NEUROGLIA MOLECULAR MECHANISMS IN PSYCHIATRIC DISORDERS

EDITED BY: Caterina Scuderi, Mami Noda and Alexei Verkhratsky
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NEUROGLIA MOLECULAR MECHANISMS IN PSYCHIATRIC DISORDERS

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Neuropsychiatric disorders have long been considered as specific dysfunctions of neuronal functions. Studies of the recent decade, however, have challenged this simplistic view, highlighting the important role played by neuroglial cells in the onset and/or progression of neuropsychiatric diseases. In the central nervous system (CNS) non-excitable neuroglia are represented by cells of ectodermal origin (astrocytes, mainly responsible for CNS homeostasis and oligodendrocytes that provide myelination and support for axons) and mesodermal origin (microglial cells that are scions of foetal macrophages entering the neural tube early in development; these cells provide for CNS defence and contribute to shaping neuronal networks).

Pathological changes of neuroglia are complex; these changes are classified into reactive gliosis (astrogliosis, activation of microglia and hypertrophy of oligodendroglial precursors), gliodegeneration with loss of function and glial pathological remodelling. Combination of these processes defines the evolution of neurological diseases in general and neuropsychiatric disorders in particular.

In this research topic we addressed the contribution of neuroglia to major neuropsychiatric pathologies including major depression, schizophrenia, and addictive disorders.

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EDITORIAL
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Editorial: Neuroglia Molecular Mechanisms in Psychiatric Disorders

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Keywords: neuroglia, astrocyte, microglia, oligodendrocyte, psychiatric disorder

Editorial on the Research Topic

Neuroglia Molecular Mechanisms in Psychiatric Disorders

Neuroglia are an extended class of cells of ectodermal (astroglia, oligodendroglia, and peripheral glial cells) and mesodermal (microglia) origin, which provide extensive homeostatic support, protection, and defense of nervous tissue (Kettenmann and Ransom, 2013; Verkhratsky and Butt, 2013; Verkhratsky and Nedergaard, 2018). Pathological potential of glial cells has been recognized since their discovery (Virchow, 1858; Andriezen, 1893; Nissl, 1899; Alzheimer, 1910), while recent decade highlighted the fundamental role of neuroglia in the progression of all types of neurological diseases (Giaume et al., 2007; Verkhratsky et al., 2013, 2014, 2016, 2017; Burda and Sofroniew, 2014; Sofroniew, 2014; Zeidan-Chulia et al., 2014; Burda et al., 2016; Pekny et al., 2016; Verkhratsky and Parpura, 2016; Ferrer, 2018). The neurogliopathology is complex and is represented by glial reactivity (activation of microglia, astrogliosis) or glial degeneration, atrophy, and loss of functions. In particular glial cells undergo prominent changes in the context of neuropsychiatric diseases; these changes are generally represented by loss and asthenia of astroglia and oligodendroglia in conjunction with microglial activation (Rajkowska and Stockmeier, 2013; Verkhratsky et al., 2015; Cobb et al., 2016; Rajkowska et al., 2018). In this research topic we addressed the contribution of neuroglia to major neuropsychiatric conditions including major depression, schizophrenia, and alcohol use disorders.

The gliocentric theory of major depression has been overviewed by Czéh and Nagy who presented recent findings on glial structural and functional abnormalities at molecular and cellular levels. Subsequently they focused on clinical studies and summarized neuroimaging as well as post-mortem molecular and histopathological evidence supporting leading role of glia in the progression of depression and related mood disorders. Notably, the depression is associated with substantial loss of macroglial cells with loss of their function, in conjunction with microglial activation, which summarily affect neuronal networks and brain function. Structural and molecular alterations of astrocytes and oligodendrocytes also lie at the core of alcohol use disorders (Miguel-Hidalgo). In particular alcohol exposure, as well as alcohol withdrawal affects glial transporters, glutamate (GABA) glutamine shuttle and gap junctions, which connect glial syncytia. The association of endothelial cells, blood-brain barrier and neurovascular unit with neuropsychiatric diseases has been discussed by Malik and Di Benedetto, who presented evidence supporting pathophysiological role of disrupted erythropoietin-producing-hepatocellular carcinoma receptors (EphR)/Ephrin signaling cascades.

Pathophysiological changes in microglia in context of environmental stress associated with neurodevelopmental and neuropsychiatric disorders are overviewed by Tay et al. In particular, they discuss the role of microglia in Nasu-Hakola disease, hereditary diffuse

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leukoencephaly with spheroids, Rett syndrome, autism spectrum disorders, obsessive-compulsive disorder and major psychiatric pathologies such as addiction, major depressive disorder, bipolar disorder, schizophrenia, eating disorders, and sleep disorders. Studies on humans have demonstrated neuroinflammation and microglial reactivity in patients with schizophrenia as well as in animal models. In the latter, the gender differences have been also described (Hui et al.); and it turned out that in males prominent differences in microglial distribution, morphology, and microglial activation may account for higher incidence of schizophrenia. Up-regulation of microglia associated genes following administration of IgG-Saporin has been demonstrated to alter a memory-associated behavior (Dobryakova et al.). The role of microglial activation and phagocytosis in Parkinson's disease and therapeutic potential of targeting microglia were reviewed by Janda et al. Of note both asphyxia and cesarean section are associated with the expression of Schizophrenia risk genes (Paparelli et al.). The link between neuregulin and β-secretase with schizophrenia is shown by Zhang et al.

Glial cells can be targets for specific therapy. Effects of electro-acupuncture on hippocampal neuroinflamamtion in rat depressed model (induced by unpredictable stress-induced depressive- and anxiety-like behavior) are presented by Yue et al. They showed that treatment with acupuncture reduce behavioral abnormalities and abrogate neuroinflammation. Quercitin neuroprotection was discussed by Gao et al.

All in all, the papers assembled in this Research Topic provide a wide picture of the importance of neuroglia in the pathogenesis of neuropsychiatric diseases and discuss possible therapeutic strategies aimed at glial cells.

AUTHOR CONTRIBUTIONS

CS, MN, and AV wrote the editorial. All authors read and approved the submitted version.

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REVIEW
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Clinical Findings Documenting Cellular and Molecular Abnormalities of Glia in Depressive Disorders

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Depressive disorders are complex, multifactorial mental disorders with unknown neurobiology. Numerous theories aim to explain the pathophysiology. According to the "gliocentric theory", glial abnormalities are responsible for the development of the disease. The aim of this review article is to summarize the rapidly growing number of cellular and molecular evidences indicating disturbed glial functioning in depressive disorders. We focus here exclusively on the clinical studies and present the *in vivo* neuroimaging findings together with the postmortem molecular and histopathological data. Postmortem studies demonstrate glial cell loss while the *in vivo* imaging data reveal disturbed glial functioning and altered white matter microstructure. Molecular studies report on altered gene expression of glial specific genes. In sum, the clinical findings provide ample evidences on glial pathology and demonstrate that all major glial cell types are affected. However, we still lack convincing theories explaining how the glial abnormalities develop and how exactly contribute to the emotional and cognitive disturbances. Abnormal astrocytic functioning may lead to disturbed metabolism affecting ion homeostasis and glutamate clearance, which in turn, affect synaptic communication. Abnormal oligodendrocyte functioning may disrupt the connectivity of neuronal networks, while microglial activation indicates neuroinflammatory processes. These cellular changes may relate to each other or they may indicate different endophenotypes. A theory has been put forward that the stress-induced inflammation—mediated by microglial activation—triggers a cascade of events leading to damaged astrocytes and oligodendroglia and consequently to their dysfunctions. The clinical data support the "gliocentric" theory, but future research should clarify whether these glial changes are truly the cause or simply the consequences of this devastating disorder.

Keywords: astrocyte, depression, magnetic resonance imaging, microglia, oligodendrocyte, oligodendroglia, PET

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INTRODUCTION

Depressive disorders are a leading cause of disease burden globally (Ferrari et al., 2013[1]). There are effective therapeutic interventions available, but the currently existing antidepressant medications are far from being optimal as they have numerous side effects and large percentage of the patients do not respond to them (e.g., Anderson, 2000;

[1]http://www.who.int/mediacentre/factsheets/fs369/en/

Montgomery et al., 2002; Millan, 2006; Rush et al., 2006; Cipriani et al., 2009). There is an urgent need for new, faster acting, more effective drugs. Understanding the pathophysiology could help us in the development of novel therapies.

Despite extensive investigations, the exact neurobiological processes leading to depression are not fully understood. According to our present comprehension depressive disorders develop as a result of interactions between genetic predispositions and environmental factors (see e.g., Halldorsdottir and Binder, 2017; Bleys et al., 2018; Zhao et al., 2018; but also Culverhouse et al., 2018). The most widely accepted classic theory regarding the underlying neuropathology is the monoamine imbalance hypothesis, which emphasizes the role of disturbed monoamine neurotransmission in the synaptic cleft (Meyer et al., 2006; Belmaker and Agam, 2008; Nikolaus et al., 2009). However, it has become evident that this monoamine theory of depression does not explain the wide spectrum of macroscopic and microscopic structural changes that have been repeatedly documented in the brains of depressed patients. Currently, there are at least a dozen of theories aiming to explain the pathophysiology and here we focus on the "gliocentric hypothesis" of depression. The aim of this review is to summarize the clinical findings documenting glial changes in mood disorders based on the *in vivo* neuroimaging and the postmortem molecular and quantitative histopathological data.

Traditionally, the term "mood disorders" included both the bipolar and the depressive disorders, but now, in the DSM-5 there are separate sections for the Bipolar Disorders (BDs) and for the Depressive Disorders. All these terms mean larger categories and include several disorders. Here, we occasionally need to use the old terminology, since the majority of the studies have been done before the DSM-5. Most of the clinical studies discussed here involved patients either with Major Depressive Disorder (MDD) or with BD.

CELLULAR EVIDENCES FOR GLIAL PATHOLOGY

Postmortem Quantitative Histopathological Data

We start with the postmortem histopathological findings since historically these were the first to reveal glial alterations in the brains of depressed individuals. The first reports were published in the late 90s. At that time, the scientific community was excited by the results of the *in vivo* imaging studies documenting reduced activity and volume shrinkage in the prefrontal cortex (PFC) of depressed patients (Drevets et al., 1997). To understand the cellular pathology behind these functional and morphological changes researchers started to carry out quantitative light microscopic analyses on postmortem PFC samples from depressed patients. The investigators expected to find changes in neuron numbers and morphology, but it turned out that in most cases the morphometric alterations affected glial cells. The first report found markedly reduced glial numbers in the ventromedial PFC (subgenual Brodmann's area 24) in the brains of patients with MDD and BD (Öngür et al., 1998). The most prominent reduction of glial cell numbers was observed in subjects with familial MDD (24%) or BD (41%; Öngür et al., 1998). In their study, Öngür et al. (1998) used schizophrenic brains as psychiatric controls which had normal neuronal and glial cell numbers. These findings evoked great interest and were followed by numerous confirmatory reports. Reduced density of glial cells was found in the dorsolateral PFC (Cotter et al., 2002) and in the rostral and caudal orbitofrontal cortex of depressed patients (Rajkowska et al., 1999). A follow-up study by Rajkowska et al. (2001) revealed similarly reduced glial density in the dorsolateral PFC of patients with BD. More recent studies confirmed the findings on decreased density of glial cells in the anterior cingulate cortex (ACC; Brodmann area 24b; Gittins and Harrison, 2011). The histological studies on glial cell loss are supported by the recent molecular findings on altered microRNA (miRNA) levels in the brains of BD patients. For example Choi et al. (2017) extracted extracellular vesicles from the PFC (Brodmann area 24) and found increased levels of miR-149 exclusively in glial cells. Since miR-149 can inhibit glial cell proliferation thus, increased miR-149 expression might contribute to the glial cell number reduction.

The first studies focused on the different subareas of the PFC, but later the investigators analyzed other limbic areas and found similar glial changes. In the amygdala, reduced glial density and glia/neuron ratio was found in MDD (Bowley et al., 2002). This reduction was more pronounced in the left hemisphere and no change was found in neuron numbers (Bowley et al., 2002). In BD subjects, who did not receive mood stabilizer medication, significantly reduced glial cell numbers were found, in contrast to the cases who were treated with lithium or valproate (Bowley et al., 2002). This later finding indicated that medication may affect glial cell numbers (see also "Experimental Evidences That Psychotropic Medication Can Affect Glial Cell Numbers" section).

In the hippocampus, the first detailed human postmortem study found no evidence for neuronal abnormality, but the packing density of glial cells was significantly increased in all hippocampal subfields of patients with MDD (Stockmeier et al., 2004). A follow up study by the same group could not find any difference in the total cell numbers of neurons and glia in any hippocampal subarea (Cobb et al., 2013), but later they reported on reduced density of astrocytes in the hilus (Cobb et al., 2016).

Obviously, these postmortem histopathological studies have their limitations (the problem of tissue preservation and finding adequate controls), thus, some scientists question their scientific soundness. But in fact most of the *in vivo* neuroimaging and preclinical experimental data support the postmortem findings. Ideally, one should carry out longitudinal studies on well-characterized patients with repeated neuroimaging investigations and in the end one should do postmortem analysis on the brains of the same subjects (Stockmeier and Rajkowska, 2004). However, so far such studies are not available.

Finally, we should note here that there are also negative findings on glial cell numbers in the literature (e.g., Damadzic et al., 2001; Cotter et al., 2005; Khundakar et al., 2011; Rubinow et al., 2016).

Postmortem Evidences for Astrocytic Abnormalities

Most of the above mentioned histopathological studies used Nissl stained brain samples which is not suitable for the identification of the different glial subtypes. The later studies used immunohistochemistry which enabled us to specify which type of glial cells are affected by the disease. Numerous studies found evidences for altered number or disrupted integrity of astrocytes in depressed patients. For example, Miguel-Hidalgo et al. (2000) studied glial fibrillary acidic protein (GFAP)-immunoreactive astrocytes in the dorsolateral PFC of MDD subjects, and found a significant reduction of areal fraction and packing density of GFAP-positive cell bodies, but only in younger (30–45 years old) patients, whereas in older subjects (46–86 years old) these glial parameters tended to be greater compared to the corresponding controls. In line with these data, reduced GFAP protein levels were reported by a western blot study analyzing PFC samples from patients with MDD (Si et al., 2004). A more recent study investigated the coverage of blood vessels by aquaporin (AQ)-4-immunoreactive astrocytes in the PFC of MDD patients and found 50% reduction in the orbitofrontal gray matter (Rajkowska et al., 2013). In the same study, the coverage of blood vessels by GFAP-positive endfeet processes did not differ between the groups (Rajkowska et al., 2013). Others reported on reduced GFAP immunoautoradiography in the white matter of the ACC (Brodmann area 24b; Gittins and Harrison, 2011). Yet another study which did a very detailed analysis on the morphology of Golgi-impregnated astrocytes found that the fibrous astrocytes (in the white matter) had significantly larger cell bodies, as well as longer, more ramified processes in depressed suicide victims in the ACC (Brodmann area 24; Torres-Platas et al., 2011). Another study found decreased area fraction and increased cell clustering of GFAP expressing astrocytes in the postmortem white matter adjacent to the dorsolateral PFC (Brodmann area 9) of BD patients (Hercher et al., 2014).

Alterations of astrocytes have also been reported in the hippocampi of depressed patients. A study focusing on the density of GFAP-positive astrocytes found reduced density of astrocytes in the dentate hilus, but not in other hippocampal subareas (Cobb et al., 2016). Furthermore, this decrease was present only in those depressed patients who were not taking antidepressant medications, but not in subjects who were medicated (Cobb et al., 2016). Another group found reduced density of S100B-immunopositive astrocytes in the hippocampal CA1 pyramidal cell layer of MDD and BD patients (Gos et al., 2013). Reduced density of GFAP-immunoreactive astrocytes was also found in the amygdala of depressed patients (Altshuler et al., 2010) and downregulation of GFAP mRNA and protein expression was reported in the thalamus and caudate nucleus of depressed suicides (Torres-Platas et al., 2016).

Since it is increasingly acknowledged that astrocytes play a number of vital roles in the CNS (e.g., maintaining synaptic homeostasis, modulating glutamate metabolism, participating in signaling between neurons and glia, neurotrophic support, etc.) thus, a theory had been put forward proposing that depressive disorders are the consequence of the disturbed astrocytic functioning (see e.g., Wang et al., 2017). The summary of astrocytic abnormalities in depressed patients is shown on **Table 1**.

Postmortem Evidences for Oligodendrocyte Abnormalities

Similarly to astrocytes, deficit of oligodendrocytes has been repeatedly documented in the PFC of MDD and BD patients. The first report was an electron microscopic study demonstrating ultrastructural evidences for apoptosis and necrosis of oligodendroglial cells in the prefrontal area 10 in BD (Uranova et al., 2001). In a follow-up study, the same group studied the numerical density of oligodendroglial cells in layer VI of the Brodmann area 9 in BD, MDD and healthy controls. They found a significantly reduced density of oligodendrocytes in BP and MDD patients (Uranova et al., 2004). Later, the same group demonstrated a prominent reduction in the number of perineuronal oligodendrocytes in layer III of the Brodmann area 9 in BP and MDD (Vostrikov et al., 2007). These findings are supported by more recent studies, using different cell counting techniques, but also reporting on reduced density of oligodendrocytes in the PFC Brodmann area 10 (Hayashi et al., 2011). Finally, a recent study found significantly decreased soma size of 2′,3′-Cyclic-nucleotide 3′-phosphodiesterase enzyme (CNPase)-immunoreactive oligodendrocytes in the ventral PFC white matter of MDD patients (Rajkowska et al., 2015). Complementing these quantitative findings, another study found reduced immunoreactivity of the myelin basic protein (an oligodendrocyte marker) in the anterior frontal cortex of depressed individuals who died by suicide (Honer et al., 1999). Another group found reduced density of S100B-immunopositive oligodendrocytes in the left hippocampal alveus of BD patients (Gos et al., 2013). More recently, a study found increased oligodendrocyte density in the postmortem white matter adjacent to the dorsolateral PFC (Brodmann area 9) of BD patients (Hercher et al., 2014). One should add here that there is a recent study which investigated the axonal myelin sheath in the genu of the corpus callosum and found that the myelin thickness was actually greater in MDD (Williams et al., 2015).

These neuroanatomical findings on oligodendroglial changes have been extended by a study which compared microarray metadata with the cytoarchitectural data. Correlation analysis between the genome-wide gene expression levels and cytoarchitectural traits revealed that 818 genes were significantly correlated with the decrease in the number of perineuronal oligodendrocytes in psychiatric subjects (Kim and Webster, 2010).

Based on these findings, it has been proposed that impaired oligodendrocyte functioning alters neuronal circuitry and by that leads to disrupted mood regulation in psychiatric disorders. For details of this concept see e.g., Edgar and Sibille (2012). The summary of oligodendrocyte abnormalities in depressed patients is shown on **Table 2**.

Postmortem Evidences for Activated Microglia

Microglial cells are the resident immune cells of the brain. Large body of evidence support the notion that neuroinflammation contributes to the pathophysiology of depression

(Rosenblat et al., 2014) and numerous scientists proposed that microglial cells play a key role in the pathogenesis of depressive disorders (Brites and Fernandes, 2015; Yirmiya et al., 2015; Singhal and Baune, 2017). But in fact, so far only a handful of postmortem studies focused on microglial cells in the brains of depressed individuals. The first study to do that was a report on the presence of activated microglia in the hippocampal CA1 area using immunohistochemical staining with the HLA-DR antibody (Bayer et al., 1999). Later Steiner et al. (2008, 2011), provided further important evidences on microglial activation in various psychiatric illnesses. The summary of microglial changes in depressed patients is shown on **Table 3**.

A more recent study analyzed Iba-1-immunoreactive microglial cells in the white matter of the dorsal ACC and found that the ratio of primed over ramified ("resting") microglia was significantly increased in depressed suicides (Torres-Platas et al., 2014). In the same study, the proportion of blood vessels surrounded by macrophages was more than twice higher

TABLE 1 | Astrocytic abnormalities.

Type of disorder	*In vivo* imaging data	Postmortem molecular data	Postmortem cellular data
Major depressive disorder	1. PET studies document reduced glucose metabolism in the PFC (Baxter et al., 1989), amygdala (Abercrombie et al., 1998; Drevets et al., 2002b), thalamus (Su et al., 2014), and lateral temporal and parietal cortex (Drevets et al., 1992)	1. Reduced GFAP protein expression in the PFC (Miguel-Hidalgo et al., 2000; Si et al., 2004), locus coeruleus (Chandley et al., 2013) and cerebellum (Fatemi et al., 2004)	1. Larger cell bodies of astrocytes and longer, more ramified processes in the ACC (Torres-Platas et al., 2011)
	2. Reduced *Glx* level detected by ^{1}H-MRS (Yüksel and Öngür, 2010; Arnone et al., 2015)	2. Reduced GFAP mRNA and protein expression in the thalamus and caudate nucleus (Torres-Platas et al., 2016)	2. Reduced areal fraction and packing density of astrocytes in the DLPFC of young subjects (Miguel-Hidalgo et al., 2000)
	3. Increased lactate in the pregenual ACC (Ernst et al., 2017)	3. Reduced expression of astrocyte specific glutamate transporter genes (EAAT1, EAAT2) in the orbitofrontal cortex (Miguel-Hidalgo et al., 2010), dorsolateral PFC (Choudary et al., 2005; Zhao et al., 2016), hippocampus (Medina et al., 2013, 2016) and locus coeruleus (Bernard et al., 2011; Chandley et al., 2013)	3. Increased areal fraction and packing density of astrocytes in the DLPFC of old subjects (Miguel-Hidalgo et al., 2000)
	4. Reduced metabotropic glutamate receptor 5 (mGluR5) binding in the PFC, cingulate cortex, insula, thalamus and hippocampus (Deschwanden et al., 2011)		4. Reduced coverage of blood vessels by AQ4-positive astrocytes in the orbitofrontal cortex (Rajkowska et al., 2013)
			5. Reduced GFAP-IR in the ACC white matter (Gittins and Harrison, 2011)
		4. Downregulation of connexin 30 and 43 expressing genes in several brain areas (Nagy et al., 2017)	6. Reduced density in the dentate hilus of non-medicated patients (Cobb et al., 2016)
		5. Reduced gene expression of a gap junction protein (GJA1) in the hippocampus (Medina et al., 2016)	7. Reduced density of S100B-positive astrocytes in the hippocampal CA1 pyramidal cell layer (Gos et al., 2013)
		6. Reduced gene expression of potassium (KCNJ10) and water channels (AQP4) in the hippocampus (Medina et al., 2016)	8. Reduced density in the amygdala (Altshuler et al., 2010)
			9. Reduced density of glutamine synthetase expressing astrocytes in specific cortical gray matter areas (Bernstein et al., 2015)
Bipolar disorder	1. Increased *Glx* and glutamate levels detected by ^{1}H-MRS (Frye et al., 2007; Yüksel and Öngür, 2010; Gigante et al., 2012; Soeiro-de-Souza et al., 2015)	1. Reduced expression of GFAP mRNA levels in the white matter of the ACC (Webster et al., 2005)	1. Increased clustering of astrocytes in the white matter adjacent to the DLPFC (Hercher et al., 2014)
	2. Increased lactate in the cingulate gyrus (Dager et al., 2004)		2. Reduced density of S100B-positive astrocytes in the hippocampal CA1 pyramidal cell layer (Gos et al., 2013)

Abbreviations: ACC, anterior cingulate cortex; AQ, aquaporin; DLPFC, dorsolateral prefrontal cortex; EAAT, excitatory amino acid transporter; GFAP, glial fibrillary acidic protein; Glx, a composite measure of glutamate and glutamine in ^{1}H-MRS studies; ^{1}H-MRS, proton magnetic resonance spectroscopy; IR, immunoreactivity; PET, Positron-Emission Tomography; PFC, prefrontal cortex.

TABLE 2 | Oligodendrocyte abnormalities.

Type of disorder	*In vivo* imaging data	Postmortem molecular data	Postmortem cellular data
Major depressive disorder	1. DTI studies report on robust reduction of fractional anisotropy in the corpus callosum and in several frontal and temporal regions (Alexopoulos et al., 2002; Taylor et al., 2004; Bae et al., 2006; Ma et al., 2007; Osoba et al., 2013; de Diego-Adeliño et al., 2014; Yamada et al., 2015; Jiang et al., 2017; Matsuoka et al., 2017) 2. Altered MTR indicating reduced myelin integrity (Kumar et al., 2004; Gunning-Dixon et al., 2008; Chen et al., 2015; Jia et al., 2017) 3. Reduced NAA/Cr ratio in the DLPFC white matter in first episode treatment-naive patients (Wang et al., 2012)	1. Reduced expression of 17 genes related to oligodendrocyte function in the temporal cortex (Aston et al., 2005) 2. Altered expression of myelin-related mRNAs and proteins in the white matter of the ventral PFC (Rajkowska et al., 2015)	1. Reduced density in the PFC (Uranova et al., 2004; Hayashi et al., 2011) 2. Reduced number in the PFC (Vostrikov et al., 2007) 3. Reduced soma size in the white matter of the ventral PFC (Rajkowska et al., 2015) 4. Reduced IR of myelin basic protein in the anterior frontal cortex (Honer et al., 1999) 5. Greater axonal myelin thickness in the genu of the corpus callosum (Williams et al., 2015)
Bipolar disorder	1. DTI studies report on reduced fractional anisotropy in the cingulum, internal capsule, posterior corpus callosum, tapetum, and occipital white matter (Lu et al., 2011, 2012; Emsell et al., 2013; Sprooten et al., 2016; Ji et al., 2017) 2. Greater mean diffusivity in the cingulum, corpus callosum, corona radiata, internal capsule, tapetum, and occipital white matter (Lu et al., 2011, 2012; Emsell et al., 2013; Sprooten et al., 2016; Ji et al., 2017) 3. Altered MTR indicating reduced myelin integrity in the anterior cingulate and subgyral white matter (Bruno et al., 2004) 4. Reduced NAA/Cr in the medial prefrontal white matter (Zhong et al., 2014)	1. Reduced expression of oligodendrocyte-related and myelination-associated genes (Tkachev et al., 2003) 2. Increased CNPase protein levels in the white matter adjacent to the DLPFC (Hercher et al., 2014) 3. Increased mRNA levels of the oligodendroglial markers (Olig1, Olig2) in the serum (Ferensztajn-Rochowiak et al., 2016)	1. Apoptosis and necrosis in the PFC (Uranova et al., 2001) 2. Reduced density in the PFC (Uranova et al., 2004) 3. Reduced number in the PFC (Vostrikov et al., 2007) 4. Reduced density of S100B-positive oligodendrocytes in the left hippocampal alveus (Gos et al., 2013) 5. Increased density in the white matter adjacent to the DLPFC (Hercher et al., 2014)

Abbreviations: CNPase, 2′,3′-Cyclic-nucleotide 3′-phosphodiesterase enzyme; Cr, Creatine; DLPFC, dorsolateral prefrontal cortex; DTI, diffusion tensor imaging; IR, immunoreactivity; MTR, magnetization transfer ratio; NAA, N-acetylaspartate; PFC, prefrontal cortex.

in depressed suicides than in controls (Torres-Platas et al., 2014). Consistent with these observations, gene expression analysis of Iba-1 and monocyte chemoattractant protein-1 (MCP-1; a chemokine involved in the recruitment of circulating monocytes), was significantly up-regulated in depressed suicides (Torres-Platas et al., 2014). Another study found greater density of Iba-1-immunoreactive cells in contact with blood vessels in the dorsal prefrontal white matter of suicide victims (Schnieder et al., 2014).

Experimental Evidences That Psychotropic Medication Can Affect Glial Cell Numbers

Ideally, a postmortem study investigating cellular changes in the brains of depressed individuals should use tissue samples from medication free patients. However, nowadays it is very difficult to get such samples, because most patients with depressive disorder or BD receive long-term treatment with various drugs. Thus, when a human postmortem study reports on altered cell numbers in specific brain areas, the next question to be answered: is this change due to the illness, or is this the result of the medication? To answer this question, several groups examined neuronal and glial cell numbers in the brains of experimental animals subjected to antidepressant, antipsychotic or mood stabilizer treatment. For example, a study treated macaque monkeys with antipsychotic drugs such as haloperidol and olanzapine (at doses producing plasma levels in the therapeutic range) and found that glial cell numbers were significantly reduced in the parietal cortex (Konopaske et al., 2007). In a follow up study, the same group reported that these glial changes affected mainly the astrocytes, since a significant loss of astrocytes were found in addition

TABLE 3 | Microglial changes.

Type of disorder	*In vivo* imaging data	Postmortem molecular data	Postmortem cellular data
Major depressive disorder	1. Increased TSPO density in the PFC, ACC and insula detected by PET scan (Setiawan et al., 2015)	1. Up-regulated gene expression of Iba-1 and MCP-1 in suicides (Torres-Platas et al., 2014)	1. HLA-DR-immunopositive activated microglia in the hippocampal CA1 area (Bayer et al., 1999)
	2. TSPO availability was higher in the ACC and insula of patients with suicidal thoughts compared to patients without such intention (Holmes et al., 2018)	2. Elevated cytokines (TNF-α, IL-1β, IL-6) and Toll-like receptors in the PFC of suicide victims (Pandey, 2017)	2. Significant microgliosis in the ACC, DLPFC and MD thalamus of suicide patients (Steiner et al., 2008)
		3. Reduced expression of genes associated with microglia and glial cell functions in the DLPFC (Pantazatos et al., 2017)	3. Significantly increased density of QUIN-positive cells in the sACC and aMCC (Steiner et al., 2008)
			4. Increased ratio of primed Iba-1-positive microglia in the white matter of the dorsal ACC in depressed suicides (Torres-Platas et al., 2014)
			5. Increased proportion of blood vessels surrounded by macrophages (Torres-Platas et al., 2014)
			6. Increased density of Iba-1-positive cells in contact with blood vessels in the dorsal PFC white matter of suicide victims (Schnieder et al., 2014)
Bipolar disorder	Elevated TSPO binding in the right hippocampus (Haarman et al., 2014)		

Abbreviations: ACC, anterior cingulate cortex; aMCC, anterior midcingulate cortex; DLPFC, dorsolateral prefrontal cortex; HLA-DR, the major histocompatibility complex II protein; Iba-1, ionized calcium binding adaptor molecule 1; MCP-1, monocyte chemoattractant protein-1; MD, mediodorsal; PET, Positron-Emission Tomography; PFC, prefrontal cortex; QUIN, quinolinic acid; sACC, subgenual anterior cingulate cortex; TSPO, translocator protein-18 kDa.

to a non-significant reduction in oligodendrocyte cell number in the antipsychotic-treated monkeys (Konopaske et al., 2008). Haloperidol and olanzapine had equivalent glia-reducing effect (Konopaske et al., 2008). A more recent study investigated the effect of lithium treatment on glial cell numbers in the PFC and dentate gyrus of mice (Rajkowska et al., 2016). Lithium is a widely used mood stabilizer and many patients with BD receive long-term lithium treatment. This experimental study found that lithium treatment can increase the total numbers of neurons and glia in the dentate gyrus and also the density of astrocytes in the PFC of mice (Rajkowska et al., 2016). Besides lithium, valproic acid is a classic choice to treat manic or mixed episodes of BD. There is increasing evidence that chronic valproic acid treatment can influence myelination and network connectivity (Rosenzweig et al., 2012). We also did an experimental study to examine the effect of an antidepressant drug (fluoxetine, a selective serotonin reuptake inhibitor) on astrocytes in an animal model for depression. We found that fluoxetine could reverse the chronic stress-induced loss of astrocytes in the hippocampus of the animals (Czéh et al., 2006). Furthermore, treatment with psychoactive agents not only affects glial cell numbers, but also influences cellular morphology. It has been shown that fluoxetine treatment can increase the plasticity of astrocytic end-feet processes and enhance their numbers (Di Benedetto et al., 2016). Similarly to that, a study using a non-human primate model of self-injurious behavior reported that naltrexone treatment can reverse astrocyte atrophy associated with self-harm (Lee et al., 2015).

