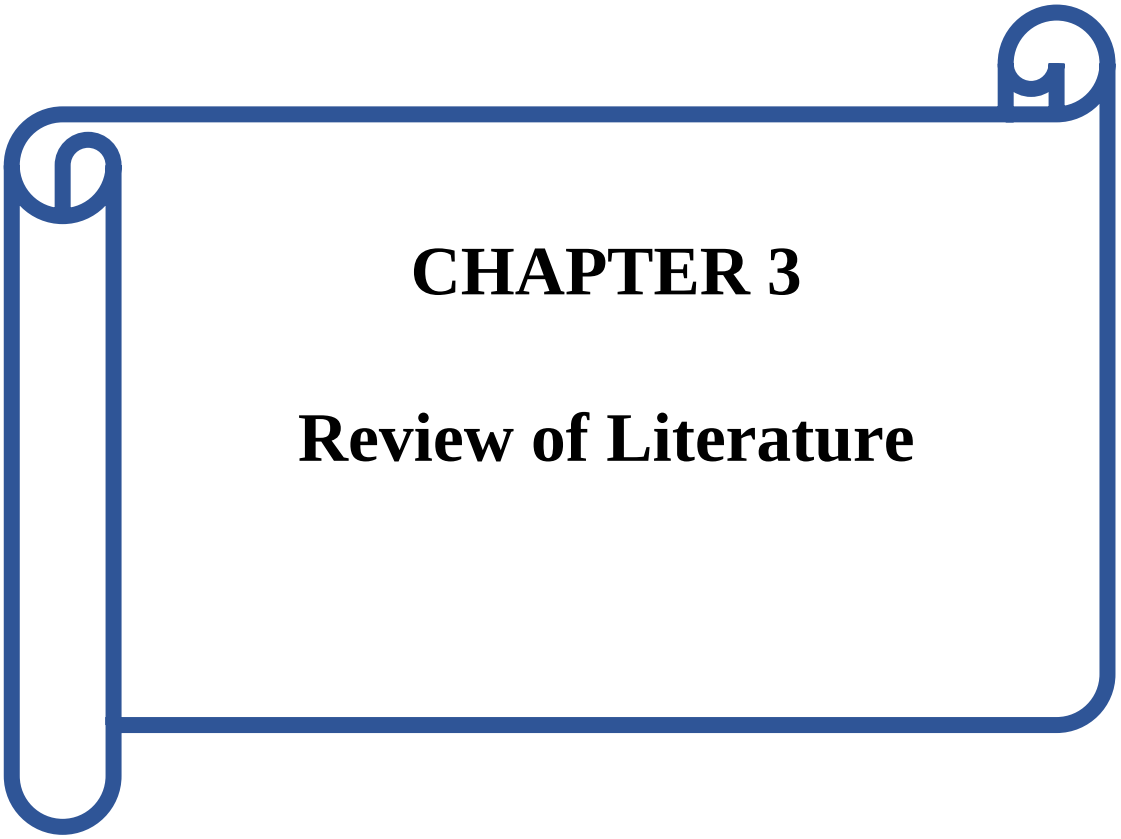

CHAPTER 3

Review of Literature

3. Review of Literature

3.1. The impacts of the SHANK3 gene mutations on the ASD

SHANK3 has been documented as one of the few proteins that directly impact synaptic activities from multiple perspectives, especially during early developmental stages. Protein interactions between SHANK3 and other postsynaptic proteins have made SHANK3 critical in the process of dendrite and spine formation, vesicle release, synaptic transmission, and synaptic plasticity. A number of SHANK3 variants have been identified in neurodevelopmental disorders, particularly in ASD [1, 2]. The mutation or deletion of SHANK3 results in different forms of synaptic dysfunctions and can even lead to altered spine morphologies, a decline in GABA-mediated synaptic transmission, and impaired long-term potentiation (LTP). As a result, these abnormalities are associated with representative autism-like behaviors [3]. Furthermore, microdeletions, microduplication, small intragenic deletions, translocations with break points and point mutations of SHANK3 have been discovered in idiopathic ASD cases [4, 5]. Fu et al. [6], in a cohort of 63,237 individuals with ASD, discovered 72 genes associated with ASD at a false discovery rate (FDR) ≤ 0.001. SHANK3 was one of the high-risk genes. Their findings revealed protein-truncating variants (PTVs) and deletion mutations in the SHANK3 gene. Consistent with studies in humans, different studies on SHANK3 mutation sites confirmed the strong genetic associations between the SHANK3 gene and ASD and individuals exhibited defects in dendrite development and morphology and axonal growth cone motility [7-10]. In addition, Waga et al., [11] identified four SHANK3 missense mutations, two deletions, and two insertions, but none in the 228 controls. SHANK3 alteration has thus been linked to ASD. Whereas a recent study elucidated that SHANK3 haploinsufficiency induces morphological and functional abnormalities in pyramidal neurons within the prefrontal cortex of a canine model. These alterations disrupted social communication patterns and resulted in autistic behaviors [12].

Conversely, the heightened expression of SHANK3 resulted not only in an elevation of postsynaptic proteins but also in an increased expression level of presynaptic proteins. It was identified as stemming from SHANK3-dependent transsynaptic signaling facilitated through the neurexin-neuroligin complex [13]. Subsequently, mutations in SHANK3 associated with ASD were observed to modify the capacity of SHANK3 to augment the

synaptic expression of both pre- and postsynaptic proteins [13].

