to humans.

5. Conclusions

Our results show a significant deposition of Aβ42 peptide and overexpression of other AD markers such as p-TAU and GFAP in the VC of WT 25-month-old mice.

We suggest that the overexpression of presenilin-2 observed in our experiments may be one of the first mechanisms involved in beta-amyloid overproduction in our model and that the mechanisms related to neuronal degeneration downstream of Aβ42 accumulation could include

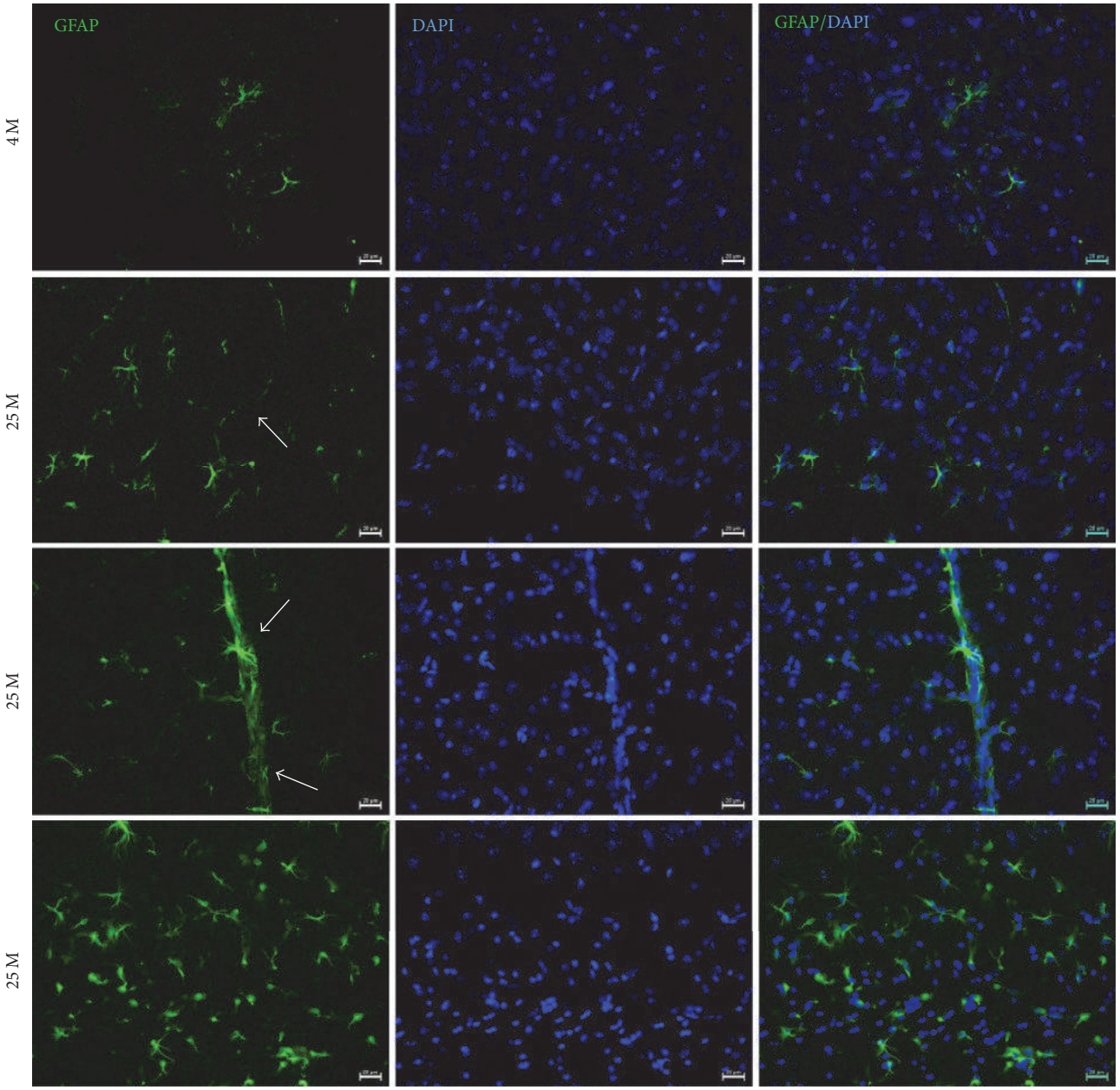

Figure 4: Double IF to detect glial fibrillary acidic protein (GFAP), an inflammation marker (green channel), in the VC of 4 M and 25 M old mice. 15 um thick brain tissue sections of VC from mice were used. DAPI stain for nuclei (DAPI) and Merge are shown. Observe GFAP overexpression and an increased number of astrocytes in the VC of 25 M old mice. Scale bar: 20 μm.

membrane-associated oxidative stress, altered Ca2+ homeostasis, altered energy metabolism, and activation of apoptosis. These findings suggest roles for an alteration of immune responses in the aging process.

Our results demonstrate that aging is possibly related to the activation of the amyloidogenic pathway, which induces Aβ42 overproduction and intracellular accumulation in visual cortex cells; furthermore, we suggest that Aβ42 accumulation affects several important mechanisms to initialize neurodegenerative processes, such as those occurring in AD, and could be related to vision loss.

Our results aid to understand the correlation between aging and the development of neurodegenerative diseases such as AD, but additional studies are needed to further investigate the effect of the Aβ42 peptide on the VC and the retina.

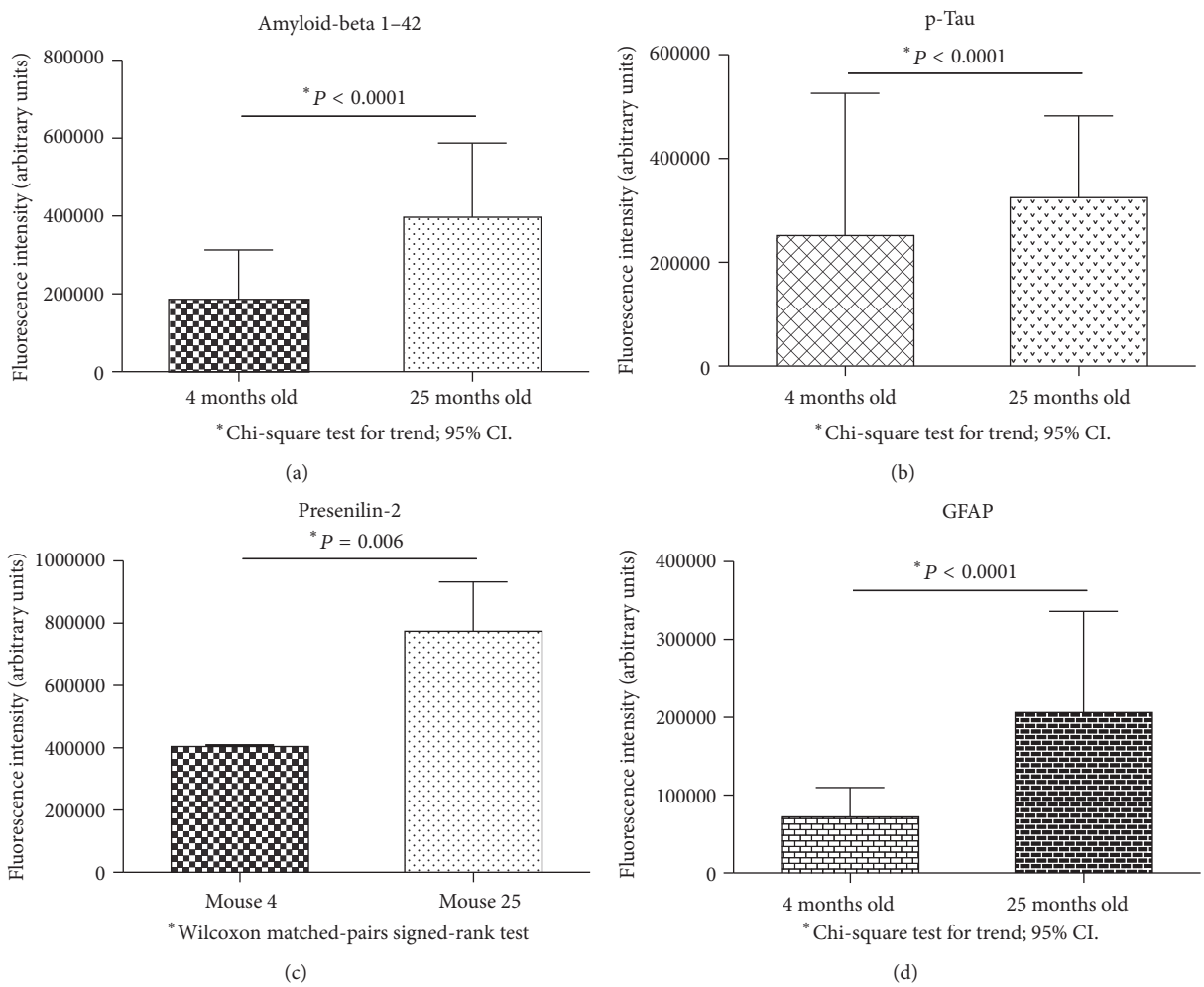

Figure 5: Fluorescence intensities quantification of Aβ42 peptide (a), p-Tau (b), presenilin-2 (c), and GFAP (d) on visual cortex of 4-month-old and 25-month-old mice. For all cases, data were significant. Data are in arbitrary units of fluorescence intensity; $n = 6$ animals per group.

Conflicts of Interest

The authors declare that there are no conflicts of interest.

Acknowledgments

This work is supported by Asociación para Evitar la Ceguera en México, "Hospital Dr. Luis Sanchez Bulnes" IAP. Thanks are due to Mariana Reyes Peña for technical support.

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Hindawi
BioMed Research International
Volume 2017, Article ID 7949582, 12 pages
https://doi.org/10.1155/2017/7949582

Review Article

Central Modulation of Neuroinflammation by Neuropeptides and Energy-Sensing Hormones during Obesity

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Received 26 May 2017; Revised 19 July 2017; Accepted 24 July 2017; Published 23 August 2017

Academic Editor: Anna Di Vito

Central nervous system (CNS) senses energy homeostasis by integrating both peripheral and autonomic signals and responding to them by neurotransmitters and neuropeptides release. Although it is previously considered an immunologically privileged organ, we now know that this is not so. Cells belonging to the immune system, such as B and T lymphocytes, can be recruited into the CNS to face damage or infection, in addition to possessing resident immunological cells, called microglia. In this way, positive energy balance during obesity promotes an inflammatory state in the CNS. Saturated fatty acids from the diet have been pointed out as powerful candidates to trigger immune response in peripheral system and in the CNS. However, how central immunity communicates to peripheral immune response remains to be clarified. Recently there has been a great interest in the neuropeptides, POMC derived peptides, ghrelin, and leptin, due to their capacity to suppress or induce inflammatory responses in the brain, respectively. These may be potential candidates to treat different pathologies associated with autoimmunity and inflammation. In this review, we will discuss the role of lipotoxicity associated with positive energy balance during obesity in proinflammatory response in microglia, B and T lymphocytes, and its modulation by neuropeptides.

1. Introduction

The first line of defense of an organism before any invasion of pathogens or tissue damage is the innate immune system. It includes physical barriers such as the skin, or specific cell types such as macrophages and complement proteins; as a whole, it modulates the inflammatory response. The inflammatory response consists of an innate cellular system and humoral responses that occur during injury, such as exposure to cold or heat, ischemia, and trauma. The inflammatory response can be divided into two types, depending on the cell type and intensity-duration of the stimulus: (1) acute inflammation, characterized by a time window of minutes to hours and by the abundant presence of neutrophils; (2) chronic inflammation, which in time extends from days up to years, and accumulation of lymphocytes in the inflamed tissue predominates. In this context, precise activation of the inflammatory response is coordinated by the involvement of various cell types including recruitment of macrophages and leukocytes, activation of endothelial cells, platelet aggregation, and release of various cytokines including interleukin 1 (IL-1), interleukin 6 (IL-6), and tumor necrosis factor alpha (TNF-α). It is through these events that the body physiologically restores the cellular homeostasis and defends the organism from external injuries [1]. However, despite the sophisticated modulation of the inflammatory response in time and space, the chronic release of inflammatory signals promotes the development of diseases such as cancer, hypertension, cardiovascular disorders, and metabolic disorders including diabetes and obesity.

The link between the immune system and the regulation of body energy metabolism has started to be understood in

the recent years. Initial studies identified selective cellular types for the immune system including pulmonary alveolar macrophages, peritoneal exudate monocytes, and polymorphonuclear leukocytes, which delegate their energy requirement to specific metabolic pathways, depending on the tissue in which they reside. For example, macrophages activate oxidative phosphorylation, whereas monocyte and polymorphonuclear leukocytes are mainly glycolytic [2]. In addition, during an inflammatory event, macrophages increase the catalysis of metabolic enzymes such as hexokinase and citrate synthase in addition to high glucose consumption [3], suggesting an increase in the glycolysis rate during phagocytosis or secretory activity. These studies established the immune system-metabolism relationship in a cellular process called "immunometabolism" [4]. Recently, research has shown that metabolic regulation not only depends on the activation of specific metabolic pathways in a cell type, but that the immune system regulates body metabolism and plays an important role in the development of metabolic disorders such as metabolic syndrome and obesity. Obesity has been characterized as an atypical form of inflammation induced primarily by the accumulation of fatty acids in tissues, altering the metabolic regulation, including liver, adipose tissue, and muscle. This type of inflammation was termed "metainflammation" or "metabolic inflammation" [5].

Positive energy balance during maternal overnutrition or obesity lead to changes in plasma and tissue specific lipidomic profile that might promote inflammation. In fact, saturated lipids have been shown to represent a group of molecules as more active candidates in promoting inflammation through their interaction with toll-like receptors TLR1 and TLR4 and by activating nuclear factor kappa B (NF-κB), a promoter of inflammatory genes [6, 7]. This type of inflammation is not limited to peripheral tissues; it extends to much more distant borders and is able to reach the CNS, promoting the development of neuroinflammation [5]. However, it is also possible that resident brain cells, such as microglia, may induce neuroinflammation independently of their peripheral activation [8]. Paradoxically, modulation of the cytokine-dependent inflammatory signal is controlled by the activation of antagonist cytokines such as IL-10, TGF-β, IL-11, and agonist receptor IL-1, among other cytokines and interleukin soluble receptors. These cytokines function as anti-inflammatory, inhibiting the activation of macrophages, T lymphocytes, and natural cytotoxic cells (NK) [1]. Recent studies have demonstrated the involvement of molecules produced in the CNS in the regulation of inflammatory and energetic metabolism, proposing that neuropeptides are synthesized by macrophages, lymphocytes, and neutrophils to regulate inflammation and metabolism [9, 10]. In this review, we will describe the signaling pathways involved in the process of metabolic inflammation in a scenario of positive energy balance and its modulation by neuropeptides.

2. Lipotoxicity Is a Mediator of Metabolic Inflammation in the CNS

Epidemiological data confirms a strong link between the increase in the level of obesity and the development of type 2 diabetes, indicating that for every kilogram of gained weight, at the population level, there is a linear increase in the diabetes rate [11]. Experimental evidence, in obese humans and animal models with obesity, suggests that the leakage of lipids from adipose tissue and ectopic accumulation of ceramides (a type of sphingolipid), acylcarnitines, diacylglycerols, and saturated fatty acids cause tissue damage to metabolically relevant organs, including the skeletal muscle, liver, pancreatic beta cells, myocardium, and brain, in an event called lipotoxicity [12–14]. The lipotoxic effect is largely determined because every organ has its own lipid profile. Hence, selective changes in lipid species in different organs may be relevant to the development of lipotoxicity. In this context, it is known that, physiologically, C18:0 type ceramides are essential for cerebellar development and C22:0 and C24:0 ceramides regulate hepatic function [12, 13], while saturated diacylglycerols and lipids take part in intracellular signaling processes in many cellular types of the body [14]. In this regard, it has been suggested that, during obesity, new lipid species, which are potentially toxic for the body's organs, are produced, including ceramides, cholesterol, saturated fatty acids, and diacylglycerols. All these species are known to inhibit insulin sensitivity in cellular cultures and animal models [15]. Saturated ceramide and lipid accumulation has even been detected in the skeletal muscle of obese humans, which correlates to insulin resistance [15]. Recent evidence has shown substantial association between lipidomic profile leading to lipotoxicity and activation of neuroinflammation.

Previously, the brain was considered an immunologically privileged organ, partly because the lack of constitutive expression of MHC class I and class II and the absence of classical antigen-presenting cells (APCs) and lymphatic vessels. However, the identification of peripheral immune system cells including B cells and T lymphocytes in genetic and nutritional models of obesity has proposed that the metabolic inflammation observed during obesity is able to colonize the CNS and promote central inflammation such as microglia activation [1]. Each of these cell populations will promote an inflammatory state through the secretion of antibodies and interleukins [16, 17]. We will now describe some of the cell populations that have been implicated in the process of neuroinflammation activation in a lipotoxic context during obesity.

2.1. Microglia. Microglia represent a selective cell type with characteristics of CNS resident macrophages, which originate from erythromyeloid progenitors derived from yolk sac cells during the embryonic stage and which subsequently colonize the brain during embryonic development [18, 19]. Physiologically, their activation is required for the proper functioning of the CNS as they positively modulate neurogenesis and synaptic plasticity in addition to acting as major APCs in the CNS [20].

The relationship between lipotoxic damage in the context of obesity and the activation of central inflammation is based on evidence showing that the exposure of high fat diet in rodents promotes inflammation in the CNS that culminates as damage to the regions of the hypothalamus, cognitive deterioration, and decreased neurogenesis [20, 21].