Adult hippocampal neurogenesis in the dentate gyrus is a cellular process that has been linked to the pathophysiology of depressive disorders (see e.g., Miller and Hen, 2015). Numerous studies document that antidepressant treatments can stimulate adult hippocampal neurogenesis and parallel to this several studies investigated the effect of antidepressant treatment on gliogenesis in the CNS of experimental animals. Several reports document increased gliogenesis in the PFC of adult rats after antidepressant or antipsychotic treatment (Kodama et al., 2004; Czéh et al., 2007). Likewise, electroconvulsive treatment stimulates gliogenesis in the PFC (Madsen et al., 2005; Öngür et al., 2007), hippocampus (Wennström et al., 2006), and amygdala (Wennström et al., 2004). These treatments stimulated the proliferation of NG2-positive glial cells. In contrast to the stimulating effect of antidepressants, lithium treatment appears to inhibit the proliferation of NG2 cells in the hippocampal dentate hilus, amygdala and corpus callosum (Orre et al., 2009).

In summary, experimental data indicate that drugs, characteristically used to treat MDD and BD, can all affect glial cell numbers, cellular morphology, gliogenesis and probably also myelination. This should be kept in mind when we interpret the postmortem clinical findings, because most brain samples

originate from individuals who received long-term medications during the course of their illness.

In Vivo Imaging Studies

In vivo neuroimaging is a valuable tool to examine glial alterations in depressive disorders. The volumetric magnetic resonance imaging (MRI) studies reveal reduced volumes of specific brain areas in depression (e.g., van Tol et al., 2010; Grieve et al., 2013). These volume changes are likely to be the gross consequence of the glial and/or neuronal cell loss. There are numerous novel *in vivo* imaging methods which can be employed to examine brain structure and function. These methods include the diffusion tensor imaging (DTI), functional magnetic resonance imaging (fMRI), proton magnetic resonance spectroscopy (^{1}H-MRS) and positron emission tomography (PET) imaging. Here, we summarize the *in vivo* clinical findings documenting glial abnormalities in patients with depressive disorders.

Astrocytic Abnormalities

Astrocytes carry out a large variety of important cellular functions for the neuronal microenvironment such as the regulation of glucose metabolism, neurotransmitter uptake (particularly glutamate, the major excitatory neurotransmitter), regulation of synaptic development and maturation and maintenance of the blood brain barrier (Kettenmann and Ransom, 2005). In PET imaging, the uptake of the radioactive ^{18}F-deoxyglucose by astrocytes is associated with neuronal activity (Magistretti and Pellerin, 1999). A well replicated finding in PET studies is the reduced glucose metabolism in the PFC of MDD patients (Baxter et al., 1989). Typically this is more pronounced in depressed periods and slightly normalized in the remission phase (Martinot et al., 1990; Drevets et al., 2002a). Mounting evidence supports the existence of abnormal glucose metabolism in other brain regions including the amygdala (Abercrombie et al., 1998; Drevets et al., 2002b), thalamus (Su et al., 2014), and the lateral temporal and parietal cortex (Drevets et al., 1992), however these later ones are less consistent.

Numerous ^{1}H-MRS studies examined glutamate, glutamine and *Glx* (a composite measure of glutamate and glutamine) levels in the context of mood disorders (reviewed by Yüksel and Öngür, 2010). These studies find a highly consistent pattern of *Glx* level reductions in MDD and elevations in BP (Yüksel and Öngür, 2010). Furthermore, the available data suggest that in depressive states reduced glutamine/glutamate ratio can be detected, whereas in mania an elevated glutamine/glutamate ratio is present (Yüksel and Öngür, 2010; Luykx et al., 2012; Arnone et al., 2015). These metabolic changes are likely to be the consequences of the altered astrocytic functioning. A recent meta-analysis based on 17 studies using ^{1}H-MRS revealed an exclusive reduction in *Glx* levels in the PFC of MDD patients (Arnone et al., 2015). The same meta-analysis reported that there was no change in glutamate levels and other metabolite levels were also not altered (Arnone et al., 2015). Other investigation found normal or slightly elevated glutamate and glutamine levels in remitted MDD (Bhagwagar et al., 2007), which suggests that *Glx* may be restored or compensated after successful treatment (Michael et al., 2003; Pfleiderer et al., 2003).

Several PET studies investigated tracer binding to the metabotropic glutamate receptor 5 (mGluR5) which is a key component of the glutamatergic system and expressed by neurons and astrocytes (Biber et al., 1999). A study by Deschwanden et al. (2011) used the radiotracer [^{11}C]ABP688 and found reduced mGluR5 binding in the PFC, cingulate cortex, insula, thalamus, and hippocampus of MDD patients. However, more recently a study using a different radiotracer, [^{18}F]FPEB, could not confirm this finding (Abdallah et al., 2017).

In case of BD patients, the picture is less ambiguous, because numerous studies consistently report on increased *Glx* and glutamate levels in several brain areas (Frye et al., 2007; Gigante et al., 2012; Soeiro-de-Souza et al., 2015). A study which involved both melancholic and non-melancholic subtypes of BD found that *Glx* and glutamate concentrations were significantly higher in the non-melancholic subtype (Frye et al., 2007). Furthermore, patients who responded to lamotrigine treatment had reduced glutamine concentration (Frye et al., 2007). Notably, up to 80% of glutamate is compartmentalized in neurons while glutamine is synthetized mainly in astrocytes (Maddock and Buonocore, 2012; Ramadan et al., 2013), hence, both astrocytic and neuronal dysfunction can lead to changes in glutamine and glutamate levels (see also "Molecular Evidences From Clinical Studies: Astrocytic Abnormalities" section).

Lactate is an important metabolite in the brain with unknown function. Astrocytes contribute to lactate metabolism in the brain (Mächler et al., 2016; Mason, 2017). Furthermore, lactate can be processed by mitochondrial oxidative metabolism (Pellerin et al., 2007) and recent theories suggest mitochondrial dysfunction in depressive disorders (Manji et al., 2012; Klinedinst and Regenold, 2015). Supporting this concept, elevated gray matter lactate was found in the cingulate gyrus of BD patients (Dager et al., 2004) and in the pregenual ACC of patients with MDD (Ernst et al., 2017).

It is well known, that astrocytic glycolysis and oxidative phosphorylation results in a clear signal change in fMRI measurements (Rossi, 2006; Attwell et al., 2010). Numerous studies document fMRI alterations in fronto-cortical areas of MDD patients (e.g., Grimm et al., 2008; Mulders et al., 2015; Davey et al., 2017), but it is not known which cell type is responsible for the alterations. In the PFC, the coverage of blood vessels by astrocytic endfeets is markedly reduced in patients with MDD suggesting that the blood flow and glucose uptake by astrocytes might be impaired (Rajkowska et al., 2013). This neuroanatomical alteration could consequently result in fMRI signal change. Plenty of evidences document that antidepressant treatment (e.g., fluoxetine, paroxetine) positively affect astrocytic function by regulating glucose metabolism (Kennedy et al., 2001; Allaman et al., 2011; Czéh and Di Benedetto, 2013) and consequently cause fMRI signal change (Harris and Reynell, 2017). Interestingly, antidepressant treatment can affect the BOLD response to positive and negative emotional stimuli differently especially in frontal brain areas (Ma, 2015).

Oligodendrocyte Abnormalities

Oligodendroglia provides support and insulation to axons by creating the myelin sheath. Consequently, oligodendrocyte pathology can cause abnormal development, demyelination or reduction of myelinated axons. Oligodendrocyte abnormalities can be investigated *in vivo* using DTI. DTI was specifically developed to investigate the integrity of white matter, where axons and myelin sheaths form longitudinal axes leading to greater water diffusion along the tracts and restricted molecular displacement perpendicularly. Fractional anisotropy (FA) measures the magnitude of directionally varying diffusion restriction effect, while mean diffusivity (MD) represents the average diffusion coefficient in all direction.

A significant number of DTI studies found robust FA reductions in the corpus callosum and in several frontal and temporal regions (e.g., Alexopoulos et al., 2002; Taylor et al., 2004; Bae et al., 2006; Ma et al., 2007; de Diego-Adeliño et al., 2014; Yamada et al., 2015; Jiang et al., 2017) indicating white matter microstructural abnormalities in depressed patients. Later studies found similar microstructural abnormalities in the anterior callosal fibers connecting bilateral frontal cortices in patients with MDD (Yamada et al., 2015; Matsuoka et al., 2017). Another study reported on significant FA deficits only in the right parietal white matter in depressed patients as well as white matter changes of specific thalamic tracts (Osoba et al., 2013).

DTI studies document abnormal white matter microstructure in BD patients as well. Untreated bipolar patients with first episode psychosis showed lower FA in several white matter tracts such as the cingulum, internal capsule, posterior corpus callosum, tapetum and occipital white matter (Lu et al., 2011). At the same time, BD patients have greater MD in the cingulum, corpus callosum, corona radiata, internal capsule, tapetum and occipital white matter including posterior thalamic radiation, inferior longitudinal fasciculus/inferior fronto-occipital fasciculus (Lu et al., 2011). Numerous follow up studies confirmed these data (Lu et al., 2012; Emsell et al., 2013; Sprooten et al., 2016; Ji et al., 2017). Similar, but more subtle white matter microstructural changes were detected in unaffected siblings as well (Sprooten et al., 2013, 2016). A recent study reported that these alterations are less pronounced in euthymic BD patients and that lithium treatment can counteract the white matter microstructural abnormalities (Haarman et al., 2016b).

There are other imaging methods to quantitatively evaluate myelin related white matter alterations. Magnetization transfer imaging is based on the interaction of unbound protons and protons bound to macromolecules. Myelin bound protons associated with protein and lipid macromolecules are not measurable by conventional MRI. During magnetization transfer imaging an off-resonance pulse is applied to partially saturate the macromolecular pool and produce contrast between tissues. This suppresses the water signal which can be measured by magnetization transfer ratio (MTR) and associated with macromolecular concentration. Hence, increased MTR in the white matter indicates remyelination, whereas decreased MTR indicates demyelination (Chen et al., 2008). Besides the gray matter MTR changes (Chen et al., 2015; Jia et al., 2017), the white matter also depicts alterations in depressive disorders. Reduced myelin integrity was suggested in late-life depression where decreased MTR was found in multiple left hemisphere frontostriatal, limbic areas, occipital white matter and in the genu and splenium of the corpus callosum (Kumar et al., 2004; Gunning-Dixon et al., 2008). Subtle abnormalities were presented in the anterior cingulate and subgyral white matter in patients with BD reflecting myelin changes and/or reduced axon density (Bruno et al., 2004).

Growing number of evidences indicate that cerebral white matter lesions contribute to the pathophysiology of depression. Recent studies demonstrate that the volume of periventricular and deep white matter lesions are differently associated with depressive symptoms (Tully et al., 2017). These are also present in suicidal behavior of MDD and BD patients (Pompili et al., 2008; Grangeon et al., 2010). Despite their similar appearance, some authors suggest that lesions in different brain areas may have distinct origin and functional consequences. Accordingly, periventricular white matter lesions have been found to have lower MTR than the ones in deep white matter, suggesting different underlying pathology and mechanisms (Spilt et al., 2006). It is argued that these white matter lesions should result in impaired connectivity of different brain regions. Lower white matter microstructural integrity (measured by DTI) and altered brain function (measured by fMRI) were found in white matter lesions (Smagula and Aizenstein, 2016). Others reported more widespread changes in white matter connectivity and lesions in BD compared to MDD (Cardoso de Almeida and Phillips, 2013). Overall, these data suggest that white matter lesions contribute to the development of depressive disorders.

^{1}H-MRS is another *in vivo* method to assess cellular integrity and functioning in the brains of psychiatric patients (see e.g., Yildiz-Yesiloglu and Ankerst, 2006; Caverzasi et al., 2012). With this method one can measure the concentration of various brain metabolites, typically, *N*-acetylaspartate (NAA), Choline (Cho), *myo*-inositol (mI), Glutamate (Glu)/Glutamine (Gln) and Creatine (Cr). *NAA* plays a role in various cellular functions such as osmoregulation, energy homeostasis and possibly also in myelin production (Moffett et al., 2007). NAA levels in the CNS provide information on the functioning of both neurons and oligodendrocytes. For example, significantly lower NAA to Cr ratio was found in the dorsolateral prefrontal white matter in first episode treatment-naive patients with MDD (Wang et al., 2012). Similarly, reduced NAA/Cr ratio was found in patients with BD in the medial prefrontal white matter (Zhong et al., 2014). These results indicate that, *in vivo* NAA changes do not reflect neuronal alterations alone, but also glial dysfunctions, both in MDD and BD.

PET is a well-established method to detect ongoing demyelination and remyelination processes *in vivo* (Stankoff et al., 2006, 2011; Wang et al., 2009; Wu et al., 2010; Tiwari et al., 2016), but to our best of knowledge so far no one investigated myelin related alterations in depressive disorders using PET imaging.

Microglial Abnormalities

Neuroinflammatory processes have been repeatedly implicated in the pathophysiology of depression. As noted earlier, microglia is a key component of the immune system and seems to contribute to the pathophysiology of depression (Eyre and Baune, 2012). Activated microglia and neuroinflammation can be studied with PET imaging, using dedicated radiopharmaceuticals targeting the translocator protein-18 kDa (TSPO). In MDD, TSPO-specific V_T (an index of TSPO density) was significantly increased by the magnitude of 30% in the PFC, ACC and insula using [^{18}F] FEPPA radiopharmaceutical (Setiawan et al., 2015). In euthymic BD, elevated TSPO binding was found by the [^{11}C] PK-11195 ligand in the right hippocampus compared to healthy controls (Haarman et al., 2014). However, another study using [^{11}C] PBR28 found no significant difference between mild-to-moderate MDD patients and controls across a range of gray matter regions (Hannestad et al., 2013). Interestingly, Holmes et al. (2018) found that TSPO availability (measured by [^{11}C] PK-11195 radioligand) was higher in the ACC and insula of MDD patients with suicidal thoughts compared to patients without such intention. They also provided the first evidence that increased TSPO may be more associated with suicidality than the MDD diagnosis itself (Holmes et al., 2018). Although the applied methodology and the binding affinity patterns of TSPO PET ligands can be different across the studies, these findings are the most compeling evidences for ongoing neuroinflammation, and for microglial activation during depressive episodes.

It has been suggested that ^{1}H-MRS can also detect activated microglia-induced metabolic changes associated with neuroinflammation in depressive states (Haroon and Miller, 2017). Inflammatory cytokines have been shown to influence glutamate metabolism in MDD, but in the literature one can find contradictory findings. The majority of studies have shown decreased *Glx* in the ACC, amygdala, hippocampus and in different subregions of the PFC (Yüksel and Öngür, 2010), while others reported increased glutamate in other brain areas (Sanacora et al., 2004). After various treatment protocols, including a serotonin reuptake inhibitor drug (citalopram, Taylor et al., 2008), electroconvulsive therapy (Zhang et al., 2013) and sleep deprivation (Murck et al., 2009) the concentration of *Glx* can normalize in the brains of MDD patients.

Studies involving BD patients have consistently reported increased *Glx* levels both in medication free and treated patients (Gigante et al., 2012). Furthermore, a recent study reported that neuronal integrity markers NAA and *N*-acetyl-aspartyl-glutamate correlated with microglia activation (measured by TSPO). Based on this, it was proposed that some microglia can induce apoptosis while others stimulate adult neurogenesis (Haarman et al., 2016a). Finally, we should emphasize that methodological heterogeneity of the ^{1}H-MRS studies (e.g., absolute concentration or ratio measurement, sample size, field strength, voxel location and geometry, etc.) may account for the contradictory results (Yüksel and Öngür, 2010).

Several studies reported that therapeutic administration of IFN-alpha (e.g., to treat viral infections) can induce profound inflammatory response and interacts with serotonin metabolism and increases glutamate which all in turn can result in depressive mood (Raison et al., 2009; Haroon et al., 2014). INF-alpha administration was associated with increased activity in the dorsal ACC and linked to impaired cognitive performance (Capuron et al., 2005). A recent resting-state fMRI study demonstrated that increased plasma concentration of C-reactive protein (a marker for inflammation) was associated with decreased connectivity between the ventral striatum and ventromedial PFC, which in turn correlated with increased anhedonia (Felger et al., 2016). Another resting-state fMRI study which used large multisite sample showed that depression can be subdivided into four subgroups by dysfunctional connectivity patterns in limbic and frontostriatal networks and also predicted the responsiveness to transcranial magnetic stimulation therapy (Drysdale et al., 2017).

MOLECULAR EVIDENCES FROM CLINICAL STUDIES

Astrocytic Abnormalities

There are numerous direct and indirect molecular evidences indicating altered astrocyte functioning in depressive disorders. Several studies reported an age-dependent reduction in the expression level of GFAP in the PFC of MDD patients (Miguel-Hidalgo et al., 2000; Si et al., 2004). Reduced expression of GFAP mRNA levels were found in the white matter of the ACC in bipolar patients (Webster et al., 2005). Reduced GFAP expression was found also in the locus coeruleus (Chandley et al., 2013) and in the cerebellum (Fatemi et al., 2004) of patients with MDD.

GFAP corresponds to GFAP—an intermediate filament protein—which has become the prototypical marker for immunohistochemical identification of astrocytes. The exact functional role of GFAP is unknown, but it has been suggested to play a role in astrocyte-neuron interactions and glial scar formation upon CNS injury (Sofroniew and Vinters, 2010) as well as in intracellular vesicle trafficking (Potokar et al., 2010).

Astrocytes are key cellular elements regulating glutamate concentrations both in the synaptic cleft and in the extracellular space (Haydon and Carmignoto, 2006). Numerous MRS studies reported on altered levels of glutamate-related metabolites in mood disorders (see "*In Vivo* Imaging Studies: Astrocytic Abnormalities" section). In line with this, a postmortem study reported reduced densities of glutamine synthetase expressing astrocytes in specific cortical gray matter areas in MDD (Bernstein et al., 2015). Glutamine synthetase catalyzes the ATP-dependent condensation of ammonia and glutamate to form glutamine, and by that plays a central role in glutamate and glutamine homoeostasis. In addition to this, several studies documented reduced expression of astrocyte specific glutamate transporter genes (EAAT1, EAAT2 or SLC1A2, SLC1A3) in the orbitofrontal cortex (Miguel-Hidalgo et al., 2010), dorsolateral PFC (Choudary et al., 2005; Zhao et al., 2016), hippocampus (Medina et al., 2013, 2016) and locus coeruleus (Bernard et al., 2011; Chandley et al., 2013) of patients with MDD. In sum,

there are ample evidences from different sources that glutamate and glutamine metabolism is impaired in depressed patients as well as in manic episodes (Öngür et al., 2008), which suggests aberrant functioning of astroglial cells in depressed patients (Haroon et al., 2017). We should also cite here a recent preclinical study which demonstrated that blockade of the astrocytic glutamate transporter (GLT-1) in the central amygdala can induce anhedonia and anxiety in rats (John et al., 2015).

Other astrocytic functions are also disturbed in depressive disorders. For example altered expression of glial gap junction proteins have been reported, indicating changes in astrocyte-astrocyte communication. Downregulation of connexin 30 and 43 expressing genes were found in several brain areas (Nagy et al., 2017), and reduced gene expression of the gap junction protein (GJA1) was reported in the hippocampus (Medina et al., 2016).

Finally, there are evidences on reduced gene expression of potassium and water channels (KCNJ10, AQP4) in the hippocampus of depressed patients (Medina et al., 2016).

Oligodendrocyte Abnormalities

The first study to document changes in oligodendrocyte-specific genes in the brains of psychiatric patients was done by Tkachev et al. (2003). They used differential display PCR, quantitative PCR and microarray analysis which provided evidences on reduced oligodendrocyte-related and myelination-associated gene expression in the brains of patients who had schizophrenia and BD. Later, Aston et al. (2005) reported on altered gene expression in the temporal cortex of MDD patients, which suggested oligodendroglial abnormalities. They used Affymetrix HgU95A microarray analysis and found a significant decrease in the expression of 17 genes related to oligodendrocyte function. Eight of these genes encode structural components of myelin (CNP, MAG, MAL, myelin oligodendrocyte glycoprotein (MOG), MOBP, PMP22, PLLP, proteolipid protein 1 (PLP1)), five other genes encode enzymes involved in the synthesis of myelin constituents (ASPA, UGT8), or regulate myelin formation (ENPP2, EDG2, TF, KLK6). SOX10, which encodes a transcription factor regulating other myelination-related genes, was also down regulated. OLIG2, a transcription factor specific for oligodendrocytes and oligodendrocyte precursors and ERBB3, which is involved in oligodendrocyte differentiation were also down regulated together with a number of genes involved in axonal growth and synaptic function (Aston et al., 2005). Overall these gene expression changes suggest disturbed neuronal communication and signal transduction mechanisms.

A recent postmortem study examined myelin-related mRNA and protein expression in the white matter of the ventral PFC in MDD patients (Rajkowska et al., 2015). Quantitative RT-PCR revealed significantly reduced expression of PLP1 mRNA and increased expression of mRNA for CNPase, MOG and oligodendrocyte transcription factors 1 (Rajkowska et al., 2015). The expression of CNPase protein was also significantly decreased in MDD. These data suggests molecular mechanisms for the degeneration of cortical axons and dysfunctional maturation of oligodendrocytes in MDD (Rajkowska et al., 2015). In BD however, increased CNPase protein levels were reported in the white matter adjacent to the dorsolateral PFC (Brodmann area 9; Hercher et al., 2014).

Interestingly, increased mRNA levels of the oligodendroglial markers, Olig1 and Olig2, have been found also in the serum of BD patients (Ferensztajn-Rochowiak et al., 2016).

Microglial Abnormalities

Numerous studies report on elevated inflammatory markers in a subgroup of patients with MDD and BD (for reviews see e.g., Rao et al., 2010; Rosenblat et al., 2014; Réus et al., 2015; Miller and Raison, 2016; Sayana et al., 2017). However, most of the studies report on cytokine abnormalities in the periphery (in the serum) of depressed patients. Direct evidences on elevated cytokines and Toll-like receptors in postmortem brain tissue of suicide victims have been reported recently by Pandey (2017). This study found significantly increased mRNA and protein expression of TNF-α, IL-1β, IL-6 and Toll-like receptors in the PFC of suicide victims (Pandey, 2017). Pantazatos et al. (2017) used next generation sequencing for whole-transcriptome profiling (RNA-seq) to identify genes, miRNA species, and molecular pathways that are altered in the dorsolateral PFC of MDD patients. They found altered immune-related gene expression in depression and suicide, and a markedly lower expression of genes associated with microglia and glial cell functions (Pantazatos et al., 2017).

SUMMARY AND FUTURE DIRECTIONS

There is ample evidence that glial abnormalities are present in the brains of depressed individuals. These glial changes affect all major glial cell types, astrocytes, microglia and oligodendrocytes and are detectable at multiple levels: at molecular, cellular and network level. Notably, we could not find any clinical studies on NG2-psoitive glia in the context of depressive disorders.

Here, we gathered the clinical observations and we did not consider the potential functional consequences, mainly because a number of recent excellent reviews discuss these issues. Astrocytes carry out a large number of vital cellular functions (e.g., Sofroniew and Vinters, 2010), their functional deficits can lead to various malfunctions, most prominently to disturbed glutamate and ion homeostasis, and to synaptic dysfunctions (see e.g., Rajkowska and Stockmeier, 2013; Jun et al., 2014; Verkhratsky and Parpura, 2016; Haroon et al., 2017; Sild et al., 2017; Wang et al., 2017). The main function of oligodendrocytes is to provide support and insulation to axons, thus, their dysfunction can lead to disrupted neuronal network connectivity and communication and consequently result in psychopathology (Menon, 2011; Edgar and Sibille, 2012). Plenty of evidences suggest neuroimmune etiology (Yirmiya et al., 2015; Miller and Raison, 2016; Haroon et al., 2017) or at least disturbed immune response regulation in a subgroup of depressed individuals (Mechawar and Savitz, 2016) and

activated microglia is one player in this complex multifaceted process.

There are two key questions that should to be clarified in the future: (1) Are these glial changes represent the cause or the consequence of the disease? (2) Do these glial abnormalities relate to each other, or are they present in different subgroup of patients? Some argue that the different glial abnormalities are connected to each other. It has been proposed that the microglia mediated inflammatory processes can damage the oligodendrocytes and disrupt glutamate homeostasis by impairing astrocytic functions (Mechawar and Savitz, 2016; Haroon et al., 2017). Prospective clinical studies collecting blood samples for biomarker analysis and combining *in vivo* neuroimaging data with postmortem histopathological analysis could help to answer these important questions. The clinical studies should also investigate putative changes affecting NG2-expressing glial cells. Preclinical studies, targeting specific glial cell types in transgenic animals (Birey et al., 2015) can also provide valuable insights.

AUTHOR CONTRIBUTIONS

BC had the concept and wrote the parts on the cellular and molecular findings. SAN wrote the parts on the *in vivo* imaging data.

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Electro-Acupuncture Alleviates Chronic Unpredictable Stress-Induced Depressive- and Anxiety-Like Behavior and Hippocampal Neuroinflammation in Rat Model of Depression

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Depression is the second leading cause of disability worldwide. The effects of clinical depression may be mediated by neuroinflammation such as activation of microglia and high levels of proinflammatory cytokines in certain brain areas. Traditional Chinese medicine techniques such as electro-acupuncture (EA) are used extensively in Asia to treat mental health disorders. However, EA has not been rigorously studied in treatment of depression. This study was designed to assess the effectiveness of EA on depressive-like behavior and explore the role of hippocampal neuroinflammation in the potential antidepressant effect of EA. In this study, we used six chronic unpredictable stressors daily in a random sequence for 10 weeks. EA were performed on "Bai-Hui" (Du-20) (+) and "Yang-Ling-Quan" (GB-34, the right side; −) acupoints by an EA apparatus (HANS Electronic Apparatus, LH202H, 2/100 Hz, 0.3 mA) for 30 min once every other day for last 4 weeks. The behavior tests including open field test and forced swimming test, which are widely used to assess depressive and anxiety-like behavior were performed on the Monday and Tuesday of the eleventh week. The results showed that 10 week of chronic unpredictable stress (CUS) caused behavioral deficits in rats and neuroinflammation in hippocampus, such as increased expression of NLRP3 inflammasome components, upregulated mRNA level of IL-1β and the protein level of IL-1β mature form (p17) and activation of microglia. Moreover, 4 weeks of EA treatment significantly attenuated behavioral deficits caused by CUS. EA's antidepressant effect was accompanied by markedly decreased expression of certain NLRP3 inflammasome components and matured IL-1β. Meanwhile, EA treatment can significantly reverse CUS-induced increases in P2X7 receptor, Iba-1, IL-18, TNFα and IL-6 expression and decreases in GFAP expression. In conclusion, EA exhibited the antidepressant effect and alleviated the hippocampal neuroinflammation.

These findings may provide insight into the role of hippocampal neuroinflammation in the antidepressant effect of EA.

Keywords: depression, EA, chronic unpredictable stress, IL-1β, P2X7 receptor, glia

INTRODUCTION

Depression is a prevalent mental health condition that affects more than 300 million people worldwide[1], and has become the leading cause of ill health and disability across the world[1]. Depression is the most common psychiatric disorder with a high mortality and morbidity rate[2]. In the past decades, preclinical studies have demonstrated that neuroinflammation, described by the increase of inflammatory cytokines in the brain, contributed to the development of depressive behavior (Yirmiya et al., 2000; Dantzer et al., 2008; Eyre and Baune, 2012). Patients with major depressive disorder (MDD) have greater pro-inflammatory cytokines in peripheral circulation and some in brain regions, such as: interleukin-6 (IL-6), IL-1β and C-reactive protein (CRP; Howren et al., 2009; Jacoby et al., 2016). Also, animal studies demonstrate that chronic stress can lead to elevated IL-1β in several brain regions, including the hippocampus, a key area implicated in the stress response and responsible for memory and emotion (Liu et al., 2013; Pan et al., 2014). Central administration of IL-1β produces several stress-like effects and pathological changes, such as the decline of adult hippocampal neurogenesis (Green and Nolan, 2012; Green et al., 2012). Treatment with IL-1β receptor antagonist reversed chronic unpredictable stress (CUS) induced depressive—like behavior (Koo and Duman, 2008, 2009; Koo et al., 2010).