Taken together, mutations within the SHANK3 gene are characterized and have the highest clinical relevance [14]. These molecular defects have been included deletions and duplications of variable size as well as disruptive translocations, or individual sequence changes such as missense, frame shift or splice site mutations [15]. While the relevance of proper SHANK3 gene dosage for normal brain function has been extensively shown with deletions or duplications leading to neuropsychiatric disorders involving ASD, the functional impact of more subtle missense mutations is less clear[16, 17].

3. 2. The N-terminus point mutations implicated in ASD

Since the pathogenicity of SHANK3 missense mutations might go beyond altering SHANK3 protein levels in excitatory synapses, numerous structural analyses of mutant SHANK3 proteins have been done to understand the origin and molecular impact of such missense mutations [18]. A plethora of different missense mutations covering most domains of the SHANK3 have been found in autistic patients [14, 19]. However, The N-terminus of SHANK3, including the SPN and ARR domains, has emerged as a hotspot of missense mutations and provided insights into the potential role of these mutations [20].

The N-terminal portion of SHANK3 has been impacted by multiple missense variants observed in ASD patients [21]. The relevance of these mutations concerning SHANK3 function and the pathogenesis of ASD remains unclear [4, 16]. Among these mutations, P141A and Q321R are de novo mutations [4, 22], whereas the rest are inherited, mainly from healthy parents (A198G, R300C and S341L) [16, 22]. Along with this, investigations revealed that the SPN domain has been affected by two mutations inherited from an epileptic father (L68P) and a mother with social phobia (R12C) mutations [16, 23].

3.2.1. Functional effects of point mutations in the SPN domain

Numerous studies have revealed that the SPN domain of SHANK3 is altered in individuals with ASD due to multiple point mutations [16, 20, 23]. Owji et al. [21] discerned 29 deleterious missense variants within the SHANK3 gene. Molecular dynamics simulations were employed, wherein mutations such as L47P and G54W within the SPN domain exhibited pronounced destabilizing effects, revealing alterations in intramolecular interactions and heightened fluctuations in the SHANK3 gene.

Furthermore, the R12C mutation constitutes a charge-neutralizing substitution situated within the β1-strand of the SPN domain, whereas the L68P mutation impacts the hydrophobic portion of this domain [20]. On the other hand, the L68P mutation exhibited higher penetrance than the R12C mutation and was not found in any healthy control [23]. The R12C mutation was initially identified in a male ASD patient and was transmitted from the mother, who has social phobia traits [16]. The patient displayed high pain threshold and low noise threshold, as well as a complete absence of language and severe mental retardation[16]. The L68P variant was found in a female ASD patient who inherited the mutation from an epileptic father and displayed developmental delay and absent speech [23].