Molecularly speaking, it is proposed that, similar to macrophages that regulate innate inflammatory activation in the peripheral system in a lipotoxic context, the activation of microglia in the brain is induced by the interaction of fatty acids with TLRs. In fact, lauric acid (C12:0) and palmitic acid (C16:0) lipids have been identified as inducing TLRs migration to lipid rafts, heterodimerization of TLR1 and TLR2 receptors, and the homodimerization of the TLR4 in macrophages [22, 23]. In addition, experimental evidence has identified the recruitment of the MyD88 protein and NADPH to these domains correlating with the production of reactive oxygen species (ROS) [22–24]. Our research group has shown that the stimulation of neurons with palmitic acid recruits the inflammatory related serine/threonine-protein kinase TANK-binding kinase 1 (TBK1) to lipid raft domains, which correlates with insulin resistance [25]. These experiments demonstrate that activation of TLR4 participates in the secretion of inflammatory cytokines via the IKK-NF-κB pathway in microglia, inducing alterations in the hypothalamus and other regions of the CNS [17, 21, 26], and potentially the recruitment of monocytes from the periphery dependent on the increase of TNF-α [27]. Taken together, exposure of saturated fatty acids favors the interaction and dimerization of TLR1, TLR2, and TLR 4 towards the lipid rafts microdomain, recruitment of NADPH oxidase and MyD88, and production of ROS, where it will be activated in parallel NF-κB by the IKK and possibly TBK1. Thus, NF-κB-dependent transcriptional activation will promote the secretion of inflammatory cytokines TNF-α, IL-β1, and IL-6 and altered metabolic profile, as we have recently proposed [28].

2.2. T Lymphocytes. The role of T lymphocytes as mediators of metabolic inflammation was initially reported in 2009. Exposure of mice to high fat diet promotes the infiltration of $CD8^+$ T lymphocytes into adipose tissue favoring the infiltration of M1 macrophages with inflammatory profile and generating insulin resistance, while its inactivation by specific antibodies represses this phenomenon [29]. Inflammatory activation in the adipose tissue of obese mice is potentiated by two possible scenarios: (1) by reducing expression of the transcriptional factor Foxp3 in T_{reg} lymphocytes [30], cells responsible for regulating the inflammatory response and suppressing autoimmune reactions [31] and (2) by activating a proinflammatory subtype of $CD4^+$ T helper cells called Th1 [32]. Activation of these molecular pathways have been widely identified in the generation of insulin resistance and type 2 diabetes mellitus in obese subjects. In fact, it has been proposed that the activation of T lymphocytes in adipose tissue is a key event and depends on the presentation of antigens by MHC class II in $CD4^+$ T cells and a costimulatory signal [27]. This mechanism has been described to promote the synthesis of IL-2, where additional interaction of TRC-MHCII is required for the activation of the coreceptor CD28. Also, the TRC-MHCII interaction recruits the Zap70 protein to the CD3 coreceptor allowing the activation of the PLCγ-PIP2-PKCθ cascade, downstream activation of ERK, and c-Fos expression. On the other hand, the CD28 coreceptor via the PI3K pathway activates MEKK and JNK allowing the production of c-jun. Both TCR and CD28 lead to the transcriptional factor AP-1 nuclear translocation, inducing the expression of IL-2 [31]. In addition, the TCR-CD28 binomial in $CD4^+$ T lymphocytes promotes PKCθ to activate the CARMA1-Bcl10-MALT1 complex by inducing the activation of NF-κB and TBK1 and the proliferation, differentiation, and production of IL-2, dependent on AP-1 [33, 34]. Thus, in a lipotoxic context, we might suggest that the interaction of $CD4^+$ T lymphocytes with an antigen-presenting cell would allow differentiation towards the Th1 subtype by altering the Th1/T_{reg} ratio towards proinflammatory, interferon-producing (INF-γ) T cells, IL-2, and TNF-α and decreased IL-10 production activity of T_{reg} cells. All this would allow the polarization of macrophages to the M1 phenotype producing proinflammatory cytokines TNF-β, IL-1β, and IL-6. On the other hand, TCR-MHCI interaction by $CD8^+$ T lymphocytes could secrete MCP1 exacerbating the recruitment of macrophages to adipose tissue and increasing inflammation.

Activation and recruitment of lymphocytes to adipose tissue during positive energy balance in obesity also cause them to migrate to more distant borders and interact with CNS cells, including microglia, as described for various pathologies such as experimental autoimmune encephalomyelitis and cerebral ischemia [33, 35]. In a summarized way they involve the attraction of T cells to the site inflamed by chemokine such as Interferon-Inducible T-Cell Alpha-Chemoattractant (I-TAC), interferon gamma-induced protein 10 (IP-10), and monokine induced by gamma interferon (MIG) [36], expression of the E and P selectins in endothelial cells that serve as anchor for their ligands in T lymphocytes, PSGL-1, and α4-integrin, facilitating the transport of lymphocytes through blood vessels (Figure 1). Finally, the ultimate barrier for the invasion of T lymphocytes into the nervous system is represented by the blood-brain barrier (BBB), which, by expressing the LFA-1 membrane protein in T cells, can bind to the ICAM-1 protein endothelial cells and cross the BBB leaving morphologically intact narrow junctions. This has been corroborated in recent studies, showing the inhibition of the expression of these adhesion proteins, reducing the infiltration of T lymphocytes into the brain during an inflammatory event [37, 38], which is presumably regulated by poly(ADP-ribose) polymerase-1 (PARP) [38]. Despite the evidence supporting the infiltration of T lymphocytes into the nervous system, molecular and cellular mediators that mediated the communication between the brain and the immune system remained unidentified. Maybe the first evidence to support CNS-peripheral immune system cross-talk was recently identified by showing that selective inflammatory stimulus (IL-1β) into the CNS and astrocytes secrete extracellular vesicles (EV), which cross the BBB and reach organs such as the liver allowing the suppression of PPARα and favoring the production of TNF-α and IL-β1 and monocyte chemoattractant protein-1 (MCP-1). Cytokines production promotes the T lymphocytes recruitment into the inflamed brain region [39] (Figure 1).

2.3. B Lymphocytes. B lymphocytes represent a cell type of the immune system, originating from hematopoietic cells

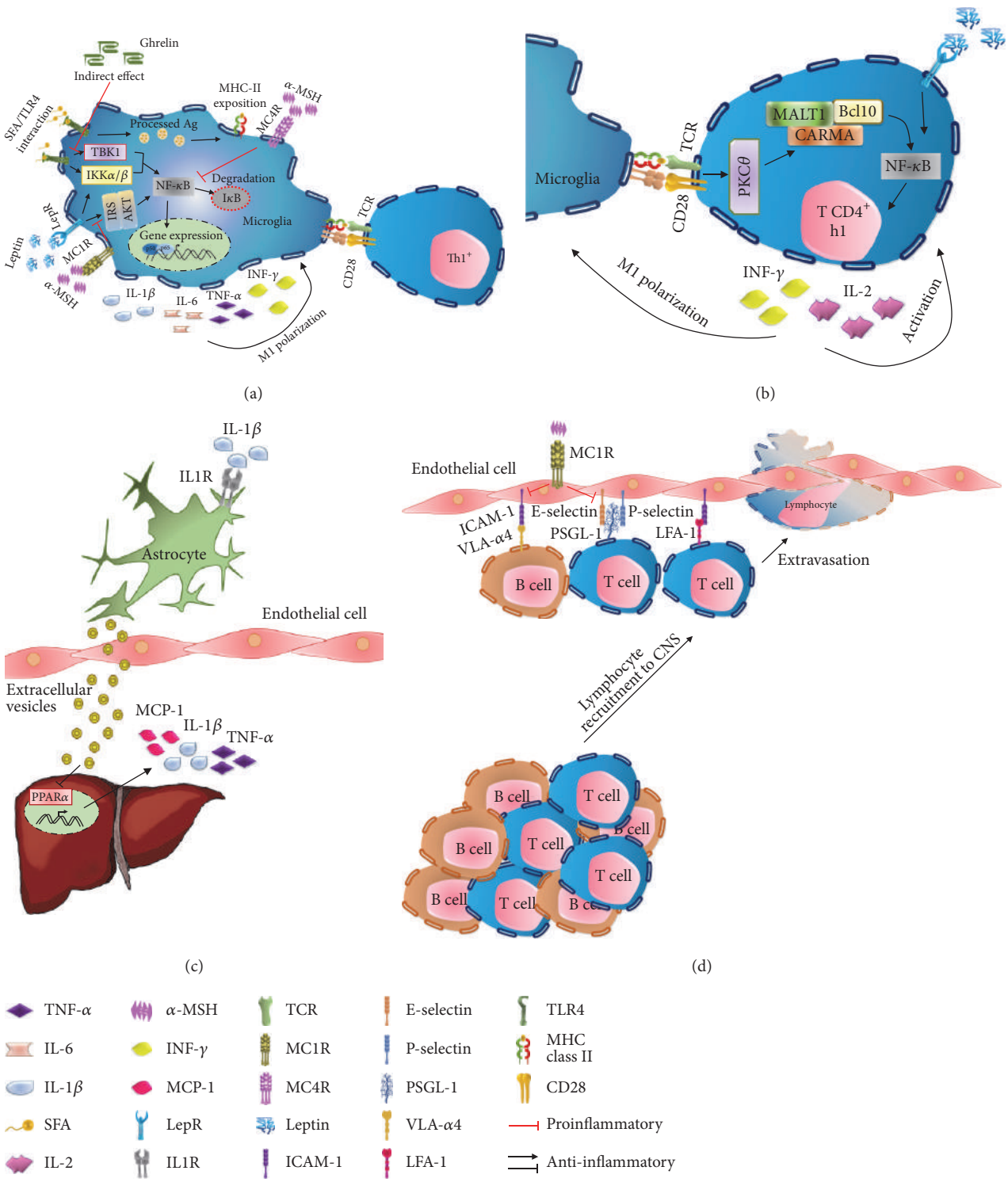

FIGURE 1: *Immunomodulatory mechanism exerted by neuropeptides in microglia exposed to a lipotoxic stimuli.* (a) *Microglia pro- and anti-inflammatory stimuli.* In microglia, fatty acids and leptin can induce cytokine secretion through TLR4/IKK/NF-κB pathway, but only leptin can activate NF-κB through LepR/IRS1/AKT pathway. Also, leptin induces MHC class II expression leading to T lymphocytes activation. Cytokines have paracrine and autocrine effects. α-MSH inhibits the IκB degradation through MC4R and by blocking the LepR pathway through MC1R. Ghrelin blocks the TLR4/IKK/NF-κB pathway activation in microglia cells by indirect effects. (b) *T lymphocyte activation.* Microglia presents the antigen to CD4+ T cells and through the receptor complex MHCII/B7-TCR/CD28 these cells proliferate to the proinflammatory phenotype Th1 which produce IL-2 and INF-γ through PKCθ-CARMA-MALT1-Bcl10/NF-κB complex and by leptin action. (c) *Astrocytes inflammatory mechanism.* IL-1β induces the secretion of extracellular vesicles which inhibits PPARα expression on hepatocytes leading to TNF-α, IL-1β, and MCP-1 production facilitating lymphocyte infiltration to CNS. (d) *Lymphocyte extravasation to CNS.* Inflammatory signals such as cytokines and CMP-1 promote the expression of adhesion proteins E-selectin, P-selectin, and ICAM-1. Lymphocytes can interact with the adhesion proteins through its own integral proteins VLA-α4, PSGL-1, and LFA-1 and cross the BBB. Melanocortins prevent T-cell infiltration by the α-MSH-MC1R interaction which blocks externalization of adhesion proteins.

that mature in bone marrow and participate in innate and adaptive immunity. Their main function is the production of antibodies against foreign antigens into the body [40]. In addition, they also function as APCs by presenting the antigens to T lymphocytes that were initially captured by their B-cell receptor and promote inflammation by secreting proinflammatory cytokines such as IL-6 and TNF-α, thus favoring the polarization of T cells into a proinflammatory phenotype [40]. The molecular mechanisms that promote the migration of B lymphocytes to the CNS are not yet fully understood; however, it has been proposed that its association with membrane proteins, such as the α-4 subunit of VLA-A, ICAM-1, and ALCAM, allows passage through the BBB [41, 42].

Unlike the role of macrophages and Th1 lymphocytes in the modulation of metabolic inflammation during obesity, the impact of B lymphocytes in this context has not yet been fully understood [43]. However, there are several reports that justify its presence and potential participation in the modulation of inflammation at the CNS level. In the first instance, B lymphocytes possess TLR capable of responding to microbial antigens in a T lymphocyte-dependent manner [44], which have been identified to actively mediate metabolic inflammation by interacting with fatty acids [23, 26]. In fact, the accumulation of antibodies of the IgG class in the microglia of the ARC nucleus has been observed in mice exposed to a hypercaloric diet, polarizing it towards the M1 phenotype through its Fc receptor [45]. B lymphocytes itself from obese mice might produce a proinflammatory IgGc class which, when administered to mice deficient in B cells, increases the production of proinflammatory cytokines, the polarization of M1 macrophages, and the activation of T lymphocytes [16]. These studies confirm that the function of B lymphocytes in a metabolic compromise scenario seems to be deleterious and promotes metabolic inflammation, leading to the belief that its inhibition could prevent this mechanism. Experimental data demonstrate that this hypothesis is partially true, since the elimination of B lymphocytes using a CD20-specific antibody in a murine model of obesity induced by high fat diet improved glucose tolerance, reduced insulin levels, and reduced the inflammatory profile in adipose tissue. However, the total elimination of B and T lymphocyte populations has no effect [46]. In this scenario, defects in B-cell function have been reported in situations of metabolic compromise as presented in diabetic and nondiabetic obese patients. Subjects with this metabolic profile show a low response to antibodies and secrete a greater amount of IL-6 and TNF-α than healthy subjects, and only obese and diabetic patients have a decrease in the production of IL-10, a key cytokine in the suppression of the immune response mediated by B cells [37, 47]. In this way, it is possible that, in a lipotoxic context, the interaction of B lymphocytes with fatty acids or their recruitment to the CNS by glial cells [48] plays a key role in metabolic inflammation, through secretion of inflammatory cytokines, the activation of $CD4^+$ T lymphocytes, and microglia polarization towards the M1 phenotype, through MHC class II mediated antigen presentation and the Fc fraction of the antibodies, respectively (Figure 1).

3. Energy-Sensing Hormones and Neuropeptides Modulate Central Inflammation

Neuropeptides are small molecules composed of amino acids, produced mainly but not exclusively by cells of the nervous system, and regulate important physiological processes, including reproduction, feeding, regulation of body weight, memory, anxiety, mood, excitement, reward, and sleep/wake stages [49]. Anti-inflammatory properties of various neuropeptides have been identified in the context of positive energy balance, which include alpha-melanocyte-stimulating hormone (MSH-α), vasoactive intestinal peptide (VIP), and neuropeptide Y (NPY) [10, 50, 51]. There is also evidence of the involvement of hormonal signals dependent on ghrelin and leptin on the modulation of an anti-inflammatory phenotype in microglia [49, 52]. In the next section, we will describe evidence of the involvement of peptides derived from prohormone proopiomelanocortin (POMC) and the ghrelin and leptin hormones as potential central modulators of microglia-dependent inflammation in a context of positive energy balance.

3.1. Peptides Derived from POMC. Melanocortins are post-translational products of the POMC gene which is expressed in the arcuate nucleus (Arc) of the hypothalamus, from which a family of opioids and melanocortins products are synthetized including β-endorphin, adrenocorticotropic hormone (ACTH), and the melanocyte stimulating α, β, and γ hormones (MSH). This neuropeptide system is unique since its regulation depends on two small endogenous proteins, the peptide-like agouti and the Y neuropeptide [53, 54].

The ability of melanocortins as anti-inflammatory agents is well documented in different models of peripheral inflammation [55]. Melanocortins exert their action through their interaction with the MC1R receptor located in immune cells innate neutrophils, macrophages, and dendritic cells also in microglia, in addition to possessing a high affinity towards MSH-α. The administration of MSH-α has shown to reduce the production of IL-1, IL-6, and TNF-α, and monocyte receptor expression is upregulated in the presence of stimuli such as lipopolysaccharides (LPS) or cytokines. At the CNS, systemic administration of MSH-α has been reported to reduce cytokine expression during cerebral ischemia and decrease inflammation at the hippocampal level by inhibiting LPS or IL-1β induced dinoprostone (PGE2) secretion. MSH-α also reduces the production of nitric oxide (NO) and prostaglandin (PG) favored by IL-1β in the hypothalamus of rats [56]. In the past decade, MC3R and MC4R receptors have been proposed as responsible for the anti-inflammatory action of melanocortins in the brain. This proposal is based on studies demonstrating that the expression of these two receptors is higher in comparison to the other members of this group [57] and that the administration of MSH-α reduces the hypothalamic production of iNOS and COX2 in rats administered with LPS and decreases the expression of TNF-α induced by LPS and INF-γ in neurons expressing the MC4R, whose effect is blocked by the administration of the MC4R antagonist [58].

Scientific evidence suggests that melanocortins exert their anti-inflammatory activity by inhibiting the transcription factor NF-κB [59] and by inducing IL-10 in microglia through the MC4R receptor. In astrocytes, both brain-derived neurotrophic factor (BDNF) and peroxisome-proliferator-activated receptor gamma (PPARγ) expression have been observed to be regulated by the MC4R-cAMP-PKA-CREB pathway [60]. On the other hand, activation of the MC1R receptor using the pharmacological agonists MS05 and MS09 is able to reduce the expression of E-selectin and VCAM, in addition to reducing the activation of NF-κB in endothelial cells exposed to TNF-α. Knowing that E-selectin and VCAM represent integral membrane proteins important for the migration of B and T lymphocytes towards the site of inflammation [41], it is proposed that the blockade of the extravasation of these cells represents an anti-inflammatory mechanism parallel to that described by melanocortins. Finally, there are reports that have shown that activation of the MC1R receptor represses the leptin-dependent inflammation in a lipotoxic context [61, 62]. Thus, it is tentative to propose that, in a lipotoxic scenario, melanocortins block the inflammatory process by four main events: (1) increase the secretion of IL-10 from the microglia, (2) decrease the activation of NF-κB, (3) block the action proinflammatory effects of leptin, and (4) prevent infiltration of lymphocytes through the BBB to the CNS.