Nowadays, all available antidepressants whose action is based on the monoamine hypothesis of depression have many side effects and limited efficacies, which limits their usages and results in poor compliance. Ultimately, many patients have turned to complementary and alternative medicine (CAM), such as acupuncture. As a modern therapeutic practice of CAM, electroacupuncture (EA) combines standard acupuncture with a gentle electrical current to stimulate acupuncture points. EA has been used successfully to treat depression in China and several other countries worldwide for its efficiency and minimal adverse effects (Ulett et al., 1998; Kim et al., 2013; Guo et al., 2016; Schroeder et al., 2017). Previous clinical and preclinical studies have showed that EA relieved depressive-like behavior in patients with depression and animal models (Ulett et al., 1998; Duan et al., 2016; Li et al., 2017). However, the underlying mechanism responsible for the antidepressant-like effects of EA remains unclear. It is thought to influence neuroinflammation in preclinical studies (Guo et al., 2014). EA has been shown to reduce neuroinflammation in animal models of neuropathic pain (Li et al., 2014). Moreover, our previous articles have confirmed that EA can improve hippocampal neurogenesis and atrophy of astrocytes in animal models of depression (Liu et al., 2007; Yang et al., 2013). EA may inhibit neuroinflammation and hence produce favorable effects to hippocampal pathological changes and depressive symptoms. Hence, it would be of interest to investigate whether hippocampal neuroinflammation is responsible for the antidepressant effects of EA.

The recently emerging evidence has suggested that the nucleotide binding and oligomerization domain-like (Nod) receptor family pyrin domain-containing 3 (NLRP3) inflammasome, the best characterized member of the intracellular Nod-like receptors (NLR) family, plays a critical role in various inflammatory diseases (Bigford et al., 2013; Parajuli et al., 2013; Meng et al., 2014; Yang F. et al., 2014). The NLRP3 inflammasome is a multiprotein complex that comprises an NLRP3 receptor, an adaptor ASC (apoptosis-associated speck-like protein containing a carboxy-terminal CARD) and an effector caspase-1 (p45). Caspase-1 is cleaved into active caspase-1 (p10), which further cleaves the inactive forms of IL-1β and IL-18 (i.e., pro-IL-1β and pro-IL-18) into their mature and active forms (Bauernfeind et al., 2009; Hanamsagar et al., 2012). MDD patients exhibited an increased level of the NLRP3 inflammasome in their peripheral blood mononuclear cells (Alcocer-Gómez and Cordero, 2014). Furthermore, in LPS or chronic stress-induced rodent models of depression, rodents displayed depressive-like behavior and activation of NLRP3 inflammasome in some brain areas such as the hippocampus (Lu et al., 2014; Zhang et al., 2014), which were decreased after standard antidepressant medications (Pan et al., 2014; Du et al., 2016).

Microglia, the resident innate immune cells in the brain, play an important role in some neurodegenerative diseases (Colonna and Butovsky, 2017). Activated microglia has been regarded as a key source of local pro-inflammatory cytokines, including IL-1β, IL-18, TNFα and IL-6, driving progressive neuron damage (Song and Wang, 2011). Recent evidence suggests that NLRP3 inflammasome was only functional in mouse brain microglia but not in astrocytes (Gustin et al., 2015). Meanwhile, activation of microglial NLRP3 inflammasome mediates IL-1β-related inflammation in the prefrontal cortex of depressive rats (Pan et al., 2014). In addition, the ATP-gated trans-membrane P2X7 receptor (P2X7R) is a non-selective cation channel that contributes to the development of inflammation via the regulation of the expression and release of inflammatory cytokines (i.e., IL-1β and TNF) from microglia (Sperlagh and Illes, 2014). Moreover, P2X7R has also received specific attention for playing an essential role in NLRP3 inflammasome activation (Di Virgilio, 2007). Two recent studies have shown further evidence that antidepressant drugs can modulate central

[1]http://www.who.int/mediacenter/factsheets/fs369/en/
[2]www.who.int

NLRP3 inflammasome activation, microglial structure and function (Pan et al., 2014; Du et al., 2016), thereby suggesting the therapeutic potential of targeting neuroinflammation to treat depression.

In this study, we sought to understand whether EA could improve behavioral deficits and decrease neuroinflammtion in rats exposed to CUS. Specifically, we examined behavioral endpoints including behavioral despair, exploration and locomotor activity, and relevant neuroinflammatory markers (NLRP3 inflammasome, proinflammatory cytokines and activation of microglia) in the hippocampus.

MATERIALS AND METHODS

Animals

Male Sprague-Dawley rats (180–200 g) were housed 2–4 per cage in an air-conditioned (22 ± 1°C) colony room maintained under a 12 h/12 h light/dark cycle with *ad libitum* access to food and water (except when indicated). Thirty-three rats were randomly divided into four groups. Group 1 included eight healthy rats which were left undisturbed in their home cages for total 10 weeks as a normal control (Group 1: Normal); Group 2 experienced only CUS for 10 weeks (Group 2: CUS, n = 9); Group 3 and Group 4 was subjected to 10-week CUS and 4-weeks sham-EA or EA treatment during the last 4 weeks of CUS (Group 3: Sham-EA, n = 8; Group 4: EA, n = 8). Then, the experiment was repeated in the other 16 SD rats, which were also randomly divided into four groups and exposed to same treatment (n = 4 per group). This study was carried out in accordance with the National Institute of Health Guide for the Care and Use of Laboratory Animals, and the protocol was approved by Animal Ethics Committee of Shanghai Medical College, Fudan University, Shanghai, China (20160225-071).

CUS Procedure

As previously described, SD rats were exposed to one of the six mild stressors daily in a random sequence for 10 weeks (Yang et al., 2013). Six different stressors were used in the experiment: cold swimming (swimming in 4°C water for 5 min), water deprivation (40 h), food deprivation (40 h), light-dark cycle reversal, Heat stress (40°C environment for 5 min) and shake stress (shaking cage for 30 min); see Supplementary Table S1.

EA Delivery

EA treatment was started in the 7th week of 10-week CUS, once every other day for 4 weeks (the latter part of the CUS period). The rats (n = 8–9 per group) were placed in wooden holders which restrained movement of the rat's body but allowed relatively free head movements when treated with EA in the first experiment. However, in the repeated experiment, the rats (n = 4 per group) were hang up with a piece of clothes when treated with EA, staying awake, convenient and safe. The rats were in transient isoflurane anesthesia (<1 min) when they got dressed. After the rats had been acclimated the holders or clothes, EA stimulation was delivered by an EA apparatus (HANS Electronic Apparatus, LH202H, 2/100 Hz, 0.3 mA) with the electrodes connected to two acupuncture needles which were inserted into "Bai-Hui" (Du-20, located above the apex auriculate, on the midline of the head) (+) and contralateral "Yang-Ling-Quan" (GB34, located near the knee joint, anterior and inferior to the small head of the fibula, in muscle peroneus longus and brevis) (−) acupoints for 30 min (**Figure 1**). EA at these two acupoints produced some antidepressant effect in our previous work and other studies (Lippert, 2007; Yang et al., 2013; Li et al., 2014; Yang L. et al., 2014). Animals allocated to the Sham-EA group were subjected to the similar procedure as EA group, but no electrical current was applied to them. Animals in Normal and CUS groups were only bounded in wooden holders or in a piece of clothes without any acupuncture needles.

Behavioral Testing

The open field test was performed in a 100 cm × 100 cm × 40 cm black Plexiglass box with a black floor as described (Redmond et al., 1997). Rats were individually placed in the center of the testing box at the beginning of the test, and then, the number of rearing behavior, the distance traveled, the center square entries and duration and the defecation in the box were recorded for 5 min and subsequently analyzed using a video-tracking system (Shanghai Mobile Datum Information Technology Company, Shanghai, China). After testing each animal, the apparatus was cleaned with 1% acetic acid to remove olfactory cues.

In the forced swimming test, individual rats were placed softly into an 18 cm diameter glass cylinder filled to 30 cm in depth with 23 ± 1°C water for 5 min. Rat's immobility and struggling behavior during the 5 min swim session were recorded and quantified by the investigators who were unaware of group assignment. Struggling was defined as vigorous movements of the forepaws breaking the water and immobility was defined when rat floated without struggling and making only those movements necessary to keep its head above the water.

Western Blot Analysis

The hippocampi of rats (n = 4 per group, from the first experiment) were ultrasonically disrupted in RIPA buffer (50 mM Tris (pH 7.4), 150 mM NaCl, 1% Triton X-100, 1% sodium deoxycholate, 0.1% sodium dodecyl sulfonate, sodium orthovanadate, sodium fluoride, ethylene diamine tetraacetic acid, leupeptin, Thermo Scientific) with protease inhibitors (PMSF, Beyotime) followed by centrifugation at 12,000× g for 20 min. Then the total protein level in the supernatants was measured using the Pierce BCA Protein Assay Kit (Thermo Scientific, Rockford IL, USA). Protein samples were separated on 12% acrylamide gels which were cut into two parts about at 45 kD band according to the protein ladders and interest proteins, then transferred to PVDF membrane (0.2 and 0.45 μm, respectively). After blocking with 5% nonfat milk in tris-buffered-saline with tween (TBST; 20 mM Tris-HCl, pH 7.5, 150 mM NaCl, and 0.05% Tween-20) for 2 h at 4°C, the membrane was blotted respectively with antibodies against Iba-1 (1:1000, Wako) and GAPDH (1:10,000, Kang Cheng)

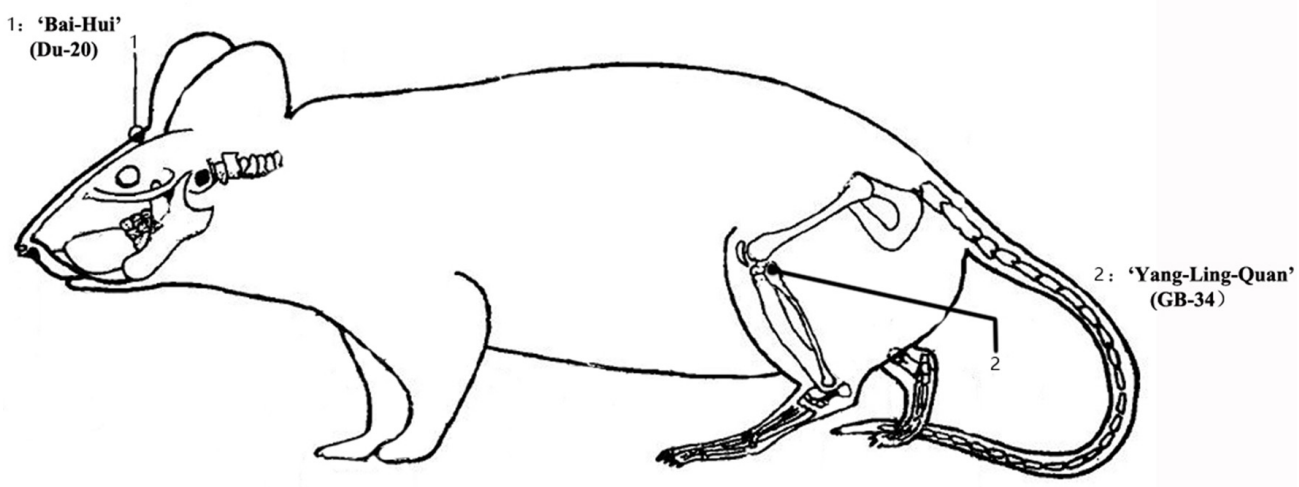

FIGURE 1 | The acupuncture points of rats.

as well as P2X7R (1:200, Santa Cruz) and GFAP (1:1000, Thermo Scientific) in sequence. For detecting the IL-1beta, ASC, Caspase-1 levels, the gels were cut into two parts about at 25 kD band and then transferred to PVDF membrane (0.2 and 0.45 μm, respectively), and blotted with antibodies against IL-1β (1: 1000, R&D System) or caspase 1 (1:200, Santa Cruz) and GAPDH (1:10,000, Kang Cheng) in sequences. Using the same procedure, we also detected NLRP3 expression levels using Cryopyrin (NLRP3, 1:200, Santa Cruz) and GAPDH (1:10,000, Kang Cheng) antibodies. And primary antibody incubation was performed at 4°C overnight.

After the blots were washed in TBST five times, the secondary antibodies (1:10,000, Earthox) were incubated for 1 h at room temperature. Western blot images were captured on an Image Quant LAS4000 mini image analyzer (GE Healthcare, Buckinghamshire, UK), and the band levels were quantified using Image J software (NIH, Bethesda, MD, USA).

Quantitative Real-Time RT-PCR

The hippocampi of rats (n = 4 per group from the first experiment) were homogenized and RNA was isolated using TRIzol reagent (Invitrogen) according to the manufacturer's instructions. The quality and quantity of the extracted RNA in each tissue was examined with spectrophotometry (Beckman DU7500). Equal amounts of RNA (2 μg/sample) isolated from the each hippocampi was reacted with M-MLV reverse transcriptase (iScriptTM cDNA Synthesis Kit, Bio-Rad, CA, USA) to generate cDNA in the following reaction: 5 μl of 5× M-MLV Reverse Transcriptase Reaction Buffer which included, in the final concentration, 1 μl of 0.5 ug/μl Oligo(dT)15 Primer (Bio-Rad), 2 μl of 10 mM dNTP Mix (Bio-Rad), 1 μl of 200 U/μl M-MLV reverse transcriptase (Bio-Rad, CA, USA), and 0.5 μl of 40 U/μl RNase inhibitor (Bio-Rad, CA, USA). Each reaction was then incubated at 37°C for 1 h. Equal amounts of cDNA (2 μl) were then used for subsequent PCR using iTap SYBR Green Master Mix (Bio-Rad, CA, USA). The $2^{-\Delta\Delta Ct}$ method [$\Delta CT = (Ct_{target} - Ct_{GAPDH})$] was then used to convert 1CT values to mRNA fold changes relative to the control group. The mRNA levels of the targets were normalized with glyceraldehyde-3-phosphatedehydrogenase (GAPDH) mRNA level to exclude effects of varying RNA amounts.

Please see Supplementary Table S2 for oligonucleotide primers specific for rat.

Immunohistochemical Analysis

The brains of rats (n = 4 per group from the second experiment) were removed and post fixed in 4% PFA at 4°C overnight and immersed in 20% sucrose (4% PFA as solvent) followed by 30% sucrose (in 0.1 M PBS). Thirty micrometer thick sections (CM1850, Leica Microsystems, Wetzlar, Germany) were blocked in 2% (wt/vol) BSA (Sigma) and then exposed overnight to the following primary antibody mixtures: anti-GFAP(Thermo, 1:1000) or anti-Iba-1(Wako, 1:1000) at 4°C. After washed five times in PBS, sections were incubated with secondary antibodies (donkey-anti-mouse, Alexa 594 conjugated, 1:1000, Invitrogen, USA; donkey anti rabbit, Alexa 594 conjugated, 1:1000, Invitrogen, USA; Hoechst, 1:1000, Beyotime, China) for 1 h at room temperature in the dark. These sections were rinsed in PBS five times 5 min each and cover slipped in the dark. Sections were imaged at 20× and 40× for analysis, using excitation wavelengths of 633 nm (blue Cy5 labeling), 543 nm (red Cy3 immunofluorescence).

Statistical Analyses

All data are analyzed using SPSS 16.0 (SPSS Inc., Chicago, IL, USA) and expressed as the mean ± standard error. The data collection and analysis were performed independently by two experimenters. The statistical significance of differences between groups was analyzed using one-way analysis of variance (ANOVA) according to the factors introduced in the experimental design. Where F ratios were significant, *post*

hoc comparisons were made using the Tukey *post hoc* test. Significance levels were set at $p < 0.05$.

RESULTS

EA Treatment Ameliorates Depressive-Like Behavior Induced by Chronic Unpredictable Stress (CUS)

In this study, the influence of long-term EA treatment on depressive-like behavior was evaluated. Experimental paradigm used in the experiment is depicted in **Figure 2A**. Except for rats in the Normal group, the other SD rats were exposed to CUS for 10 weeks, and treated with EA, Sham-EA every other day or no treatment during the last 4 weeks of CUS. Ten weeks of CUS exposure induced significantly depressive and anxiety-like behavior, indicated by more immobility time ($F_{(3,29)} = 13.311$, $p < 0.001$) and less struggling behavior ($F_{(3,29)} = 30.946$, $p < 0.001$) in FST (**Figures 2B,C**), less rearing behavior ($F_{(3,29)} = 28.364$, $p < 0.001$), shorter distance traveled ($F_{(3,29)} = 8.207$, $p < 0.001$), less center square entries ($F_{(3,29)} = 13.899$, $p < 0.001$) and less center square duration ($F_{(3,29)} = 3.008$, $p < 0.01$) in OFT (**Figures 2D–G**). Although CUS exhibited a tendency to increase the defecation of rats in OFT, there are no significant difference between CUS and Normal group ($F_{(3,29)} = 0.726$, $p = 0.0513$, **Figure 2H**).

After 4 weeks of EA treatment, rats exhibited less immobility time ($F_{(3,29)} = 13.311$, $p < 0.001$) and more struggling behavior ($F_{(3,29)} = 30.946$, $p < 0.001$) in FST as compared to rats that were only exposed to CUS or treated with Sham-EA simultaneously, which indicated that EA alleviated behavioral despair (**Figures 2B,C**). Furthermore, rats treated with EA also exhibited more rearing behavior ($F_{(3,29)} = 28.364$, $p < 0.001$) and traveled longer distances ($F_{(3,29)} = 8.207$, $p < 0.001$), spent more time on the center square ($F_{(3,29)} = 3.008$, $p < 0.001$), more frequently entered the central square ($F_{(3,29)} = 13.899$, $p < 0.001$) in OFT (**Figures 2D–G**), which indicated that EA increased exploration and the locomotor activity in CUS rats as well. But there is no significant difference in defecation among all four groups ($F_{(3,29)} = 0.726$, $p = 0.198$, **Figure 2H**). These results suggest EA treatment could reverse the CUS-induced depressive- and anxiety-like behavior, substantiating the evidence for antidepressant-like and anxiolytic effects of EA treatment.

CUS-Induced Increase in IL-1β and NLRP3 Inflammasome in the Hippocampus Is Reversed by EA Treatment

Western blotting analysis showed that the levels of matured IL-1β (p17) in the hippocampus were significantly increased in rats belonging to the CUS and Sham-EA groups, respectively, as compared to the Normal group ($F_{(3,12)} = 137.2$, $p < 0.001$, **Figure 3A**). EA treatment significantly decreased the level of matured IL-1β as compared with the Sham-EA group or CUS group ($p < 0.001$, **Figure 3A**). Likewise, EA also downregulated the mRNA level of IL-1β which increased in the CUS group ($F_{(3,12)} = 122.5$, $p < 0.001$, **Figure 3B**). Interestingly, although there was no significant difference in the protein level of pro-IL-1β (p31) in the hippocampus across all experimental groups ($F_{(3,12)} = 0.7327$, $p = 0.5522$, **Figure 3C**), the relative mRNA level of IL-1β was significantly enhanced by exposure to CUS ($p < 0.001$, **Figure 3B**). The results hinted that CUS may also upregulate the cleavage of IL-1β except for the transcription of IL-1β.

Thus, NLRP3 inflammasome components, including NLRP3, ASC and caspase-1, in the hippocampus were also evaluated by western blotting. As shown in **Figure 4A**, the hippocampal ASC protein level significantly increased in the CUS and Sham-EA groups as compared to the Normal group ($F_{(3,12)} = 52.88$, $p < 0.001$). The levels of caspase-1 (p45; $F_{(3,12)} = 23.76$, $p < 0.001$) and cleaved-caspase-1 (active caspase-1, p10; $F_{(3,12)} = 188.8$, $p < 0.001$) in the hippocampus were also significantly increased in the CUS and Sham-EA groups as compared to the Normal group (**Figures 4B,C**). Simultaneously, EA treatment showed a significant decrease in hippocampal ASC protein expression ($p < 0.001$, **Figure 4A**) as well as caspase-1 (p45; $p < 0.001$, **Figure 4B**) as compared to the Sham-EA group and CUS group. The level of cleaved-caspase-1 (p10) in the hippocampus also exhibited a slight decrease in the EA group as compared to the CUS group ($p < 0.05$, **Figure 4C**). However, NLPR3 protein expression in the hippocampus did not differ significantly among the four experimental groups ($F_{(3,12)} = 1.425$, $p = 0.2838$, **Figure 4D**).

CUS-Induced Changes of P2X7R, Iba-1 and GFAP Expression in the Hippocampus Is Regulated by EA Treatment

Given that P2X7R is one of the vital factors for NLRP3 inflammasome activation (Lu et al., 2014), we also observed the expression of P2X7R in the hippocampus. As shown in **Figures 5A,D**, the hippocampal P2X7R mRNA ($F_{(3,12)} = 49.35$, $p < 0.001$) and protein levels ($F_{(3,12)} = 45.26$, $p < 0.001$) were significantly increased in the CUS and Sham-EA groups as compared to the Normal group. EA treatment also significantly reduced the hippocampal P2X7R mRNA ($p < 0.001$) and protein levels ($p < 0.001$) as compared to the CUS or Sham-EA groups.

A primary source of CNS inflammatory cytokines is the activated microglia cells (Meng et al., 2014). Here, we used the microglia marker, Iba1, to evaluate microglia activation. As shown in **Figures 5B,E,G**, after exposure to CUS, the mRNA ($F_{(3,12)} = 14.91$, $p < 0.001$) and protein levels ($F_{(3,12)} = 32.89$, $p < 0.001$) of hippocampal Iba1 in the hippocampus were significantly increased as compared to the Normal group. Meanwhile, EA treatment significantly decreased the mRNA and protein level of Iba1 in the hippocampus (**Figures 5B,E**). Interestingly, Sham-EA treatment also significantly downregulated the mRNA level of Iba1 ($F_{(3,12)} = 14.91$, $p < 0.01$) but not the protein level ($F_{(3,12)} = 14.91$, $p = 0.062$) as compared with the CUS group (**Figures 5B,E**).

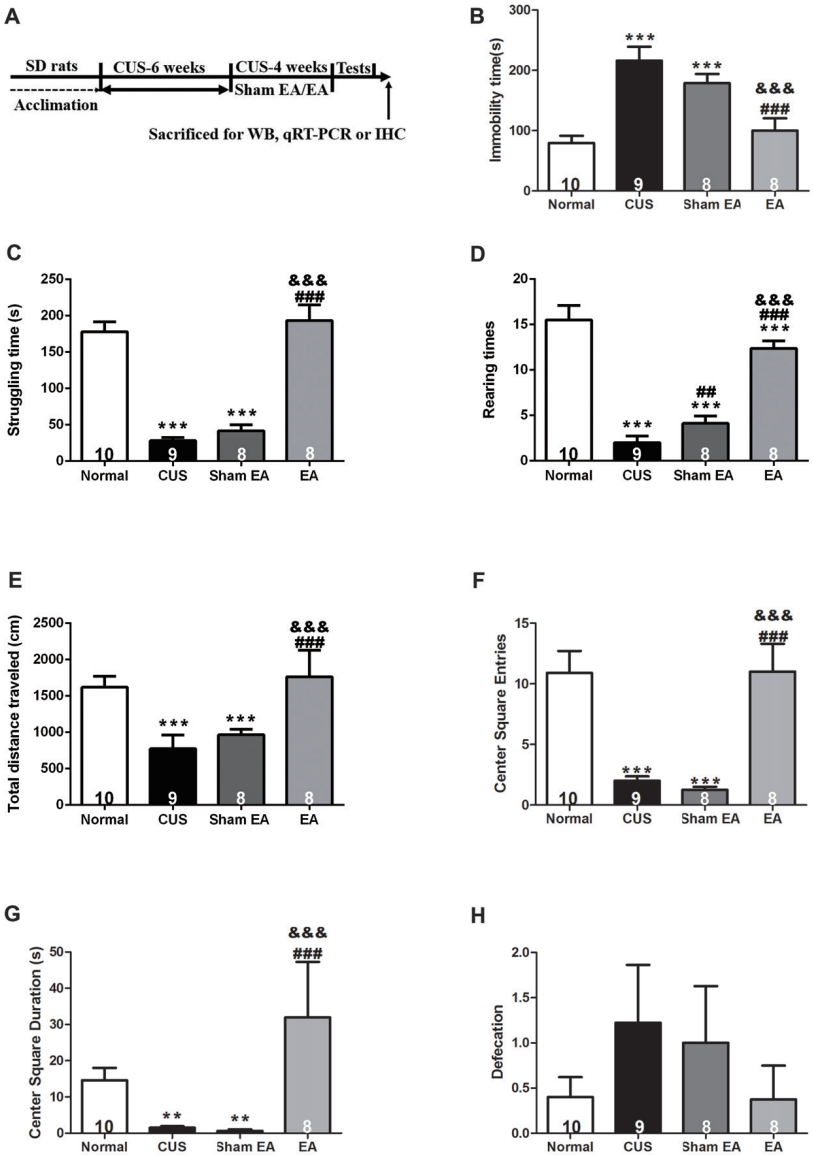

FIGURE 2 | Electro-Acupuncture (EA) alleviates depressive-like behavior induced by chronic unpredictable stress (CUS) in Sprague-Dawley (SD) rats. **(A)** Experimental paradigm. Forty-nine male SD rats were randomly divided into four groups and treated as shown above. Behavioral indicators were then assessed, including. **(B)** Immobility time, **(C)** Struggling time in forced swimming test (FST). **(D)** Rearing numbers. **(E)** Total distance traveled in open field test (OFT). **(F)** Center square entries. **(G)** Center square duration and **(H)** Defecation. All data are expressed as the mean ± SEM (n = 8–10 per group). ** $p < 0.01$, ***$p < 0.001$, compared with Normal group; $^{\#\#}p < 0.01$, $^{\#\#\#}p < 0.001$, compared with CUS group; $^{\&\&\&}p < 0.001$, compared with Sham EA group.

In addition to IL-1β, we also assessed the transcription of other proinflammatory cytokines, such as, IL-18, TNF1β and IL-6 in the hippocampus, using real-time quantitative PCR. The results exhibited that exposure to CUS induced significant upregulation of the mRNA level of IL-18 ($F_{(3,12)}$ = 32.65, $p < 0.001$), TNF1x003B1; ($F_{(3,12)}$ = 12.51, $p < 0.001$) and IL-6 ($F_{(3,12)}$ = 52.28, $p < 0.001$) in the hippocampus (**Figures 6A–C**). Meanwhile, the mRNA level of IL-18, TNF1x003B1; but not IL-6 in the hippocampus was cut down significantly in the EA group as compared with the CUS group and Sham-EA group (**Figures 6A–C**). The results give further support to the supposition that CUS-induced neuroinflammation in the hippocampus was alleviated by EA treatment.

In addition, astrocytes have been regarded as the source of certain proinflammatory cytokines. However, there is increasing evidence of astrocytic atrophy or dysfunction in depressive disorders. Hence, we also evaluated astrocyte levels using the astrocyte marker, GFAP. As shown in **Figures 5C,F,H**, both GFAP mRNA ($F_{(3,12)}$ = 78.82, $p < 0.001$) and protein expression ($F_{(3,12)}$ = 27.75, $p < 0.001$) in the hippocampus were significantly decreased in the CUS group as compared to the Normal group. As compared to the CUS group, Sham-EA

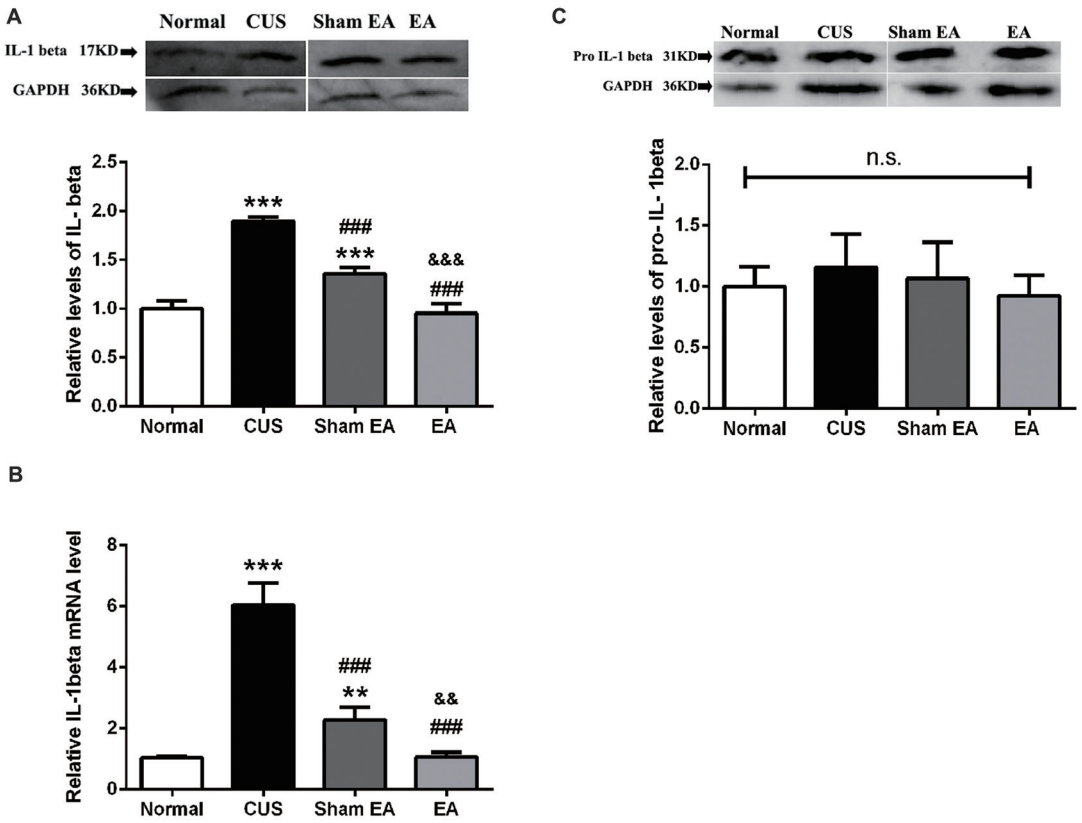

FIGURE 3 | EA downregulates mRNA level of IL-1beta and protein level of mature IL-1beta (p17), which were upregulated by CUS in hippocampi of rats. After behavior tests, rats' hippocampi were collected and analyzed for protein level of **(A)** mature IL-1β (p17), **(C)** pro-IL-1β (p35), and **(B)** the mRNA level of IL-1β using western blotting and quantitative PCR. Results are expressed as the mean ± SEM (n = 4 per group. $^{**}p < 0.01$, $^{***}p < 0.001$, compared with Normal group; $^{\#\#\#}p < 0.001$, compared with CUS group; $^{\&\&}p < 0.01$, $^{\&\&\&}p < 0.001$, compared with Sham EA group; n.s, not significant.

treatment significantly increased hippocampal GFAP protein and the mRNA level (**Figures 5C,F**). EA Treatment only significantly increased the GFAP mRNA level, but not the protein level, when compared to the Sham-EA and CUS groups (**Figures 5C,F**).