Interestingly, Woike et al. [24] revealed that ASD patient-derived missense mutations lead to an opening conformation through the unfolding of the SPN domain by the L68P variant. However, It was shown that the R12C mutation reduces the binding affinity of the SPN domain to Rap1 approximately by a factor of 11 and effectively abolishes binding to H-Ras, Rap1a and Rap1b in 13 pulldown assays [20]. The prior state was elucidated by the engagement of the R12 residue within the SPN domain in the establishment of an ion-ion pair with E37 of Ras, which can no longer be formed upon R12C substitution. While the L68P mutation showed a similarly disruptive behavior in the interaction with H-Ras, Rap1a and Rap1b, this mutation was predicted to primarily affect the structure of the SPN domain and how it interacts with the ARR domain [20]. The latter was discussed as a potential cause for the increased interaction of L68P mutant SHANK3 with the ARR domain ligand sharpin [25]. Notably, a very recent study demonstrated that SHANK3 can bind actin directly via the SPN domain, which is regulated by a conformational switch of the SPN and ARR domain [26]. Thereby, the interaction of the SPN domain with the ARR domain represents an autoinhibited “closed” state of SHANK3, which prevents binding to actin [26]. At the cellular level, it was observed that the overexpression of the SHANK3 R12C mutant in primary hippocampal neurons led to a less significant augmentation in miniature excitatory post-synaptic currents (mEPSCs) in comparison with neurons overexpressing the SHANK3 WT [9]. Additionally, the R12C mutant increased F-actin levels and the spine density in hippocampal primary neurons less efficiently than SHANK3 WT upon overexpression, leading to intermediate effects on spine structure and synaptic transmission [9]. The

experimental observations by using rat SHANK3 N52R constructed mutant destabilized the closed conformation of SHANK3 and distrupted the intramolecular interactions between SPN and ARR domains and triggered open-up conformation in the molecule [26, 27].

Collectively, The N-terminal SPN-ARR is folded in a closed conformation [20], and this fold has been shown to be stabilized. Conversely, numerous studies revealed that missense mutations in the ARR domain may also lead to changes in this conformation [2, 27].

3.2.2. Functional effects of point mutations in the ARR domain

Previous studies provided evidence that mutation-induced structural changes serve as a starting point for a chain of defects acting on multiple levels, from altered intracellular protein dynamics to changes in biochemical signal transduction [18, 26]. Among the ARR domain mutations, the Q321R mutation is located within this N-terminal SHANK3 fragment[18]. The latter represents a de novo mutation found in a female ASD patient displaying social and language deficits with limited reciprocal conversation, social withdrawal, verbal repetitive behaviors, as well as severe inattention and irritability [22]. On a molecular level, overexpression of SHANK3 Q321R mutant in immature hippocampal neurons has been reported to culminate in a substantial reduction of F-actin levels in dendritic spines compared to overexpressed SHANK3 WT [9]. This suggests that the Q321R mutation interferes with the ability of SHANK3 to regulate F-actin levels in dendritic spines, resulting in an alteration of spine structure and function[9]. Furthermore, Q321R mutant SHANK3 displays enhanced interaction with the ARR domain ligand sharpin [25]. Recently, SHANK3Q321R/Q321R mice were shown to exhibit reduced excitability of hippocampal CA1 pyramidal neurons, corroborating the role of SHANK3 in the regulation of synaptic transmission [28].

However, the majority of mutations are uncommon; the R300C mutation has been identified 15 times in a database containing exomes from 64,000 predominantly healthy individuals (ExAC database), indicating that it could be a polymorphism instead of a harmful mutation. Nevertheless, another investigation demonstrated that overexpression of SHANK3 WT in hippocampal neurons led to a significant heightening in mEPSC frequency. In contrast, three missense mutations, including R300C, resulted in an elevation in mEPSC frequency, albeit not to the same extent as observed with SHANK3

WT [9]. Arons et al. [13] demonstrated that mutations associated with ASD in SHANK3 (R300C, Q321R) disrupt SHANK3's function in synaptic transmission signaling. Unlike the overexpression of SHANK3 WT, these mutants were unable to elevate the levels of postsynaptic Homer1. Moreover, neurons expressing these mutant variants of SHANK3 exhibited a notable reduction in the size of the total recycling pool of synaptic vesicles at presynaptic sites, which interacted with dendritic profiles, in comparison to neurons expressing SHANK3 WT [13].