3.2. Ghrelin. It is a peptide of 28 amino acids secreted mainly by the stomach and duodenum, although it is also produced by neurons in the arcuate nucleus [63]. However, overproduction of ghrelin in the hypothalamus promotes food consumption and increases body weight [64]. Ghrelin seems to induce acute peripheral insulin resistance independent of growth hormone (GH), cortisol, and basal serum free fatty acids [65] and both insulin and ghrelin exert regulatory effects on each other [66, 67]. Two types of ghrelin, des-acyl-ghrelin (DAG) and acyl-ghrelin (AG), are known to regulate food intake and growth hormone secretion and influence glucose homeostasis, neuroprotection, memory, immunity, and neuroinflammation [63, 68]. Its main function is to act as an orexigenic signal by antagonizing the effects of leptin via the NPY/Y_1R axis, through its interaction with the growth hormone secretagogues receptor (GHSR) in NPY and AgRP neurons.

Obesity is known to promote an imbalance in the hormonal profile of obese individuals. Changes in the AG/DAG ratio in obese and metabolic abnormal Italian children compared with normal weight children have been reported [69]. The authors found a 81% increase in AG in obese and metabolic abnormal children when compared with healthy children [69]. Also, recently it has been documented that AG concentrations are higher in plasma of obese patients (435 pg/mL) than nonobese patients (167 pg/mL) [70]. Although it has been stated that during obesity ghrelin plasma levels are decreased in obese individuals as a compensatory mechanism to reduce appetite [71, 72], it only refers to total ghrelin in plasma, given that AG depicts 10% of total ghrelin. In this context, decreasing levels of this hormone may be potentially related to a decrease in DAG concentration [70]. In fact, diet induced obesity (DIO) in mice by high fat diet exposure leads to 15% increase in preproghrelin mRNA-producing cells than control [73]. In addition, both DIO and ob/ob mice model had normal plasma levels of ghrelin which correlates with a decrease in DAG plasma levels [73]. It is known that DAG is metabolized to AG by action of the ghrelin O-acyltransferase (GOAT); not only does the importance of this enzyme lie in its ability to acetylate the unacetylated form of ghrelin, but it has been reported that knocking down the GOAT gene protects mice from obesity induced diet, improves insulin sensitivity, and reduces adiposity when fed HFD and high glucose diet [74]. Furthermore, there is a positive correlation between body mass index (BMI) and GOAT concentration in obese patients, where BMI > 50 had increased concentrations (+34%) compared with normal weight controls [75]. These evidences suggest that an alteration in the AG/DAG ratio related to GOAT activity is potentially important to contribute to metabolic alterations observed during obesity and diabetes. This proposal is tested in recent reports showing that decreasing AG plasma levels associates with positive effects in metabolic disorders, such as decreasing postprandial glucose levels and improvement of insulin sensitivity in overweight patients with type 2 diabetes [76, 77].

On the other hand, the anti-inflammatory and neuroprotective properties of ghrelin have been demonstrated in experimental cord injury (SCI) models, where the administration of ghrelin inhibits the activation of the p38 AMPK/NF-κB pathway followed by the release of the factor of nerve growth (proNGF). These data were corroborated in in vitro models showing that ghrelin stimulation prevented the activation of the AMPK and JUN signaling pathway in addition to reducing the production of ROS in microglia stimulated with LPS [78]. Other studies demonstrated that the intracerebroventricular administration of ghrelin reduces the mRNA expression of the proinflammatory cytokines IL-1β, IL-6, TNF-α, INF-γ, and iNOS in the blood of rats subjected to a 70% calorie restriction by one week [79]. However, it appears that this mechanism is independent of the GHSR1α receptor, since this receptor is not expressed in the resident microglia of the brain and spinal cord or in primary culture. In this context, ghrelin might potentially act by blocking the expression of the matrix metalloproteinase 3 (MMP-3) on dopaminergic stressed cells [80]. In addition to this, ghrelin has been proposed as a neuroprotective agent by decreasing the production of proinflammatory cytokines, IL-1β, IL-6, TNF-α, iNOS, and ROS, by microglia in models of amyotrophic lateral sclerosis, neurotoxicity, neuronal death induced by kainic acid, experimental autoimmune encephalomyelitis, Parkinson's, and Alzheimer's [49, 81]. In addition, it blocks the activation of the microglia and reduces infiltration of T lymphocytes towards the spinal cord against a challenge with LPS [78, 82]. Ghrelin also prevents the differentiation of a proinflammatory T-cell subtype, termed Th17, by blocking the activation of the mTOR/STAT3 pathway [83]. In relation to diseases closely related to metabolism, ghrelin has been linked to attenuation in the activation of the TLR4/MyD88/TRAF6/NF-κB pathway and cell death in pheochromocytoma cells (PC12) in a model of diabetic

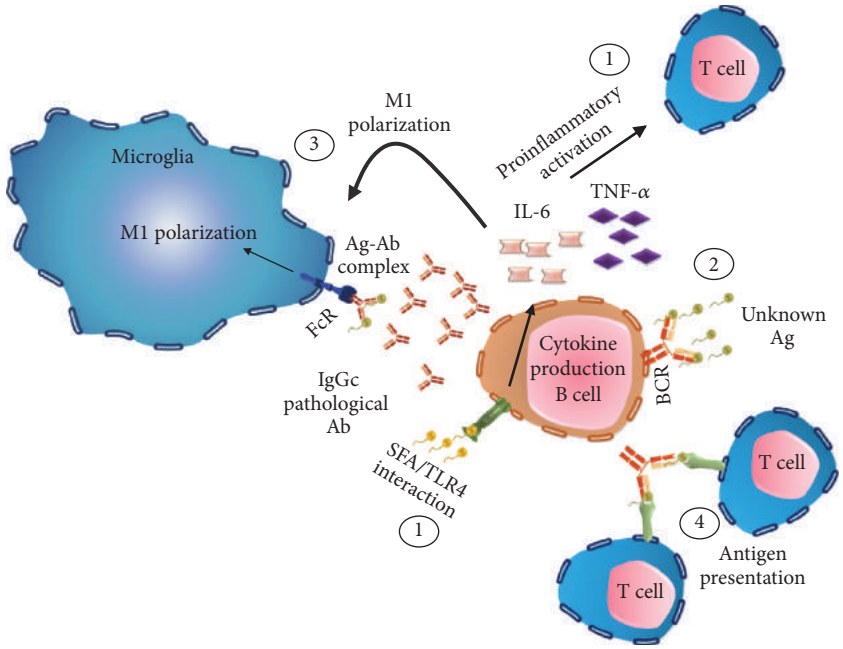

Figure 2: *B lymphocytes regulate neuroinflammation*. (1) Saturated fatty acids induce the secretion of proinflammatory cytokine through the interaction with the TLR4 located in B lymphocytes, favoring the polarization of lymphocytes and microglia activation to a proinflammatory phenotype. (2) Production of a pathogenic IgG class antibody (Ab) regulated by an unknown antigen (Ag). (3) Microglia M1 polarization trough the Ab-Fc receptor interaction. (4) B cells receptor (BCR) mediated antigen presentation.

encephalopathy [84]. Taken together, these data allow us to hypothesize that ghrelin plays an important role in the regulation of metabolic inflammation in the CNS by modulating the secretion of proinflammatory cytokines by microglia.

3.3. Leptin. Leptin is a peptide hormone with a molecular structure similar to interleukins, composed of 146 amino acids, its synthesis is primarily in adipocytes, and it travels through the circulation to reach the CNS, where it interacts with the leptin receptor (LepR) located in hypothalamic neurons and regulates food intake through an anorexigenic signal [85]. It is also involved in hematopoiesis, angiogenesis, and glucose metabolism and has a proven role in cells innate and adaptive immune system [85]. In normal human patients, leptin plasma levels are around 21.6 pg/mL, whereas obese patients show higher concentrations (61.9 pg/mL) [70] Experimental data show that the LepR has the ability to activate signaling pathways associated with inflammatory profiles including those of IL-6, the JAK-STAT, and MAPK-PI3K pathway, regulates the production of IL-2 and INF-γ in Th1 lymphocytes, and reduces the production of anti-inflammatory IL-10 cytokine [86]. Furthermore, it appears that leptin and swelling ratio is positive feedback type as systemic injection of LPS to mice increases the concentration of mRNA in adipose tissue leptin mice and induces the secretion of IL-1β; besides TNF-α and IL-1 cytokines regulate the expression of leptin. Additionally, the inflammatory effect exerted by leptin is dependent on modulation at the level of adhesion molecules expression such as ICAM-1 and VLA2 in $CD4^+$ cells, preventing proliferation of suppressor cells of the immune response type T_{reg} Foxp3 [31, 87]. The role of this hormone on the proinflammatory action of B lymphocytes seems limited to increase phosphorylation STAT3, a crucial mechanism in the production of TNF-α, accompanied by low phosphorylation in AMPK, crucial for the activation of E47 through phosphorylation of p38 AMPK; and inhibiting apoptosis in a mice model exposed to HFD [88]. The proinflammatory effects of leptin on microglia are by nature proinflammatory and induce secretion of IL-1β cytokine-dependent stimulation with LPS, by a mechanism independent caspase 1, IL-6 by the pathway IRS1-PI3K-AKT-NF-kB and TNF-α, and CINC-1 MIP-2 chemokines [89]. In fact, IL-6 has proved to have a sensitizing action to leptin in hypothalamic neurons in obese animals by exposure to high fat diet [90]. It has been observed that in microglia deficient mouse leptin (ob/ob) there is a downregulation in genes integrin-alpha X (Iigax), NALP3, and molecule F4/80, important for correct development of the inflammatory response and T-cell differentiation regulated by APC [91] (Figure 2).

4. Potential Treatments for Inflammation in Metabolic Related-Diseases

Obesity is a worldwide health problem showing failure in pharmacologic and therapeutic interventions to ameliorate its metabolic complications. Diet might show the first potential avenue to modulate this pandemic. It is widely reported that polyunsaturated fatty acids (PUFAs) had beneficial effects on several metabolic related-diseases, such as obesity. For instance, omega-3 fatty acids (ω-3 PUFAs) inhibit mammary tumor progression in obese mice [92], like wise

this fatty acids has anti-inflammatory effects in the adipose tissue and hypothalamus [93, 94], and protect against insulin resistance and dyslipidemia by suppressing the activation of TLR4 [95, 96]. The key receptors involve in this beneficial effects are the G protein-coupled receptor (GRP), specifically the selective GRP40 and GRP120, which has been proposed has possible therapeutic targets for insulin resistance and metabolic inflammation [97, 98]. In fact, administration of a GRP40 agonist (Yhhu4488) promotes high expression of glucagon-like peptide-1 (GLP-1), decreased fasting blood glucose level, improved β-cell function and lipid homeostasis in type 2 diabetic ob/ob mice [99]. Also, GRP120 stimulation by a selective agonist improved glucose tolerance, decreased hyperinsulinemia, increased insulin sensitivity and decreased hepatic steatosis in a DIO mice model [100]. Of note, the role of GRP40 and GRP120 might be potentially relevant given that both show high expression within the hypothalamus and the combined activation of both receptors results in better metabolic outcomes [98].

Another possible neuroinmmunometabolic target are the kinases TBK1 and IKKε related to inflammatory pathways. These proteins had been reported to participated in the development of insulin resistance and diabetes [25, 101]. In addition, we proposed recently that TBK1 may have a significant role in the microglia-mediated neuroinflammation observe during obesity [28]. In fact, it has been demonstrated that the administration of an specific TBK1-IKKε inhibitor (amlexanox), reduces weight, insulin resistance, fatty liver and inflammation, as well as increased energy expenditure [102]. Together, these data suggest that dual-specificity inhibitors of IKKε and TBK1 may be effective therapies for metabolic disease in an identifiable subset of human patients [103].

Finally, the ghrelin system might represent another possible molecular target for immunomodulation. The administration of des-acyl-ghrelin analog (AZP531) prevent dysregulation of glucose homeostasis in C57BL/6J mice exposed to a HFD [104]. Furthermore, chronic exposure to an inhibitor of AG secretion (CF801) decreased weight gain and adiposity without affecting caloric intake [105]. More recently, a synthetic triterpenoids has been proposed as a potential therapeutic agent to treat diabetes and obesity., due to its ability to inhibit ghrelin acylation by the human isoform of GOAT (hGOAT), these compounds function as covalent reversible inhibitors of hGOAT [106]. Thus, blocking proinflammatory signals through GRPs or nuclear factors inhibitor such as TBK1, and reducing AG plasma levels, might be potential pharmacologic treatments to obesity and metabolic disorders.

5. Conclusions

We contemplate that the activation inflammation associated central lipotoxicity in a scenario of positive energy balance is dependent on time and intensity of the stimulus. At early times of ingestion of a high fat diet, lipids interact with toll-like receptors (SFA-TLR4) activating inflammatory pathway MyD88/IKK/NF-κB and possibly the TBK1 protein, initiating secretion of proinflammatory cytokines IL-1β, IL-6, TNF-α and INF-γ. Meanwhile, in a parallel scenario, increased inflammation at the level of adipose tissue promotes increased concentration of leptin in the plasma and by promoting the expression of adhesion proteins on the cells of the BBB, as ICAM-1, VLA-2 and ALCAM, cells might recruit peripheral immune into the CNS by the action of IL-1β. In particular, leptin increase might sensitize microglia subsequent to proinflammatory stimuli and will induce expression of MHC class II and expression of IL-1β. At late, cells such as B and T lymphocytes and macrophages could infiltrate the CNS, where microglia would serve as an APC cell to T-cell and by TCR-CD28/MHCII interaction, might recruit the complex CARMA1-Bcl10 -MALT1, allowing activation NF-kB and IL-2 secretion. In this state T cells could be placed in a state of Th1 type secreting cell or inflammatory cytokines. Likewise, a reduction would be expected in the production of IL-10 because of the action of the INF-γ and SFA on T_{reg} cells. B cells attracted to the CNS begin producing IgG toxic antibodies that will accumulate within microglia and will be able to act as ACP with T cells. This intricate network of cells and cytokines form a positive feedback loop that amplifies the effect initiated by increasing dietary lipids. At this level, neuropeptides as ghrelin and POMC may represent potential modulators of inflammation based on their characteristic of being anti-inflammatory. Ghrelin can block the TLR4/MyD88/TRAF6/NF-KB pathway activated in microglia by SFA and decrease the activity of the AMPK and JUN, important for the production of IL-2. POMC derived peptides attenuate secretion of proinflammatory cytokines via MC4R-cAMP-PKA-CREB, which induces the release of IL-10. While MC1R receptor activation reduces the expression of the adhesion proteins E-selectin and VCAM, and reduce the activation of NF-kB in endothelial cells exposed to TNF-α (Figure 2). Overall, neuropeptides in the CNS modulate inflammation and migration of peripheral cells into the CNS via the BBB and may represent a molecular node during positive energy balance as is the obesity and maternal overnutrition.

Conflicts of Interest

The authors declare no conflicts of interest.

Acknowledgments

The authors thank Dr. Sergio Lozano from Scientific Publications Support, Faculty of Medicine, Autonomous University of Nuevo Leon, for reading and commenting on the manuscript. This work was funded by National Council of Science and Technology in Mexico (CONACYT) (255317 and 261420) and ISN CAEN Award for Alberto Camacho.

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Hindawi
BioMed Research International
Volume 2017, Article ID 6823209, 12 pages
https://doi.org/10.1155/2017/6823209

Research Article

Peripheral Inhibitor of AChE, Neostigmine, Prevents the Inflammatory Dependent Suppression of GnRH/LH Secretion during the Follicular Phase of the Estrous Cycle

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Received 3 May 2017; Revised 6 July 2017; Accepted 16 July 2017; Published 15 August 2017

Academic Editor: Daniele Tomassoni

The study was designed to test the hypothesis that the inhibition of acetylcholinesterase (AChE) activity at the periphery by Neostigmine (0.5 mg/animal) will be sufficient to prevent inflammatory dependent suppression of the gonadotropin-releasing hormone (GnRH)/luteinising hormone (LH) secretion in ewes in the follicular phase of the estrous cycle, and this effect will be comparable with the systemic AChE inhibitor, Donepezil (2.5 mg/animal). An immune/inflammatory challenge was induced by peripheral administration of lipopolysaccharide (LPS; 400 ng/kg). Peripheral treatment with Donepezil and Neostigmine prevented the LPS-induced decrease ($P < 0.05$) in LHβ gene expression in the anterior pituitary gland (AP) and in LH release. Moreover, Donepezil completely abolished ($P < 0.05$) the suppressory effect of inflammation on GnRH synthesis in the preoptic area, when pretreatment with Neostigmine reduced ($P < 0.05$) the decrease in GnRH content in this hypothalamic structure. Moreover, administration of both AChE inhibitors diminished ($P < 0.05$) the inhibitory effect of LPS treatment on the expression of GnRH receptor in the AP. Our study shows that inflammatory dependent changes in the GnRH/LH secretion may be eliminated or reduced by AChE inhibitors suppressing inflammatory reaction only at the periphery such as Neostigmine, without the need for interfering in the central nervous system.