DISCUSSION

The present study demonstrated that 10 weeks of CUS-induced depressive- and anxiety-like behavior, which are described as a dramatic increase in the immobility time in FST and a significant decrease in the rearing frequency and total distance traveled as well as less central square entries and duration in OFT (**Figure 2H**). Meanwhile, EA treatment ameliorated the depressive- and anxiety-like behavior. In addition, EA inhibited the increase of the level of hippocampal IL-1β protein and its' convertase (active caspase 1) caused by CUS. Likewise, the protein levels of ASC and pro-caspase 1 p45 (inactive caspase-1), two important components of NLRP3 inflammasome, which significantly increased in CUS rats, were also diminished by EA treatment. Moreover, CUS caused the significant upregulation of the P2X7R mRNA and protein levels, which is accompanied by microglia activation and astrocytic atrophy. EA treatment also alleviated this CUS-induced hippocampal pathology.

CUS Induces Depressive-Like Behavior and EA Exhibits the Antidepressant-Like Effect

CUS induced in animal models is often regarded as one of the strongest animal models of depression because of its good predictive validity (Henn and Vollmayr, 2005), face validity and construct validity. After exposure to CUS, the mice or rats will exhibit some depressive-like behavior, such as behavioral despair, anhedonia, less exploration and less locomotion (Hazra et al., 2017; Jett et al., 2017). In our experiments, we also evaluated the depressive-like behavior in an open field test (OFT) and forced swimming test (FST). The present study showed that CUS induced an obvious decrease in exploration and locomotion in OFT and less struggling and more immobile behavior in FST. Like many antidepressants (Liu et al., 2008; Mutlu et al., 2012), EA also significantly relieved the depressive-like behavior induced by CUS. The results underpin the antidepressant-like effects of EA consistent with the previous studies (Liu et al., 2007; Duan et al., 2016; Li et al., 2017).

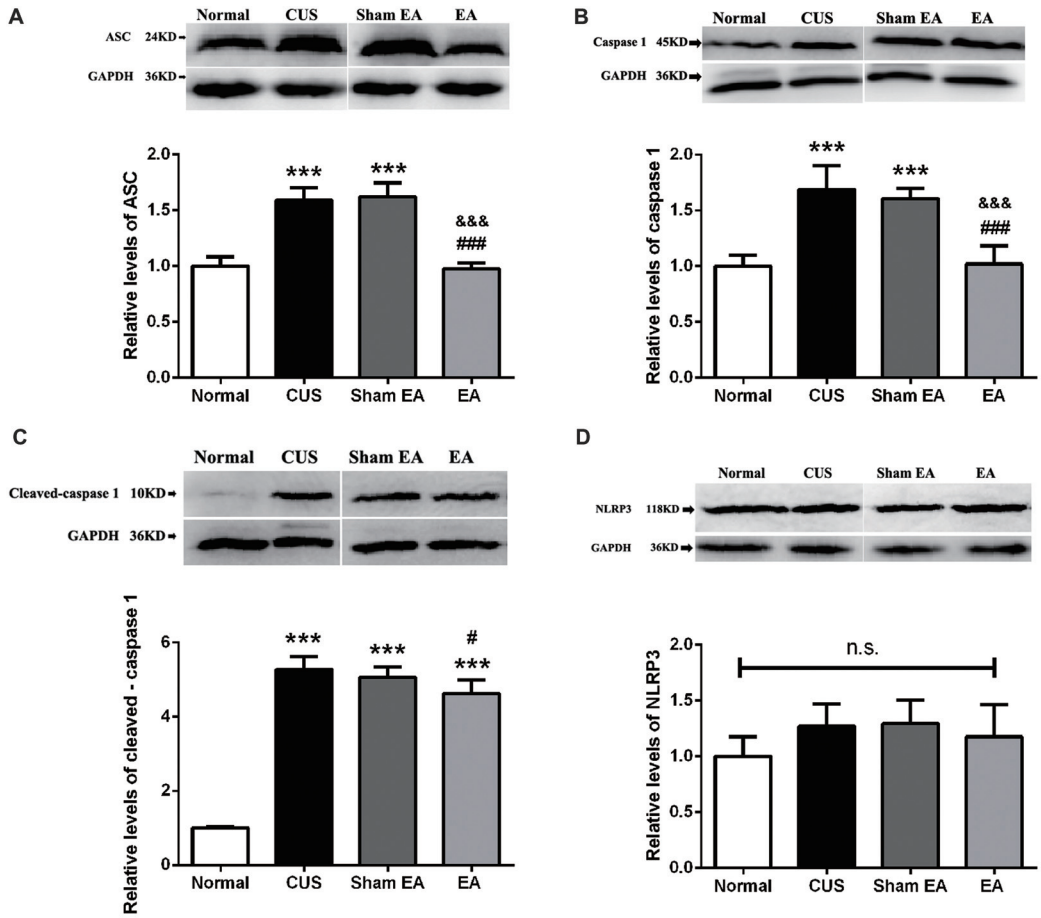

FIGURE 4 | EA decreases the protein level of ASC, caspase 1 (p45) and cleaved-caspase 1 (p10) but not NLRP3 in hippocampi of CUS rats. After behavior tests, rats' hippocampi were collected and analyzed for levels of **(A)** ASC, **(B)** caspase 1 (p45), **(C)** cleaved-caspase 1 (p10) and **(D)** NLRP3 using western blotting. Results are expressed as the mean ± SEM (n = 4 per group). $^{***}p < 0.001$, compared with Normal group; $^{\#}p < 0.05$, $^{\#\#\#}p < 0.001$ compared with CUS group; $^{\&\&\&}$ compared with Sham-EA group; n.s., not significant.

CUS Upregulates IL-1β and NLRP3 Inflammasome

Evidence is increasing that psychological and physical stressors could activate immune and inflammation processes, contributing to depressive symptoms (Iwata et al., 2013). Proinflammatory cytokine IL-1β in some brain areas of the limbic system, such as the prefrontal cortex and hippocampus of depressive rats, was implicated in the pathophysiology of depression (Pan et al., 2014). The result that exposure to CUS increased hippocampal matured IL-1β (p17) is consistent with these findings. Recent research has also indicated that activation of NLRP3 inflammasome signaling, a pivotal mediator of IL-1β function (Haneklaus et al., 2013), contributes to depression (Iwata et al., 2016). Additionally, several other studies have also reported the change of NLRP3 inflammasome components in rodents exposed to CUMS, LPS stimulus or estrogen deficiency (Lu et al., 2014; Zhang et al., 2014; Xu et al., 2016). In their studies, they observed the overexpression of certain components of NLRP3 inflammasome, such as NLRP3, ASC and caspase-1. In accordance with their results, our results also showed an increase in the expression of ASC, one of the components of NLRP3 inflammasome and caspase-1 (p45 and p10), the effector and the product of NLRP3 inflammasome, respectively, after exposure to CUS. Taken together, it implies that CUS upregulates the expression of NLRP3 inflammasome components and induces the activation of NLRP3 inflammasome, indicated by the increase of caspase-1 (p10). Additionally, the other important question for the field is the implication of inflammasome in antidepressant-like effect of EA. Our results exhibited that EA significantly inhibited the upregulation of components and products of NLRP3 inflammasome. In accordance with our results, other studies have indicated that the classical antidepressant, fluoxetine, and some monomeric compounds extracted from the traditional Chinese herb, such as L-Menthone and Icariin, can inhibit the activation or upregulation of NLRP3 inflammasome as well as confer their antidepressant effects (Xue et al., 2015; Du et al., 2016).

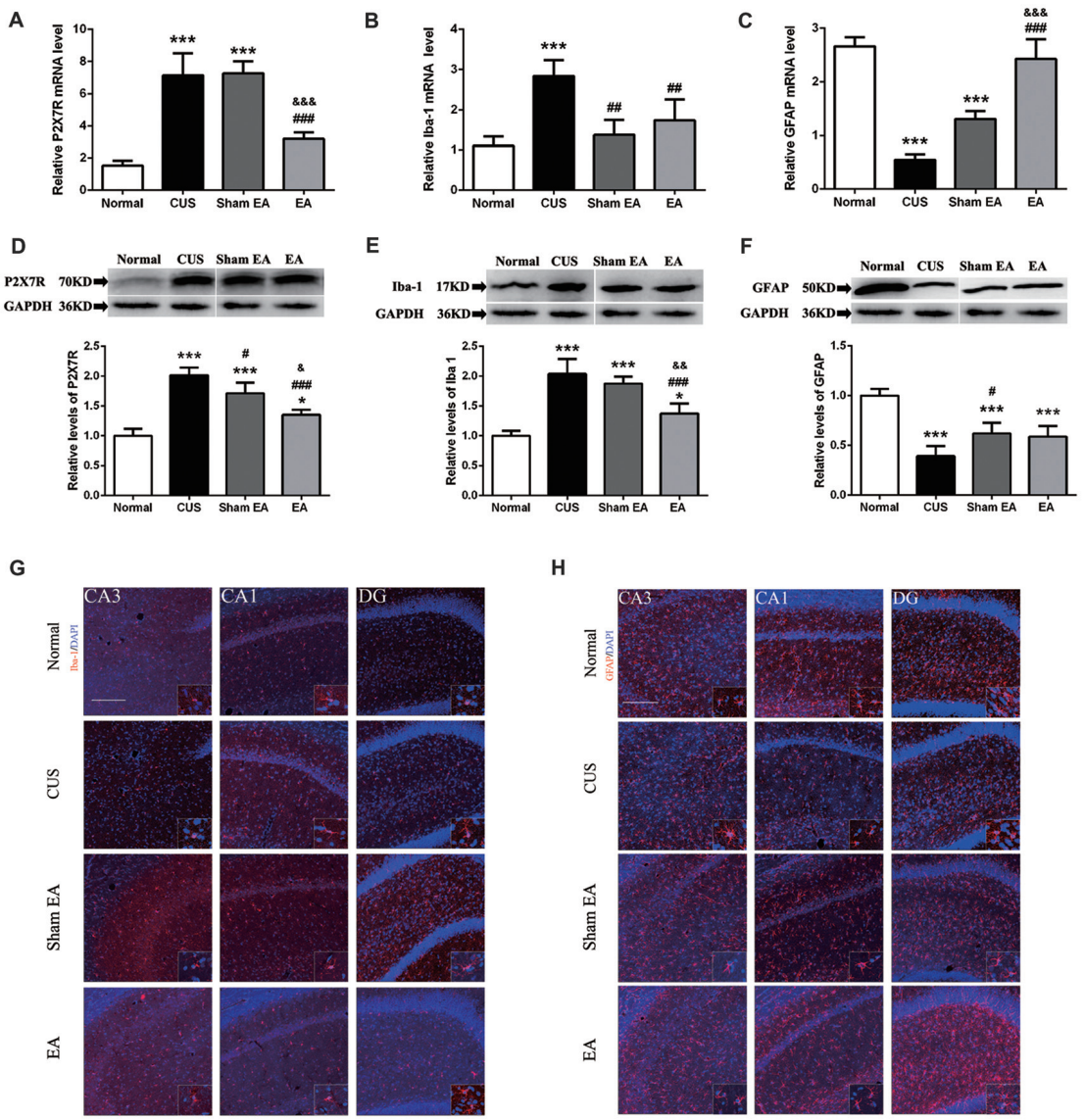

FIGURE 5 | Effect of EA on gene and protein expression of P2X7R, GFAP and Iba-1 in hippocampi of CUS rats. The relative levels of protein corresponding to **(A)** P2X7R, **(B)** GFAP and **(C)** Iba-1 were assessed using an immunoblotting method. Results were normalized to GAPDH. Levels in Normal rats were arbitrarily assigned a value of 1.0. The relative levels of mRNAs encoding for **(D)** P2X7R, **(E)** GFAP and **(F)** Iba-1 were assessed using quantitative PCR. Results were normalized to GAPDH. All values are the means ± SEM ($n = 4$). $^{*}p < 0.05$ and $^{***}p < 0.001$, compared with Normal group. $^{\#}p < 0.05$, $^{\#\#}p < 0.01$, $^{\#\#\#}p < 0.001$, compared with CUS group. **(G,H)** Immunofluorescence staining of hippocampal sections. GFAP and Iba-1 expression in the CA1, CA3, dentate gyrus (DG) of hippocampus detected by immunofluorescence in Normal rats and rats exposed to CUS with/without EA or Sham EA treatment. Representative images show GFAP/Iba-1 (red) and DAPI (blue). $^{\&}p < 0.05$, $^{\&\&}p < 0.01$, $^{\&\&\&}p < 0.001$, compared with Sham EA group.

EA Reversed CUS-Induced IL-1β-Related Microglia Activation Which May Be Mediated by P2X7-NLRP3 Signaling

Two recent articles from the Ronald S. Duman laboratory and our group have indicated that extracellular ATP-P2X7R signaling may mediate the stress-induced neuroinflammation, possibly via NLRP3 inflammasome-dependent IL-1β mature and microglia activation (Iwata et al., 2016; Yue et al., 2017). The experiment indicated that chronic accumulation of stress (10 weeks) further induced upregulation of P2X7R, IL-1beta and microglia activation. In addition, NLPR3 and P2X7R primarily localized in microglia provide further support for a tight relationship between P2X7R, NLRP3 and microglia activation (Gustin et al., 2015). Moreover, the IL-1beta mature and release was regarded as the key marker of proinflammatory activation of microglia (Song and Wang, 2011). Likewise, activated microglia was taken as the source of local synthesized cytokines in the brain (Song and Wang, 2011; Harry and Kraft, 2012). However,

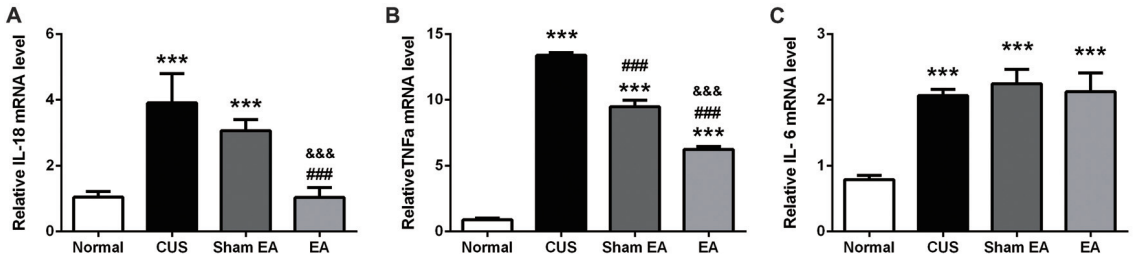

FIGURE 6 | EA downregulates mRNA level of IL-18, TNFα, which were upregulated by CUS in hippocampi of rats. After behavior tests, rats' hippocampi were collected and analyzed for mRNA level of **(A)** IL-6, **(B)** IL-18, and **(C)** TNF α using quantitative PCR. Results are expressed as the mean ± SEM (n = 4 per group. $^{***}p < 0.001$, compared with Normal group; $^{###}p < 0.001$, compared with CUS group; $^{&&&}p < 0.001$, compared with Sham EA group.

the research focus on microglia in depression presented contradictory results (Yirmiya et al., 2015; Santos et al., 2016). The results exhibited that CUS induced the increase of mature IL-1β, the upregulation of P2X7R, NLRP3 inflammasome and microglia activation, which raises the possibility that upregulation of P2X7R-NLRP3 inflammasome signaling may be correlated with IL-1β-related microglia activation in CUS rats. The above discussion further raises another important question relating to the implication of P2X7R and microglia activation in the effect of EA and other antidepressants. A previous study in which trifluoperazine and paroxetine suppressed P2X7-mediated IL-1β secretion from lipopolysaccharide (LPS)-primed human CD14+ monocytes has shed some light on the question (Dao-Ung et al., 2015). In addition, our previous research has confirmed that knockout P2X7R (P2X7-null or P2X7-/- mice) displayed an antidepressant phenotype after exposure to CUS. Simultaneously, the antagonist of P2X7R also can impede the depressive-like behavior induced by CUS (Iwata et al., 2016; Yue et al., 2017). All of these results give more support to the hypothesis that downregulation of P2X7R expression may mediate the antidepressant effects of EA. Interestingly, sham-EA treatment also diminished the mRNA and mature form of IL-1β like EA, although to a less extent. In clinic, acupuncture, by sticking needles into skin to some specific acupoints with some manual operation but not electric current also has efficacy on many diseases to a certain extent like EA (Li et al., 2012; Ma et al., 2012; Zhang et al., 2017). Sham-EA may be regarded as a low-intensity form of therapeutic needling which also have a little efficacy on some diseases, such as pain (Adrian White, 2009; White and Cummings, 2009). S Consistent with these findings, the results also shed some light on the relieving effect of Sham-EA on neuroinflammation, especially, IL-1β expression and mature in hippocampus.

EA Reversed CUS-Induced Microglia Activation and Astrocytic Atrophy

As is common knowledge, microglia is the main source of pro-inflammatory cytokines in the brain (Harry and Kraft, 2012). The pro-inflammatory cytokines are suggested to be involved in the pathophysiology of MDD (Yirmiya et al., 2000; Dantzer et al., 2008; Song and Wang, 2011; Eyre and Baune, 2012). The activation of microglia may have detrimental effects on neurons by expressing and synthesizing pro-inflammatory cytokines such as IL-1β, which induces neuro-inflammation and eventually induces the death of neurons under these conditions (Brown and Vilalta, 2015; Dao-Ung et al., 2015). In our present study, we showed that CUS induced the dramatic activation of microglia, which was in accordance with the previous studies indicating that diversiform stress including: environmental, psychological and chronic stress activated microglia (Hinwood et al., 2012; Liu et al., 2015; McKim et al., 2016). However, EA treatment did not significantly alleviate the microglial activation in the hippocampus, although the microglia activation was slightly inhibited in the EA group, which implies that EA might also regulate other glia cells. Interestingly, our present results showed that EA treatment could reverse astrocytic atrophy induced by CUS in mRNA levels. Increasing evidence has unmasked the controversy that numerical alterations of astrocytes in the front limbic systems are tightly connected with depression, as implied in post-mortem studies of patients with MDD (Wang et al., 2017). Many animal studies also indicated that psychological and physiological stress can induce the dysfunction or atrophy of astrocytes (Wilhelmsson et al., 2006; Gosselin et al., 2009; Liu et al., 2009; Zhang et al., 2015; Cobb et al., 2016). The present results provide insight into the role of astrocytic atrophy in depression and the antidepressant effect of EA.

AUTHOR CONTRIBUTIONS

JY, NY, BL and G-CW conceived and designed the experiments. NY performed the experiments. NY, LY, Y-LW, Q-QH, H-JH, JW and RY analyzed the data. All of the authors discussed the results. JY, QL and NY wrote and modified the manuscript. All authors reviewed the manuscript.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fnmol.2018.00149/full#supplementary-material

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REVIEW
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BACE1-Dependent Neuregulin-1 Signaling: An Implication for Schizophrenia

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Schizophrenia is a chronic psychiatric disorder with a lifetime prevalence of about 1% in the general population. Recent studies have shown that Neuregulin-1 (Nrg1) is a candidate gene for schizophrenia. At least 15 alternative splicing of NRG1 isoforms all contain an extracellular epidermal growth factor (EGF)-like domain, which is sufficient for Nrg1 biological activity including the formation of myelin sheaths and the regulation of synaptic plasticity. It is known that Nrg1 can be cleaved by β-secretase (BACE1) and the resulting N-terminal fragment (Nrg1-ntf) binds to receptor tyrosine kinase ErbB4, which activates Nrg1/ErbB4 signaling. While changes in Nrg1 expression levels in schizophrenia still remain controversial, understanding the BACE1-cleaved Nrg1-ntf and Nrg1/ErbB4 signaling in schizophrenia neuropathogenesis is essential and important. In this review paper, we included three major parts: (1) Nrg1 structure and cleavage pattern by BACE1; (2) BACE1-dependent Nrg1 cleavage associated with schizophrenia in human studies; and (3) Animal studies of Nrg1 and BACE1 mutations with behavioral observations. Our review will provide a better understanding of Nrg1 in schizophrenia and a potential strategy for using BACE1 cleavage of Nrg1 as a unique biomarker for diagnosis, as well as a new therapeutic target, of schizophrenia.

Keywords: schizophrenia, β-secretase (BACE1), neuregulin-1 (NRG1), erb-b2 receptor tyrosine kinase 4 (ErbB4), signaling pathway

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INTRODUCTION

Schizophrenia is a hereditary, disabling mental disorder that affects ∼1% of the general population. The etiology of schizophrenia is complicated and is influenced by more than genetics alone. Other factors such as neurotransmitter imbalance, abnormal neuronal development, infection, and neuronal inflammation are also possible mechanisms (Schultz et al., 2007).

Abbreviations: AD, Alzheimer Disease; AKT, serine/threonine kinase 1; ALIC, anterior limb of the internal capsule; ASEM, anti-saccade eye movements; BACE1, β-secretase; DISC1, disrupted in schizophrenia 1; EGF, epidermal growth factor; EMT, eye movement test; ErbB4, erb-b2 receptor tyrosine kinase 4; Erk, extracellular regulated MAP kinase; ERP, event-related potential; HAP, haplotype; Ig, immunoglobulin; nACC, nucleus accumbens; NMDA, N-methyl-D-aspartic acid; NRG1, Neuregulin-1; NRG1-CRD, NRG1-intracellular domain; Nrg1-ctf, Nrg1 C-terminal fragment; Nrg1-ntf, Nrg1 N-terminal fragment; NRG3, Neuregulin-3; PI3K, phosphatidylinositol-4,5-bisphosphate 3-kinase; PPI, pre-pulse inhibition; PSD95, postsynaptic density protein 95; SPEM, smooth pursuit eye movements; TACE, tumor necrosis factor-α-converting enzyme; UF, uncinate fasciculus; vHPC, ventral hippocampus.

Schizophrenia is characterized by several major clinical symptoms such as positive symptoms (hallucinations and delusions), negative symptoms (emotional blunting, and social withdrawal), and cognitive impairments (attention, performance, and working memory). *NRG1* was one of the 108 schizophrenia-associated genes identified in 2014 (Schizophrenia Working Group of the Psychiatric Genomics Consortium, 2014), and it attracted much attention due to its role in regulation of neuronal migration and myelination. *NRG1* is widely distributed in the frontal cortex, midbrain, and cerebellum (Rieff et al., 1999; Liu et al., 2001; Stefansson et al., 2003), and significantly associated with endophenotypes of schizophrenia via regulating myelination (Chen et al., 2006), neuronal migration (Ghashghaei et al., 2006), and function of neurotransmitter receptors (Liu et al., 2001; Hahn et al., 2006). Nrg1 can be cleaved by the proteolytic enzyme, BACE1. The BACE1-cleaved Nrg1-ntf plays roles in brain function via activation of ErbB receptor signaling pathways (Luo et al., 2011). Since most studies have compared the total Nrg1 levels between schizophrenia and healthy controls, it is critical to know whether the specific activity of BACE1 in cleavage of Nrg1 plays an important role in schizophrenia. In this review, we provide a summary and perspective on information of BACE1 involvement in Nrg1 regulation in schizophrenia according to recent clinical and preclinical discoveries, presented in three sections: (1) Nrg1 structure and cleavage pattern by BACE1; (2) BACE1-dependent Nrg1 cleavage associated with schizophrenia in human studies; (3) Animal studies of Nrg1 and BACE1 mutations with behavioral observations.

Neuregulin-1 Structure and Cleavage Pattern by BACE1

The neuregulin family includes four proteins (Nrg1, Nrg2, Nrg3, and Nrg4), encoded by their respective genes, which are widely expressed in various tissues including brain, heart, and breast. In general, Nrg1 can be divided into three major isoforms from alternative splicing. Type I Nrg1 has alternative names such as acetylcholine receptor inducing activity, differentiation factor, or neuregulin. Type II Nrg1 is also called glial growth factor, while type III Nrg1 is also known as sensory and motor neuron-derived factor. There are common structures between Nrg1 isoforms, such as Ig domains, EGF domains, a transmembrane region and unequal length of intracellular domain (Falls, 2003). Due to alternative splicing effect, Nrg1 is also divided into type alpha and beta based on the difference between the 5th and 6th cysteine amino acid in the EGF-like domain, whereas the beta variant has higher affinity for its downstream ErbB receptors (Wen et al., 1994; Burgess et al., 1995) (**Figure 1**).

Nrg1-induced cellular responses are mostly mediated by binding to tyrosine kinase receptors in the ErbB family. The ErbB family includes ErbB1, ErbB2, ErbB3, and ErbB4 receptors. Nrg1-mediated ErbB2 receptor activation requires the participation of ErbB3 or ErbB4 to form heterodimers (Bublil and Yarden, 2007). ErbB3 on its own lacks tyrosine kinase activity, so the activation of ErbB3 is dependent on heterodimer formation with other ErbB receptors (Falls, 2003). Nrg1 performs most of its functions via binding to both ErbB3 and ErbB4, while Nrg3 can only bind to ErbB4 (Zhang et al., 1997).

Both human and animal studies have shown that BACE1-cleaved Nrg1-ntf plays roles in brain function via activation of ErbB receptor signaling pathways. BACE1 cleaves type I and III Nrg1 at its position between the region of EF and ME residues and releases soluble fragments of Nrg1. BACE1, together with ADAM17 or ADAM10 which is also called TACE was involved in successive release of the EGF-like domain of NRG1 type III two membrane-bound structures, which has been generated by an initial BACE1 dependent proteolytic cleavage (Horiuchi et al., 2005). NRG3, another substrate of BACE, was considered as a compensation for loss of NRG1 and cleaved to produce EGF-domain through juxtacrine interactions with ErbB4 receptor like NRG1-CRD on axon of neuron (Vullhorst et al., 2017). These fragments bind to the ErbB4 receptor at its EGF-like domain, thereby activating ErbB receptors involved in Nrg1/ErbB signaling pathways that ultimately increase ERK and AKT phosphorylation, which are necessary for cell survival, synaptic development, glutamatergic transmission (Krivosheya et al., 2008; Mei and Xiong, 2008), and remyelination (Hu et al., 2006; Luo et al., 2011). The remain fragment of NRG1 cleavage is called NRG1-CTF, which can be further processed by γ-secretase to release the NRG1-ICD that participated to enhance synaptic plasticity for the development of cortical neurons (Bao et al., 2004; Chen et al., 2010). In addition, it is speculated that the expression of NRG1-CTF might be regulated by antipsychotic drugs, as the same effect on NRG1 precursor (Hashimoto et al., 2004; Barakat et al., 2010) (**Figure 2**).

Nrg1/ErbB signaling pathways are important in the regulation of the central nervous system, particularly in regulation of neuronal migration, myelination and glutamatergic networks. For example, during cortical development, neuronal Nrg1 reacts with ErbB4 in glial cells to promote cerebral cortical neurons and cerebellar granule cell migration (Schmid et al., 2003). By blocking ErbB in glial cells, both radial glia formation and neuronal migration, were impaired (Rio et al., 1997). Another important function of Nrg1/ErbB signaling is helping myelin formation. The dysfunctions of myelination have been reported in the PNS of schizophrenic patients (Chavarria-Siles et al., 2016; Stedehouder and Kushner, 2017). The activation of Nrg1/ErbB has involved both formation of myelin and development myelination via axonal signaling in Schwann cells, such as Nrg1-type III which is interacting with ErbB2 and ErbB3 (Boerboom et al., 2017; Miyamoto et al., 2017). In addition, Nrg1 is required for post-injury remyelination in later adulthood (Stassart et al., 2013). Some reported indicated that Nrg1 can regulate Schwann cell development to promote myelination (Michailov et al., 2004; Nave and Salzer, 2006), as well as affect oligodendrocyte proliferation or differentiation (Fernandez et al., 2000; Flores et al., 2000). For example, the development of oligodendrocytes was paused at the pre-oligodendroblast stage in ErbB2-null mice, which indicated that Nrg1/ErbB was an essential integrant in the final step of oligodendrocyte differentiation (Park et al., 2001). Lastly, Nrg1/ErbB signaling can directly affect glutamatergic systems by regulating the expression and function of N-methyl-D-aspartate (NMDA) receptors with

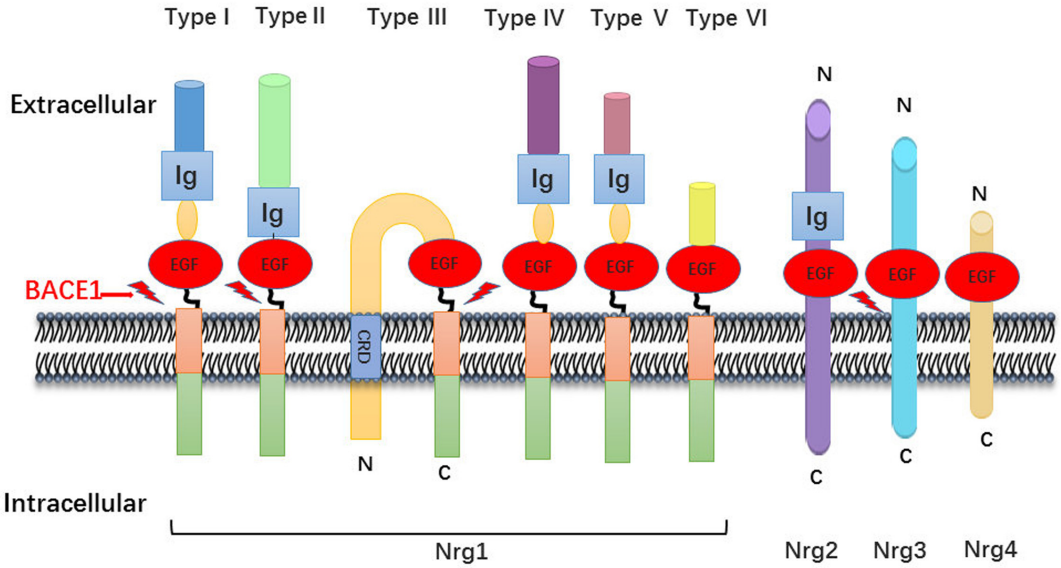

FIGURE 1 | Neuregulin-1 isoforms and membrane location. Nrg1 has six different forms, which distinguish mainly from their N-terminal peptide. All six isoforms own an EGF-like domain and contain the Ig domain excluding type III Nrg1. There is a cysteine rich domain (CRD) embedded in the lipid bilayer in the type III Nrg1, which leaves N-terminal side tethered on the membrane. As described, the other family member Nrg2, Nrg3, Nrg4 has the similar domain anchor once membrane.

brain region specificity. For example, in the prefrontal cortex, Nrg1 may promote NMDA receptor type 1 subunit endocytosis and inhibit NMDA receptor-mediated activity in prefrontal cortical pyramidal neurons (Yarden and Sliwkowski, 2001). Nrg1 type β significantly increased levels of NMDA receptor type 2C subunit in the cerebellum (Harrison and Law, 2006), while also reversing the long-term potentiation in the hippocampal CA1 region through regulation of AMPA endocytosis (Kwon et al., 2005). Importantly, Nrg1 and Nrg1/ErbB signaling regulate several processes of neurodevelopment that play extremely critical roles in schizophrenia neuropathology.