Furthermore, P141A is a novel mutation found in an autistic patient; P141A is situated in the contact surface between SPN-ARR domains [4]. Notably, the SHANK3 P141A mutant has been revealed to have a dramatic effect as it disrupted the intramolecular interaction between SPN-ARR domains, causing open-up conformational [24, 27]. On the other hand, mutations in SHANK3 have been mostly associated with ASD [14]; the L270M variant was reported in a family where carriers of this variant exhibit a somewhat divergent phenotypic spectrum. Most remarkable is the spectrum of challenging behavior, including ADHD and learning difficulties [27]. Noteworthy, the SHANK3 L270M mutation is positioned within the hydrophobic part of the ARR domain without displaying significant characteristics of an unfolding state. However, it is speculated that it slightly alters the surface of the Ank repeats in a way that is incompatible with binding the protein partners of SHANK3 [27].

3.3. SHANK3 protein partners

Recently, there has been a research focus on the missense mutations identified in autistic patients that have been found to be situated in the N-terminal of SHANK3 [18, 25, 27, 29]. However, the functional relevance of these mutations is still unclear due to the N-terminal domains being much less studied compared with the other SHANK3 domains and motifs [18]. These mutations in the SHANK3 N-terminal can have severe consequences on synaptic functioning, so understanding the N-terminal mechanisms will be key to elucidating how these mutations lead to a disease phenotype. The SHANK3 N-terminal domains have been shown previously to interact with other important proteins involved in signalling cascades and have direct implications on the dynamics and organisation of the actin cytoskeleton and abnormal dendritic spine morphology and function[15, 20, 27]. Two significant SHANK3 point mutations associated with ASD are the R12C and the L68P mutations. The R12C mutation has been shown to inhibit the

SPN domain interaction with Ras-family GTPases, and the L68P mutation has been linked with an unfolding event in the SPN domain structure [2, 20, 30]. Neurons expressing these mutations of SHANK3 exhibit dysfunctional dendritic spines with observations of impaired growth cone motility, impaired β1-integrin inhibition and a reduction in spine density and maturation [9, 18, 20]. Furthermore, several recent studies have begun to clarify the significance of the intramolecular interaction between SPN-ARR domains. Thus, it was observed that the loop structure between SPN and ARR serves as a specific site for the interaction with CaMKIIα, which partially conceals the Ras binding site. αCaMKII, a crucial element in synaptic plasticity and learning processes, plays a pivotal role in decoding synaptic Ca2+ oscillations, regulating calcium levels, PSD integration, and shaping the morphology of dendritic spines [31]. Interestingly, several missense mutations lead to an opening conformation, such as P141A or N52R variants, upon disrupting the contact area on the side of the Ank domain, resulting in impaired binding to αCaMKII [2, 25, 27]. The physiological relevance of this interaction may be that Shank3 acts as a negative regulator of the αCaMKII signalling pathway, which is prominently involved in synaptic plasticity. One prediction here would be that SHANK3 deficiency (or the presence of mutants L68P, N52R, or P141A) would lead to a higher basal activity of αCaMKII due to the loss of the inhibiting effect of the Shank3 N-terminus [27]. Conversely, earlier research indicated that intramolecular interaction prevents α-Fodrin from accessing its location on the ARR domain in SHANK3 [25], whereas SHANK3 P141A significantly boosted the connecting of α-Fodrin to SHANK3 [27]. A potential scenario might be that the open conformation facilitates α-Fodrin binding, which increases actin linkage and enhances integrin activation. Taken together, these mutants might change the flexible conformation of SHANK3, impairing the capacity to coordinate cytoskeletal aggregation and signaling dysregulation. The physiological activity of the SHANK3-actin connection influences dendritic protrusion morphology in neuron cells and ASD-related characteristics *in vivo* [32]. Notably, SHANK3 loss-of-function mutations have been associated with neurodevelopmental diseases such as ASD [2].