1. Introduction

An immune/inflammatory challenges caused by the bacterial or viral infection could be one of the reasons of reproductive disorders in both humans and animals [1]. It is postulated that the interaction between the immune and neuroendocrine systems may occur at all levels of the neurohormonal system of hypothalamic-pituitary-gonadal (HPG) axis controlling the female reproductive process. A particularly important role in the communication between these two systems is played by the hypothalamus, the part of the brain responsible for the integration and processing of signals from the nervous, endocrine, and immune systems, what is essential for maintaining the homeostasis. The hypothalamus plays a key role in the control of reproduction in females by tonic release of gonadotropin-releasing hormone (GnRH) to the hypothalamic-pituitary portal circulation. In turn, GnRH regulates the secretion of luteinising hormone (LH) and follicle-stimulating hormone (FSH) from the gonadotropic cells in the anterior pituitary gland (AP) [2].

It was previously reported that both acute and prolonged inflammation induced by peripheral administration of bacterial endotoxin-lipopolysaccharide (LPS) may disturb the secretion of GnRH and LH [3, 4]. The study on ewes in the follicular phase of the estrous cycle showed that inflammation interrupted the preovulatory estradiol increase and delayed

or blocks the subsequent LH and FSH surges [5]. This suppressive effect of inflammation on the gonadotropins secretion seems to be mediated via proinflammatory cytokines reaching the hypothalamic area during immune challenges [6]. Interleukin- (IL-) 1β and tumor necrosis factor α (TNFα) may represent the major proinflammatory cytokines mediating the LPS-induced suppression of GnRH and LH release, whereas the role of IL-6 in this process seems to be marginal [6–8].

One of the endogenous mechanisms involved in the regulation of immune response and cytokine secretion is the cholinergic anti-inflammatory pathway. It had been previously described that the cholinergic anti-inflammatory pathway could be activated by stimulation of the vagus nerve thereby increasing the acetylcholine (ACh) secretion [9]. This anti-inflammatory mechanism could be also activated by pharmacological blockade of the acetylcholinesterase (AChE) activity, the enzyme responsible for the degradation of ACh. In vitro studies revealed that ACh acting probably via nicotinic receptor CHRNA7 reduced LPS-stimulated release of proinflammatory cytokines, including IL-1β, IL-6, and TNFα [10]. In vivo study also showed that blockade of AChE activity reduced synthesis of IL-1β during peripheral inflammation in mouse [11] and sheep [12] hypothalamus. Moreover, our previous study on ewes showed that the activation of the cholinergic anti-inflammatory pathway by Rivastigmine may abolish the inhibitory effect of LPS administration on the GnRH/LH secretion and reduced the release of stress markers such as cortisol and prolactin [13]. However, Rivastigmine, AChE inhibitor used in this study, exhibits the systemic action; therefore, it blocks the AChE activity both in the brain parenchyma and in the periphery, because it easily crosses the blood-brain barrier (BBB). Therefore, it could not be concluded whether and to what extent the observed reduction of IL-1β synthesis in the central nervous system (CNS) and changes in hormone secretion resulted from the inhibition of the AChE activity in the CNS or the reduction in peripheral levels of proinflammatory cytokines. The results of experiments performed on mice suggest that only the reduction of circulating concentration of proinflammatory cytokines under certain conditions may be sufficient to significant inhibition of LPS-induced synthesis of IL-1β in the CNS [11]. This study suggests that, to disturb the functioning of CNS, the blood level of immune mediators has to enrich a critical level. Therefore, the reduction of proinflammatory cytokine concentration below this critical value may block the transmission of the inflammatory signal into the brain parenchyma. These all suggest that the activation of the cholinergic anti-inflammatory pathway only in the periphery may be sufficient to stop excessive increase in the concentration of proinflammatory cytokines in the blood, which in turn may be sufficient to reverse the negative effects of immune stress on the GnRH/LH, without providing the AChE inhibitor and direct interference in the CNS. Therefore, in the present study we used two AChE inhibitors differing in the ability to cross the BBB: Donepezil which greatly cross the BBB and Neostigmine which does not penetrate the BBB.

The present study tested the hypothesis that the inhibition of AChE activity at the periphery by Neostigmine will be sufficient to prevent the LPS-induced suppression of GnRH/LH secretion in ewes in the follicular phase of the estrous cycle, and this effect will be comparable with the systemic action of Donepezil.

2. Materials and Methods

2.1. Animals. The studies were performed on adult, 2-year-old Blackhead ewes during the reproductive season (September-October). The ewes were maintained in good conditions; that is, their body condition was estimated at 3 in a five-point scale [14] and the animals were acclimated to the experimental conditions for one month. The ewes had constant visual contact with each other in order to avoid isolation stress. The animals were fed a constant diet of commercial concentrates with hay and water available ad libitum, according to the recommendations proposed by the National Research Institute of Animal Production for adult ewes [15].

In order to best standardize experimental conditions the stage of the estrous cycle of ewes were synchronized by the Chronogest® CR (Merck Animal Health, Boxmeer, Netherlands) method using an intravaginal sponge impregnated with 20 mg of a synthetic progesterone-like hormone. All ewe had Chronogest CR sponges placement for 14 days. Following sponge removal, the ewes will receive an intramuscular injection of 500 iu pregnant mare's serum gonadotropin (PMSG) (Merck Animal Health, Boxmeer, the Netherlands). The experimental procedure was performed 24 h following PMSG injection. In treated animals, the immune stress was induced by the intravenous (iv.) injection of LPS from *Escherichia coli* 055:B5 (Sigma-Aldrich, St. Louis, MO, USA) in a dose of 400 ng/kg, dissolved in saline (0.9% w/v NaCl) (Baxter, Deerfield, IL, USA) at a concentration of 10 mg/L.

All procedures were performed with agreement of the Local Ethics Committee of Warsaw University of Life Sciences-SGGW.

2.2. Experimental Procedures. Venous catheters were implanted into the jugular vein on the day prior to the experiment. Ewes ($n = 36$) were randomly divided into six experimental groups (Table 1). Jugular blood samples (6 ml) were taken for measurement of the peripheral hormone at 15 min intervals beginning 2 h before the iv. administration of LPS or an equivalent volume of saline injection and continuing for 3 h. Half hour prior to LPS/saline treatment the animals were slowly intravenously treated with saline (groups 1 and 2) or suitable AChE inhibitor (groups 3, 4, 5, and 6) (Table 1). After the blood collection, the animals were immediately euthanized (3 h after LPS or saline administration) and the brains were rapidly removed from the skulls. From the ovine brains four hypothalamic structures were dissected due to their involvement in the GnRH-ergic activity. In the hypothalamus of sheep GnRH-ergic neurons did not form dense clusters, but they spread from brain septum and the horizontal diagonal band of Broca, through the preoptic area (POA), anterior hypothalamus (AHA), and medial basal hypothalamus (MBH) [2]. However, most of GnRH neurons have their pericarions located in the POA; therefore, it plays a

Table 1: The scheme of the experiment.

Group	Number of animals	Experimental treatment I (iv.)	Dose [mg/animal]	Experimental treatment II (iv.)	Dose [ng/kg]
1: control	6	NaCl	0	NaCl	0
2: LPS treated	6	NaCl	0	LPS	400
3: Donepezil treated	6	Donepezil	2.5	NaCl	0
4: Neostigmine treated	6	Neostigmine	0.5	NaCl	0
5: Donepezil + LPS treated	6	Donepezil	2.5	LPS	400
6: Neostigmine + LPS treated	6	Neostigmine	0.5	LPS	400
Total amount of animals	*36*				

pivotal role in GnRH synthesis. The majority of GnRH-ergic neurons send their axonal projection to the median eminence (ME) where GnRH is released to the hypophyseal portal system [2]. The hypothalamic structures such as the POA, AHA, MBH, and ME were dissected according to stereotaxic atlas of the sheep brain [18] as it was described elsewhere [13]. Landmarks were the mammillary body, median eminence, and optic chiasm. The depths of the cuts were 2 to 2.5 mm for MBH and 2.5 to 3 mm for AHA and POA. All tissues were frozen immediately after collection in liquid nitrogen and then will be stored at −80°C.

2.3. Assays

2.3.1. Radioimmunoassay for LH. The plasma LH concentration was assayed with a double-antibody RIA using anti-ovine-LH and anti-rabbit-γ-globulin antisera and ovine standard (teri.oLH, Tucker Endocrine Research Institute), according to Stupnicki and Madej [19]. The assay sensitivity was 0.3 ng/ml and the intra- and interassay coefficients of variation were 8% and 11.5%, respectively.

2.3.2. Radioimmunoassay for FSH. The concentration of FSH was determined by double-antibody radioimmunoassay (RIA) using anti-ovine-FSH (teri.anti-oFSH) and anti-rabbit-γ-globulin antisera, according to L'Hermite et al. [20]. The anti-FSH, as well as the FSH standard (teri. oFSH-and teri. FSH ig), was kindly supplied by Dr. Reichert Jr. (Tucker Endocrine Research Institute LLC, Atlanta, Georgia, USA). The assay sensitivity was 1.5 ng/ml and the intra- and interassay coefficients of variation were 3.5% and 11.3%, respectively.

2.3.3. Radioimmunoassay for Prolactin. The plasma prolactin concentration was assayed by a radioimmunoassay double-antibody method, using specific antiovine prolactin and anti-rabbit-γ-globulin antisera according to Wolińska et al. [21]. The prolactin standard for iodination was obtained according to the method described by H. Kochman and K. Kochman [22]. The assay sensitivity for prolactin was 2 ng/ml, and the intra- and interassay coefficients of variation were 9% and 12%, respectively.

2.3.4. Radioimmunoassay for Cortisol. The cortisol concentrations were determined by radioimmunoassay (RIA) according to Kokot and Stupnicki [23], using rabbit anticortisol antisera (R/75) and an HPLC-grade cortisol standard (Sigma-Aldrich, St. Louis, MO, USA). The assay sensitivity was 1 ng/ml and the intra- and interassay coefficients of variation for cortisol were 9% and 12%, respectively.

2.3.5. ELISA Assay for the GnRH Concentration in the POA. The concentrations of GnRH in the POA were determined with a commercial GnRH ELISA kit (BlueGene Biotech Co., Ltd., China) dedicated for sheep. All stages of GnRH analysis were performed according manufacturer's protocol. The tissues were homogenized in 400 μl of phosphate buffered saline (0.02 M). Then homogenates were subjected to two freeze-thaw cycles to further break the cell membranes. After that, the homogenates were centrifugated for 15 min at 1500 ×g in 4°C. The supernatants were aliquoted and stored until assay in −80°C. All steps in the assays were performed according to the manufacturer's instructions. The incubation of plates and absorbance measurement at 450 nm were performed using a VersaMax reader (Molecular Devices LLC, Sunnyvale, California, United States). The assay sensitivity was 1.0 pg/ml. The values of GnRH concentration were normalised to total protein content in each sample assayed using Bradford method.

2.3.6. Determining the Relative Gene Expression. Total RNA from the hypothalamic structure and AP were isolated using the components of NucleoSpin® RNA/Protein Kit (MACHEREY-NAGEL Gmbh & Co., Düren, Germany) according to a manufacturer's instruction. The purity and concentration of isolated RNA were spectrophotometrically quantified by measuring the optical density at 230, 260, and 280 nm in a NanoDrop 1000 instrument (Thermo Fisher Scientific Inc., Waltham, USA). The RNA integrity was verified by electrophoresis using 1% agarose gel stained with ethidium bromide (Sigma-Aldrich, St. Louis, MO, USA). Maxima™ First Strand cDNA Synthesis Kit for RT-qPCR (Thermo Fisher Scientific Inc., Waltham, USA) was used to prepare cDNA synthesis. As a starting material for this PCR synthesis 2 μg of total RNA was used.

Real-time RT-PCR was carried out using HOT FIREPol EvaGreen® qPCR Mix Plus (Solis BioDyne, Tartu, Estonia) components and HPLC-grade oligonucleotide primers synthesised by Genomed (Poland), according to the method described elsewhere [16]. Specific primers for determining

the expression of housekeeping genes and the genes of interest were chosen based on our previous studies (Table 2). One tube contained 4 μl PCR Master Mix (5x), 14 μl RNase-free water, 1 μl primers (0.5 μl each, working concentration was 0.25 μM), and 1 μl cDNA template. The tubes were run on the Rotor Gene 6000 (Qiagen, Duesseldorf, Germany). The following protocol was used: 95°C in 15 min for activating Hot Star DNA polymerase and finally the PCR including 30 cycles at 95°C in 10 sec for denaturation, 60°C in 20 sec for annealing, and 72°C in 10 sec for extension. After the cycles, a final melting curve analysis under continuous fluorescence measurements was performed to confirm the specificity of the amplification.

Relative gene expression was calculated using the comparative quantification option [24] of the Rotor Gene 6000 software version 1.7 (Qiagen, Dusseldorf, Germany). Three housekeeping genes were examined: glyceraldehyde-3-phosphate dehydrogenase (GAPDH), β-actin (ACTB), and histone deacetylase 1 (HDAC1). The mean expression of these three housekeeping genes was used to normalise the expression of the analysed genes. The results are presented in arbitrary units, as the ratio of the target gene expression to the mean expression of the housekeeping genes.

2.3.7. Western Blot Assays for GnRHR Expression in the AP. Before electrophoresis, the protein concentrations of samples isolated previously from the AP using the NucleoSpin RNA/Protein Kit (MACHEREY-NAGEL Gmbh & Co., Düren, Germany) were quantified using a Protein Quantification Assay Kit (MACHEREY-NAGEL Gmbh & Co., Düren, Germany). The appropriate volume of molecular grade water (Sigma-Aldrich, St. Louis, MO, USA) was added to a volume of sample containing 50 μg of total protein to bring the total sample volume to 20 μl. Next, 19 μl of Laemmli buffer (Sigma-Aldrich, St. Louis, MO, USA) and 1 μl of β–mercaptoethanol (Sigma-Aldrich, St. Louis, MO, USA) were added. Such mixtures were boiled for 3 min. Electrophoresis was then performed in the presence of molecular weight markers (Spectra Multicolor Broad Range Protein Ladder, Thermo Fisher Scientific Inc., Waltham, MA, USA). Denatured samples and molecular weight standards were loaded onto 4–12% polyacrylamide gels and subjected to electrophoresis in a Tris-glycine running buffer using the Protean II xi Cell (Bio-Rad Laboratories, Inc., Hercules, CA, USA) according to the manufacturer's instructions. Next, proteins were transferred in Tris-glycine blotting buffer to polyvinylidene difluoride membranes (Immobilon™-P (0.45 μm), Merck KGaA, Darmstadt, Germany) using the Trans-Blot® SD Semi-Dry Transfer Cell (Bio-Rad Laboratories, Inc., Hercules, CA, USA) for 30 min at 20 V. The membranes were blocked for 1 h at room temperature in blocking buffer made up of Tris buffered saline at pH 7.5 with 0.05% Tween-20 (TBST) (Sigma-Aldrich, St. Louis, MO, USA) containing 3% bovine serum albumin fraction V (Sigma-Aldrich, St. Louis, MO, USA). Next, membranes were incubated overnight at 4°C with the following primary antibodies: goat anti-GnRHR polyclonal antibody (cat. number sc-8682, Santa Cruz Biotechnology Inc., Dallas, USA) and mouse anti-ACTB monoclonal antibody (cat. number sc-47778, Santa Cruz Biotechnology Inc., Dallas, USA) dissolved in blocking buffer at dilutions of 1 : 500 and 1 : 1000, respectively. After washing three times, membranes were incubated with the following secondary HRP conjugated antibodies: donkey anti-goat IgG-HRP (cat. number sc-2304, Santa Cruz Biotechnology Inc., Dallas, TX, USA) and goat anti-mouse IgG1 heavy chain (HRP) (cat. number ab97240, Abcam, Cambridge, UK) dissolved in blocking buffer at a dilution of 1 : 10,000. After washing three times, the membranes were visualised using chromogenic detection with a Pierce 1-step TMB-blotting substrate solution (Thermo Fisher Scientific, Waltham, MA, USA). After visualisation, the membranes were dried and scanned using an Epson Perfection V370 Photo scanner (Seiko Epson Corporation, Suwa, Japan). Densitometric analysis of the scanned membrane was performed using the software ImageJ (Research Services Branch, National Institute of Mental Health, Bethesda, MD, USA).

2.4. Statistical Analysis of Data. The results of hormones concentration are presented as the mean ± SEM. All experiments consisted of a baseline period when no treatment was given (2 to 0.5 h before) and a period after treatment (1 to 3 h after). To identify treatment effects, the mean values for the baseline and treatment periods were obtained. To compare the baseline period when no treatment was given and a period after treatment, the obtained data were compared with use of Student's t-test for dependent samples ("repeated measures"). Statistical significance was defined as $P < 0.05$.

The results of blood hormones concentration obtained only after treatment period, GnRH content in the POA, GnRHR protein expression, and all examined genes expression were analysed using a two-way ANOVA, the examined factors were inflammatory state and AChE inhibitor treatment (Donepezil or Neostigmine). Before ANOVA was conducted its two assumptions were checked: normality (Shapiro-Wilk's test) and homogeneity of the variances (Levene's test). When a significant treatment by time interaction was observed, a post hoc analysis was conducted to identify treatment effects. Fisher's least significant difference post hoc test was used to compare precompared with posttreatment values. Statistical significance was defined as $P < 0.05$.

The statistical analysis was performed using the STATISTICA 10 software (StatSoft Inc., Tulsa, OK, USA).