NRG3, a paralog of *NRG1*, was also reported its risk variants associated with clinical symptoms and cognitive function (Kao et al., 2010; Diez et al., 2014). Moreover, genetic modified mice of Nrg3 also exhibit behaviors consistent with psychotic disorders (Hayes et al., 2016). Nrg3 is a critical mediator in the assembly of cortical inhibitory circuits and balance of ex-inhibition, which is hypothesized as pathophysiology schizophrenia (Bartolini et al., 2017). As the closest NRG1 homolog, NRG2 is involved in increasing susceptibility to schizophrenia from human study through interaction with other NRG and ERBB (Benzel et al., 2007). Nrg2 has also been involved in the modulation of schizophrenia-liked behaviors in animal studies (Yan et al., 2017). In addition, *in vitro* studies showed that Nrg2 plays roles in dopamine system regulation, bidirectional mediation of GABAergic synaptogenesis and maturation of glutamatergic synapse in network integration of newborn neurons (Oh et al., 2015; Yan et al., 2017). Comparing other members of NRG family, there are no direct evidence of Nrg4 linked to schizophrenia. The function of Nrg4 has been mainly reported in modulating of energy metabolism and the development of obesity-associated disorders (Wang et al., 2014; Jiang et al., 2016).

Human Studies: BACE1-Dependent NRG1 Cleavage in Schizophrenia

As a member of the neuregulin family, NRG1 is a key molecule involved in normal brain development. Limited human studies on NRG1 in schizophrenia have shown that the structure and biological process of NRG1 is associated with disease susceptibility as well the clinical phenotypes. In this section, we will focus on the function of BACE1-dependent NRG1 cleavage in schizophrenia clinical studies.

NRG1 Proteolysis in Schizophrenia

BACE1 has at least 15 well-known physiological substrates, as numerous novel substrates were identified by means of different screens (Kuhn et al., 2012; Zhou et al., 2012). Activity of BACE1 has cell-, tissue-, and substrate-specificity. Early studies of postmortem schizophrenia brains showed no differences of BACE1 protein levels in Brodmann's area (BA) 6 compared to control subjects (Dean et al., 2008). It is suggested that only measuring BACE1 protein levels in the brain might not be sufficient to show BACE1-specific activity in cleaving NRG1 in schizophrenia. Later, the same research group reported a positive correlation between the levels of BACE1 and full-length NRG1 precursor in the BA6 brain region of healthy control group. This positive relationship between BACE1 and NRG1 was not observed in the schizophrenic group; however, a reduction of the NRG1-CTF was observed in this brain region (Barakat et al., 2010). Using human postmortem brain tissue, an independent research group further found brain region-specific changes of NRG1 cleavage in schizophrenic patients with a great increase in the ratio of NRG1-NTF to full length NRG1 in the BA9 region (Marballi et al., 2012). However, to our knowledge, there

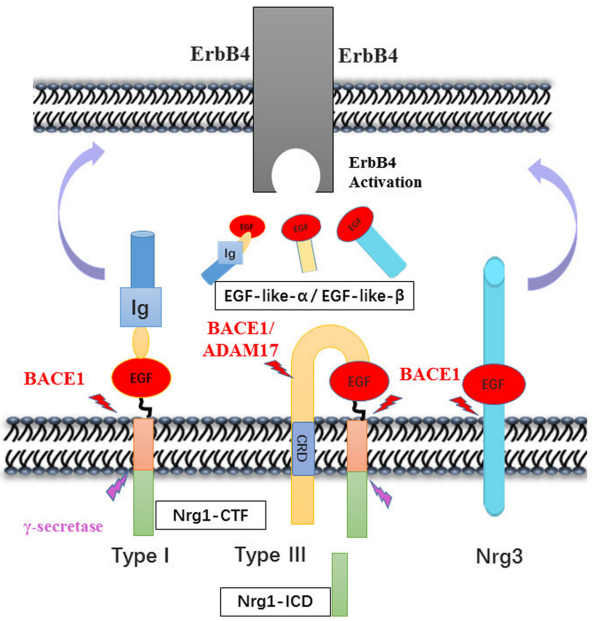

FIGURE 2 | Neuregulins proteolytic cleavage pattern by BACE1. The enzymatic mapping shows that the cleavage of Nrg1 is mediated by BACE1 at between Glu-Phe and Met-Glu. Then, type I Nrg1 releases its N-terminal fragment to the extracellular space, in which the Nrg1-ntf binds to ErbB4 receptor on nearby cells. Whereas type III Nrg1 was cleave by BACE1 remaining tethered on the lipid bilayer via its hydrophobic Cys-rich domain. The second may cut may occur by either BACE1 or ADAM10/17 cleavage and release the EGF-domain that signal through ErbB4 in adjacent glial cells. Nrg3 was also cleaved by BACE1, producing the EGF-domain to activity its downstream pathway. The Nrg1-CTF was cleaved by γ-secretase in the cytosolic space, producing C-terminal peptide Nrg1-ICD that may regulate neuron development.

is no study of BACE1-dependent NRG1 cleavage activity in living patients with schizophrenia.

Genetic Studies of *NRG1* in Schizophrenia

In contrast, the genetic studies of *NRG1* in schizophrenia have been extensively investigated. While most human genetic studies indicated that variants of *NRG1* might increase risk to psychiatric disorders including schizophrenia, there are still reports from various studies with controversial results.

NRG1 was discovered as a prime candidate gene for schizophrenia by Stefansson et al. (2002) who used Systematic linkage disequilibrium (LD) mapping of 8p12–21 in an Icelandic study. Since then, other reports from different countries have been published, such as studies from Japan (Fukui et al., 2006), China (Yang et al., 2003), Scotland (Thomson et al., 2007), India (Kukshal et al., 2013), Italy (Squassina et al., 2010), Denmark (Ingason et al., 2006), Pakistan (Naz et al., 2011), Finland (Turunen et al., 2007), and Sweden (Alaerts et al., 2009). However, different haplotypes of NRG1 were found from various studies. For instance, in the Icelandic population, SNP haplotype in the 5′ region of *NRG1* (HAP_{ICE}: SNP8NRG221533, SNP8NRG241930, SNP8NRG243177) was identified with linkage of schizophrenia risk (Stefansson et al., 2002), while in the Scottish population, a significant association between *NRG1* (HAP_{ICE}) and schizophrenia was detected by PCR (Stefansson et al., 2003). In Japan, researchers failed to replicate the association between *NRG1* and schizophrenia in a large Japanese population, while no association between *NRG1* and schizophrenia was also reported in a large Danish sample (Ingason et al., 2006; Ikeda et al., 2008). In addition, a novel haplotype of the *NRG1* gene was found to confer risk of schizophrenia susceptibility in Chinese Han, but not in the Icelandic/Scottish population (Li et al., 2004). This suggests that stratification and phenotypic heterogeneity may have constrained detection of genetic associations. Other variations or haplotypes located in *NRG1* were also associated with schizophrenia using different SNPs tagging, analysis methods, sample size, and populations. Using association analysis method, one study showed variants in *NRG1* (rs2919381) and *ERBB4* might contribute to susceptibility to schizophrenia in Japanese population (Shiota et al., 2008). Evidence for *NRG3* (rs1937970 and rs677221) as a susceptibility gene for schizophrenia was identified in Chinese Han population (Wang Y.C. et al., 2008). Using LD method, the haplotype 221121 of *NRG1* and its six SNPs were associated to schizophrenia in Indian population (Kukshal et al., 2013). In a study of Northern Swedish Isolated Population, five SNPs located in the second intron of *NRG1* were found with schizophrenia association also by LD method (Alaerts et al., 2009). Variants of NRG1 can be detected genetic association with schizophrenia in different periods and features of patients, which can further confirm these risks to disease.

NRG1 and Schizophrenia Clinical Categories

Schizophrenia symptoms are typically classified under four broad categories: positive symptoms, negative symptoms, disorganization, and cognitive dysfunction (van Os and Kapur, 2009). *NRG1* is considered as a risk gene for schizophrenia, and variants of it are associated with schizophrenia clinical symptoms. Bakker et al. (2004) divided schizophrenic patients into two groups based on their chronic idiopathic negative symptoms as deficit group (negative symptoms) and non-deficit group by the Schedule for the Deficit Syndrome (Carpenter et al., 1988). They found *NRG1* (SNP8NRG221533) was related to the non-deficit schizophrenia subtype only in Caucasian population (Bakker et al., 2004). Later, a study tested three SNPs (SNP8NRG 221132, SNP8NRG241930, and SNP8NRG 243177) in Hungarian population and found only SNP8NRG241930 was related to cognitive and hostility factors by PANSS in non-deficit schizophrenia (Rethelyi et al., 2010). Another case-control study in Caucasian population showed several haplotypic variants of *NRG1* (SNP8NRG221533 SNP8NRG241930 SNP8NRG243177 MS478B14-848 MS420M9-1395) had "protective" effects on age of onset and positive symptoms of schizophrenia (Papiol et al., 2011), which is consistent with the findings in other investigations (Kim et al., 2006; Alaerts et al., 2009). Recently, in a study of Iranian population, Yoosefee et al. (2016) found the G allele of rs2439272 might be significant association with negative symptoms especially in male participants and increased risk of developing schizophrenia.

NRG1 and Neurophysiological Endophenotypes of Schizophrenia

Endophenotypes are thought to be more stable and homogenous than clinical syndromes. Neurophysiological endophenotypes of schizophrenia are characterized by a series of biological and behavioral traits, such as changes in cognitive function, PPI, EMT, ERP and neuroimaging (Braff and Light, 2005). For example, deficits of the inhibition function in patients with schizophrenia were suggested by many studies (Turetsky et al., 2007). The impaired inhibition function can be expressed as changes of PPI, ASEM, SPEM, P50 auditory evoked potential suppression, P300 event-related brain potential, and more. Here, we will discuss the relationship between *NRG1* gene and a few specific endophenotypes of schizophrenia.

NRG1 and PPI

Pre-pulse inhibition is a neurological phenomenon that has been widely used for detecting inhibitory sensory motor gating of the startle reflex, and it is recognized as one of the schizophrenic endophenotypes (Cadenhead et al., 2000; Kumari et al., 2005). There have been several clinical studies that suggest a relationship between *NRG1* gene and PPI. One study demonstrated the lowest level of PPI in Caucasians and African Americans schizophrenia subjects who also carried the homozygous A allele (*NRG1* rs3924999) (Hong et al., 2008). Another study showed that carrying *NRG1* risk genotype variations (SNP8NRG241930, rs6994992, rs2439272 rs10503929 and rs3924999) was related to reduced PPI in healthy subjects (Roussos et al., 2011). These reports suggest that individuals with *NRG1* phenotype might be associated with attenuation of PPI, regardless of if they are healthy populations or patients with schizophrenia. While the underlying mechanisms involving *NRG1* genotype in PPI are unknown, studies implicated that Nrg1 regulates NMDA receptors in specific brain regions that could induce PPI reduction and contribute to schizophrenia-like symptoms (Javitt and Lindsley, 2001; Gu et al., 2005; Hahn et al., 2006). Thus, glutamate signaling may be a potential target for the relationship between *NRG1* and PPI.

NRG1 and ERP

The brain's gating function refers to the capacity to filter out duplicated or redundant stimuli (Freedman et al., 1996). ERP, the measurement of brain response to a specific sensory, cognitive, or motor event, is a schizophrenic endophenotype. Using electroencephalography, several waveforms have been found to be related to ERP, such as N100, P50, and P300 (Hall et al., 2007). While P300 reflects attentive resource allocation to the relevant stimulation, P50 sensory gating reflects the filtering process to irrelevant stimulus in the early stage of brain attentive function (Polich and Kok, 1995; Wan et al., 2008). Studies of patients with schizophrenia demonstrated that NRG1-induced AKT phosphorylation is associated with P50 suppression observed in first-episode patients with schizophrenia. This finding suggests that the PI3K/AKT system may be involved in the impaired sensory gating observed in schizophrenia (Keri et al., 2010). In concert with this finding, a study of acoustic startle response and P50 in patients with schizophrenia showed greater S2 response amplitude and deficit of P50 suppression in patients with schizophrenia than in controls. However, no correlations between PPI and P50 suppression were found in either patients with schizophrenia or control groups (Storozheva et al., 2016), suggesting different mechanisms underlie specific schizophrenia endophenotypes. Regarding investigation of the relationship between *NRG1* gene and ERP in schizophrenia, a study found a significant linkage between SNP8NRG221533 and P300 latency, showing individuals carrying more C alleles had greater P300 latency delay (Bramon et al., 2008). However, there was no significant association between *NRG1* SNPs (SNP8NRG221533, SNP8NRG241930, and SNP8NRG243177) and P50 suppression observed in a large schizophrenia endophenotype study (Shaikh et al., 2011). A recent meta-analytic review concluded that P50 suppression, P300 amplitude, and P300 latency may serve as viable endophenotypes for schizophrenia (Earls et al., 2016). Therefore, whether *NRG1* is related to specific schizophrenic endophenotypes might need further investigations.

NRG1 and eye movement deficits

Eye movement deficits, particularly in SPEM and ASEM, are important endophenotypes in patients with schizophrenia (Meyhofer et al., 2015; Wan et al., 2017). While a number of studies demonstrated 50–80% of patients with schizophrenia have impaired SPEM compared to 8% of healthy individuals (Lencer et al., 2003; Ettinger et al., 2004), few studies have investigated genetic association of eye movement deficits with *NRG1*. A study of *NRG1* genotypes with eye movement deficits in 113 patients with schizophrenia and 106 age-matched healthy controls found no relationship between *NRG1* genotype (SNP8NRG222662, SNP8NRG243177) and ASEM or SPEM task performance (Haraldsson et al., 2010). Consistent with Haraldsson's study, two studies in Korea also found no associations between *NRG1* (rs35753505G, rs4623364G; rs6994992T rs3924999A) and ASEM or SPEM abnormality (Pasaje et al., 2011; Kim et al., 2012). However, the result in healthy subjects showed interaction between *NRG1* and eye movement deficits. One study found SNP8NRG243177 in healthy young males was related to SPEM by using the root-mean-square error method (Smyrnis et al., 2011), while another study showed a significant effect of *NRG1* rs3924999 genotype on ASEM amplitude gain, but not to SPEM or other variables of ASEM, in 114 healthy Caucasian subjects (Schmechtig et al., 2010), suggesting *NRG1* genotypes may affect visuospatial sensorimotor transformations in general and could be a potential mechanism underlying impaired eye movements in patients with schizophrenia.

NRG1 and neuropathology

Some of the major schizophrenia pathological characters are brain atrophy (Harvey et al., 1993; Lim et al., 1996), reduction of whole brain volumes (Gaser et al., 2004), and abnormality in density as well as integrity in diverse brain areas (Burns et al., 2003; Kubicki et al., 2003; Sun et al., 2003; Wang et al., 2004). As NRG1 plays critical roles in myelination, there is an

increase in an attention to the *NRG1* gene variant association with neuropathology in patients with schizophrenia.

A reduction of white matter density and integrity in the ALIC and prefrontal subgyrus in *NRG1* (SNP8NRG243177) carriers was first reported in 2008 (McIntosh et al., 2008), while the SNP8NRG221533 genotype of *NRG1* was reported as affecting medial frontal white matter microstructure (Winterer et al., 2008). Later, studies on SNP8NRG221533 in schizophrenia showed that the *NRG1* variation was related to decreased anterior cingulum fractional anisotropy (Wang et al., 2009), lower volume of internal capsule (Cannon et al., 2012), and reduced volume of left UF (Voineskos et al., 2013). In addition to white matter, studies also explored the effect of *NRG1* variation on gray matter volume. For example, two studies suggested that *NRG1* (rs35753505) was significantly associated with gray matter volume reduction (Knickmeyer et al., 2014; Thirunavukkarasu et al., 2014), while another investigation found a significant association between SNP8NRG222662 (rs4623364) and reduced volume of left superior temporal gyrus cortex (Tosato et al., 2012). However, whether *NRG1* genetic variations directly cause brain structural and functional changes in schizophrenia remains unclear and further studies in schizophrenic patients with neuroimaging in combination with other disease-specific biomarkers would be helpful.

In summary, human studies demonstrated that *NRG1* as a schizophrenia-linked candidate gene plays an important role in the pathological process of schizophrenia through its effect on brain function. Together, findings provide evidence to support an important role of NRG1 in neurodevelopment and susceptibility to schizophrenia (**Table 1**).

Preclinical Research: *Nrg1* and *BACE1* Gene Modified Animal Models

While human studies have demonstrated that NRG1 plays critical roles in schizophrenia, preclinical research using gene knockout or mutant mice have provided some valuable evidence

TABLE 1 | Effect of *NRG1* on schizophrenia in human studies.

	Features	Results	Reference
Protein expression in the brain	N-terminal	The level of NRG1-NTF was increased in BA9 of schizophrenia	Marballi et al., 2012
	C-terminal	The level of NRG1-CTF was decreased in BA6 of schizophrenia	Barakat et al., 2010
	Full length	The level of full-length NRG1 was lower in BA9 of schizophrenia; No changes in BA6 of schizophrenia	Barakat et al., 2010; Marballi et al., 2012
Genetic association	HAP_{ICE}	SNP8NRG221533, SNP8NRG241930, SNP8NRG243177 was reported positive association in Scottish population, negative association in Japanese population, Danish population, Chinese population	Stefansson et al., 2003; Li et al., 2004; Ingason et al., 2006; Ikeda et al., 2008
	Novel haplotypes and SNPs	The haplotype 221121 (rs35753505-rs6994992-rs1354336-rs10093107-rs3924999-rs11780123) in India population; Rs7017348, rs6468061, rs7014221, rs7014410, rs17601950 in northern Swedish Isolated population; Rs2919381 in Japanese population; $HAP_{China\ 1}$, $HAP_{China\ 2}$ and $HAP_{China\ 3}$ in Chinese population	Li et al., 2004; Shiota et al., 2008; Alaerts et al., 2009; Kukshal et al., 2013
Clinical Categories	Non-Deficit	SNP8NRG241930 in Hungary population; SNP8NRG221533 in Caucasian population	Bakker et al., 2004; Rethelyi et al., 2010
	Protective effect	SNP8NRG221533, SNP8NRG241930, SNP8NRG243177, MS478B14-848, MS420M9-1395 in Caucasian population	Papiol et al., 2011
	Negative symptom	Rs2439272 in Iranian population	Yoosefee et al., 2016
Endophenotypes	PPI	SNP8NRG241930, rs6994992, rs2439272 rs10503929 and rs3924999 in Greek healthy males; Rs3924999 in Caucasians and African Americans	Hong et al., 2008; Roussos et al., 2011
	ERP	SNP8NRG221533 in Maudsley Family	Bramon et al., 2008
	EMT	SNP8NRG243177 related to SPEM in healthy young males; Rs3924999 related to ASEM in healthy Caucasian	Schmechtig et al., 2010; Smyrnis et al., 2011
	Neuroimaging	White matter: SNP8NRG243177 related to reduction white matter in ALIC and prefrontal subgyrus; SNP8NRG221533 related to medial frontal white matter microstructure; decreased anterior cingulum fractional anisotropy; lower volume of internal capsule; lower volume of left UF Gray matter: Rs35753505 related to gray matter volume reduction; SNP8NRG222662 related to lower volumes of left superior temporal gyrus cortex	McIntosh et al., 2008; Winterer et al., 2008; Wang et al., 2009; Cannon et al., 2012; Tosato et al., 2012; Voineskos et al., 2013; Knickmeyer et al., 2014; Thirunavukkarasu et al., 2014

BA, Brodmann's Area; HAP, Haplotypes; SNP, Single Nucleotide Polymorphism; PPI, Pre-Pulse Inhibition; ERP, Event-Related Potential; EMT, Eye Movement Test; SPEM, Smooth Pursuit Eye Movements; ASEM, Anti-Saccade Eye Movements; ALIC, Anterior Limb of the Internal Capsule; UF, Uncinate Fasciculus.

TABLE 2 | The effect of *Nrgl* and *Bacel* mutation on schizophrenia-like genotypes in mice.

Genotyping	Feature of mice	Behaviors	Pathology	Reference
TM-Nrg1$^{+/-}$	Transmembrane region deletion in heterozygous mice	Impaired PPI, increased spontaneous activity	Fewer NMD A receptor level ; Disturbance glutamatergic and dopaminergic neurotransmission in different ages	Stefansson et al., 2002; Newell et al., 2013
Ig-Nrg1$^{+/-}$	Mutation in *Nrg1* immunoglobulin-like domain in heterozygous mice	Reduced activity in open field, running wheel and T-maze, decreased latent inhibition with clozapine treatment		Rimer et al., 2005
Overexpressing *Nrg1-type I*	*Nrg1-type I* overexpressed in 11-month-old mice	Impaired spatial working memory	Altered hippocampal oscillatory, lower carbachol-induced epileptiform activity	Deakin et al., 2012
Overexpressing *Nrg1-type III*	*Nrg1-type III* overexpressed in mice	Sensorimotor gating deficits; Altered salient memories	Disrupted from vHPC to nACC circuit projections; Disrupted cortical-amygdala neural circuits	Nason et al., 2011; Jiang et al., 2013
Overexpressing *Nrg1-I* *V*INSE-tTA	Selectively *Nrg1-W* overexpressed in a neuronal specific manner mice	Impaired sensorimotor, discrimination memory and social behaviors	Abnormal synaptic, imbalance ex-inhibitory in PFC	Papaleo et al., 2016
Overexpressing *Nrg1-ntf*β	N-terminal fragment overexpressed in mice	Reduced spontaneous alternations, impaired contextual fear conditioning	Deceased NMDA receptors	Luo et al., 2014
Bacel$^{-/-}$	*Bace1* gene knock out mice	Impaired PPI, working memory and social recognition; Spontaneous hyperactivity	Accumulation of intact Nrgl; Impaired process of myelination; Disturbed NRGl/ErbB4 signaling pathway; Disturbed NRG1/AKT signaling pathway	Hu et al., 2006, 2008; Willem et al., 2006; Savonenko et al., 2008; Seshadri et al., 2010

TM, Transmembrane; Ig, Immunoglobulin; vHPC, Ventral Hippocampus; nACC, Nucleus Accumbens; PFC, Prefrontal Cortex.

TABLE 3 | Effects of antipsychotic drugs on expression of Nrg1 and ErbB4 signaling.

	Subjects	Drugs	Dosage	Treatment duration	Nrg1/ErbB4	Reference
Human studies	PBL cells	Clozapine/ Haloperidol	2 μM/500 nM	3 weeks	Up/No changes	Chana et al., 2009
	Onset patients	Risperidone/ Quetiapine	(533.33±71.45)mg/ day/(544.62±63.85) mg/day	4 weeks	Up/Up	Zhang et al., 2008
Animal studies	Rat	Haloperidol/ Risperidone/ Clozapine	1 mg/kg i.p./1 mg/kg i.p./ 10 mg/kg i.p.	4 weeks	Up/Up/Down	Wang Y.C. et al., 2008
	Monkey	Haloperidol	0.125–0.25 mg/mL/day	8 weeks	No changes	Shibuya et al., 2010
	Mice	Haloperidol	2 mg/kg/day	12 weeks	Down	Hahn et al., 2006
	Rat	Aripiprazole/ Olanzapine/ Haloperidol	UN	12 weeks	Down/Down/Down	Pan et al., 2011

Up, Up Regulation; Down, Down Regulation; PBL, Peripheral Blood Lymphocytes; UN, Unknown.

of association between Bace1 and Bace1-Nrg1 cleavage and schizophrenia by behavioral studies as well as pharmacological investigations.

Mice with Mutated *Nrg1* Develop Schizophrenia-Like Behaviors

During the last decades, several types of Nrg1 transgenic mice have been developed to explore the effect of Nrg1 on behaviors, as well as the underlying mechanisms. One of which is a mouse model of heterozygous transmembrane domain Nrg1 mutant (TM-*Nrg1*$^{+/-}$). The TM-*Nrg1*$^{+/-}$ mice develop dysfunctional NMDA receptors in the forebrain, impaired PPI, and increased spontaneous activity that clozapine treatment was able to reverse (Stefansson et al., 2002). Another feature of TM-*Nrg1*$^{+/-}$ mice was age- and brain region-related alternations of NMDA and D_2 receptor levels which cause selective disturbance of glutamatergic and dopaminergic neurotransmission in the animals (Newell et al., 2013). A mouse

model with a different mutation of *Nrg1*, a heterozygous mutation in Nrg1 immunoglobulin-like domain (Ig-*Nrg1*$^{+/-}$), displayed schizophrenia-like behaviors, particularly suppression of open field, running wheel, and T-maze. The Ig-*Nrg1* $^{+/-}$ mice were more sensitive to clozapine treatment (Rimer et al., 2005). Additionally, animal models of overexpression with different Nrg1 isoforms also developed schizophrenia-liked behaviors. For example, 11-month-old mice with overexpression of *Nrg1-type I* showed impaired hippocampal-dependent spatial working memory and oscillations (Deakin et al., 2012), while *Nrg1-type III*-overexpressed transgenic mice developed sensorimotor gating deficits with changes in the activity of circuit projections from the vHPC to the nACC (Nason et al., 2011). Disrupted cortical-amygdala neural circuits have also been observed in similar transgenic mice, leading to altered processing of salient memories (Jiang et al., 2013). A novel transgenic mouse model of overexpressed *Nrg1-type IV* (Nrg1-IV/NSE-tTA) also exhibited impaired sensorimotor function, discrimination memory, and social behaviors. The Nrg1-IV/NSE-tTA mice also expressed disrupted dendritic development, synaptic pathology, and excitatory-inhibitory imbalance in the prefrontal cortex, which may be mediated by ErbB4 and the downstream signal target, PIK3-p110δ (Papaleo et al., 2016). Interestingly, overexpression of secreted Nrg1 by Bace1 cleavage (Nrg1-ntf$_\beta$) in mice was sufficient to cause schizophrenia-like phenotypes. The abnormal behaviors were Nrg1-ntf$_\beta$-specific since turning off the Nrg1-ntf$_\beta$ expression genetically can reverse the schizophrenia-like behaviors in the mouse model (Luo et al., 2014). Lines of evidence suggested that gain-off function mutations in Nrg1 are also risk factors for schizophrenia. According to these *Nrg1* genetic models, it is possible that dysfunction of NRG1 or NRG1/ErbB4 signaling may affect neural development and synaptic plasticity by disturbance of glutamatergic or GABAergic systems implicated in schizophrenia. We therefore summarized that schizophrenia-like behaviors are related to various *Nrg1* mutations (**Table 2**).

Mutation of Bace1 Mice Show Schizophrenia-Like Behaviors

As a transmembrane protease, BACE1 is important for several disease-related substrates, including beta amyloid peptide production in AD and NRG1 in schizophrenia (Wang et al., 2013). In addition to BACE1 cleavage of a series of types of Nrg, including Nrg1-type I, Nrg1-type III, and Nrg3, BACE1 also cleaves the β2 subunit of voltage-gated sodium channels (Na_v1, β2) (Corbett et al., 2013) that participate in regulation of neuronal development and maintenance of normal brain function. Studies of *Bace1*$^{-/-}$ mice showed reduction of myelination, deficits in cognitive performance, and impaired emotional activity (Harrison et al., 2003; Hu et al., 2006). Moreover, the *Bace1*$^{-/-}$ mice showed seizure-like genotype with increased expression of $Na_v1β2$ in hippocampal areas, which is related to hyperactivity and elevated excitability of hippocampal neurons (Hu et al., 2010). Together, results suggest the possible relationship between BACE1 and dysfunctions of the brain such as schizophrenia, epileptic seizures, and AD.

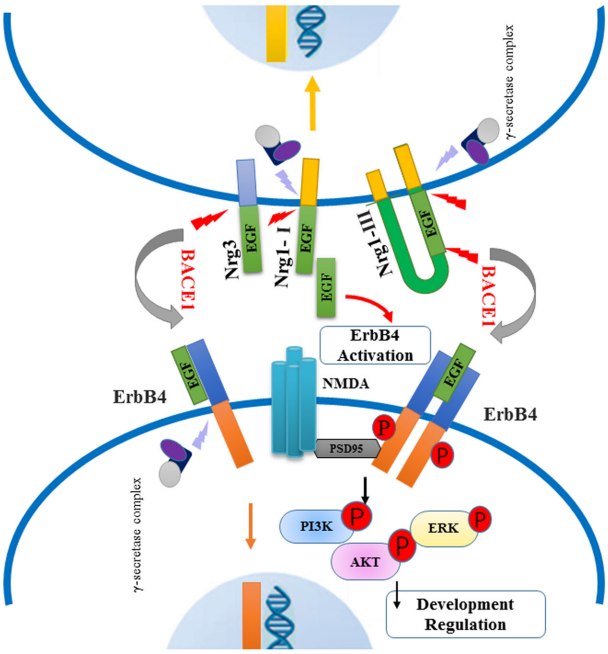

FIGURE 3 | Schematic of BACE1-dependent NRG1/ErbB4 signaling pathway involving in the pathogenies of schizophrenia. Neuregulins (type I, type III Nrg1 and Nrg3) are cleaved by BACE1 and release their EGF-domain into in the extracellular space, through binding the ErbB4 receptors to activate downstream signaling pathway. The NRG1/ErbB4 signaling in neurons can exert an effect on NMDA receptors interacting with PSD-95, which lead to the phosphorylation of PI3K-AKT and ERK molecules. Abnormal NRG1/ErbB4 signaling pathway may contribute to impaired myelination and synaptic function. Meanwhile, intracellular fragment of Nrg1 and ErbB4 are cut off by γ-secretase complex, producing the peptide into the nuclear to regulate neuron development.