Woike et al. [27] unveiled a novel SHANK3 missense mutation (L270M) situated within the AR R domain, observed in individuals displaying an ADHD-like phenotype. Functional analysis elucidated that this mutation resulted in a reduction in δ-catenin

binding. Thereby, the plausible interpretation of the SHANK3 L270M mutation subtly modifies the surface characteristics of the ARR domain in a manner incompatible with δ-catenin binding. Herein worth noting that loss-of-function mutations in the CTNND2 gene that encodes δ-catenin have been linked to an ADHD phenotype [33]. Consequently, the L270M mutation may have contributed to the relatively milder ASD phenotype [27].

These disease-related mutations indicate that single point mutations in the SHANK3 N-terminal can have severe consequences on synaptic functioning so understanding the N-terminal mechanisms will be key to elucidating how these mutations lead to a disease phenotype. Overall, individual molecular and cellular consequences of these ASD-associated missense mutations have been explored in some detail; however, studies providing a link between mutation-induced structural perturbations of SHANK3 and their functional contribution to corresponding synaptic phenotypes are largely missing.

3.4. Interactions between genetic alterations in synaptic proteins and brain immune activation

The genetic alterations of synaptic proteins may drive the brain's immune system toward an activated state defined by the term “immune-synaptopathy. Dysfunctional synapses/circuits may signal to other brain cells endowed with immune abilities, such as microglia and astrocytes, through “alert messages,” which in turn activate the immune system. Whether this activation might represent an attempt to cope with defective synapses/circuits is an intriguing possibility that is worth addressing [34].

An interesting study showed that mice heterozygous for SHANK3, differently from their full knock-out (KO) counterpart, did not display ASD-related behavioral deficits. However, these animals started showing similar circuit and behavioral alterations when acutely challenged with LPS at P60 [35]. It indicates that inflammation may modify the expression of key molecular drivers of behavior and mimic the molecular consequences triggered by genetic defects in synaptopathy genes. Altogether, these data clearly showed that immune system activation, even if it occurs in adulthood, in a highly vulnerable genetic background may unleash social behavior traits [35]. Alterations in the expression levels of synaptic proteins may also impact the immune system. For example, the early loss of Shank3 in the nucleus accumbens, which affects mouse behavior, causes an

imbalance of inflammatory mediators, as demonstrated by transcriptomic analysis of medium spiny neurons of the nucleus accumbens [35]. Atanasova et al. [36] investigated gene-environment interactions in ASD using a two-hit mouse model combining Shank3Δ11−/− and maternal immune activation (MIA). Their findings demonstrated that Poly I: C in the two-hit mice can unravel an ASD core symptom that was not present in the SHANK3Δ11−/− mice alone. The two-hit mice exhibited social behavior deficits, with an unexpected increase in postsynaptic density (PSD) proteins at excitatory synapses in specific brain regions. While the specific pathways behind the exacerbated phenotype remain unclear.

3.5. Signaling pathways involved in neuroinflammation

Several signaling pathways are involved in the neuronal inflammatory response. Primarily, we will focus on nuclear factor-κB (NF-κB) and mitogen-activated protein kinase (MAPK) signaling cascades, which all play key roles in inflammatory responses [37].

3.5.1. NF-κB signaling pathway:

The Nuclear kappa factor B (NF-κB) route plays a significant role in the processes of apoptosis, immunological activation, and inflammation. Activation of NF-κB is inducible by inflammatory cytokines, markers of infection, or stress-activated protein kinases [38]. More than 500 genes related to inflammation have been shown to be activated by the NF-κB family, which in turn triggers the release of cytokines and chemokines necessary for inflammation [39]. Some of the proinflammatory cytokines such as IL-6, IL-12, and TNF-α form common target genes for activated NF-κB [40], creating a positive feedback loop that, if NF-κB becomes abnormally or continuously active, has the potential to cause chronic and excessive inflammation [39]. The neurological system seems to be where the ubiquitous functions of NF-κB in neuroprotection, apoptosis, and inflammation are most pronounced [41]. The consequences of NF-κB activation on gene expression vary depending on the stimulus that activates it, the type of cell that it targets, and the microenvironment [42]. According to previous research, NF-κB activation in glial cells may result in the loss of neurons, but activation in neurons may increase the likelihood of their survival [42]. The NF-κB is a complex player in the etiology of the targeting pathway of ASD, an indication of neuroinflammation that turns out to be

crucial both as a marker for the illness treatment and as a selector for therapeutic interposition.