3. Results

3.1. Effect of AChE Inhibitors and LPS Injection on LH, FSH, Prolatin, and Cortisol Release. Both Donepezil and Neostigmine treatment prevented the LPS-induced decrease ($P < 0.05$) in plasma concentrations of LH. Moreover, in animals treated with Neostigmine and LPS the plasma concentration of LH was higher ($P < 0.05$) than in the control group (Figure 1(a)). In contrast, the peripheral concentrations of FSH were unaffected by all treatments (Figure 1(b)). Endotoxin injection increased ($P < 0.05$) the concentration of stress markers: cortisol (Figure 2(a))

Table 2: All genes analyzed by real-time PCR are listed with their full name and abbreviation.

GenBank acc. number	Gene	Amplicon size [bp]	Forward/reverse	Sequence 5′ →3′	References
NM_001034034	*GAPDH* *glyceraldehyde-3-phosphate dehydrogenase*	134	forward reverse	AGAAGGCTGGGGCTCACT GGCATTGCTGACAATCTTGA	[16]
U39357	*ACTB* *beta actin*	168	forward reverse	CTTCCTTCCTGGGCATGG GGGCAGTGATCTCTTTCTGC	[16]
BC108088.1	*HDAC1* histone deacetylase 1	115	forward reverse	CTGGGGACCTACGGGATATT GACATGACCGGCTTGAAAAT	[16]
NM_001009397	*GnRHR* *gonadotropin-releasing hormone receptor*	150	forward reverse	TCTTTGCTGGACCACAGTTAT GGCAGCTGAAGGTGAAAAAG	[17]
U02517	*GnRH* *gonadotropin-releasing hormone*	123	forward reverse	GCCCTGGAGGAAAGAGAAAT GAGGAGAATGGGACTGGTGA	[17]
X52488	*LHB* *luteinizing hormone beta-subunit*	184	forward reverse	AGATGCTCCAGGGACTGCT TGCTTCATGCTGAGGCAGTA	[17]
X15493	*FSHB* *follicle stimulating hormone beta-subunit*	131	forward reverse	TATTGCTACACCCGGGACTT TACAGGGAGTCTGCATGGTG	[17]
NM_001009306	*PRL* *prolactin*	131	forward reverse	CCTCTCCTCGGAAATGTTCA AGGACTTCATGGTGGGTCTG	[17]

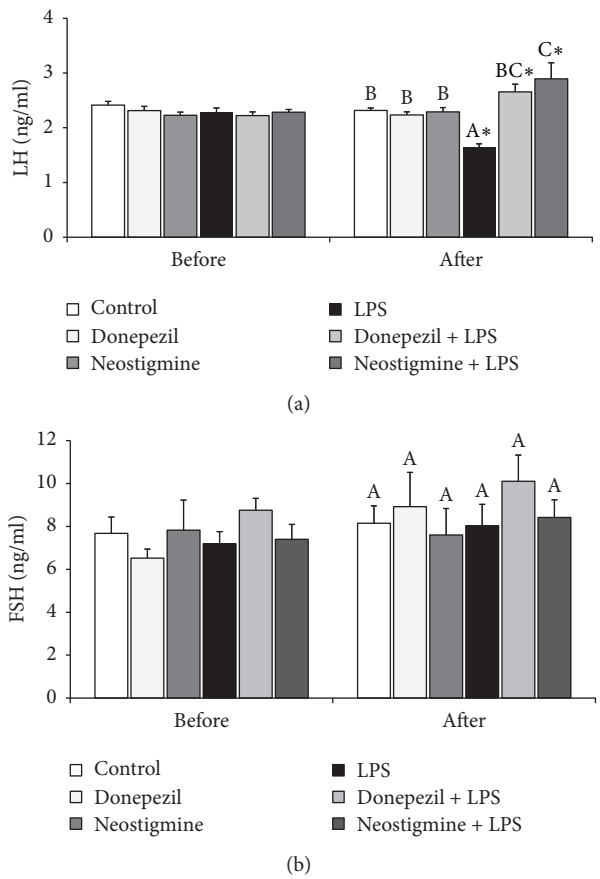

Figure 1: Effect of lipopolysaccharide (LPS; 400 ng/kg; iv.) and acetylcholinesterase inhibitors: Donepezil (2.5 mg/animal; iv.) and Neostigmine (0.5 mg/animal; iv.) injections on blood concentration of luteinising hormone (LH) (a) and follicle-stimulating hormone (FSH) (b) concentration in the blood plasma. The data are presented as the mean value ± SEM. All experiments consisted of a baseline period when no treatment was given (2 to 0.5 h before) and a period after treatment (1 to 3 h after). ∗: asterisk indicates statistically significant differences between the period when no treatment was given and a period after treatment according to Student's t-test for dependent samples ("repeated measures"). The results of blood hormones concentration obtained only after treatment period were analysed using a two-way ANOVA. Different capital letters indicate significant differences according to a two-way ANOVA followed by Fisher's post hoc test. Statistical significance was defined as $P < 0.05$.

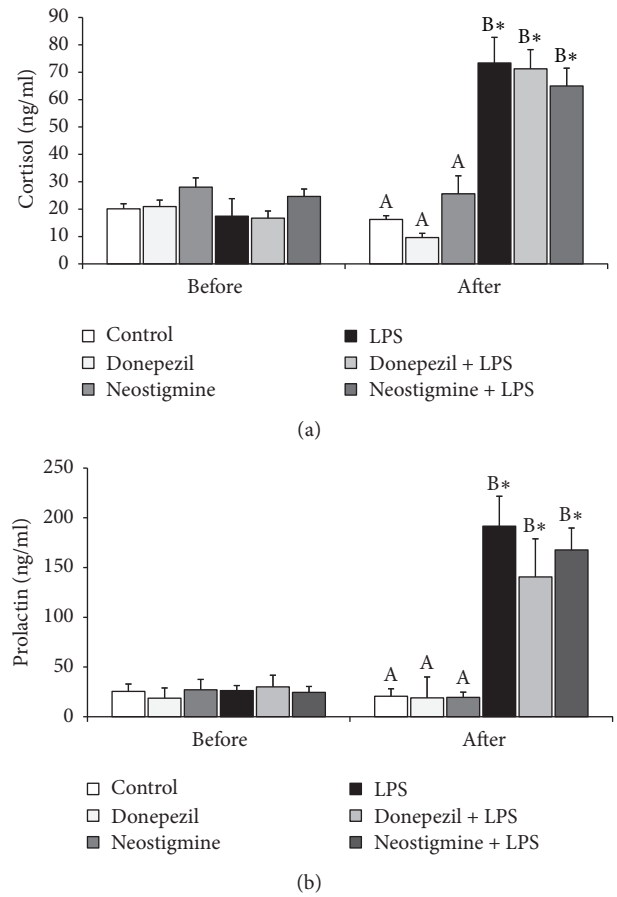

Figure 2: Effect of lipopolysaccharide (LPS; 400 ng/kg; iv.) and acetylcholinesterase inhibitors: Donepezil (2.5 mg/animal; iv.) and Neostigmine (0.5 mg/animal; iv.) injections on blood concentration of stress markers: cortisol (a) and prolactin (b) concentration in the blood plasma. The data are presented as the mean value ± SEM. All experiments consisted of a baseline period when no treatment was given (2 to 0.5 h before) and a period after treatment (1 to 3 h after). ∗: asterisk indicates statistically significant differences between the period when no treatment was given and a period after treatment according to Student's t-test for dependent samples ("repeated measures"). The results of blood hormones concentration obtained only after treatment period were analysed using a two-way ANOVA. Different capital letters indicate significant differences according to a two-way ANOVA followed by Fisher's post hoc test. Statistical significance was defined as $P < 0.05$.

and prolactin (Figure 2(b)), and these increases were not influenced by the AChE inhibitors treatment.

3.2. Effect of AChE Inhibitors and LPS Injection on GnRH Content in the POA. Endotoxin treatment decreased ($P < 0.05$) the content of GnRH in the POA. Preceding injection of Donepezil completely abolished this suppressory effect of inflammation on the GnRH content in the POA, when pretreatment with Neostigmine reduced ($P < 0.05$) the negative effect of inflammation on the GnRH content in the POA, but it stayed significantly lower compared with the control group (Figure 3).

3.3. Effect of AChE Inhibitors and LPS Injection on GnRHR Protein Expression in the AP. Inflammation reduced ($P < 0.05$) expression of GnRHR in the AP of ewes but the preceding injection of Donepezil and Neostigmine abolished the inhibitory effect of LPS treatment on the expression of this receptor (Figure 4).

3.4. Effect of AChE Inhibitors and LPS Injection on the Gene Expression in the Hypothalamus and AP. Endotoxin treatment decreased ($P < 0.05$) the level of GnRH mRNA only in the ME, but preceding injection of both AChE inhibitors prevented this effect of inflammation. It is worth mentioning that the gene expression of GnRH was not

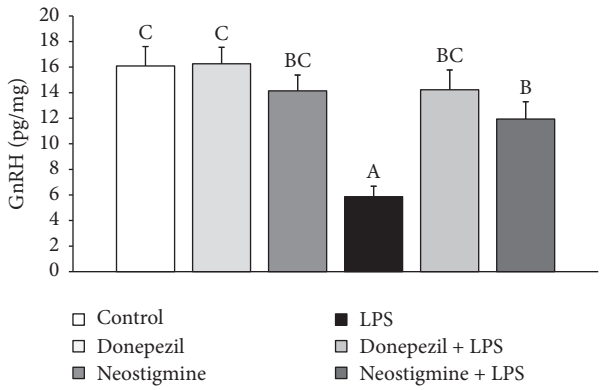

Figure 3: Effect of lipopolysaccharide (LPS; 400 ng/kg; iv.) and acetylcholinesterase inhibitors: Donepezil (2.5 mg/animal; iv.) and Neostigmine (0.5 mg/animal; iv.) injections on blood the content of gonadotropin-releasing hormone (GnRH) in the hypothalamus of ewes during the follicular phase of the estrous cycle. The data are presented as the mean value ± SEM. The results were analysed using a two-way ANOVA. Different capital letters indicate significant differences according to a two-way ANOVA followed by Fisher's post hoc test. Statistical significance was defined as $P < 0.05$.

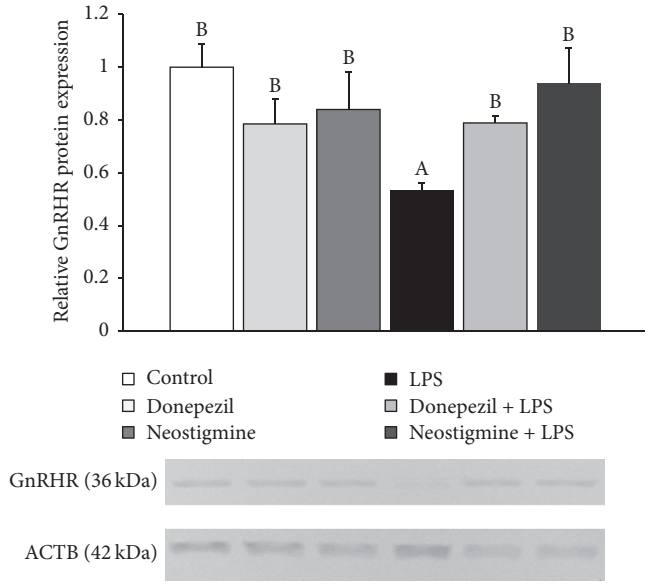

Figure 4: Effect of lipopolysaccharide (LPS; 400 ng/kg; iv.) and acetylcholinesterase inhibitors: Donepezil (2.5 mg/animal; iv.) and Neostigmine (0.5 mg/animal; iv.) injections on the relative protein expression (mean ± SEM; $n = 6$ animals per group) of gonadotropin-releasing hormone receptor (GnRHR) in the anterior pituitary of ewes during the follicular phase of the estrous cycle. The data are presented as the mean value ± SEM. The results were analysed using a two-way ANOVA. Different capital letters indicate significant differences according to a two-way ANOVA followed by Fisher's post hoc test. Statistical significance was defined as $P < 0.05$.

affected by any treatment in other hypothalamic structures analysed (Table 3).

In the AP, the inflammation decreased the gene expression of GnRHR and pretreatment with both AChE inhibitors did not influence the effect of inflammation on this receptor gene expression. On the other hand, preceding injection of Donepezil and Neostigmine diminish ($P < 0.05$) suppressory effect of inflammation on the LHβ mRNA expression in the AP. No effect of any treatment on the gene expression of FSHβ was determined. It was also determined that LPS injection stimulated ($P < 0.05$) gene expression of prolactin in the AP, and neither Donepezil nor Neostigmine influenced the level of prolactin mRNA (Table 4).

4. Discussion

The present study showed that peripheral AChE inhibitor, Neostigmine, the same as Donepezil, suppressed inhibitory effect of acute inflammation on LH release and LHβ gene expression in the AP in ewes during the follicular phase of the estrous cycle. Moreover, in animals treated together with Neostigmine and LPS the circulating level of LH was higher than in the control group. The study supports the results of our previous experiment on ewes which showed that systemic AChE inhibitor successfully reduced negative effect of inflammation on LH secretion in the follicular phase ewes [13]. On the other hand, no effect of either AChE inhibitors or acute immune stress was found upon the circulating concentration of FSH. This also supports the results of previous studies indicating that acute inflammation did not influence the FSH release in both anoestrous [25] and follicular phase ewes [13]. However, other studies on ewes showed that the potency of LPS to affect the secretion of FSH may be dependent upon the duration of the inflammatory stimuli, because prolonged exposition of ewe on the action of bacterial endotoxin was found to disturb FSH release [3, 5]. It is worth mentioning that, except duration, the effect of endotoxin on gonadotropins secretion could be also dependent on the circulating concentration of ovarian steroids. It was found that endotoxin delayed the time to an experimentally induced LH surge in ovariectomized ewes but did not alter surge amplitude, duration, or incidence. This effect of LPS on the LH surge was dependent upon the moment when endotoxin was introduced relative to the onset of the estradiol signal. When endotoxin was administered early in the initial period of estrogen sensitivity, it blocked the LH surge in most ewes, but when endotoxin was administered after the period of estrogen sensitivity, the level of LH remained unaffected [26].

The changes in the endocrine activity of the AP seem to be a repercussion of events occurring at the level of hypothalamus. The study showed that preceding administration of AChE inhibitors reduced the suppressory action of acute inflammation on GnRH synthesis in the POA. However, our results suggest that in the follicular phase of the estrous cycle inflammation suppresses the GnRH synthesis at the posttranscriptional level, because no effect of LPS treatment on the gene expression of GnRH was found in the hypothalamic structures containing pericarya of GnRH neurons. This observation in the follicular phase ewes is generally consistent with the characteristic of GnRH mRNA synthesis. The previous study showed that the ratio of amount of GnRH

Table 3: Effect of lipopolysaccharide (LPS; 400 ng/kg; iv.) and acetylcholinesterase inhibitors: Donepezil (2.5 mg/animal; iv.) and Neostigmine (0.5 mg/animal; iv.) injections on the relative gene expression (mean ± SEM; $n = 6$ animals per group) of gonadotropin-releasing hormone (GnRH) in the hypothalamus of ewes during the follicular phase of the estrous cycle. POA: the preoptic area; AHA: the anterior hypothalamus; MBH: the medial basal hypothalamus; ME: the median eminence; control: group injected with saline; Don.: group treated with Donepezil; Neo.: group injected with Neostigmine; LPS: group which received the endotoxin injection; Don. + LPS: group treated with both Donepezil and LPS; Neo. + LPS: group treated with both Neostigmine and LPS. In all hypothalamic structures gene expression data were normalised to the average relative level of gene expression in the control ewes, which was set to 1.0. Different capital letters indicate significant ($P < 0.05$) differences according to a two-way ANOVA followed by Fisher's post hoc test.

Structure	GnRH relative gene expression					
	Control	Don.	Neo.	LPS	Don. + LPS	Neo. + LPS
POA	1 ± 0.1^{A}	0.9 ± 0.1^{A}	0.9 ± 0.2^{A}	0.8 ± 0.2^{A}	0.9 ± 0.1^{A}	1 ± 0.2^{A}
AHA	1 ± 0.1^{A}	1.1 ± 0.1^{A}	0.8 ± 0.2^{A}	0.7 ± 0.1^{A}	0.9 ± 0.1^{A}	0.9 ± 0.1^{A}
MBH	1 ± 0.1^{A}	0.8 ± 0.1^{A}	0.8 ± 0.2^{A}	1.1 ± 0.1^{A}	1 ± 0.1^{A}	1.1 ± 0.2^{A}
ME	1 ± 0.2^{B}	1.3 ± 0.2^{B}	1 ± 0.2^{B}	0.1 ± 0.1^{A}	0.7 ± 0.1^{B}	1.2 ± 0.2^{B}

Table 4: Effect of lipopolysaccharide (LPS; 400 ng/kg; iv.) and acetylcholinesterase inhibitors: Donepezil (2.5 mg/animal; iv.) and Neostigmine (0.5 mg/animal; iv.) injections on the relative gene expression (mean ± SEM; $n = 6$ animals per group) of gonadotropin-releasing hormone receptor (GnRHR), luteinizing hormone β-subunit (LHβ), follicle-stimulating hormone β-subunit (FSHβ), and prolactin (PRL) genes in the anterior pituitary of ewes during the follicular phase of the estrous cycle. control: group injected with saline; Don.: group treated with Donepezil; Neo.: group injected with Neostigmine; LPS: group which received the endotoxin injection; Don. + LPS: group treated with both Donepezil and LPS; Neo. + LPS: group treated with both Neostigmine and LPS. The gene expression of each gene was normalised to the average relative level of gene expression in the control ewes, which was set to 1.0. Different capital letters indicate significant ($P < 0.05$) differences according to a two-way ANOVA followed by Fisher's post hoc test.