Whether there are any specific effects of Bace1 cleavage of Nrg1 on animal behavior is still in question. Several studies of *Bace1* knockout mice have found reduction of Nrg1-type I and type III β1 levels, elevated full length Nrg1, and diminished activation of Akt in the brain (Willem et al., 2006), along with a delayed process of myelination and reduced myelin thickness (Hu et al., 2006, 2008). This suggests that BACE1-dependent cleavage of Nrg1 may regulate myelination and myelin sheath thickness by mediating phosphorylation of Akt. As myelin and oligodendrocyte function could affect neuronal connectivity, the dysfunction of myelination may well be related to the neuropathogenesis of schizophrenia (Nave and Ehrenreich, 2014). Additionally, the *Bace1*$^{-/-}$ mice treated with a glutamatergic psychostimulant showed impaired PPI, working memory, and social recognition, as well as spontaneous hyperactivity as schizophrenia-like behaviors. Decreased spine density in hippocampal pyramidal neurons was also observed in *Bace1*$^{-/-}$ mice via NRG1/ErbB4 signal pathway regulation (Savonenko et al., 2008), suggesting that disturbed NRG1/ErbB4 signaling pathways in the *Bace1*$^{-/-}$ mouse model may contribute to the pathophysiology of schizophrenia. There was a decreased DISC1 expression reported in *Nrg1*$^{-/-}$ knockout

mice, as well as in *Bace1*$^{-/-}$ mice, which might be linked to impaired NRG1/AKT signal pathway (Seshadri et al., 2010). As described above, animal studies suggest that BACE1 might be involved in the pathology of schizophrenia via cleaving substrates to stimulate the downstream signal pathway (**Table 2**).

Nrg1 and Antipsychotic Treatment

The mechanism of antipsychotics is complicated, and includes binding with DA, 5-HT, H1, M1, and α receptors. In addition, some antipsychotics are selective for specific symptoms. For instance, risperidone works better on positive symptoms while others like aripiprazole can improve the severity of negative symptoms (Komossa et al., 2011; Maher and Theodore, 2012). While many studies focus on the effect of antipsychotic treatment on the alteration of *NRG1* gene expression in animal models, there are few human reports in this field due to the ethical issues and method limitation.

A clinical study in Chinese Han patients indicated that exposure to risperidone and quetiapine for 4 weeks could increase the NRG1 expression of peripheral blood lymphocytes of first episode schizophrenia (Zhang et al., 2008). Another study showed that clozapine treatment elevated *NRG1* expression in human fetal brain aggregates, which was not yet observed in a haloperidol-treated group (Chana et al., 2009). These human studies suggest that different antipsychotic treatments may cause differential effects on expression of *NRG1*. Results from animal studies also indicate that the duration of antipsychotics also contributed to various changes of *Nrg1*. The levels of Nrg1 and ErbB4 receptors in rat prefrontal cortex and hippocampus were increased by treatment with haloperidol for 4 weeks (Wang X.D. et al., 2008), while an 8-week haloperidol treatment showed no effect on Nrg1 levels in mice (Shibuya et al., 2010). Furthermore, a 12-week haloperidol treatment experiment reduced the ErbB4 activation (Hahn et al., 2006), as well as expression of Nrg1 and ErbB4, in the brains of mice (Pan et al., 2011). Overall, these studies suggest that not only type of antipsychotics, but also duration of antipsychotic treatment, may be a crucial factor to change *Nrg1* expression, while also considering the brain region-specific effects of antipsychotics (**Table 3**).

BACE1 inhibitor as a therapeutic strategy to improve cognitive in AD has been challenging. Both safety and efficacy are questionable. *In vitro*, inhibition of BACE1 can cause adverse side effects during synaptic developmental stages (Kamikubo et al., 2017). However, there are almost no reports on psychotic symptoms from BACE1 inhibitor clinical trials rather than improved cognitive function in AD patients (Kennedy et al., 2016; Timmers et al., 2017). We speculated that the current available BACE1 inhibitors might be made for targeting on APP which has different cleavage site than other substrates as NRG1. Further investigations on substrate-dependent BACE1 cleavage activity are needed.

In the future, exploring the dynamic changes of BACE1-dependent NRG1 cleavage process in biological samples from schizophrenic patients would be important. It will provide new insights into how BACE1-dependent NRG1 proteolytic processing could contribute to the pathophysiology of schizophrenia, and help to discover the underlying biomarker of schizophrenia, which is essential for early diagnosis of the disorder disease and effective medical treatment.

CONCLUDING REMARKS

Neuregulin, especially Nrg1, plays a major role as the psychological substrate of BACE1. Numerous lines of evidence support the hypothesis that Nrg1 can contribute to the pathophysiology of schizophrenia. Both, human and animal research, suggest that BACE1-dependent Nrg1 cleavage and NRG1/ErbB4 signaling may play specific roles in schizophrenia, as summarized in **Figure 3**. Several BACE1 inhibitors have entered into phase I studies, and at least one of these inhibitors has advanced to phase III human trails. Due to various BACE1 substrates, it is helpful to investigate their role and further illustrate the function of Nrg1 downstream signaling pathways in schizophrenia. It is important for understanding the biological mechanism of BACE1 together with its substrates Nrg1, and further exploring effective and specific inhibitor drugs for schizophrenia, not interfering other biological progress, which could provide possible therapeutic strategies for this psychiatry disorder. In future studies, it will be important to investigate BACE1, Nrg1-related molecular pathways, and neural circuits in endophenotypes resembling features of schizophrenia.

AUTHOR CONTRIBUTIONS

RL: Initiated research topic and discussed literatures and hypothesis within the review topic. Some editing as well. ZZ: Wrote major part of the review. JH: Wrote some part of the review. YS: Edited manuscript.

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Prenatal Immune Challenge in Mice Leads to Partly Sex-Dependent Behavioral, Microglial, and Molecular Abnormalities Associated with Schizophrenia

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Epidemiological studies revealed that environmental factors comprising prenatal infection are strongly linked to risk for later development of neuropsychiatric disorders such as schizophrenia. Considering strong sex differences in schizophrenia and its increased prevalence in males, we designed a methodological approach to investigate possible sex differences in pathophysiological mechanisms. Prenatal immune challenge was modeled by systemic administration of the viral mimic polyinosinic-polycytidylic acid (Poly I:C) to C57BL/6 mice at embryonic day 9.5. The consequences on behavior, gene expression, and microglia—brain immune cells that are critical for normal development—were characterized in male vs. female offspring at adulthood. The cerebral cortex, hippocampus, and cerebellum, regions where structural and functional alterations were mainly described in schizophrenia patients, were selected for cellular and molecular analyses. Confocal and electron microscopy revealed most pronounced differences in microglial distribution, arborization, cellular stress, and synaptic interactions in the hippocampus of male vs. female offspring exposed to Poly I:C. Sex differences in microglia were also measured under both steady-state and Poly I:C conditions. These microglial alterations were accompanied by behavioral impairment, affecting for instance sensorimotor gating, in males. Consistent with these results, increased expression of genes related to inflammation was measured in cerebral cortex and hippocampus of males challenged with Poly I:C. Overall, these findings suggest that schizophrenia's higher incidence in males might be associated, among other mechanisms, with an increased microglial reactivity to prenatal immune challenges, hence determining disease outcomes into adulthood.

Keywords: microglia, schizophrenia, immune challenge, behavior and cognition, gene expression, electron microscopy, morphology and physiology, ultrastructure change

INTRODUCTION

Schizophrenia is a chronic and severe psychiatric disorder that is 1.4 times more frequently diagnosed in males than females, over the course of late adolescence or early adulthood (Picchioni and Murray, 2007). Male patients show an earlier age of onset, decreased social functioning, and worsened negative but reduced depressive symptoms as compared with females (Abel et al., 2010). Sex differences in antipsychotic responses were also identified, with better improvement of negative symptoms measured in men, and of affective symptoms and cognitive functions in women (Abel et al., 2010). However, sex differences in cellular and molecular mechanisms remain largely undetermined.

The disease is related to genetic (Schizophrenia Working Group of the Psychiatric Genomics Consortium, 2014; Siegert et al., 2015; Richards et al., 2016) and environmental factors (Dean and Murray, 2005; Gallagher et al., 2016), and likely triggered through a complex interplay between the two (Davis et al., 2016; Mandelli et al., 2016). Epidemiological studies have implicated prenatal infection and immune genes variations, notably of complement component 4 linked to microglial refinement of neuronal circuits, in the development of schizophrenia (Müller et al., 2015; Estes and McAllister, 2016; Hudson and Miller, 2016; Sekar et al., 2016; Srinivas et al., 2016). Animal models of maternal immune activation (mIA) using the viral mimic Poly I:C, or other immune stimuli, display neurobehavioral impairments affecting motor control, anxiety, sociability, memory, and sensorimotor gating that are reminiscent of schizophrenia symptoms (Jones et al., 2011; Meyer, 2014). Poly I:C is an agonist of Toll-like receptor (TLR)3, a pattern recognition receptor that allows myeloid cells, including microglia in the brain, to detect local changes in homeostasis by recognizing double-stranded viral RNA (Alexopoulou et al., 2001). Important insights into the pathophysiology of schizophrenia were provided by mIA models, involving neuroinflammation, oxidative stress, neuronal dysfunction, neurotransmitter imbalance, and neurogenesis, among others mechanisms (Lanté et al., 2007; Meyer et al., 2008; Bitanihirwe et al., 2010; Mattei et al., 2014; Manitz et al., 2016).

Microglia, immunes cells that are required for brain development, plasticity, and homeostasis (Tian et al., 2017), were also implicated in schizophrenia, based on evidence from both human and animal studies. In human, exacerbated inflammation and microglial reactivity were reported in patients with schizophrenia, or individuals at ultra-high risk of psychosis (Müller et al., 2015; Najjar and Pearlman, 2015; Bloomfield et al., 2016; Laskaris et al., 2016). In a Poly I:C mouse model, microglia were found to show an increased density as well as reduced process arborization (Juckel et al., 2011), while in a Poly I:C rat model, microglia released increased levels of pro-inflammatory IL-1β and TNFα, and displayed reduced phagocytic activity *ex vivo* (Mattei et al., 2014, 2017). These microglial changes and associated impairments of adult hippocampal neurogenesis and sensorimotor gating in rats were rescued by treatment with the tetracycline derivative minocycline (Mattei et al., 2014, 2017). In a two-hit mouse model combining prenatal Poly I:C with peripubertal stress, microglial alterations, and behavioral abnormalities were similarly normalized by pre-treatment with minocycline (Giovanoli et al., 2016a). Clinical studies have further reported a significant decrease of positive and negative symptoms in schizophrenia patients that received minocycline as an add-on treatment to antipsychotics (Miyaoka et al., 2008, 2012; Levkovitz et al., 2010).

Considering the sex differences in microglial density and morphology described during early postnatal development, as well as in maturation, immune reactivity, and physiological functions across postnatal development, adolescence, and adulthood (Schwarz et al., 2012; Bolton et al., 2014, 2017; Hanamsagar et al., 2017), we hypothesized that microglia could be crucial determinants of sex differences in schizophrenia. In the present study, the effects of mIA on microglia (density, distribution, morphology, ultrastructure), behavior, inflammation, and oxidative stress were compared between male and female offspring at adulthood. Poly I:C was injected into pregnant C57BL/6 mice at embryonic day (E)9.5 (Meyer et al., 2008; Hsiao et al., 2012; Khan et al., 2014; Zhu et al., 2014; Giovanoli et al., 2016b). The prefrontal cortex, hippocampus, and cerebellum, regions where structural and functional alterations were mainly described in schizophrenia patients (Harrison, 2004; Salgado-Pineda et al., 2007; Picard et al., 2008), were selected for analysis.

Our results revealed increased microglial clustering, reduced arborization area, increased cellular stress, and interactions with synapses in hippocampus of male offspring exposed to prenatal Poly I:C. These microglial alterations were accompanied by impaired sensorimotor gating and anxiety-like behavior, alongside inflammation in whole cerebral cortex and hippocampus. Female offspring instead displayed increased microglial contacts with myelinated axons upon prenatal Poly I:C. Sex differences in microglial density and cell body circularity were additionally observed in hippocampus under steady-state and Poly I:C conditions, while both sexes showed increased microglial process area, together with exacerbated stereotypic behavior and impaired sociability.

MATERIALS AND METHODS

Animals

All experiments were approved and performed under the guidelines of Université Laval's animal ethics committees and the Canadian Council on Animal Care's. Animals were housed at 22–25°C under a 12-h light–dark cycle with free access to food and water. Experimental animals were generated through mating of C57BL/6 mice from Charles River (St-Constant, QC, Canada). C57BL/6 intruders were also acquired from Charles River (St-Constant, QC, Canada).

Experimental Groups

Viral infection was simulated by injecting Poly I:C potassium salt dissolved in doubled-distilled water (5 mg/kg; Sigma-Aldrich, P9582, St. Louis, MO, USA) intraperitoneally (i.p.) into pregnant mice at E9.5. The pups were weaned at postnatal day (P)21-P22. A vehicle control group was injected with sterile saline.

Two to five animals per sex (combination of saline and Poly I:C challenged) were housed together until the onset of experiments at P60. Sixteen litters were used in total for behavioral testing and post-mortem analyses. No significant difference in weight was observed between saline and Poly I:C challenged animals between P60 and P80. The weight ranges of male and female animals for behavioral experiments were 20–26 and 18–23 g in all groups, respectively. Mice with developmental problems (e.g., eye not opened) were excluded from the studies. The numbers of animals used in each experiment are detailed below.

Behavioral Testing

Tests were performed between 9:00 a.m. and 5:30 p.m. under background noise of ∼50 db and light intensity of ∼50 lux. All behaviors except marble burying, SHIRPA, and prepulse inhibition (PPI) were recorded with the ANY-maze system (version 4.8, Stoelting, Wood Dale, USA). In total, two cohorts of mice were used for two different sets of paradigms. The first cohort sequentially performed marble burying, open field, novel and spatial object recognition, elevated plus maze, and the three-chambered social interaction test from P60 to P80. In total, 16 saline-exposed animals (8 males and 8 females) and 12 Poly I:C-exposed animals (6 males and 6 females) were used in these tests. The second cohort underwent SHIRPA and PPI from P60 to P70. In total, 17 saline-exposed animals (9 males and 8 females) and 14 Poly I:C-exposed animals (7 males and 7 females) were used in SHIRPA and PPI. Detailed testing procedures can be found in the Supporting methods. SHIRPA showed no significant difference in all the parameters measured upon prenatal Poly I:C (Table S1), indicating that the viral mimic does not induce long-term neurological deficits.

Fluorescent Immunohistochemistry and Confocal Microscopy

Forty-eight hours after the social interaction test, at P80-P90, 16 saline-exposed animals (8 males and 8 females) and 12 Poly I:C-exposed animals (6 males and 6 females) from the first cohort were anesthetized with ketamine (80 mg/kg)/xylazine (10 mg/kg) (i.p.) and transcardially perfused with ice-cold phosphate buffered saline (PBS). Left-brain hemispheres were fixed in 4% paraformaldehyde (PFA; EMS, Hatfield, PA, USA) at 4°C overnight and cut with a cryostat at 30 μm longitudinally. Longitudinal sections containing Bregma 0.36 to 1.00, based on the stereotaxic atlas of Paxinos and Franklin (4th edition), were processed for immunofluorescence staining and confocal imaging. Sections were incubated in 0.1 M citrate buffer at 90°C during 8–10 min for antigen retrieval. After the slides had cooled down, they were washed and blocked in 10% donkey serum (with 0.3% Triton X-100 in PBS) for 1 h at room temperature. All primary and secondary antibodies were diluted in the same blocking buffer. Sections were incubated with IBA1 antibody (1:1000, #019-19741, Wako) at 4°C overnight, rinsed in PBS, and then with an Alexa Fluor 568 secondary antibody (A10040, Thermo-scientific, Waltham, MA, USA) for 2 h at room temperature. Sections were washed in PBS, counter-stained with DAPI (1:20000, Thermo-scientific), and mounted with anti-fading media (H-1000, Vector Laboratories, Burlington, Ontario, Canada) under a glass coverslip.

Using a Quorum WaveFX Spinning disc confocal microscope, microglial imaging was performed in ventromedial prefrontal cortex, hippocampal dentate gyrus (DG), and cerebellum of five to six mice per experimental group. Z-stacks were acquired at 20x magnification with an ORCA-R2 camera (Hamamatsu, 1344 × 1024 pixels) in two areas covering the ventromedial prefrontal cortex, two areas covering the hippocampal DG, and three areas of cerebellum (one image for vermis and two images for cortex). Each stack contained ∼30 slices (1 μm each) and focus stacking was performed using Volocity software (Version 5.4, PerkinElmer, Woodbridge, Ontario, Canada).

Analyses of Microglial Density, Spacing, Clustering, and Morphology

Quantitative analysis was conducted to assess the density, spacing, clustering, and morphology of microglia in all the images. The analysis was performed blind to the experimental conditions with ImageJ software (National Institutes of Health) as previously described (Tremblay et al., 2012; Milior et al., 2016). To determine cellular density and spacing, the center of each microglial cell body was marked with a dot using the paintbrush tool. The "analyze particles" function was used to automatically record cell numbers and spatial coordinates, in order to determine the nearest neighbor distance for each cell with the "nearest neighbor distance" plugin. Cellular density was determined by dividing the total number of cells by the total surface area of the acquired pictures measured in mm^2 for each animal. A spacing index was calculated as the square of the average nearest neighbor distance multiplied by microglial density per animal. Clusters comprising two and more microglial cells closer than 12 μm one from another were counted. A morphological index was calculated using the formula: soma area/arborization area. The larger the value, the greater the soma size was in relation to the arbor size. To analyze morphology, a total of 10–15 microglial cells per animal were analyzed. Every IBA1-immunopositive microglia in a particular picture was analyzed before moving on to the next picture as to not introduce selection bias. For each microglia, the soma area was determined by drawing a line around the cell body by using the freehand selection tool. The arborization area was determined with the polygon selection tool to connect the most distal extremities of each process. Soma and arborization areas were calculated in pixels and converted into micrometers. Cell body circularity was determined using the "shape descriptors" measurement tool in Image J and was expressed in arbitrary units. The observer was blinded to the experimental conditions throughout the analysis.

Molecular Analyses Using Quantitative Real-Time PCR

The right hemispheres from five animals per experimental group of the first cohort were dissected into cerebral cortex, hippocampus, and cerebellum regions. Tissue was homogenized in QIAzol lysis reagent (#79306, Qiagen, Hilden, Germany) and total RNA was extracted according to the manufacturer's

protocol. Subsequently, 1 μg of total RNA was reverse transcribed into cDNA using the iScript cDNA synthesis kit (#170-8891, BioRad, Hercules, CA, USA). Real-time PCR was performed with the SsoAdvanced universal SYBR Green supermix kit (BioRad) in a Lightcycler 480II (Roche, Basel, Switzerland). The sets of primers used are listed in Table S2. Relative expression was calculated with the $2^{-\Delta\Delta CT}$ method using *Gapdh* for normalization as previously described (Yuan et al., 2006).

Tissue Preparation and Immunoperoxidase Staining for Electron Microscopy

For electron microscopy, a separate cohort of three males and three females per experimental group (either exposed to saline or Poly I:C at E9.5) was anesthetized with sodium pentobarbital (80 mg/kg, i.p.) and perfused with 3.5% acrolein and 4% PFA (Bisht et al., 2016a) at P80-P90. Fifty-micrometer thick transverse sections from Bregma 2.12–1.64, based on the stereotaxic atlas of Paxinos and Franklin (4th edition), cut with a vibratome, were processed as previously described (Bisht et al., 2016b). Briefly, transverse sections were washed in PBS, quenched, and processed for IBA1 immunostaining. They were blocked and incubated overnight in primary antibody, incubated with goat anti-rabbit secondary antibody conjugated to biotin (#111-065-003, Jackson ImmunoResearch, West Grove, PA, USA) for 1.5 h, and then with ABC Vectastain (1:100, Vector Laboratories, #PK-6100), followed by diaminobenzidine (0.05%) and hydrogen peroxide (0.015%). The sections were post-fixed in 1% osmium tetroxide, dehydrated in ethanol, and embedded with Durcupan resin between ACLAR films (EMS) at 55°C for 72 h. Areas of interest were cut at 65–80 nm using an ultramicrotome (Leica Ultracut UC7). Ultrathin sections were collected on mesh grids and examined at 80 kV with a FEI Tecnai Spirit G2 transmission electron microscope.

Analyses of Microglial Ultrastructural Features

Ultrastructural observations were conducted at the tissue–resin border, where the penetration of antibodies and staining intensity is maximal (Tremblay et al., 2010b). Profiles of neurons, synaptic elements, microglia, astrocytes, oligodendrocytes, and myelinated axons were identified according to established criteria (Peters et al., 1991). Microglia showing well-characterized signs of oxidative stress, including condensed, electron-dense cytoplasm and nucleoplasm, cytoplasmic shrinkage, dilated Golgi apparatus and endoplasmic reticulum, as well as mitochondrial alteration, and very weak immunostaining for IBA1 were referred to as "dark" microglia (Bisht et al., 2016b). To measure the density of dark microglia, the square-mesh grids were sequentially imaged at the lowest magnification (440x) under our microscope to systematically determine the total number of grid squares enclosing neuropil tissue from the DG polymorphic layer. Its total surface area was calculated at high precision by multiplying the number of grid squares containing that layer by the area of a single grid square, as previously explained in details (Bisht et al., 2016b). A schematic representation of all grid squares included in the analysis was drawn for each animal. The grid squares were afterward rigorously screened for the presence of dark microglia as previously described (Bisht et al., 2016b). Dark microglia's density was expressed as numbers per mm^2 of tissue surface. The analysis was performed blind to the experimental conditions. For quantitative analysis of IBA1-stained processes from "typical" microglia, ∼75 profiles per animal were randomly captured at 6,800x using an ORCA-HR digital camera (Hamamatsu; 10 MP). The area, perimeter, and shape descriptors "circularity" and "solidity" were used to assess changes in morphology with ImageJ. Direct contacts with synaptic clefts and myelinated axons were counted for each microglial process profile. Vacuoles associated with autophagy or phagocytosis, and endosomes containing cellular materials in the process of being digested (termed "cellular" inclusions) were also counted on a microglial process profile basis (Tremblay et al., 2010a). The analysis was performed blind to the experimental conditions.

Statistics

Data were analyzed using Prism (GraphPad, Version 5). Two-way ANOVAs with Bonferroni *post-hoc* tests were used to determine interactions between Poly I:C exposure and sex effects in all groups unless otherwise specified. An online Grubbs' test calculator (GraphPad Software, https://www.graphpad.com/quickcalcs/Grubbs1.cfm) was used to determine significant outliers in all experiments and the outliers were removed from the datasets. Sample size (n) refers to animals in all experiments, except for the ultrastructural analyses of IBA1-immunopositive microglial processes where it refers to individual profiles as previously published by our group (Milior et al., 2016). $p < 0.05$ was considered statistically significant. All reported values are mean ± standard error of the mean (S.E.M.).

RESULTS

Poly I:C Alters Microglial Distribution and Morphology, Especially in Male Hippocampus

The combined findings from previous studies demonstrate various changes in microglial density, morphology, phagocytic activity, gene and protein expression within several brain regions of Poly I:C-challenged rodent models (Ribeiro et al., 2013; Mattei et al., 2014, 2017; Van den Eynde et al., 2014; Zhu et al., 2014; Eßlinger et al., 2016; Manitz et al., 2016) and schizophrenia patients (Müller et al., 2015; Najjar and Pearlman, 2015; Bloomfield et al., 2016; Laskaris et al., 2016). Also, prenatal Poly I:C treatment at E9 has been shown to suppress the effects of environmental enrichment on the increase of microglial density in mouse cerebral cortex and hippocampus during adulthood (Buschert et al., 2016). However, unaltered microglial density and morphology were also reported among the DG and hippocampus CA1 of adult mice exposed to a prenatal Poly I:C challenge (Giovanoli et al., 2015, 2016b). To study further microglial alterations and their possible sex-differences induced by prenatal Poly I:C, extensive morphological analyses were performed in male vs. female adult (P80-P90) offspring exposed to Poly I:C at

E9.5. The prefrontal cortex, DG, and cerebellum, associated with structural and functional alterations in schizophrenia patients, were examined.

Microglial density, spacing index, cell body area, and cell body circularity were relatively unchanged across experimental groups, in the three examined regions (**Figure 1** and Figures S1, S2). However, ANOVA showed a significant Poly I:C x sex interaction for spacing index in the DG [$F_{(1, 18)} = 4.45$, $p = 0.0491$] (**Figure 1F**). ANOVA also showed a main effect of Poly I:C on microglial arborization area [$F_{(1, 18)} = 7.10$, $p = 0.0158$], with significant Poly I:C x sex interaction [$F_{(1, 18)} = 5.73$, $p = 0.0278$] in DG, while *post-hoc* analysis revealed a reduced arborization area in Poly I:C-exposed males only ($p < 0.01$, **Figure 1I**). When a morphological index was computed by dividing soma area by arborization area (Tremblay et al., 2012), a ratio which is susceptible to increase in reactive microglia (Streit et al., 1999), a main effect of Poly I:C was identified in the DG [$F_{(1, 18)} = 5.94$, $p = 0.0254$], without Poly I:C x sex interaction. In addition, *post-hoc* analysis revealed that the increase was significant in Poly I:C challenged males only ($p < 0.05$, **Figure 1H**). Sex differences in microglial distribution and morphology were further identified, with a significant effect of sex on microglial

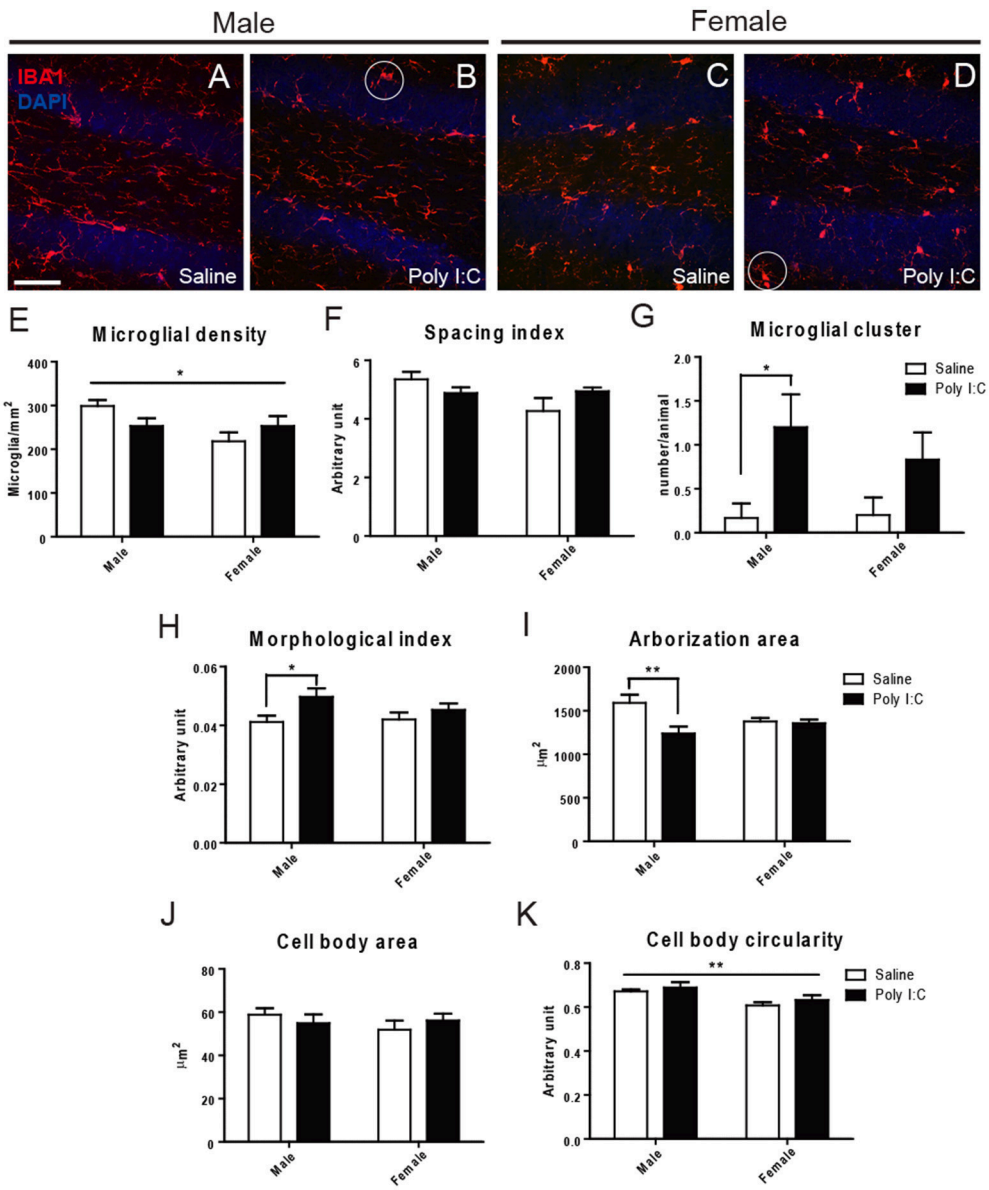

FIGURE 1 | Effects of Poly I:C on microglial distribution and morphology in hippocampal DG. **(A–D)** Low magnification (20x) pictures showing the density of IBA1-stained microglial cells. Examples of clustering are encircled in **(B,D)**. **(E–K)** Microglial density, spacing index, clustering, morphological index, arborization area, cell body area, and cell body circularity are shown. In **(E,K)**, the asterisk refers to comparison between sexes. $n = 5$–6 mice per sex and group. Scale bar: 50 µm. $^{*}p < 0.05$, $^{**}p < 0.01$.

density [$F_{(1, 18)} = 4.54$, $p = 0.0472$] (**Figure 1E**) and cell body circularity [$F_{(1, 18)} = 10.71$, $p = 0.0042$] in the DG (**Figure 1K**). An increased clustering of microglia, in which neighbor cells lose their territorial organization and become close to one another, was also identified in animals exposed to Poly I:C (**Figures 1B,D**). ANOVA showed a main effect of Poly I:C on the number of clusters in the DG [$F_{(1, 18)} = 4.54$, $p = 0.0472$], without Poly I:C x sex interaction, and *post-hoc* analysis indicated that the change occurred in males selectively ($p < 0.05$, **Figure 1G**).