Increased expression and enriched NF-κB signaling has been found in some studies performed on the peripheral blood samples and postmortem brains of ASD patients, as well as in studies on animal models [42]. Several theories have emerged concerning the involvement of NF-κB molecular signaling as a nexus for multiple intricate pathways in autism. Inflammatory cytokines, generated by the immune system in both acute and chronic contexts, play pivotal roles in responses. Elevated cytokine expression, coupled with the activation of NF-κB/iNOS, has been identified in the monocytes of individuals with ASD. This interconnected mechanism may contribute to the manifestation of neuroinflammation in ASD [39].

The biological and physiological immune system's protective effects of nuclear factor kappa B activation are currently being outweighed by the detrimental effects of nuclear factor kappa B malfunction. Considerable progress has been made in comprehending the primary function and operation of NF-κB [43]. Various mechanisms implication of the activation of NF-κB inflammatory signaling pathway in the etiology of ASD possibility due to the increasing burden of oxidative levels. Overall, it can be said that the connection between microglia substance and expression in different brain locations elucidates the prospective role of NF-κB in distinguishing the inflammation region of the brain on ASD [44]. Of note, NF-κB signaling can also be initiated downstream of the MAPK cascade [45].

3.5.2. Mitogen-activated protein kinase (MAPK) signaling pathway

The MAPK signaling cascade is activated by cytokines, mediators of stress, and inflammatory markers, such as ligands of Toll-like receptors [46]. Through a series of phosphorylation cascades, MAPK proteins transduce and propagate extracellular signals within the cell [47]. During inflammation, MAPK signaling is mainly induced by Toll-like receptor activation, resulting in the phosphorylation of transcription factors [48]. Once phosphorylated, these transcription factors translocate to the nucleus and transcribe genes encoding proinflammatory cytokines, such as IL-6 and TNF-α [49]. Within the CNS, IL-6 signaling forms a positive feedback loop through the activation of MAPK signaling, which is reported to promote neurogenesis [50]. Microglial activation can also induce the MAPK signaling cascade, promoting a neuroinflammatory environment that

contributes to neurodegeneration [51].

The ERK/MAPK signaling is known to play a critical role in brain development, as well as in learning, memory, and cognition [52]. Therefore, it may be possible that MIA, through excessive IL-17A expression in the fetal brain, might impact ASD symptoms through the dysregulation of this pathway. Importantly, mutations in ERK-pathway genes have been observed in patients with ASD [53], and the dysregulation of ERK signaling has been observed in animal models and the lymphocytes of ASD patients [54].

In a study conducted by Rosina et al. [55] to identify the expression of the MAPK pathway in patients affected by moderate and severe idiopathic autism, it was discovered that there was an increase in the activity of MAPK pathway in these patients by analyzing the peripheral blood at the protein level, which showed the impact of perturbed MAPK signaling in the etiology of ASD. Therefore, a well-regulated MAPK is necessary for normal neurodevelopment, whereas perturbation of the MAPK pathway results in neurodevelopmental disorders, including ASD [56].

During an inflammatory response, NF-κB and MAPK signaling pathways can all be activated by proinflammatory cytokines [46].