Gene	Anterior pituitary					
	Control	Don.	Neo.	LPS	Don. + LPS	Neo. + LPS
GnRHR	1 ± 0.1^{BCD}	1.2 ± 0.2^{BCD}	1.4 ± 0.2^{D}	0.5 ± 0.1^{A}	0.9 ± 0.2^{ABC}	0.7 ± 0.2^{AB}
LHβ	1 ± 0.1^{C}	0.9 ± 0.1^{BC}	1 ± 0.1^{C}	0.5 ± 0.1^{A}	0.9 ± 0.1^{BC}	0.8 ± 0.1^{BC}
FSHβ	1 ± 0.1^{A}	0.8 ± 0.1^{A}	0.8 ± 0.2^{A}	1.1 ± 0.1^{A}	1 ± 0.1^{A}	1.1 ± 0.2^{A}
PRL	1 ± 0.2^{A}	1.1 ± 0.2^{A}	1 ± 0.2^{A}	1.6 ± 0.1^{B}	1.7 ± 0.1^{B}	1.7 ± 0.2^{B}

nuclear mRNA to GnRH cytoplasmic mRNA is 1 : 2.5 and 1 : 1.5, respectively, depending on the study [27, 28]. Therefore, a greater amount of nuclear transcript provides a steady flow of GnRH mRNA to the cytoplasm, and it is generally postulated that changes in the amount of GnRH mRNA in the perikaryons are rather dependent on this mRNA turnover, both rapid accumulation and fast degradation. It should be noted that the effect of inflammation on the GnRH mRNA expression in the hypothalamus of sheep may be influenced upon the circulating concentrations of estradiol. The study on ewes during anestrous season, when the level of estradiol is presumably low, showed that endotoxin-induced inflammation decreased the transcription of GnRH mRNA in the POA [29]. Moreover, previous study showed that central action of potent proinflammatory cytokine, IL-1β, may be responsible for the suppression of the translational efficiency of GnRH mRNA in the rat [30] and sheep [8] hypothalamus. Therefore, it seems that in the present study the decrease found in the content of GnRH in the POA in LPS treated ewes may result from reduced translation of this decapeptide, and in turn the ability of AChE inhibitors to blockade this negative effect on the GnRH synthesis may result from the suppression of the level of central cytokines.

In the present study both Neostigmine and Donepezil treatment completely abolished LPS-induced decrease in the content of GnRH mRNA in the ME, where GnRH nerve terminals are located. This observation supports the results of previous studies, which showed that immune stress may reduce the GnRH mRNA transport to the nerve terminals, thus reducing the amount of GnRH mRNA in the ME [29], but this effect may be restrained by Rivastigmine [13]. Because it is supposed that the storage of the GnRH mRNA in the nerves terminals may be an element of the system supporting the secretion of this decapeptide, the ability of AChE inhibitors to restore the amounts of GnRH mRNA in the ME could have a profound positive influence on the GnRH secretion. This may be one of the mechanisms responsible for the protective action of the AChE inhibitors on the GnRH/LH secretion during an immune/inflammatory challenge. It was described that inflammation disturbs GnRH release in ovariectomized ewes decreasing GnRH pulse amplitude without affecting the GnRH pulse frequency [31], but our study showed that Rivastigmine not only reduced the suppressory effect of inflammation on the GnRH release but also even stimulated this neurohormone secretion into the cerebrospinal fluid of ewes in the follicular phase of the estrous cycle [13].

The ability of Neostigmine and Donepezil to block the effect of inflammation on GnRH secretion in the hypothalamus may primarily result from attenuation the inflammatory signal from periphery to the brain parenchyma. As it was mentioned above, it is considered that the main mechanism

via endotoxin-induced inflammation disturbing the GnRH secretion in the hypothalamus is the central action of inflammatory cytokines. In our previous study, it was found that peripheral administration of Rivastigmine inhibited the LPS-induced synthesis of IL-1β in and gene expression of IL-1 receptors in the hypothalamus of ewe [12]. However, the action of Rivastigmine as well as Donepezil is systemic; these compounds inhibit the AChE activity and lead to elevation of ACh concentration in both the peripheral and central tissues [32]. However, the effectiveness of Donepezil in the prevention of inflammatory dependent changes in the GnRH/LH secretion in the follicular phase does not surprise because obtained results are concomitant with those described in our study with the use of Rivastigmine [13]. The fact that pretreatment with Neostigmine also abolished suppressive effect of LPS treatment on the GnRH/LH secretion suggests that the inhibition of proinflammatory cytokines secretion in the peripheral tissues by Neostigmine is sufficient to block the transition the inflammatory signal into the brain parenchyma, which was previously described by Pollak et al. [11]. This shows that peripheral and systemic AChE inhibitors characterize similar effectiveness in the prevention of inflammatory dependent distribution of GnRH/LH secretion and suggests that pivotal mechanism in the pathophysiology of neuroendocrine disorders occurring during an immune challenge is the elevation of peripheral proinflammatory cytokines concentration to the level necessary to transition of the information about the ongoing peripheral inflammation into the brain.

The proceeding injection of both AChE inhibitors prevented inflammatory dependent decrease in the expression of GnRHR protein but did not significantly influence on the GnRHR gene expression. During inflammatory condition, reduced expression of GnRHR in the AP may result from decreased secretion of the hypothalamic GnRH. It was described that GnRH is one of the most potent regulators of its own receptor expression. This decapeptide activates the transcriptional activity of its own receptor gene through multiple pathways, including cAMP-, PKC-, and Ca^{2+}-dependent signal transduction pathways [33]. It is worth mentioning that the effect of GnRH on the expression of its own receptor in the AP is closely dependent upon character of its action. When this neurohormone is released in the pulsatile fashion, it maintains steady-state concentrations of GnRHR mRNA and numbers of GnRH receptors in the pituitary gonadotropes. But in contrast to the effects of pulsatile GnRH, continuous infusion of GnRH leads to a desensitization of gonadotropes and reduction in the number of GnRHR [34]. However, the suppression of GnRHR gene expression during inflammation may be also caused by proinflammatory cytokines and stress because it was shown that both IL-1β and corticotropin-releasing hormone (CRH) suppressed GnRHR expression [30, 35, 36]. The fact that preceding injection of AChE inhibitors does not allow the reduction of GnRHR expression in the AP during an immune/inflammatory challenge may have a profound importance for the reactivity of the AP because the factor determining ability and strength of the pituitary gonadotropes response to GnRH is the amount of GnRHR [34].

In the present study, neither Donepezil nor Neostigmine treatment influenced the circulating concentration of the stress markers: cortisol and prolactin which excludes that the effect of AChE injection on the GnRH/LH secretion as well as GnRHR protein expression results from the attenuation of stress reaction induced by an immune/inflammatory challenge. The stimulatory effect of immune response on the release of cortisol and prolactin has been previously described in sheep [13, 25, 37]. Cortisol is considered as an important inhibitor of the HPG axis activity, targeting the LH release [37]. However, the suppressive effect of cortisol on the release of LH release depends on the reproductive status of ewes. The ovarian steroids, particularly estradiol, enable the cortisol suppression of LH pulse frequency in sheep. Whereas cortisol seems to minimally affect LH release in the ovariectomized ewes devoid of gonadal steroids [38, 39]. Moreover, it was found that the other components of the HPA axis such as CRH and arginine vasopressin may inhibit the pulsatile GnRH/LH secretion [40]. Also prolactin may suppress LH secretion. It is known that the physiological states associated with elevated prolactin blood concentration (i.e., pregnancy, pseudopregnancy, postpartum, and lactation); the LH secretion is always decreased [41]. Circulating prolactin crosses the blood-cerebrospinal fluid barrier and reaches the brain parenchyma; therefore, the prolactin dependent inhibition of LH release could result from its action on the GnRH secretion. The results of in vitro studies conducted on the GT1 neuronal cell line showed that prolactin directly inhibits GnRH release and possibly gene expression in these cells [41]. In our previous study Rivastigmine treatment decreased the plasma concentration of these hormones in ewes but not to the control values [13]. However, the background of this Rivastigmine action was not completely clear because ACh is considered to be a stimulant of cortisol [42] and prolactin [43] release, as well as the hypothalamus-pituitary-adrenal (HPA) axis activator [44]. It was speculated that the reduction of cortisol and prolactin release might result from the analgesic action of ACh inhibitors modulating the inflammatory pain [45] and decreasing the production of proinflammatory cytokines which are also able to stimulate the activity of the HPA axis [46]. The present study suggests that the stress-reducing effect of AChE inhibitors may be not universal property of all these compounds or may depend upon used dose of the drug. However, obtained results support the current view about no pivotal role of cortisol in the inhibition of the HPG axis during inflammatory conditions. This observation is generally consistent with the study which showed that the activation of the HPA axis is not essential for reproductive disorders during endotoxin-induced inflammatory challenges [37].

From one hand, it seems that our present study also negatively tested our hypothesis formulated in the study with the usage of Rivastigmine claiming that stimulating effect of the AChE inhibitor on the GnRH secretion during immune stress may result from the accumulation of ACh in the brain. This thesis was justified because the regions of the brain that exhibit cholinergic activity have projections to the POA and therefore may regulate GnRH neuron activity [47, 48]. Moreover, in vitro experiment performed

on rat hypothalamic tissue cultures demonstrated that ACh stimulated GnRH release [49]. An in vitro study performed on rat hypothalamic neurons and GT1-7 line cells showed that ACh modulated GnRH release in an enhanced way and acted through different cholinergic receptor subtypes to exert stimulatory and inhibitory effects [50]. However, in our study animals treated only with Donepezil or Neostigmine did not show any changes in the GnRH/LH secretion which suggests that maintaining of undisturbed GnRH/LH secretion in animals concomitant treated with LPS rather results from the anti-inflammatory action of this AChE inhibitors than simply from accumulation of ACh in the hypothalamus. On the other hand, the reactivity of the brain tissues during inflammatory condition may be changed. Therefore, certainly it cannot be stated that ACh does not play some role in the AChE inhibitors action on the secretion of GnRH and/or LH during inflammation because in this physiological state it may influence the responsiveness of both the hypothalamic and AP tissues on the ACh action. It was previously found that LPS influences the profile of ACh receptors which may change responsiveness of the cells on the action of this neurotransmitter [51]. In our study we determined that in animals treated with Neostigmine and LPS the mean circulating concentration of LH was higher than in the control group, but this effect was not parallel to the changes in the GnRH content in the POA. This suggests that the LH secretion is enhanced by the peripheral factors reaching directly the AP. Although the ex vivo study suggested that stimulatory action of ACh on the LH secretion from the AP is indirect and is targeted on the stimulation of the hypothalamic GnRH release [52], more present study showed that the ACh receptors are present and active in the majority of AP cells [53]. Therefore, it cannot be excluded that at least partially the stimulatory effect of Neostigmine and LPS treatment on the LH secretion may result from the accumulation of ACh in the blood which directly affects the secretory activity of AP.

In summary, our study showed that peripheral inhibitor of AChE activity, Neostigmine, effectively abolished the suppressive effect of acute inflammation on the GnRH/LH secretion and this effect was generally similar to the systemic action of Donepezil. This indicates that inflammatory dependent changes in the GnRH/LH secretion may be eliminated or reduced by the compounds suppressing inflammatory reaction only at the periphery without the need for interfering in the CNS. Our study suggests that AChE inhibitors not capable of crossing the BBB might potentially be used in the therapy of inflammatory induced neuroendocrine disorders.

Conflicts of Interest

All of the authors have declared that there are no conflicts of interest regarding this work.

Acknowledgments

This research was supported by the funds granted by the National Science Centre based on Decision no. DEC-2013/11/B/NZ9/01848.

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Hindawi
BioMed Research International
Volume 2017, Article ID 1549194, 14 pages
https://doi.org/10.1155/2017/1549194

Review Article

Proximate Mediators of Microvascular Dysfunction at the Blood-Brain Barrier: Neuroinflammatory Pathways to Neurodegeneration

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Received 28 April 2017; Accepted 9 July 2017; Published 14 August 2017

Academic Editor: Giuseppe Donato

Current projections are that by 2050 the numbers of people aged 65 and older with *Alzheimer's disease* (AD) in the US may increase threefold while *dementia* is projected to double every 20 years reaching ~115 million by 2050. AD is clinically characterized by progressive dementia and neuropathologically by neuronal and synapse loss, accumulation of amyloid plaques, and neurofibrillary tangles (NFTs) in specific brain regions. The preclinical or presymptomatic stage of AD-related brain changes may begin over 20 years before symptoms occur, *making development of noninvasive biomarkers essential*. Distinct from neuroimaging and cerebrospinal fluid biomarkers, plasma or serum *biomarkers* can be analyzed to assess (i) the presence/absence of AD, (ii) the risk of developing AD, (iii) the progression of AD, or (iv) AD response to treatment. No unifying theory fully explains the neurodegenerative brain lesions but *neuroinflammation* (a lethal stressor for healthy neurons) is universally present. Current consensus is that the earlier the diagnosis, the better the chance to develop treatments that influence disease progression. In this article we provide a detailed review and analysis of the role of the blood-brain barrier (BBB) and damage-associated molecular patterns (DAMPs) as well as coagulation molecules in the onset and progression of these neurodegenerative disorders.

1. Introduction

1.1. Innate Immune Activation in CNS and Neurodegeneration. Often described as a double-edged sword [1, 2] or Janus-faced [3, 4], *neuroinflammation* is a host defense system for prompt recovery from various acute conditions in the CNS, both infectious and sterile [5–7]. In these situations, it is usually short-lived, accomplishing its task and setting the stage for repair and recovery. However, if prolonged and chronic it may also play detrimental roles leading to neurodegenerative processes. The innate immune system, in simple terms, consists of both pro- and anti-inflammatory "factors" and in the CNS responds to genetic influences, protein aggregates and abnormal cell constituents, injury-released mediators from neurons, and mechanism suppression that would otherwise control neuroinflammation. Systemic infection or injury causes an inflammatory response that transmits information to the brain, directing a metabolic and behavioral cascade known as "sickness behavior" [8]. As mentioned, in the brain this innate immune activation is short-lived, prompt, and well-organized but may be prolonged in sepsis or polytrauma that results in the systemic inflammatory response syndrome (SIRS) [9, 10]. With infection, pathogen-associated molecular patterns, such as the prototype endotoxin or lipopolysaccharide (LPS), bring about activation of surface pattern recognition receptors (PRRs) on immune and other cells for a robust inflammatory response mediated primarily via the Toll-like receptor (TLR) family, of which thirteen are now known but only eleven in humans [6, 11, 12].

The principal cells of the innate immune system, circulating monocytes or macrophages, collectively peripheral

immune white blood cells (piWBC), exist in various phenotypes beyond the "classically activated" M1 (γ-interferon-exposed) or "alternatively activated" M2 macrophages following interleukin-4 or interleukin-13 treatment [14]. Similar evidence exists for microglia within the CNS [15]. Numerous publications and reviews have identified positive and negative roles for microglia in the neuroinflammation that accompanies trauma and neurodegeneration [16–18]. Without question microglia, the brain's resident macrophages, are vital players in early development and in the innate immune response within the brain. However, our focus here is on the interplay between the systemic innate immune response to injury and possible mechanisms that implicate endothelial cells (ECs) of the BBB as both *target and source* of inflammatory reactions within the brain that promote, amplify, and sustain neuroinflammation that progresses to degeneration.

1.2. "Danger" or "Damage" Theory. Theorized by Matzinger in the late 1990s [19], the "danger" or "damage" theory of immunity challenged the dominant self/non-self-basis of immunology. It is based on danger or "alarm" signals that come from the body's own cells and began gaining acceptance in the early 2000s with publication of the EMBO Workshop on Innate Danger Signals and HMGB1 held in February 2006 in Milan [20], although not without vigorous opposition. The key point of the danger theory, in contrast to self/non-self-discrimination, is that self-constituents can also trigger an immune response, if they become damaged or are "dangerous." This is fundamental to our understanding of how the peripheral innate immune system might activate the ECs of the BBB to orchestrate neuroinflammation that eventually becomes unregulated and uncontrolled.

1.3. Alzheimer's Disease (AD). AD is a chronic neurodegenerative disease responsible for 60 to 70% of cases of all dementia [21–23]. In 2015, approximately 48 million cases of AD were diagnosed worldwide, according to the World Health Organization. The Alzheimer's Association states that about 5 million Americans currently live with AD, and this number is projected and expected to reach about 13.5 million by 2050 [24]. According to the Centers for Disease Control (CDC), in the US, AD is the sixth leading cause of death killing about 94,000 people annually. The annual costs of care to the US are projected to rise from $226 billion in 2016 to $1.1 trillion by 2050, with Medicare and Medicaid paying 70 percent of these costs. Consequently, early, preclinical diagnosis and developing new therapeutic targets to delay AD onset by only five years by 2025 could save an estimated $935 billion over the following 10 years.

Typically beginning in people over the age of 65, late-onset AD (LOAD), the early clinical indicators of AD may include memory loss worsening over time, behavioral signs such as extreme or rapid swings in mood, judgment or disorientation deficits, and problems with language. These initial symptoms are often mistaken for normal aging, further delaying proper diagnosis of AD. Ultimately, bodily functions are progressively lost, leading to death usually by pneumonia. The average life expectancy for an LOAD patient following diagnosis is approximately between 3 and 9 years. In addition to LOAD, accounting for 95% of patients, several genetic mutations exist that cause early onset or familial AD (fAD). Whether LOAD or genetic early onset fAD, neuropathologic hallmarks are extracellular amyloid and neuritic plaques and intracellular neurofibrillary tangle (NFT) formation.