Poly I:C Increases Dark Microglia's Density in Males, While Inducing Sex-Differences in Typical Microglia

In the adult offspring (P80-P90) exposed to prenatal Poly I:C, we subsequently conducted EM analyses to determine the density of "dark" microglia, a newly-defined phenotype that displays several ultrastructural features of cellular stress, weak immunoreactivity for IBA1, extensive contacts with pre- and post-synaptic elements, as well as strong immunoreactivity for CD11b in processes encircling synaptic elements (Bisht et al., 2016b) (**Figures 2A,B**). CD11b is an essential component of complement receptor 3 mediating microglial pruning of axon terminals (Schafer et al., 2012). ANOVA first revealed a main effect of Poly I:C on the density of dark microglia [$F_{(1, 8)} = 8.37$, $p = 0.0201$], without significant Poly I:C x sex interaction, and *post-hoc* analysis identified a selective increase in males ($p < 0.01$, **Figure 2F**).

Typical microglia, which are strongly immunoreactive for IBA1 and focally contact (rather than encircle) synaptic elements, were also analyzed (Tremblay et al., 2010a; Bisht et al., 2016b) (**Figures 2C–H**). ANOVA identified a main effect of Poly I:C on microglial process area [$F_{(1, 977)} = 5.14$, $p = 0.0236$], perimeter [$F_{(1, 977)} = 6.63$, $p = 0.0102$], and contacts with myelinated axons [$F_{(1, 977)} = 5.02$, $p = 0.0253$]. Poly I:C x sex interaction was not significant for microglial process area, perimeter, and contacts with myelinated axons. However, the increase of perimeter and contacts with myelinated axons were found to be significant in Poly I:C-treated female offspring after *post-hoc* analysis (both $p < 0.05$, **Figure 2K** and Figure S3A), without any sex difference for the increase of process area (**Figure 2J**). Interestingly, ANOVA also revealed a significant sex x Poly I:C interaction for the number of cellular inclusions within microglial processes [$F_{(1, 977)} = 5.10$, $p = 0.0242$], with Poly I:C resulting in opposite trends between the sexes (**Figure 2L**). Microglial process circularity, contacts with synaptic clefts, and number of vacuoles were unchanged (Figures S3B–D).

Poly I:C-Induced Microglial Alterations Are Accompanied by Behavioral Deficits in Males

To characterize sex differences in behavioral outcome associated with microglial alterations, the offspring having received Poly I:C challenge at E9.5 was subjected during adulthood (P60-P90) to extensive testing of motor control, anxiety, sociability, memory, and sensorimotor gating.

Marble burying was first performed to distinguish abnormal repetitive behavior (see **Figures 3A–D** for representative images). ANOVA revealed a significant effect of Poly I:C [$F_{(1, 23)} = 25.84$, $p < 0.0001$] and *post-hoc* analysis showed that adult offspring from both sexes display increased repetitive behavior after prenatal Poly I:C exposure ($p < 0.01$, **Figure 3E**).

With the open field (see Figures S4A,B for representative images), no changes in locomotion or exploratory activity were measured in the Poly I:C-exposed offspring at adulthood (Figures S4C–F). However, ANOVA showed main effects of Poly I:C [$F_{(1, 23)} = 12.02$, $p = 0.0027$] and sex [$F_{(1, 23)} = 7.39$, $p = 0.0141$] on anxiety-like behavior, in males only, based on their dropping of significantly more fecal boli (*post-hoc* analysis; $p < 0.05$, Figures S4G–I). To measure anxiety more selectively, the animals were afterwards tested with the elevated plus maze (**Figure 4A**). ANOVA showed a main effect of Poly I:C on the time spent [$F_{(1, 23)} = 10.52$, $p = 0.0036$] and number of entries in the open arms [$F_{(1, 23)} = 7.32$, $p = 0.0126$]. Although Poly I:C x sex interactions were not significant, *post-hoc* analysis revealed that adult male offspring exposed to prenatal Poly I:C spent significantly less time ($p < 0.01$, **Figure 4C**) and entered the open arms less frequently ($p < 0.01$, **Figure 4D**) than male offspring receiving saline, as shown in the representative plots (**Figure 4B**), thus indicating enhanced anxiety-like behavior. No changes in time spent in closed arms, entry into closed arms, total distance traveled, and total immobile time were identified between groups (Figures S5A–D).

Using the three-chamber social interaction test (**Figure 5A**), ANOVA showed a main effect of Poly I:C on sociability [$F_{(1, 23)} = 14.35$, $p = 0.0010$], measured as the propensity to interact with a novel intruder, without Poly I:C x sex interaction. Similar to our findings with marble burying, *post-hoc* analysis revealed that Poly I:C reduces sociability in offspring from both sexes ($p < 0.05$, **Figure 5B**). Also, social novelty (or social memory), defined as the ability to recognize a second intruder, was not affected by prenatal Poly I:C (**Figure 5C**).

To further characterize our mIA model, novel and spatial object recognition memory was next assessed (Figure S6A). However, no deficit in novelty or spatial memory was observed in the adult offspring exposed to prenatal Poly I:C (Figures S6B,C).

Since PPI is considered a translatable model of sensorimotor deficits consistently measured in schizophrenia patients (Swerdlow et al., 2016), we lastly performed acoustic PPI in adult offspring having received Poly I:C according to the protocol described in the Supporting method. ANOVA showed main effects of Poly I:C on PPI at 76 dB [$F_{(1, 27)} = 7.06$, $p = 0.0131$], and of sex at all the prepulses tested 73 dB:[$F_{(1, 27)} = 9.38$, $p = 0.0049$], 76 dB:[$F_{(1, 27)} = 12.54$, $p = 0.0015$], 79 dB: [$F_{(1, 27)} = 6.17$, $p = 0.0195$], 82 dB: [$F_{(1, 27)} = 8.59$, $p = 0.0068$], 85 dB: [$F_{(1, 27)} = 8.59$, $p = 0.0068$], with Poly I:C x sex interaction showing significance only at 76 dB [$F_{(1, 27)} = 7.07$, $p = 0.0130$]. *Post-hoc* analysis additionally revealed that Poly I:C induces deficits at 76 dB ($p < 0.01$), 79 dB ($p < 0.05$), and 85 dB ($p < 0.05$) only in male offspring (**Figure 6A**). After averaging PPI values from all the prepulse-pulse trials (**Figure 6B**), ANOVA further showed main effects of sex [$F_{(1, 27)} = 13.50$, $p = 0.0010$] with significant Poly I:C x sex interaction [$F_{(1, 27)} = 5.26$, $p = 0.0299$].

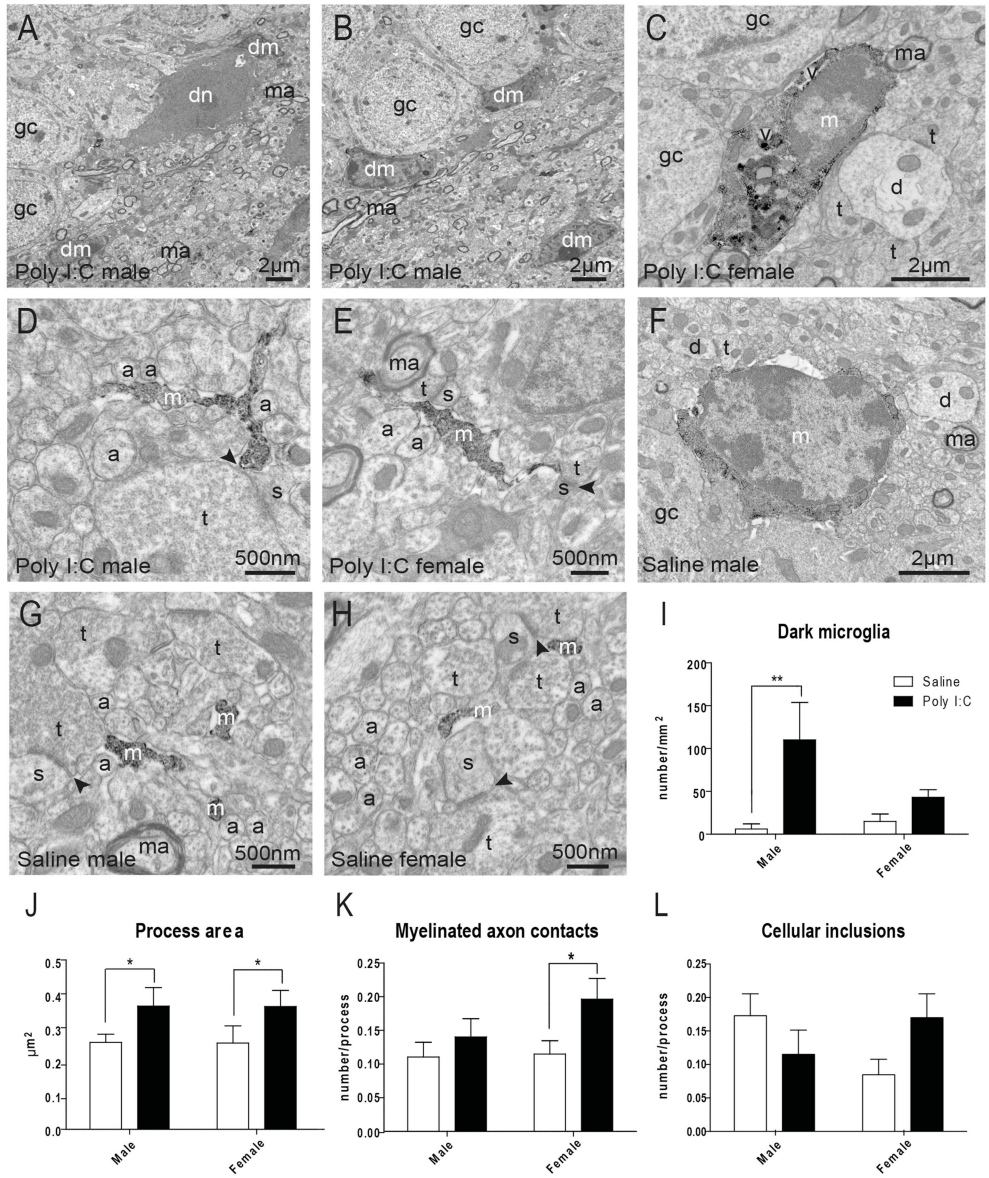

FIGURE 2 | Effects of Poly I:C on microglial ultrastructure in hippocampal DG. **(A–E)** Examples of cell bodies and processes from dark microglia (dm) vs. typical microglia (m) that show strong immunoreactivity for IBA1 and directly contact dark neurons (dn) with features of cellular stress. **(F–H)** Examples of IBA1-stained microglial cell body and processes in saline-exposed offspring of both sexes. **(I)** Quantification of dark microglia's density in the polymorphic layer. **(J–L)** Microglial process area, number of contacts with myelinated axons (ma), and number of cellular inclusions in the polymorphic layer. $n = 3$ animals (dark microglia) or 224–296 profiles (IBA1-stained processes) in three mice per sex and group. Arrowhead, synaptic cleft; a, unmyelinated axon; d, dendritic spine; gc, granule cell; t, axon terminal; v, vacuole. $^{*}p < 0.05$, $^{**}p < 0.01$.

Post-hoc analysis lastly revealed a significant reduction of PPI in male offspring after Poly I:C exposure ($P < 0.05$, **Figure 6B**), indicating that Poly I:C may result in this schizophrenia-like behavior specifically in males.

Poly I:C Also Exacerbates Inflammation in Male Cerebral Cortex and Hippocampus

To provide molecular insights into the sex differences in microglia and behavior, qRT-PCR was conducted in cerebral cortex, hippocampus and cerebellum of male and female adult offspring (P80-P90) quantifying expression levels of genes related to inflammation, oxidative stress, and microglial homeostatic phenotype that is altered in the human disease (Chen et al., 2012; Ferretti et al., 2014; Colonna and Wang, 2016; López González et al., 2016).

Gene expression ratios are provided in **Figure 7** and Tables S3–S5. In the whole cerebral cortex, ANOVA identified main effects of Poly I:C on *Sod1* [$F_{(1, 14)} = 10.28$, $p = 0.0063$],

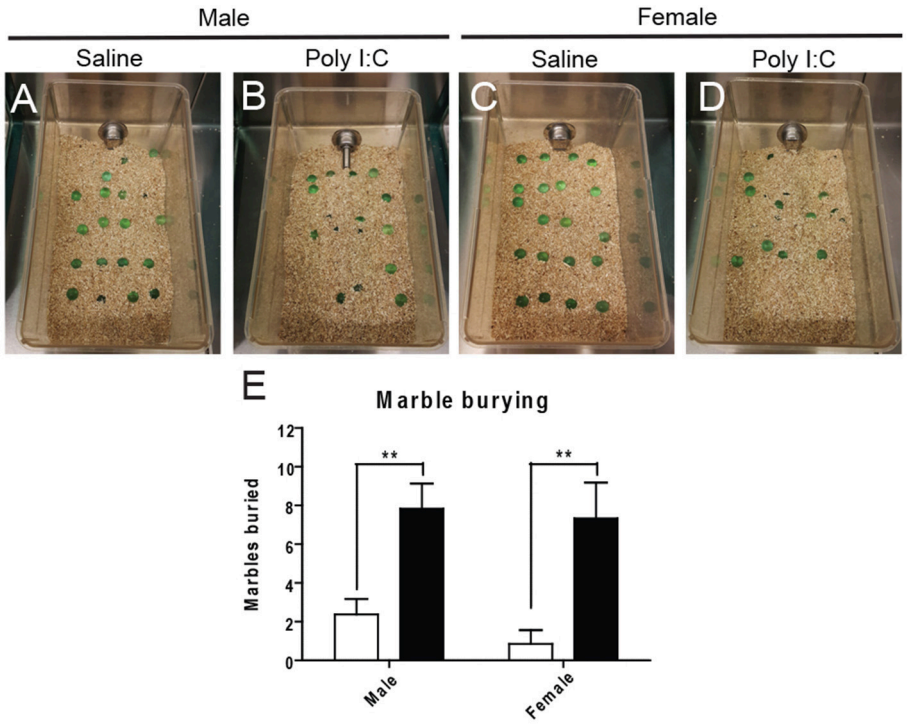

FIGURE 3 | Effects of Poly I:C on repetitive behaviors. Representative pictures of the experimental cages after marble burying are shown in **(A–D)**. Numbers of marbles buried as quantified in **(E)**. $n = 6$–8 mice per sex and group. $^{**}p < 0.01$.

Ptgs2 coding for COX-2 protein [$F_{(1, 16)} = 4.66$, $p = 0.0465$], and *Il1β* [$F_{(1, 14)} = 5.71$, $p = 0.0316$], and of sex on *Il1β* [$F_{(1, 8)} = 6.69$, $p = 0.0323$], and *Nox2* [$F_{(1, 16)} = 4.56$, $p = 0.0485$], without significant Poly I:C x sex interaction identified. In particular, *post-hoc* analysis revealed that Poly I:C significantly increases inflammatory *Ptgs2* ($p < 0.05$) (**Figure 7A**) and *Il1β* ($p < 0.05$) (**Figure 7B**) and reduces anti-oxidant *Sod1* ($p < 0.01$) (**Figure 7C**) in male offspring, while increasing oxidant *Nox2* ($p < 0.05$) (**Figure 7D**) in female offspring. Consistent with these results in cortex, ANOVA identified a main effect of Poly I:C [$F_{(1, 16)} = 4.82$, $p = 0.0433$] on *Ptgs2* in hippocampus, without Poly I:C x sex interaction, and *post-hoc* analysis indicated that males exposed to Poly I:C are particularly vulnerable, showing increased inflammatory *Ptgs2* in hippocampus ($p < 0.05$) (**Figure 7E**). A main effect of sex was also identified for the phagocytosis-related gene *Trem2* [$F_{(1, 16)} = 4.64$, $p = 0.0468$] in hippocampus (**Figure 7F**), which might suggest its contribution to the opposite changes in phagocytosis between sexes (**Figure 2I**). A main effect of Poly I:C was shown for the anti-inflammatory *Ym1* [$F_{(1, 16)} = 11.40$, $p = 0.0038$] and inflammatory *Il1β* [$F_{(1, 15)} = 4.98$, $p = 0.0414$] in cerebellum, without Poly I:C x sex interaction, and *post-hoc* test showed reduced expression of both genes in males only ($p < 0.05$) (**Figures 7G,H**). No changes in the homeostatic genes *Cx3cr1* and *Cx3cl1* were identified in all brain regions investigated. These data suggest that prenatal exposure to Poly I:C exacerbates inflammation and induces oxidative imbalance, mainly to the cerebral cortex and hippocampus of males.

DISCUSSION

The present findings show that Poly I:C-induced prenatal immune challenge at E9.5 triggers behavioral impairments accompanied by exacerbated inflammation, oxidative stress, and microglial alterations, particularly in males. Sex differences in Poly I:C models have remained largely undetermined due to the use of mixed sexes and different timing of exposure or species across studies (Meyer et al., 2008; Mattei et al., 2014; Zhu et al., 2014; Aavani et al., 2015). Our behavioral characterization in the two sexes revealed that male offspring exposed to Poly I:C at E9.5 have additional deficits, compared with females, affecting anxiety and sensorimotor gating, in agreement with the previous human (Aleman et al., 2003; McGrath et al., 2004) and animal studies measuring sensorimotor gating and sociability (Bitanihirwe et al., 2010; Xuan and Hampson, 2014).

In our analyses, pro-inflammatory gene levels were found to increase in the cerebral cortex and hippocampus of male offspring exposed to Poly I:C at E9.5, indicating long-lasting effects of prenatal infection into adulthood. Chronic inflammation has been reported in schizophrenia patients and mIA models (Garay et al., 2013; Khandaker et al., 2013; Mattei et al., 2014, 2017) and was associated with pronounced behavioral impairment (Cunningham et al., 2009; Mattei et al., 2014, 2017; Harrison et al., 2015; Müller et al., 2015). The sex-dependent increase of inflammation that we measured in the hippocampus of Poly I:C-challenged male offspring is consistent with previous rodent studies reporting sex differences in microglial reactivity

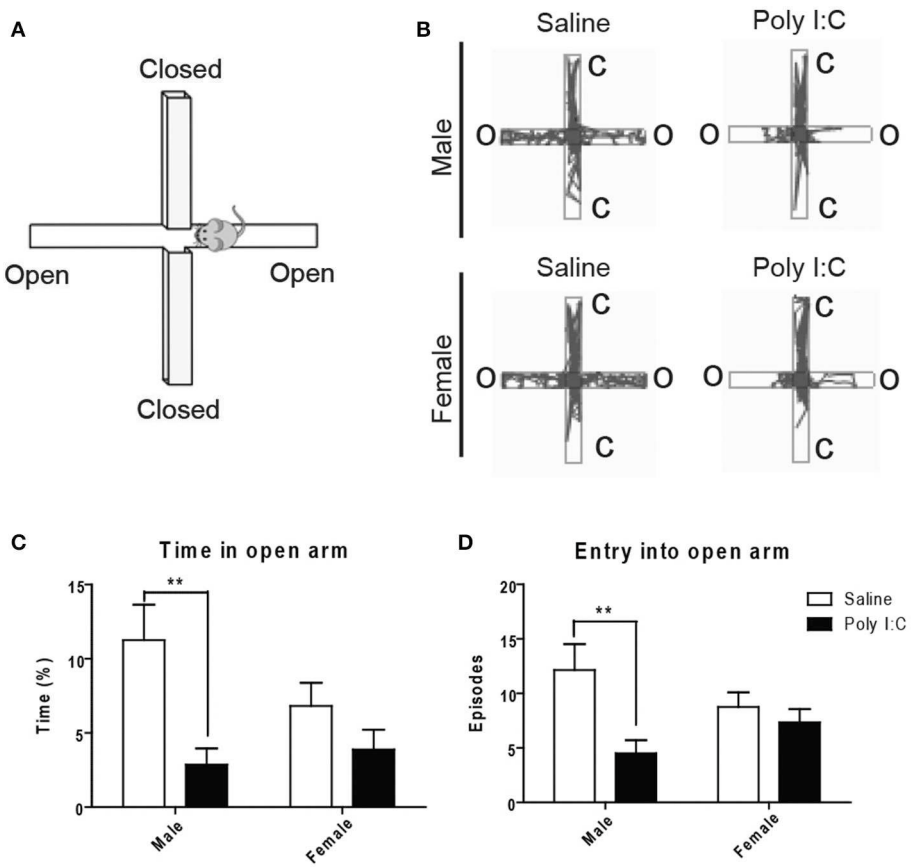

FIGURE 4 | Effects of Poly I:C on anxiety-like behavior measured in the elevated plus maze. **(A)** Orientation of the elevated plus maze platform. **(B)** Representative motion plots in males and females from saline and Poly I:C injection groups. **(C,D)** Anxiety level was determined by measuring the time spent and number of entries in the open arm during testing. $n = 6$–8 mice per sex and group. C, closed arm; O, open arm. $^{**}p < 0.01$.

upon immune challenge (Ho et al., 2015; Hanamsagar et al., 2017) and during aging (Mangold et al., 2017). While oxidative stress was proposed to underlie the social memory and neurocognitive deficits of schizophrenia patients and mIA models (Bošković et al., 2011; Flatow et al., 2013; Emiliani et al., 2014; Gonzalez-Liencres et al., 2014; Hardingham and Do, 2016), our gene expression analysis suggested an increase in the cerebral cortex of males receiving Poly I:C challenge.

In line with the recent findings of sex differences in microglial density, especially of amoeboid cells, reported in DG during early postnatal development (Schwarz et al., 2012; Bolton et al., 2014, 2017; Laskaris et al., 2016; Hanamsagar et al., 2017), in our analyses, reduced arborization area was measured in DG of adult male offspring after E9.5 Poly I:C challenge. Reduced number of microglial processes was previously reported at adulthood in DG of another schizophrenia model, the male Gunn rat (Liaury et al., 2012), suggesting altered surveillance of the brain parenchyma. A recent study by Bolton and colleagues revealed that microglia in DG display enlarged cell bodies with thinner and longer processes in P30 male mice that were prenatally-challenged by air pollution, suggesting that microglia in this hippocampal region are primed after the prenatal immune challenge (Bolton et al., 2017). Consistent with this finding, Bolton and colleagues had previously shown that high fat diet consumption during adulthood increases gene expression levels of the microglia/macrophage markers *Cd11b*, *Cx3cr1,* and *Tlr4* in hippocampus of male offspring prenatally-challenged by air pollution (Bolton et al., 2014). In our study, cell body area remained unchanged across experimental groups, while cell body circularity was decreased in females under both Poly I:C and steady-state conditions. We also found microglial clusters that were more prevalent in the DG of males exposed to Poly I:C at E9.5. Microglial clustering without concomitant increase in density—a feature which however decreased in females under both Poly I:C and steady-state conditions—indicates the occurrence of neuropil areas unsampled by microglia, which could result in impaired response to damage. Similarly, microglial density was previously shown to remain unaltered upon E9 Poly I:C challenge, when measured at adulthood, in the DG of male CD1 mice (Buschert et al., 2016). These microglial clusters also raise the intriguing possibility of self-renewing microglia locally proliferating after Poly I:C stimulation (Bruttger et al., 2015).

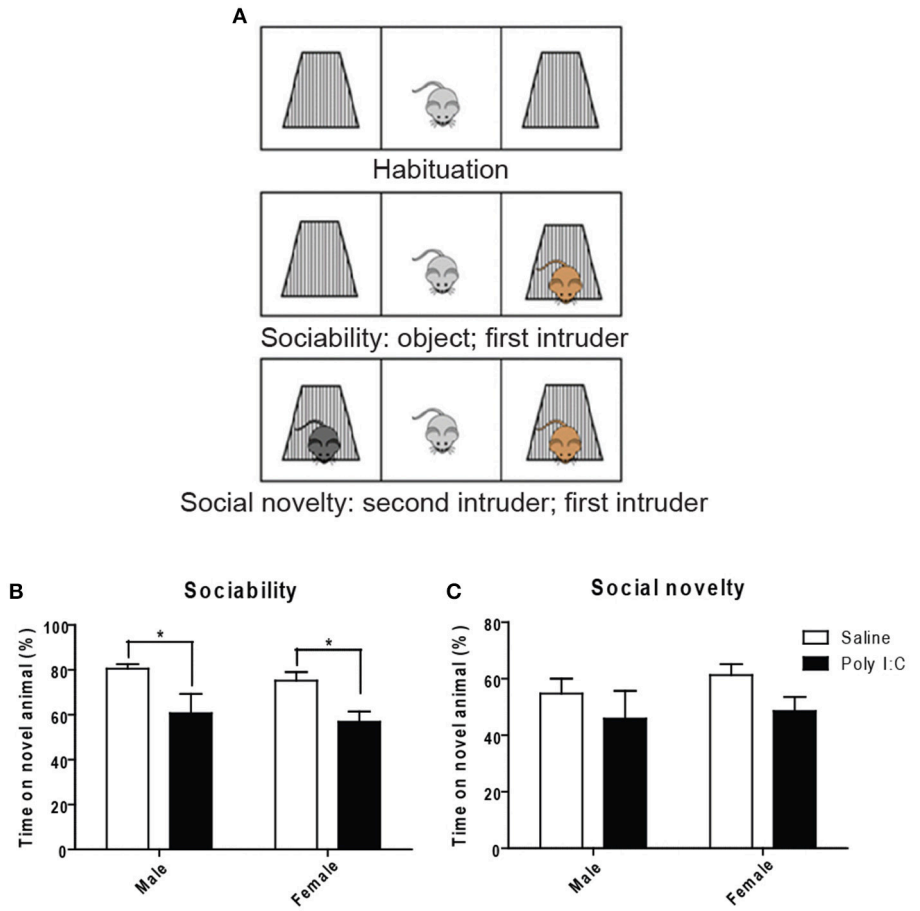

FIGURE 5 | Effects of Poly I:C on sociability and social novelty assessed with the three-chambered social interaction test. **(A)** Schematic representation of the procedure for testing (modified from http://med.stanford.edu). Quantification of sociability is shown in **(B)** and social novelty is shown in **(C)**. $n = 6$–8 mice per sex and group. $^{*}p < 0.05$.

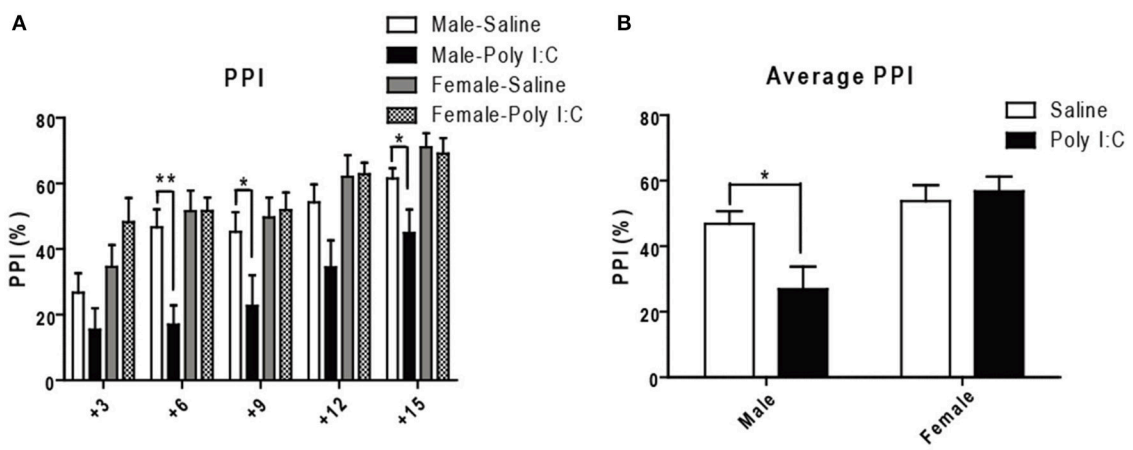

FIGURE 6 | Effects of Poly I:C on sensorimotor gating activity under prepulse inhibition paradigm. **(A)** Sensorimotor gating was determined by calculating percentage reduction of startle amplitude using five prepulses (3–15 dB higher than background noise) administered before the 120 dB startle tone. **(B)** The average reduction of startle amplitude is shown. $n = 7$–9 mice per sex and group. $^{*}p < 0.05$, $^{**}p < 0.01$.

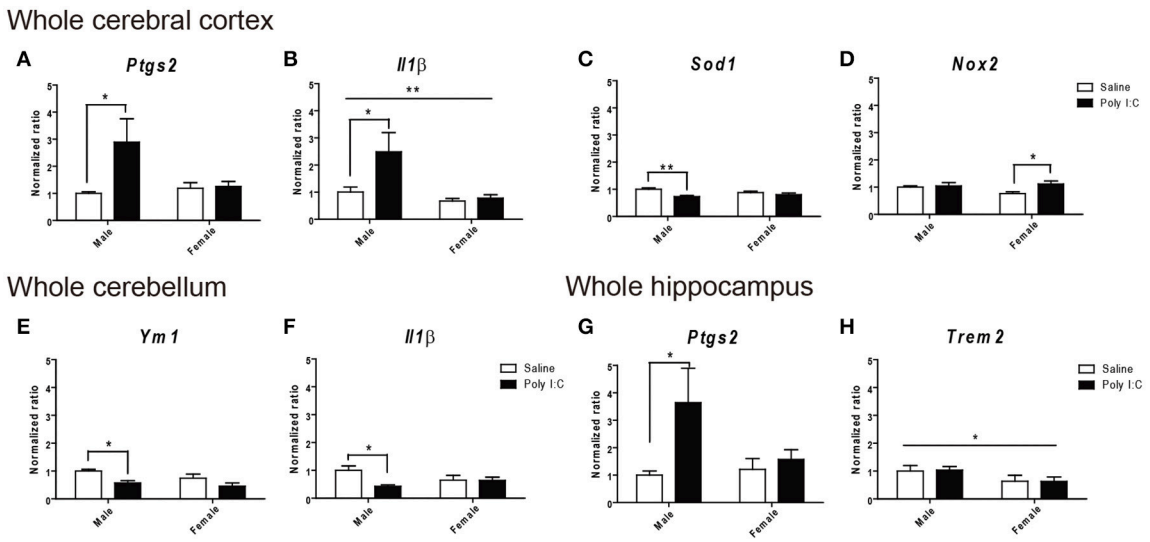

FIGURE 7 | Effects of Poly I:C on gene expression in whole cerebral cortex, cerebellum, and hippocampus. Significant changes in gene expression levels of **(A–D)** *Ptgs2*, *Il1β*, *Sod2,* and *Nox2* were measured in the cerebral cortex, **(E,F)** *Ym1* and *Il1β* in the cerebellum, and **(G–H)** *Ptgs2* and *Trem2* in the hippocampus. $n = 4$–5 mice per sex and group. $^{*}p < 0.05$, $^{**}p < 0.01$.