3.6. Cytokines and chemokines drive neuroinflammation in ASD

Irregular cytokine and chemokine profiles have been recorded in the cerebrospinal fluid (CSF), blood, and brain of those with ASD [57-59]. Plasma samples from male ASD subjects revealed elevated levels of IL-1β, IL-5, IL-8, IL-12, IL-13, and IL-17 compared to controls [60]. In another multiplex cytokine screen, similar differences were found in IL-1β, IL-12, and IL-17, but also IL-6, in individuals with ASD compared with age-matched typically developing children [61]. A recent meta-analysis conducted with 1,393 patients with ASD found higher concentrations of IL-6 in the blood, as well as increased concentrations of proinflammatory cytokines IL-1β, TNF-α, and interferon-γ (IFN-γ) within the periphery [62]. Within the CNS, localized cytokine and chemokine variances have been reported. Higher levels of IL-6, IL-8, TNF-α, and IFN-γ are observed in the frontal cerebral cortex of ASD patients compared to age-matched control cortices [63]. Moreover, evidence of elevated TNF receptor I levels in the CSF of ASD children further indicates inflammation not just in the periphery but also within the CNS [64]. Although inflammatory markers may differ depending on genetic predisposition, when screening panel results are normalized to parental cytokine expression, IL-8 expression was still

seen to be elevated in children with ASD [65]. These cytokine profiles have been found to differ in males and females, indicating that testosterone may impinge on the inflammatory processes underlying ASD [66].

Microglia are activated in multiple brain regions of young adults with ASD [67]. Increased pro-inflammatory cytokines in blood and cerebrospinal fluid (CSF) and increased microglia number and activation in the postmortem dorsolateral prefrontal cortex (DLPFC) provide strong evidence of neuroinflammation in ASD [68]. In addition, changes were observed in the expression levels of pro-inflammatory (CD68 and IL-1β) and anti-inflammatory genes (IGF1 and IGF1R) in gray- and white-matter tissues of ACC in males with ASD [69]. Current studies have shown that the gene expression of anti-inflammatory cytokine IL-37 and pro-inflammatory cytokines IL-18 and TNF increases in the amygdala and dorsolateral prefrontal cortex of children with ASD [70]. In addition, IL-38 is decreased in the amygdala of children with ASD [71].

Chemokines, as a subset of cytokines that guide cell migration, are mainly divided into two categories: CXC chemokines and CC chemokines (including CCL2 (MCP-1), CCL3 (MIP-1α), CCL4 (MIP-1β), CCL5 (RANTES), CCL11 (Eotaxin)). CCL2 is conformably elevated in the brain and blood of individuals with autism and has been extensively studied. CCL2 is produced by microglia and astrocytes in the CNS, and in turn, CCL2 regulates the reactivity, migration, and proliferation of microglia [72, 73]. In the offspring of maternal exposure to CAF (cafeteria diet) diet or Poly (I: C) inoculation, CCL2 signaling disrupts social behavior by microglia morphology [74]. Flavonoid methoxy luteolin, a peptide neurotensin (NT) inhibitor, reduced the gene expression and release of proinflammatory cytokines IL-1β, CCL2, and CCL5 in human microglia [75]. All data support cytokines and chemokines as essential mediators in neuroinflammation and autism-like behaviors. Many studies reported activation of astrocytes in postmortem ASD brains [72, 76, 77]. Reactive astrocytes are the major source of releasing cytokines. The macrophage chemoattractant protein (MCP-1), which is in charge of monocyte/macrophage recruitment to the areas of inflammation, and pro-inflammatory cytokine interleukin-6 (IL-6), are altered in cortical and subcortical white matter in ASD [76]. The expression of the translocator protein 18 kDa (TSPO), which is a marker for brain inflammation, and the amount of activated microglia in the frontal cortex and cerebellum are increased in reactive astrocytes in ASD [78]. Monocyte chemoattractant

protein-1 (MCP-1/CCL2) is a chemokine that has been reported to be elevated in the brain and blood of ASD cases [76, 79]. CCL2 is produced by astrocytes and microglia in the brain and is necessary for the proliferation, migration, and activation of microglia and astrocytes [80, 81]. The elevated level of CCL2 could also increase blood–brain barrier (BBB) permeability and allow more T-lymphocytes to enter the brain during neuroinflammation [82]. Multifocal perivascular lymphocytic cuffs are associated with astrocyte blebs that represent a cytotoxic reaction to lymphocyte attack, suggesting a dysregulation in cellular immunity that could damage astrocytes in ASD brains [83]. However, many studies reported immune system dysfunction.

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