Amyloid beta (Aβ) is the principal constituent of plaques, and soluble levels increase in the blood, in both AD patients and transgenic mouse models, early in the disease [25–29]. Transgenic mice have been generated which mimic some of the features of AD based on amyloid precursor protein, tau, both, or other mutations. In the brain Aβ aggregates promote a chronic neuroinflammatory response mediated by activated microglia and astrocytes and microvascular ECs [30–32]: amyloid plaques $\rightarrow$ NFTs $\rightarrow$ neuroinflammation. Beginning in 1992 this had prompted creation of the amyloid metabolic cascade hypothesis [33] and since then much debate has ensued with some continuing pros [34, 35] but many more cons [34, 36–38] after it has been critically reexamined. Unfortunately, since LOAD is not associated with genetic mutations information from transgenic animal models cannot be fully extrapolated to the bulk of human AD pathology. Furthermore, microglial activation and other aspects of parenchymal neuroinflammation along with oxidative stress—reactive oxygen species (ROS) and nitric oxide (NO) formation—can actually precede neuronal damage [39–41] prior to AD histopathologic lesions.

Consequently, the pathogenesis of AD remains poorly understood although major risks to develop the disease are believed to be genetic, even for LOAD, and this includes alleles of apolipoprotein E [42, 43]. However, other nongenetic or epigenetic risk factors may be as or more significant for the 95% LOAD patients especially traumatic brain injury (TBI) but also hypertension, type 2 diabetes mellitus, and a number of modifiable factors including smoking [44]. Because of the failure of randomized clinical trials (RCTs) based on the amyloid hypothesis and using recruitment of AD patients with established symptoms, recent emphasis has been placed by a number of panels and working groups on developing tests to diagnose AD prior to symptom development [45]. Unfortunately, a number of current standard tests are quite invasive, expensive, and poorly tolerated including the sampling and analysis of cerebrospinal fluid for β-amyloid or tau proteins [46]. In addition to diagnostic screening of at-risk populations prior to symptom development blood-based biomarkers can also be useful for detecting and monitoring efficacy of therapeutic candidates on the disease progression and as safety markers to detect and monitor potential side effects of drug candidates at the earliest time possible. Therefore, the discovery of equally effective and highly predictive blood-biomarkers is now becoming a major priority in AD therapeutic trial research. Antecedent TBI may be particularly appealing for biomarker screening studies since numerous studies identify it as the most prominent nongenetic risk factor for LOAD development [47].

1.4. Parkinson's Disease (PD). In addition to AD, PD is also intimately associated with neuroinflammation, together with its neuropathologic hallmarks of Lewy bodies (LBs) in dopaminergic (DA) neurons and their degeneration in the

substantia nigra pars compacta (SNpc) [7, 48]. Similar to AD, PD is a slowly evolving, long-term neurodegeneration of the CNS but one affecting primarily motor pathways [49–51]. PD, also known as the "shaking palsy," consists of signs that include shaking (tremor), rigidity, and slowness of movement (bradykinesia), which generally become manifest over time. Dementia may appear late in the advanced stages of PD along with anxiety and depression, although in significant numbers these neuropsychiatric manifestations may actually precede motor signs. As with AD the pathogenesis of PD is unknown but like AD genetics plays a significant role in a number of cases. Other factors that may play prodromal roles in its development include TBI and exposure to certain pesticides. In contrast to AD, nicotine contained in tobacco smoke seems to have a beneficial and protective effect.

The most evident symptoms of PD affecting motor functions are the results of the cell death of DA neurons in the midbrain SNpc. The causes of neuronal cell death in the SNpc are not well understood but appear to involve the accumulation of aggregated proteins such as α-synuclein into LBs within these neurons [49, 51]. Recent data show that over 50 million cases of PD were diagnosed globally with a death toll of over 100,000 worldwide. As with AD, there is no effective cure or treatment to halt progression but symptomatic treatments with L-Dopa and DA agonists or deep brain stimulation exist to help restore motor functionality in individuals whose quality of life has been greatly impaired by disease progression. Diagnosis of PD is primarily based on signs and symptom presentation with the aid of neuroimaging techniques to rule out other possible disorders.

Again, the focus of many studies and reviews is parenchymal neuroinflammation, wherein microglia and astrocytes lead to progressive death of SNpc DA neurons [52]. In this regard, microglia are viewed as initiating inflammatory responses with slower responding astrocytes amplifying them [53]. Important to these studies, given the availability of transgenic models, are the roles of PD-associated genes and neuroinflammation [54]. These include not only the α-synuclein gene but also *parkin*, mutations which are the most common cause of recessively inherited PD [52]. In this regard, variations in another gene, the leucine-rich repeat kinase 2 (LRRK2) gene, have been found in both familial and sporadic PD, which appears to play crucial roles in peripheral inflammation, since LRRK2 is abundant in piWBC. Studies show that giving the prototypic PAMP, LPS, to LRRK2 mutant mice increases cytokine production in their microglia compared to wild-type (WT) mice [52].

As already discussed, innate immune signaling from the periphery to the brain is usually transient, and no evidence exists that this leads to permanent brain tissue damage. However, when signaling is prolonged then parenchymal neuroinflammatory changes become obvious. Just what underlies the mechanisms for this prolongation is the critical question. Perhaps less studied in this regard is the role transmigration of piWBC across the BBB in neuroinflammation plays with neurodegenerative diseases [55]. As with AD and other neurodegenerative diseases, close interplay of the systemic immune system and PD progression is known. Crosstalk between underlying molecular mechanisms of sepsis and SIRS is likely to lead to better understanding of the CNS and innate immune system relationship that should help to clarify PD pathogenesis. Important here is that like AD systemic infection may contribute to PD progression and even its etiology [52].

1.5. Amyotrophic Lateral Sclerosis (ALS). ALS, also known as *Lou Gehrig's or motor neuron disease*, is a neurodegenerative disorder characterized by a progressive loss of control of voluntary movements and muscle weakness and atrophy of extremity and trunk skeletal muscles, as well as muscles of the neck, face, and tongue. This is caused by the degeneration of upper and lower motor neurons in the spinal cord and brainstem [56, 57]. ALS patients may experience (depending on the stage of the disease) muscle stiffness, pain, and atrophy with progressive weakness that ultimately may impair speaking, swallowing, and eventually breathing. The pathogenesis of ALS is unknown in the vast majority of cases, sporadic (sALS), with only a small minority attributed to genetic mutations, fALS [58]. The typical age of onset is in the 50s while much younger onsets are also seen especially in familial (fALS) cases [59].

As in AD and PD ample evidence exists for neuroinflammation and peripheral inflammation in both sALS and fALS [60–62]. More than 25 years ago piWBC were identified within the spinal cords of ALS patients [63]. Based on this and antibodies found Appel and colleagues initially implicated autoimmunity in ALS pathogenesis [64–66]. Autoimmunity is less recognized today but mention has already been made of TLRs and the advanced glycation end products receptor (RAGE [11, 67] in innate immunity and these have been found to be increased in spinal cords of ALS patients and in SOD1 transgenic mice, as reviewed [68, 69]. Subsequent efforts have focused on both reactive ROS and the innate immune system, which appears inextricably linked to this devastating disease [68, 70–72]. One proposed mechanism of ALS (which incorporates genetic mutations of RNA binding proteins, mitochondrial disfunction, and ROS/inflammation) suggests that over time the ability of the cells to be safeguarded against the genetic mutation due to increasing ROS and resulting inflammation is significantly decreased [73, 74]. Either due to an inability to fully neutralize ROS (which results in oxidative DNA damage) and/or due to impaired mitochondrial function [75], the end result is the death of the most sensitive cells such as neurons, especially motor neurons. Like other neurodegenerative disorders no treatment currently exists to cure or halt progression of ALS. Current pharmacological therapies aim at reducing symptoms and improve both live span and the quality of life of the patients. Also, as in other neurodegenerative diseases, the earliest detection prior to onset of symptoms is the key to accomplish these goals.

1.6. Chronic Traumatic Encephalopathy (CTE). CTE is a progressive neurodegenerative disease found most commonly in subjects (generally athletes practicing contact sports) with a history of repetitive TBIs resulting from either symptomatic or asymptomatic (subconcussive) hits to the head. Martland first described a dementia syndrome in former boxers that often was accompanied by parkinsonian and cerebellar motor

signs and which was initially called the "punch-drunk" syndrome [76]. Then beginning in 2005 Omalu and colleagues began reporting neuropathologic findings in former US professional football players [77, 78]. CTE symptoms generally appear 8 to 10 years after cessation of repeat bouts of mild TBI [79]. Initial symptoms include disorientation, disattention, dizziness, and frequent headaches, usually migrainous in type. As the disease progresses, additional symptoms become apparent emphasizing erratic behavior and emotional instability and including memory loss. During the later stages of the disease patients become affected by progressive slowing and parkinsonian muscular movements, tremors, worsening dementia, speech impairment (dysarthria), and difficulty in *swallowing*. Currently clinical diagnosis is difficult and diagnosis is dependent on postmortem neuropathologic examination. As with other neurodegenerations there is no effective treatment available for CTE. Neuropathologically, like AD CTE is a *tauopathy* although criteria have recently been established which distinguish it from AD and other tauopathies in brain tissue, inasmuch as NFTs have been observed in perivascular epicentres in the frontal neocortex whereas in the most severe cases they affect widespread brain regions [80].

To date more than 150 brains have been examined at the Boston University CTE Center and a consensus has been developed by Dr. McKee and other neuropathologists on the criteria for CTE diagnosis [81]. As a part of this a distinctive pattern of perivascular phosphorylated tau distinguished CTE from other tauopathies. In addition to the NFTs, in specific areas as per the consensus report, widespread neuroinflammation exists [1, 82, 83]. As with other neurodegenerations, the lack of distinct biomarkers for CTE is a major roadblock to be overcome for the development of effective preclinical screening tests and prognostic assessments of CTE following TBI.

1.7. BBB Dysfunction and Neurodegeneration. The term BBB refers to a dynamic functional interface between the blood circulation and the neural tissue in the CNS which protects the brain (long considered an immunologically privileged site due to the existence of the BBB) from harm and maintains the brain's homeostasis through a tight regulation of what comes in and out of the brain's environment. Originally depicted as a standalone specialized multicellular structure formed by brain microvascular ECs connected by tight junctions, a thick basement membrane, and juxtaposed *astrocytic* endfeet, the BBB has now become an integral part of a more complex biological system known at the neurovascular unit which represents a more elaborate and encompassing structure beyond the historical core BBB. In fact, neurons, microglia, and pericytes are members of the neurovascular unit since they interact with core elements of the BBB and its microvascular components leading to functional interplay of central and peripheral cells (including immune leukocytes) which influence and modulate the barrier functions and its physiological responses. These include pathophysiological conditions such as CNS and peripheral inflammation. As such the brain's status as an immune privileged organ is being reexamined and "BBB dysfunction" (essentially a universal feature associated with animal models of preclinical TBI [84] and a critical characteristic of neuroinflammation) can now be extended beyond the tissue or cellular pathophysiology of the BBB components to encompass the entire neurovascular unit [85–89].

Beyond microglial and astrocytic activation, although not as well appreciated, are the activation and transmigration of blood-borne and activated circulating immune leukocytic cells (piWBC) into the CNS in AD, PD, ALS, and other neurodegenerative disorders all associated with robust neuroinflammation [16, 90–95]. Although inflammatory responses in neurodegenerative diseases denote both glial activation and piWBC transmigration, the relationship between these two different inflammatory pathways is clearly far from being understood. What appears to be critical in both, however, is the dysfunction of the BBB/neurovascular unit system. Appreciated in AD, PD, and ALS [96–98], this is also becoming more recognized in the context of CTE as well [99], and, in this regard, BBB breach may persist for years after TBI [100], which consequently negatively impacts the entire neurovascular unit. However, the precise factors governing the initial disruption of the BBB following TBI that lead to neurodegeneration have not fully been identified.

1.8. DAMPs and Coagulation Molecules. Central molecules of the two most potent host defense systems that form a *nexus* at the crossroads of innate immunity are high mobility group box protein 1 (HMGB1) and thrombin. HMGB1 is a nonhistone nuclear protein with dual functions: within cells, it is localized primarily to the nucleus where it binds and bends DNA and plays a role in transcriptional regulation [101]. Once outside the cell it can serve as a proinflammatory cytokine and as a late mediator of sepsis [102]. Beyond infections, HMGB1 has roles during trauma and sterile inflammation, such as in SIRS, where it orchestrates key events including piWBC recruitment and induction to secrete inflammatory cytokines [103, 104]. In addition, once outside cells, HMGB1, also known as *amphotericin*, promotes motility of cells as well as axonal nerve growth and has been found to be essential for brain development [105]. Increasingly, HMGB1, DAMPs, and the "danger" hypothesis are being explored in the CNS and its disorders [105].

1.9. HMGB1 and BBB Dysfunction. HMGB1 is released by innate immune cells in response to bacterial LPS or by endogenous TNF and other proinflammatory cytokines from innate immune cells. Externally located HMGB1 binds to PRRs such as TLR2 and TLR4 [11, 106] as well as RAGE [107, 108]. Evidence indicates that engagement of TLRs is needed for further cytokine production and release while activation of RAGE by HMGB1 induces piWBC recruitment [108]. Of interest, earlier studies indicated that RAGE was also required for neurite outgrowth by *amphotericin* in the developing nervous system [105, 109].

Increased circulating HMGB1 from peripheral systemic inflammation can activate one or more of its receptors such as TLR2 or TLR4 or RAGE on microvascular ECs [110], in a *target-based* approach. Based on recent published evidence, a mechanism by which HMGB1 can influence piWBC

recruitment is by its formation of a heterocomplex with the homeostatic chemokine CXCL12 on these cells [111]. This heterocomplex appears to act more potently on the CXCR4 receptor than on CXCL12 alone and CXCR4 expression on ECs of the BBB has been shown to enhance transmigration of piWBC [112]. Thus, the *HMGB1-CXCL12-CXCR4* axis may represent a new "player" in the transEC migration of piWBC in BBB/neuroinflammation leading to neurodegeneration.

1.10. Coagulation Cascade: Thrombin and BBB Dysfunction. Thrombin is the proinflammatory serine protease essential as the ultimate protease in the coagulation pathway, and as prothrombin it circulates at micromolar concentrations. By activation of a small family of G-protein-coupled receptors, known as PARs (proteinase-activated receptors) [113], thrombin has been found to have extensive roles within developing nervous system and following injury or degeneration outside of its pivotal position in coagulation [114, 115]. We postulated that both HMGB1 and thrombin may play a significant role in BBB disruption since both are proinflammatory and both are known to disrupt vascular barriers in other tissues [110, 116–121].

In an attempt to explain thrombin's effect on brain edema Guan and colleagues injected thrombin stereotactically into rat caudate nuclei and found extravasation of Evans Blue dye [122]. They also found that in addition adding thrombin to EC cell cultures increased expression of matrix metalloproteinase-2, which was proposed to occur by activating PAR1. In similar experiments, Garcia's group and others showed thrombin-mediated disruption of several microvascular barriers via a PAR1 mechanism [110, 116–121]. In a recent study by Festoff et al., both thrombin and HMGB1 can directly impair BBB integrity in vitro [123].

Over the last decade it has become increasingly appreciated that inflammation and coagulation are linked evolutionary defense systems [124, 125], a fact that is slowly becoming recognized in the CNS as well. In this regard, TBI, ischemic and hemorrhagic stroke are characterized by increased levels of intraparenchymal thrombin and HMGB1 as well as BBB dysfunction [126, 127]. In the brain cell low concentrations of thrombin act through its principal receptor, PAR1, to induce neuroprotection [115]. In contrast, at higher concentrations thrombin causes brain damage [128] where it appears to act via PAR4 [129–131]. Thrombin directly affects the activity of multiple cell types and regulates a variety of biological functions, including inflammation, leukocyte migration, and vascular permeability through PAR activation [132–135]. Furthermore, direct links also exist between thrombin and HMGB1: HMGB1 is involved in a number of systemic vascular diseases [136, 137] and is also increased in stroke [105, 138], while both HMGB1 and thrombin are released in various neurologic conditions and HMGB1 promotes coagulation [139]. Of interest, in TBI, the critical nongenetic antecedent event in AD, PD, and CTE, HMGB1 and thrombin—post-TBI coagulopathy [140, 141]—are both increased. Taken together, these observations raise the possibility that HMGB1 and thrombin participate during neuroinflammatory situations such as occurs post-TBI/CTE as well as in AD, PD, and ALS, which contribute to BBB dysfunction and transendothelial migration of piWBC.

One particular linkage topic that relates to the BBB as potentially revealing new therapeutic targets in AD is Aβ transport in and out of the brain. A number of reports have emphasized RAGE and the low density receptor related protein (LRP-1) in this capacity [26, 86]. Most consider that RAGE is the primary transporter of Aβ from blood to brain, while LRP-1 mediates transport of Aβ the opposite way. Consequently, a therapeutic approach might focus on interrupting RAGE binding to Aβ, and an oral, small-molecule inhibitor of RAGE, Azeliragon (TTP488), for mild AD entered Phase 3 trials in the US and Canada in 2015 (STEADFAST).

Furthermore, HMGB1 is quite susceptible to changes in redox state, both ROS and NO. Within the nucleus HMGB1 contains two DNA-binding HMG box domains (N-terminal A and central B). Recent evidence indicates that HMGB1 also normally translocates to the mitochondrion, where it affects mitochondrial quality control [142]. In normal brain cells the cysteines of the A-box (Cys23, Cys45) and B-box (Cys106) are reduced (-SH) allowing HMGB1 to bind DNA and translocate to/enter mitochondria. Reduced, nuclear, and mitochondrial HMGB1 can be actively secreted from macrophages and dendritic cells [142, 143]. In addition, HMGB1 can be released from *exploding* necrotic cells, while typically apoptotic cells retain HMGB1, which is tightly attached to hypoacetylated chromatin. Because of this, HMGB1 is not usually released from apoptotic cells and does not induce inflammation [144]. In neuroinflammation following TBI and stroke or in neurodegeneration, the damaged neural cell becomes oxidized and disulfide (S-S) bridges are formed between Cys23 and Cys45 while Cys106 can remain -SH; in this situation HMGB1 is proinflammatory. If it becomes completely oxidized; however, an additional S-S is formed with Cys23 and Cys45 and now HMGB1, as *amphotericin*, can promote regeneration; that is, it stimulates nerve growth [109, 145]. HMGB1 and TBI are actively being investigated including increased brain expression [146] and plasma levels associated with outcome after injury [147]. The relationship between HMGB1 and mitochondria, perhaps the HMGB1 fraction translocated to these organelles, is being established. HMGB1 and other DAMPs such as mitochondrial DNA [148], and other mitochondrial DAMPs released from mitochondria by trauma and other stimuli [9], can figure critically in development of neuroinflammation, as in systemic inflammation [10], leading to neurodegeneration.

Outside of the cell oxidized HMGB1 is known to ligate three different PRRs, all of which are expressed on the surface of cerebrovascular ECs. These include TLR2 and TLR4 [149–151] as well as RAGE [107, 152, 153]. Each of these PRRs binds a variety of ligands, besides HMGB1, most of which are critical in determining vascular complications of different diseases such as diabetes and atherosclerosis [67, 154]. Both TLR and RAGE ligation leads to NFκB activation that is sustained, and this in turn increases PRR expression, as well as TNF production [107]. This ensures that the inflammatory signal is maintained and amplified [67, 154]. Signal transduction through TLRs involves the Toll/IL-1 receptor

(TIR) domain (TIR) [155] and has both MyD88-dependent and independent pathways. MyD88 is essential for induction of inflammatory cytokines triggered by all TLRs, while a MyD88-independent pathway is specific for TLR4 and TLR3.

Thrombin has also been associated with ROS and, in particular, ROS-mediated membrane lipid peroxidation (MLP) [156, 157]. Prothrombin, like TM, is produced by astrocytes in normal brain [158] and both thrombin and prothrombin have been found to be associated with plaques and NFTs in AD brains [159]. We have recently shown that MLP is critical in neurodegeneration, both AD and PD, and involves thrombin and PAR1 [160]. In turn, ROS is clearly increased in association with neurodegeneration [161]. Furthermore, in support of the BBB in AD as source of proinflammatory factors, reports have shown an increase in thrombin and other proinflammatory factors in AD ECs [162].

Consequently, inhibiting RAGE might also produce beneficial results apart from Aβ transport by interrupting HMGB1 signaling through this receptor. Such activity would be additive in this context since HMGB1 also binds to and signals via TLR2 and TLR4 as well [106]. Recent interpretations suggest that HMGB1-RAGE is instrumental for piWBC infiltration [104, 111], whereas HMGB1-TLR4 may be responsible for cytokine production [11, 163].

1.11. Blood Markers of Microvascular Damage. The chondroitin sulfate proteoglycan, thrombomodulin (TM), is ubiquitously present on the surface of ECs [125]. We also found TM on mouse astrocyte surfaces [164] where it was functionally active and similar to the EC molecule. TM is an endogenous anticoagulant, one of three natural anticoagulant mechanisms, since it binds thrombin with high affinity and also the circulating zymogen protein C (PC) to form activated PC (APC) which then inactivates factors Va and VIIIa to stop coagulation [13, 125]. Beyond braking clotting, APC can also dampen inflammation, which it accomplishes in several ways: (1) by inhibiting expression of tissue factor (TF) and release of proinflammatory cytokines by monocytes; (2) by blocking expression of leukocyte adhesion molecules; and (3) by inhibiting neutrophil chemotaxis and cytoprotection [13, 125]. In this regard, recombinant TM (rTM) is cytoprotective since it binds thrombin, preventing its activation of PARs on neural or immune cells [165, 166]. We found it enhanced recovery after spinal cord injury in rats [167] and proposed at the time that the most attractive mechanism was binding of thrombin by rTM preventing its activation of specific PARs. However, using a slightly different rTM others found similar results that they attributed to APC's effects on activation and inhibition of leukocyte migration [168], as reviewed [169]. Important to note here is that thrombin is a potent inducer of microvessel hyperpermeability that is mediated by Rho kinase-dependent myosin light chain-2 phosphorylation and Ca^{2+} Influx through the Na^{+}/Ca^{2+} exchanger. These interactions ultimately activate the contractile mechanism of the endothelium leading to the physical opening of the interendothelial clefts and loss of BBB integrity [170, 171]. Furthermore, APC possesses various cytoprotective functions which, in addition to antiapoptotic and anti-inflammatory activities, include endothelial barrier stabilization. These cytoprotective activities seem to require both the endothelial protein C receptor (EPCR) and a subpopulation of PAR1, whereas APC elicits cytoprotective signaling through cleavage of these atypical PAR1 receptors leading to the activation of Rac1 signaling which promotes endothelial barrier protective responses [172, 173].

1.12. TM: Multifunction or Do-All Receptor at the BBB. Beyond anticoagulation, TM also functions as a natural anti-inflammatory agent [174] that has been attributed not to its thrombin and PC binding domain (known as TMD2/3) but to its NH2 terminal C-type lectin-like domain (TMD1) [125, 175, 176]. A separate explanation for the anti-inflammatory effects of rTM, apart from APC generation to inactivate Factors V and VIII, came from studies showing that TMD1 binds HMGB1 very tightly [177]. This path-finding study, and subsequent other novel ones in which the D1 domain was "knocked in" to produce transgenic mice lacking this C-type lectin-like domain (called $TM^{LeD/LeD}$ mice) [178], suggested that more than one anti-inflammatory mechanism might account for TM's effects [179]. Mechanistically, TMD1 binding to HMGB1 would prevent its engagement of RAGE [180] and/or TLR2/TLR4 [106]. Because of this Esmon [13] considered TM a "do-all" receptor bridging the nexus—the crossroads—of coagulation and inflammation (innate immunity). His schematic for TM showing functions discussed above is shown in Figure 1.

However, TM's anti-inflammatory mechanism may be more complicated since, in addition to HMGB1, TMD1 also actively binds to the carbohydrate Lewis Y (Le^{y}) antigen in LPS [175]. By binding to the Le^{y} antigen, rTMD1 is able to block the interaction of LPS with CD14 and/or TLRs, reducing subsequent LPS-induced inflammatory reactions and thereby suppressing downstream inflammatory signaling [176]. Consequently, in addition to thrombin binding and APC activation TM provides anti-inflammatory regulation via TMD1 binding of both HMGB1 and the Le^{y} antigen.

Considerable evidence exists that blood levels of soluble TM (sTM) and von Willebrand factor (vWf) can serve as surrogate markers for microvascular damage [181, 182]. Although few in number, several studies have also evaluated the plasma levels of sTM levels in different CNS diseases such as AD [183] and multiple sclerosis (MS) patients [184] suggesting that sTM is potentially a good marker to assess brain (BBB) microvascular EC damage.

More recently, in our efforts to develop a validated marker for conversion of MCI to AD we measured both sTM antigen (TM-Ag) and a functional assay (TMa) [185, 186] for TM activation of PC to APC. We found significant increases above age-matched controls when MCI and AD levels are grouped. However, MCI sTM levels were, in fact, greater than in AD patients. But when TM-Ag was analyzed specifically the following relationship was found: AD > MCI > control [123]. In addition to thrombin, the prototypic DAMP alarmin, HMGB1, dramatically enhanced in vitro BBB permeability to several molecular weight dextran whether at 3 or 6 hr incubations at ng/mL concentrations. Others have shown that

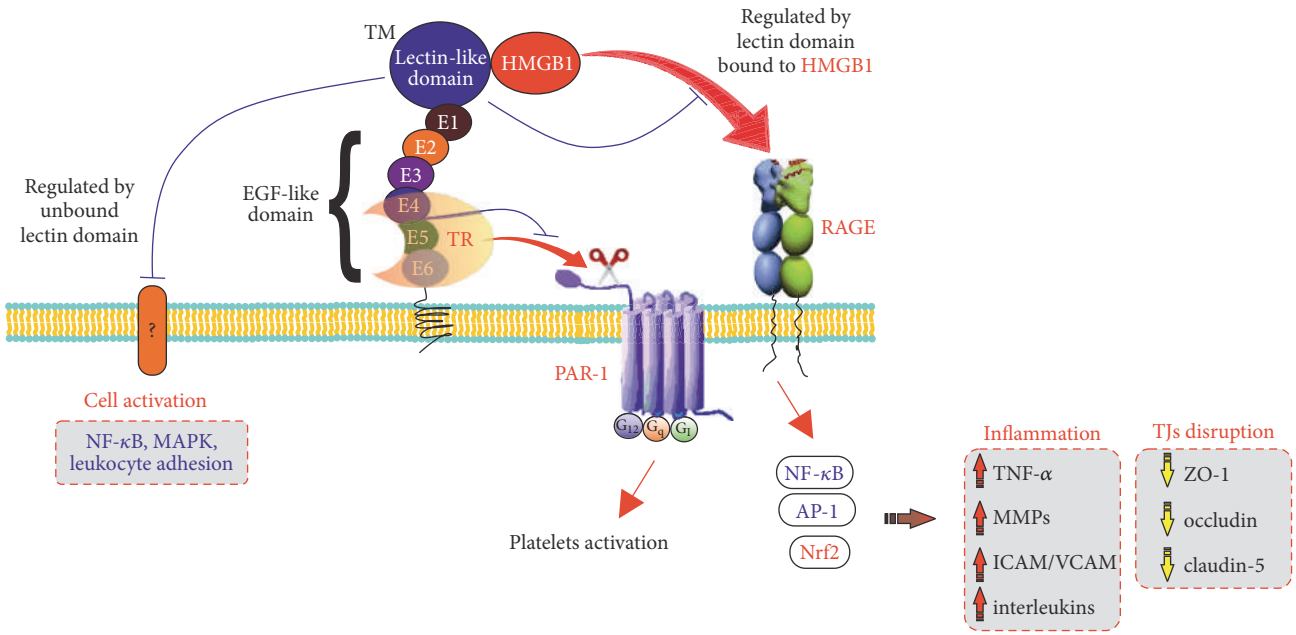

Figure 1: TM as a multifunctional or "Do-All" receptor, after Esmon [13] in considering roles at nexus of coagulation and innate immune inflammation at the BBB.

in rats whether BBB dysfunction was due to experimental stroke or TBI, a neutralizing monoclonal antibody (mAb) to HMGB1 prevented the BBB dysfunction [126, 127]. This same group has shown more recently that anti-HMGB1 mAb provides neuroprotection in a rat model of PD by attenuating the BBB breach in this disease [187]. We directly correlated such direct effects with levels of several of these molecules present in sera from AD, MCI, and control samples and we found direct correlation with both sRAGE and HMGB1 with Aβ [123].

Figure 2 represents our current concepts as to how *coag-inflammatory* molecules such as DAMPs, thrombin, and TM might interact with CNS injury, cytokines, and other proinflammatory molecules and Aβ in the breach of the BBB/neurovascular unit leading to neuroinflammation and, ultimately, neurodegeneration. Incorporated within this concept is how we might use this information to develop relatively noninvasive blood-based biomarkers to diagnose these conditions *before* the onset of symptoms or signs and to develop new therapeutic targets to prevent evolution of these disorders after initial injuries develop.

2. Conclusions

The BBB and greater neurovascular unit might function as both *source* and *target* of inflammatory factors since over the last 15 years studies have shown that the cerebral microcirculation is in an "activated proinflammatory" state in neurodegenerative diseases such as AD [188]. One such target may be TM since we found that as with relapsing remitting MS patients [184] increased levels of sTM occur in sera of AD and MCI patients compared with controls [123], clearly a source of this anti-inflammatory/anticoagulant. In turn, when HMGB1 binds to *target* RAGE and/or TLRs on brain microvascular ECs they are *source* of proinflammatory agents by releasing TNF and other cytokines [188].

An increased understanding of the role of HMGB1 and other DAMPs, along with thrombin/PARs in the activation and transendothelial migration of piWBC contributing to neuroinflammation in AD, PD, and all neurodegenerative diseases as well as neurotrauma, may allow discovery of novel therapeutic targets and treatment strategies. Not only might these facilitate treatment to halt progression in these poorly treated and currently not curable diseases, but also they might aid in detecting the conversion from minimal deficit or preclinical condition to disease in other neurological disorders that display BBB dysfunction that lead to the migration of inflammatory cells into the CNS. Noteworthy to mention here is also the fact that inflammatory diseases of the gastrointestinal tract such as intestinal inflammatory bowel diseases, which includes Crohn's disease and ulcerative colitis, can affect the CNS leading to behavioral symptoms and cognitive dysfunction [189]. This further emphasizes the potential impact of peripheral inflammation on the CNS.

Abbreviations

ALS: Amyotrophic lateral sclerosis
AD: Alzheimer's disease
Aβ: Amyloid beta
APC: Activated PC
BBB: Blood-brain barrier
CTE: Chronic traumatic encephalopathy
DAMPs: Damage-associated molecular patterns
EC: Endothelial cell

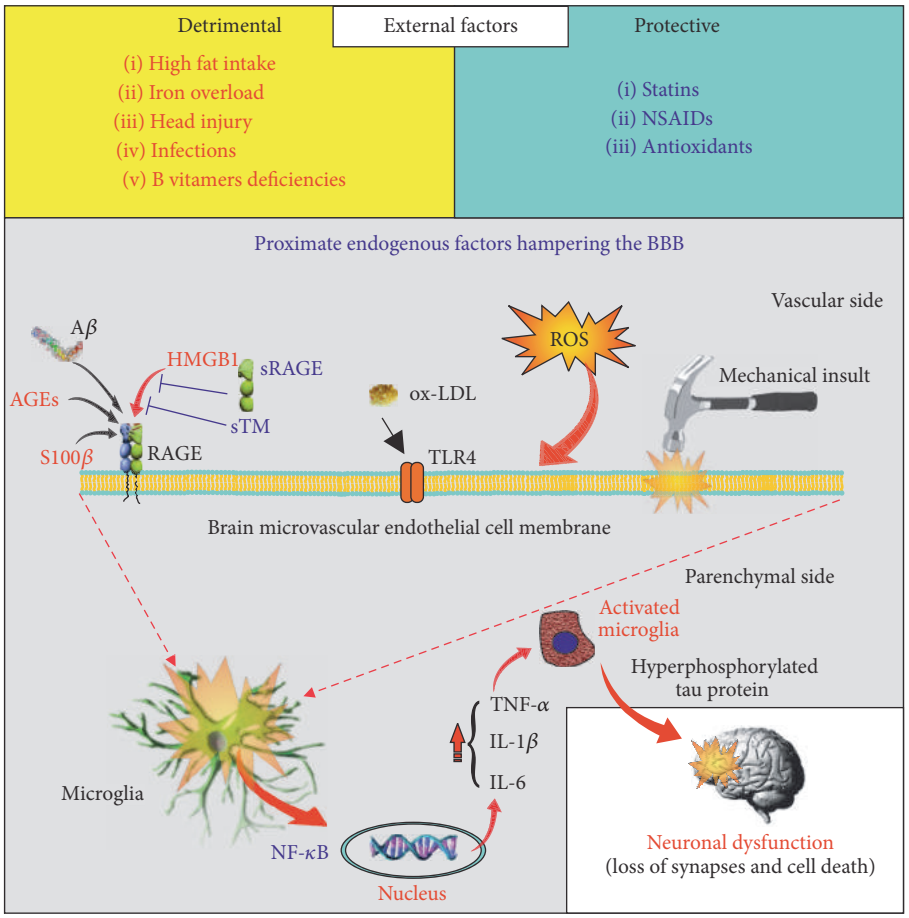

Figure 2: Schematic based on data, both ours and others, where proximate factors generated from external sources, including dietary, infections, and injury, act on brain microvascular ECs to cause a "breach" of the BBB/NVU. The proximate factors include molecules associated with innate immune activation (DAMPs and Aβ) while agents that can interdict these factors include sTM and sRAGE. The initial and subsequent episodes of the dysfunction can "fan the flames" of neuroinflammation within the brain with microglial (and astrocytic) activation at transcriptional levels resulting in mitochondrial dysfunction, tau hyperphosphorylation, and aggregation, synapse loss, and neuronal cell death.

HMGB1: High mobility group box protein 1
LBs: Lewy bodies
LPS: Lipopolysaccharide
LRP-1: Low density receptor related protein
LRRK2: Leucine-rich repeat kinase 2
MCI: Mild cognitive impairment
MLP: Membrane lipid peroxidation
MS: Multiple sclerosis
NFT: Neurofibrillary tangles
PAR: Protease activated receptor
PD: Parkinson's disease
PRRs: Pattern recognition receptors
SIRS: Systemic inflammatory response syndrome
SNpc: Substantia nigra pars compacta
sRAGE: Soluble receptor for advanced glycation end products
TBI: Traumatic brain injury
TLR: Toll-like receptor
TM: Thrombomodulin
TNF: Tumor necrosis factor
WBC: White blood cells.

Conflicts of Interest

Barry W. Festoff is the founder and president of PHLOGISTIX LLC; Ravi K. Sajja and Luca Cucullo have no conflicts of interest.

Authors' Contributions

Barry W. Festoff, Luca Cucullo, and Ravi K. Sajja equally contributed to the concept, manuscript drafting, revision, and approval. All authors read and approved the final manuscript.

Acknowledgments

This work was supported by NIH/NIDA R01-DA029121-01A1 and Alternative Research Development Foundation grants

received by Luca Cucullo, the Kansas Bioscience Authority, Digital Sandbox KC, and PHLOGISTIX LLC (received by Barry W. Festoff).

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