Ultrastructural analyses further revealed a significant increase in the density of dark microglia extensively interacting with synapses in DG of males receiving Poly I:C stimulus. These cells dowregulating IBA1 were recently found to be associated with pathological states and to display well-characterized signs of cellular stress (Bisht et al., 2016b). This finding suggests that dark microglia could remodel neuronal circuits in a dysfunctional manner in schizophrenia, a hypothesis that warrants further investigation. Processes from typical microglia strongly positive for IBA1 also showed an increased area, in both sexes, supporting the process thickening that was previously reported in Gunn rat (Liaury et al., 2012). In females, unexpectedly, not only were microglial process area and perimeter increased, but also their number of contacts with myelinated axons, which may indicate their involvement with the removal of injured axons (Lafrenaye, 2016). Microglial phagocytic activity was additionally assessed by counting the number of cellular inclusions per microglial processes. In our model, Poly I:C challenged males showed a trend toward reduced phagocytic activity (less cellular inclusions), which is consistent with previous studies (Mattei et al., 2014, 2017). Females displayed an opposite trend (more cellular inclusions) after Poly I:C challenge, supporting the differential microglial responses to prenatal infection between sexes. Whether all of these microglial ultrastructural changes are beneficial or detrimental, and underlying the behavioral alterations, requires further investigation.

These changes were observed in the DG, an hippocampal region that is involved in learning and contributes to spatial and episodic memory by separating activity patterns in the entorhinal cortex, and encoding a large number of inputs (Leutgeb et al., 2007; Bakker et al., 2008). It is also one of the main regions where neurogenesis persists in the adult brain (Sierra et al., 2014). Computational models and human studies identified a pattern separation deficiency in schizophrenia patients suggesting that DG dysfunction (Das et al., 2014; Faghihi and Moustafa, 2015; Martinelli and Shergill, 2015) might underlie their impairment of declarative memory (Das et al., 2014). The proposed mechanisms include a high enrichment of *Tmem108*, a schizophrenia-susceptibility gene, in DG granule cells which results in impaired glutamatergic transmission in mice (Jiao et al., 2017), the reduction of glutamate signaling measured specifically in DG of schizophrenia patients (Stan et al., 2015), and alteration of adult hippocampal neurogenesis that was reported both in animal models and patients (Reif et al., 2006; Walton et al., 2012). These findings suggest that the DG is a region primarily affected in schizophrenia progression and we hypothesize that microglial alterations in the DG may represent one of the factors leading to its dysfunction in schizophrenia patients. Further insights into the pathophysiological mechanisms will be provided by investigating how compromised neuron-microglia interactions in DG result in sex-dependent behavioral deficits.

Overall, our results indicate that schizophrenia's higher incidence in males might be associated, among other mechanisms, with an increased microglial reactivity to prenatal immune challenges. Additional studies are warranted to elucidate, in both sexes, microglial implication with the exacerbated inflammation and oxidative stress, the pathological remodeling of neuronal circuits, and behavioral impairments in schizophrenia and other neuropsychiatric disorders associated with prenatal immune challenges.

AUTHOR CONTRIBUTIONS

CWH and M-ÈT: designed the study; CWH, AS-P, HEH, YR, and M-ÈT: conducted experiments and analyzed data; SSH, GNL, and

LS: provided expertise and resources; CWH, GNL, LS, and M-ÈT wrote the manuscript.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fnmol.2018.00013/full#supplementary-material

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frontiers in Molecular Neuroscience

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Perinatal Asphyxia in Rat Alters Expression of Novel Schizophrenia Risk Genes

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Epidemiological studies suggest that obstetric complications, particularly those related to hypoxia during labor and delivery, are a risk factor for development of schizophrenia. The impact of perinatal asphyxia on postnatal life has been studied in a rodent model of global hypoxia, which is accompanied by cesarean section birth. This asphyxia model shows several behavioral, pharmacological, neurochemical, and neuroanatomical abnormalities in adulthood that have relevance to schizophrenia. Further, it is suggested that schizophrenia has a strong genetic component, and indeed novel candidate genes were recently identified by a genome-wide association study. Here, we examined alteration in the novel schizophrenia risk genes, *CNNM2*, *CSMD1*, and *MMP16* in the brains of rats undergoing cesarean section with or without global hypoxia. The brain regions studied were the prefrontal cortex, striatum, and hippocampus, which are all relevant to schizophrenia. Risk gene expression was measured at three time periods: neonatal, adolescence, and adulthood. We also performed an *in vitro* analysis to determine involvement of these genes in CNS maturation during differentiation of human neuronal and glial cell lines. *Cnnm2* expression was altered in the brains of asphyxia model rats. However, *Csmd1* and *Mmp16* showed altered expression by exposure to cesarean section only. These findings suggest that altered expression of these risk genes via asphyxia and cesarean section may be associated, albeit through distinct pathways, with the pathobiology of schizophrenia.

Keywords: asphyxia, cesarean section, perinatal, schizophrenia, risk genes, animal model

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INTRODUCTION

Schizophrenia is a mental disorder with an often chronic course, presenting with various symptoms including delusions, hallucinations, and impaired cognition. Multiple risk factors incorporating genetic susceptibility are associated with development of schizophrenia, indicating the underlying complexity of this debilitating condition. Epidemiological studies suggest that among many risk factors, obstetric complications, particularly those related to hypoxia during labor and delivery, are factors for increasing risk of schizophrenia (Dalman et al., 1999; Geddes et al., 1999;

Zornberg et al., 2000; Cannon et al., 2008). Moreover, another line of investigation suggests a strong genetic component to schizophrenia. This is exemplified by a recent Genome-Wide Association Study (GWAS) that identified a number of genetic elements that predispose to schizophrenia (Gershon et al., 2011; Schizophrenia Psychiatric Genome-Wide Association Study [GWAS] Consortium, 2011; Aberg et al., 2013; Cross-Disorder Group of the Psychiatric Genomics Consortium, 2013; Ripke et al., 2013). On top of these separate viewpoints (i.e., environmental risk vs. genes), researchers highlight the likelihood of interplay between environmental risk and genes on predisposition to complex diseases such as schizophrenia (Ibi and Gonzalez-Maeso, 2015).

The impact of perinatal asphyxia on postnatal life has been studied using a rodent model of global hypoxia during cesarean section birth, known as the asphyxia model. There are several types of asphyxia models according to the time of asphyxia exposure. One model which employed 15 (±1) min asphyxia in the perinatal period has demonstrated several behavioral, pharmacological, neurochemical, and neuroanatomical abnormalities in adulthood, which are relevant to schizophrenia. For instance, the model has shown increased spontaneous locomotor activity and hypersensitivity to injection of apomorphine, amphetamine, and cocaine (Bjelke et al., 1991; Wakuda et al., 2008; Galeano et al., 2013). The same model has provided some evidence of impaired prepulse inhibition, implicated as a proxy measure for dysfunctional information processing underlying symptoms of schizophrenia (Fendt et al., 2008; Laplante et al., 2012). In addition, other studies that used the model have displayed a significant increase in catechol-*O*-methyltransferase (COMT) mRNA expression (Wakuda et al., 2015), and an increase in dopamine (DA) transporter density (Brake et al., 2000; El-Khodor and Boksa, 2000). In line with these findings, increased number of dopaminergic neurons has also been reported in the asphyxic rat brain (Bjelke et al., 1991). On the other hand, the other model which employed longer asphyxia (19–20 min) in the perinatal period has impairment in the GABAergic system in the striatum (Str) (Capani et al., 2009) and shown abnormal habituation memory in adulthood, which relevant to neurodevelopmental disorders (Saraceno et al., 2016). In this study, we placed importance on dopaminergic abnormalities in the 15 (±1) min asphyxia models. Because the abnormalities were in accordance with well-established findings in schizophrenia patients (Mohr and Ettinger, 2014). Therefore, we used the 15 min asphyxia rat as an animal model of schizophrenia. It has also been reported that cesarean section itself is associated with schizophrenia (Byrne et al., 2000), but so far this finding is inconsistent (Cannon et al., 2002). In rats, cesarean section (hereafter "C-section") birth is sufficient to produce some abnormalities in the dopaminergic system, including increased spontaneous locomotor activity, hypersensitivity to amphetamine injection, and an increase in DA transporter density (Boksa and El-Khodor, 2003). Therefore, in this study, we also considered C-section as a potential exogenous insult.

As outlined above, previous asphyxia models have focused on the dopaminergic system. However, the process of asphyxia may also compromise other neural systems. Accordingly, we have previously found that expression of neuregulin 1 (*NRG1*), a schizophrenia risk gene (DeRosse et al., 2012), is significantly decreased in asphyxia-induced rats (Wakuda et al., 2015). The protein encoded by *NRG1* plays a role in regulation of synaptic plasticity and neurotransmission (Mei and Xiong, 2008). Thus, these studies suggest that a process induced by asphyxia during the perinatal period may involve alterations in not only the dopaminergic but also wider neural regulatory systems, and further, that these alterations may be mediated by genes.

Recently, novel five schizophrenia loci were identified by the Schizophrenia Psychiatric Genome-Wide Association Study [GWAS] Consortium (2011). This study provided high quality genetic data using substantial sample sizes and an optimal experimental design (Schizophrenia Psychiatric Genome-Wide Association Study [GWAS] Consortium, 2011). Therefore, we determined if these novel schizophrenia risk genes show altered expression in asphyxia-induced rats. Among these five novel schizophrenia risk genes, we focused on *Cnnm2*, *Csmd1*, and *Mmp16* in this study. Because, risk variants of *CNNM2*, *CSMD1*, and *MMP16* are suggested to be involved in one of symptoms of schizophrenia, cognitive impairment (Koiliari et al., 2014; Rose et al., 2014; Morton et al., 2017). We measured gene expression at three periods: neonatal (postnatal day 1, P1), adolescence (5-week-old, 5W), and adulthood (12-week-old, 12W). These time frames are generally used for animal models of schizophrenia and correspond to the timing of asphyxia and/or C-section event, onset, and the chronic stage of schizophrenia in humans (Beninger et al., 2002; Baharnoori et al., 2009; Fatemi et al., 2009). We analyzed expression levels of schizophrenia risk genes in rat brain tissue, specifically, the prefrontal cortex (Pfc), Str, and hippocampus (Hip), which are suggested to be critically involved in schizophrenia (Bolkan et al., 2016). Little is known about whether expressions of *Cnnm2*, *Csmd1*, and *Mmp16* are involved in maturation of the central nervous system (CNS). Hence, we also examined expressions of the novel schizophrenia risk genes during the course of differentiation and maturation of neural and glial cell lines to confirm involvement of the genes (Schizophrenia Psychiatric Genome-Wide Association Study [GWAS] Consortium, 2011) in CNS maturation.

MATERIALS AND METHODS

Animals and Induction of Perinatal Asphyxia

All animal experiments were performed in accordance with the Guide for Animal Experimentation at the Hamamatsu University School of Medicine. Intrauterine anoxia was induced in rats delivered by C-section according to a previously described method (Wakuda et al., 2008, 2015). Pregnant female Sprague-Dawley rats (Japan SLC, Hamamatsu, Japan) within the last day of gestation were anesthetised by diethyl ether, and hysterectomised. The uterus, including fetuses, was placed in a water bath at 37°C to induce 15 min of asphyxia, which is associated with 100% survival. After delivery, the umbilical cord was ligated, and the pups left to recover on a heating pad for

at least 40 min. Rats that had delivered normally were used as surrogate mothers. Each surrogate mother received four vaginally delivered pups from another surrogate mother, four C-section-delivered, and four asphyxia-exposed pups. One day after birth (P1), brain tissue was collected from anesthetised male rats. Other male rats were housed three per cage in a temperature- and humidity-controlled colony room, maintained on a 12-h light/dark cycle (07:00 to 19:00 h light on) and with food and water provided *ad libitum*, and brain tissue was collected under anesthesia at 5 and 12 weeks after birth. The animals were divided into three groups based on their delivery: vaginal delivery (V group: $n = 7$), C-section (C group: $n = 6$), or C-section with 15 min of perinatal asphyxia (A group: $n = 8$). The Pfc, Str, and Hip were dissected on ice and used for the quantitative real-time reverse-transcription-polymerase chain reaction (qRT-PCR) analysis. The brain regions were defined according to the atlas of Paxinos and Watson (Wakuda et al., 2015).

Cell Culture and Neuronal and Glial Differentiation

The SK-N-SH cell line was cultured in α-MEM (Nacalai Tesque, Kyoto, Japan) containing 10% (v/v) FBS (Life Technologies, Tokyo, Japan), penicillin (100 U/ml), and streptomycin (100 μg/ml) at 37°C in 5% CO_2. The medium was changed every 2–3 days. The MO3.13 cell line was cultured in DMEM (Sigma–Aldrich) containing 10% (v/v) FBS (Life Technologies), penicillin (100 U/ml), and streptomycin (100 μg/ml) at 37°C in 5% CO_2. Medium was changed every 2–3 days. To induce differentiations, SK-N-SH cells were treated with 10 μM ATRA (Maris and Matthay, 1999; Wainwright et al., 2001) (Sigma–Aldrich) and MO3.13 cells were treated with 100 nM PMA (McLaurin et al., 1995) (Sigma–Aldrich). DMSO (0.1%; Sigma–Aldrich) was used as the negative control for both differentiation inductions.

RNA Isolation and Quantitative Real-Time Reverse-Transcription-Polymerase Chain Reaction (qRT-PCR)

Total RNA was isolated from brain tissue (including Pfc, Str, and Hip) and cell lines using TRIZOL Reagent (Invitrogen, Carlsbad, CA, United States). Total RNA was transcribed to cDNA using the SuperScript III First-Strand Synthesis System (Invitrogen). qRT-PCR was performed using SYBR Green (Qiagen, Hilden, Germany). Relative quantification of the rat genes: ancient conserved domain-containing protein 2 (Cnnm2), CUB and Sushi multiple domains 1 (Csmd1), and matrix metallopeptidase 16 (Mmp16); and the human genes: neuron-specific enolase (NSE), myelin basic protein (MBP), CNNM2, CSMD1, and MMP16 were determined using the delta-delta C_T method (Bookout and Mangelsdorf, 2003). The constitutive gene, Gapdh/GAPDH, was used as the internal control. mRNA levels were expressed as fold change relative to the V group. The primer sequences for qRT-PCR were as follows: Cnnm2 sense, 5′-TTGTCAGCAG GACAGAGGTG-3′; antisense, 5′-GTCGCTCCGACTGAGAG AGT-3′; Csmd1 sense, 5′-ATCATTACCAGGGCACCAG-3′; antisense, 5′-TTTTCATGGCCAGCATAGC-3′; Mmp16 sense, 5′-AGCTTTCGTCCACAAGGAAA-3′; antisense, 5′-CCTTGA GGATGGATCTTGGA-3′; Gapdh sense, 5′-GACATGCCGC CTGGAGAAAC-3′; antisense, 5′-AGCCCAGGATGCCCTTT AGT-3′; NSE sense, 5′-AGGCCAGATCAAGACTGGTG-3′; antisense, 5′-CACAGCACACTGGGATTACG-3′; MBP sense, 5′-ATGGCTAGACGCTGAAAACC-3′; antisense, 5′-AGGGGC AAGTGGGATTAAAG-3′; CNNM2 sense, 5′-GAAGCCATCC TGGACTTCAA-3′; antisense, 5′-CTCCCCTTCAAACACTG GAA-3′; CSMD1 sense, 5′-CTGCCATTCTGGTTCCTTTC-3′; antisense, 5′-CTGTTTTCATGCCCAGCATA-3′; MMP16 sense, 5′-AATCTCCTCAGGGAGCATTTGTA-3′; antisense, 5′-TCC AGGTTCTACCTTGAGTATCTG-3′; GAPDH sense, 5′-ATCA GCAATGCCTCCTGCAC-3′; and antisense, 5′-TGGCATGGA CTGTGGTCATG-3′.

Statistical Analysis

Quantitative gene expression in brain tissue was analyzed using a two-way multivariate analysis of variance (two-way MANOVA) and analysis of variance (ANOVA). In this model, the three separate brain areas (Pfc, Str, and Hip) in each rat were not independent and mutually correlated, and were therefore together treated as dependent variables to allow for applying two-way MANOVA. ANOVA followed by Tukey's *post hoc* test was applied for analyzed gene expressions in individual group (V vs. A, C vs. A and V vs. A). For *in vitro* studies, two-tailed unpaired *t*-tests were used after no violation of the equal variance assumption was confirmed by *F*-test. All statistical analyses were set at a two-tailed α level of 0.05 for significance. The statistical analysis software, SPSS (version 12.0 J; SPSS, Inc., Chicago, IL, United States), was used for analyses.

RESULTS

Gene Expression in Three Brain Regions at Different Time Periods Following Hypoxic Insult at Birth

Expression levels of these genes in Pfc, Str, and Hip in the V group ($n = 7$), C group ($n = 6$), and A group ($n = 8$) at three time points (P1, 5W, and 12W) were measured by qRT-PCR (Supplementary Table S1). First, we determined if gene expression levels were altered in C-section rats compared with the V group. Two-way MANOVA with a grouping factor (V and C groups) and time point (P1, 5W, and 12W) as the fixed factors, and gene expression in three brain regions (Pfc, Str, and Hip) as the dependent variables, was performed separately for each gene. This analysis identified no significant grouping effect ($F_{3,31} = 0.54$, $P = 0.65$) for *Cnnm2* expression, suggesting there is no difference in gene expression between the C and V groups. Thus, these two groups of rats (V and C groups) were amalgamated to yield a single control group in subsequent analyses. However, gene expression in the remaining two genes, *Csmd1* and *Mmp16*, was found to differ according

TABLE 1 | Cnnm2 in three brain regions at different time periods following hypoxic insult at birth.

Gene name	Brain region	VC group			A group		
		P1	5W	12W	P1	5W	12W
Cnnm2	Pfc	0.95 ± 0.06	1.05 ± 0.05	0.92 ± 0.07	0.61 ± 0.06	0.72 ± 0.04	0.86 ± 0.09
	Str	0.93 ± 0.05	1.00 ± 0.05	1.13 ± 0.05	0.54 ± 0.04	0.90 ± 0.06	1.07 ± 0.08
	Hip	0.88 ± 0.05	1.17 ± 0.08	0.92 ± 0.04	0.68 ± 0.05	1.09 ± 0.04	0.97 ± 0.09

Quantitative real-time reverse-transcription-polymerase chain reaction (qRT-PCR) quantification of Cnnm2expression in prefrontal cortex (Pfc), striatum (Str), and hippocampus (Hip) at neonatal (postnatal day 1, P1), adolescence (5-week-old, 5W), and adulthood (12-week-old, 12W). Values are expressed as mean ± SEM.

to the presence or absence of C-section. There was a significant grouping effect ($F_{3,31} = 17.94$, $P < 0.001$ and $F_{3,31} = 10.81$, $P < 0.001$, respectively) for *Csmd1* and *Mmp16*. These results indicate a moderate effect of C-section on expression of these two genes.

Following the MANOVA results comparing gene expression between the V and C groups, we next determined if *Cnnm2* gene expression differed between the A group and a combined control rat group (called the "VC group") (**Table 1**). Two-way MANOVA revealed a significant grouping effect ($F_{3,55} = 8.76$, $P < 0.001$), and an overall difference in *Cnnm2* expression between asphyxia-induced and control rats, with *Cnnm2* expression significantly lower in the A group compared with the VC group. Subsequent univariate analyses found group differences present in Pfc ($P < 0.001$) and Str ($P < 0.001$), but not Hip ($P = 0.18$). In addition, a significant time × group interaction was detected in Str ($P = 0.019$), but not Pfc ($P = 0.100$) by two-way ANOVA. This difference in quantified *Cnnm2* mRNA was apparent immediately after asphyxia (i.e., at P1) in striatal tissue, but became less marked afterward (at 5W and 12W) (**Figure 1B**). In contrast, as indicated by the non-significant time × group interaction in Pfc, decreased gene expression in asphyxia rats was persistent across all three time points, although the difference was minimized at 12W (**Figure 1A**). In supporting of this, when we analyzed *Cnnm2* expression in individual group (V vs. A, C vs. A, and V vs. A) by ANOVA, group differences in Pfc (V vs. A, $P < 0.001$, Tukey's *post hoc* test; C vs. A, $P = 0.008$, Tukey's *post hoc* test) and Str (V vs. A, $P = 0.018$, Tukey's *post hoc* test; C vs. A, $P = 0.004$, Tukey's *post hoc* test) were also found (Supplementary Figures S1A,B).

Initial analyses revealed that *Csmd1* and *Mmp16* gene expression differed between the C and V groups, that is, due to the presence or absence of C-section. This finding of differing gene expression in association with C-section led us to speculate that C-section itself could be considered to be an exogenous insult, and may play a role in altering brain expression of these two genes. Further, we found no additional influence of asphyxia on expression levels of either gene, when the A group (asphyxia + C-section) and C group (C-section only) were compared ($F_{3,34} = 0.49$, $P = 0.69$ and $F_{3,34} = 0.678$, $P = 0.57$; for *Csmd1* and *Mmp16* expression, respectively). Because there was no difference in gene expression between rats that had received a C-section, we combined the two rat groups (A and C groups) to yield one C-section group (CS group). Next, we compared gene expression levels between the V and CS groups (**Table 2**).

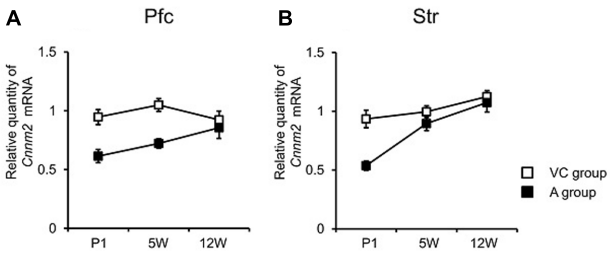

FIGURE 1 | *Cnnm2* expression of different brain regions at different time periods following hypoxic insult at birth. quantitative real-time reverse-transcription-polymerase chain reaction (qRT-PCR) quantification of *Cnnm2* expression in prefrontal cortex (Pfc) **(A)** and striatum (Str) **(B)** at neonatal (postnatal day 1, P1) adolescence (5-week-old, 5W), and adulthood (12-week-old, 12W). Relative *Cnnm2* expression was obtained by normalizing to *Gapdh* from the same cDNA. Results are calculated as a ratio of V group expression. Error bars represent mean ± SEM (VC group, $n = 13$; A group, $n = 8$).

As for *Csmd1*, two-way MANOVA revealed a significant grouping effect ($F_{3,55} = 12.104$, $P < 0.001$). Univariate analyses found group differences in Str ($P < 0.001$) and Hip ($P = 0.011$), but not Pfc ($P = 0.085$). Subsequent ANOVA showed a significant time × group interaction in Hip ($P = 0.007$), but not Str ($P = 0.128$). As implicated by the non-significant time × group interaction, increased *Csmd1* expression in the CS group in Str was relatively constant across all three time points, although the difference was somewhat minimized at 5W (**Figure 2A**). In Hip, elevated *Csmd1* expression was present at P1 (i.e., immediately after C-section), but became less marked afterward (i.e., at 5W and 1W) (**Figure 2B**). We also found group differences of *Csmd1* expression in individual group in Str (V vs. A, $P = 0.002$, Tukey's *post hoc* test; V vs. C, $P = 0.002$, Tukey's *post hoc* test) and Hip (V vs. C, $P = 0.017$, Tukey's *post hoc* test) by ANOVA (Supplementary Figures S2A,B). There is no group difference between V and A in Hip. It may due to low statistical power because of small sample size.

With regard to *Mmp16*, two-way MANOVA revealed a significant grouping effect ($F_{3,55} = 4.60$, $P = 0.006$). Subsequent univariate analyses indicated that the group difference was present in Str only ($P = 0.009$), while two-way ANOVA detected a significant time × group interaction in Str ($P = 0.016$). *Mmp16* expression in Str began at a higher level in the CS group than the V group at P1, but the difference became less marked afterward (**Figure 3**). We also found group difference of *Mmp16* expression

TABLE 2 | Csmd1 and Mmp16 expression in three brain regions at different time periods following C-section at birth.

Gene name	Brain region	V group			CS group		
		P1	5W	12W	P1	5W	12W
Csmd1	Pfc	1.00 ± 0.08	1.00 ± 0.09	1.00 ± 0.15	2.46 ± 0.58	1.07 ± 0.04	0.86 ± 0.09
	Str	1.00 ± 0.03	1.00 ± 0.12	1.00 ± 0.08	1.55 ± 0.11	1.14 ± 0.08	1.34 ± 0.07
	Hip	1.00 ± 0.06	1.00 ± 0.05	1.00 ± 0.05	1.94 ± 0.24	1.19 ± 0.09	0.92 ± 0.08
Mmp16	Pfc	1.00 ± 0.06	1.00 ± 0.08	1.00 ± 0.17	2.70 ± 0.72	1.15 ± 0.11	0.95 ± 0.09
	Str	1.00 ± 0.07	1.00 ± 0.10	1.00 ± 0.04	1.69 ± 0.19	0.97 ± 0.06	1.16 ± 0.06
	Hip	1.00 ± 0.06	1.00 ± 0.05	1.00 ± 0.04	1.55 ± 0.27	1.13 ± 0.08	1.43 ± 0.45

Quantitative real-time reverse-transcription-polymerase chain reaction quantification of Cnnm2expression in prefrontal cortex (Pfc), striatum (Str), and hippocampus (Hip) at neonatal (postnatal day 1, P1), adolescence (5-week-old, 5W), and adulthood (12-week-old, 12W). Values are expressed as mean ± SEM.

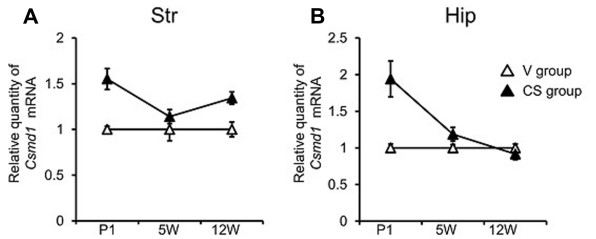

FIGURE 2 | Csmd1 expression of different brain regions at different time periods following C-section at birth. qRT-PCR quantification of Csmd1 expression in Str **(A)** and hippocampus (Hip) **(B)** at neonatal (postnatal day 1, P1) adolescence (5-week-old, 5W), and adulthood (12-week-old, 12W). Relative Csmd1 expression was obtained by normalizing to Gapdh from the same cDNA. Results are expressed as a ratio of V group expression, resulting in a V group ratio of 1. Error bars represent mean ± SEM (V group, $n = 7$; CS group, $n = 14$).

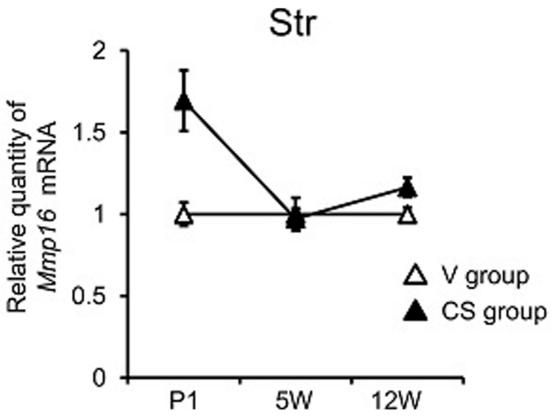

FIGURE 3 | Mmp16 expression of different brain regions at different time periods following C-section at birth. qRT-PCR quantification of Mmp16 expression in Str at neonatal (postnatal day 1, P1) adolescence (5-week-old, 5W), and adulthood (12-week-old, 12W). Relative Mmp16 expression was obtained by normalizing to Gapdh from the same cDNA. Results are expressed as a ratio of V group expression, resulting in a V group ratio of 1. Error bars represent mean ± SEM (V group, $n = 7$; CS group, $n = 14$).

in individual group in Str (V vs. C, $P = 0.012$, Tukey's *post hoc* test) by ANOVA (Supplementary Figure S3). There is no group difference between V and A, may due to low statistical power because of small sample size.

Altered Expression of Novel Schizophrenia Risk Genes during Differentiation of Neuronal and Glial Cell Lines

To investigate involvement of these genes in CNS development, we measured mRNA expression during neuronal differentiation and glial maturation in human cell lines. The human neuroblastoma cell line, SK-N-SH, was treated with ATRA (10 μM) to induce neuronal differentiation. After 72 h treatment, gene expression of the neuronal differentiation maker, NSE, was clearly increased ($P < 0.001$, two-tailed unpaired *t*-test) (Supplementary Figure S4A). Additionally, the human oligodendrocytic cell line, MO3.13, was treated with PMA (100 nM) to induce oligodendrocytic maturation. After 96 h treatment, gene expression of the oligodendrocytic differentiation maker, MBP, was substantively increased ($P < 0.001$, two-tailed unpaired *t*-test) (Supplementary Figure S4B).

We then measured *CNNM2*, *CSMD1*, and *MMP16* expression during the differentiation processes. During neuronal differentiation, *CNNM2* and *MMP16* expression were significantly increased ($P < 0.001$ and $P = 0.031$, respectively) (**Figure 4A**). *CSMD1* was undetectable in both undifferentiated and differentiated SK-N-SH cells. During oligodendrocytic maturation, *CNNM2* and *MMP16* expression were significantly decreased (both $P < 0.001$), while *CSMD1* expression was significantly increased in MO3.13 cells ($P < 0.001$) (**Figure 4B**).

DISCUSSION

Here, we investigated the schizophrenia risk genes, *CNNM2*, *CSMD1*, and *MMP16* (Schizophrenia Psychiatric Genome-Wide Association Study [GWAS] Consortium, 2011), and identified alterations in gene expression in the asphyxia rat model for schizophrenia. In addition, we examined the influence of C-section on gene expression. Interestingly, we found that among the three genes examined, *Cnnm2* was related specifically

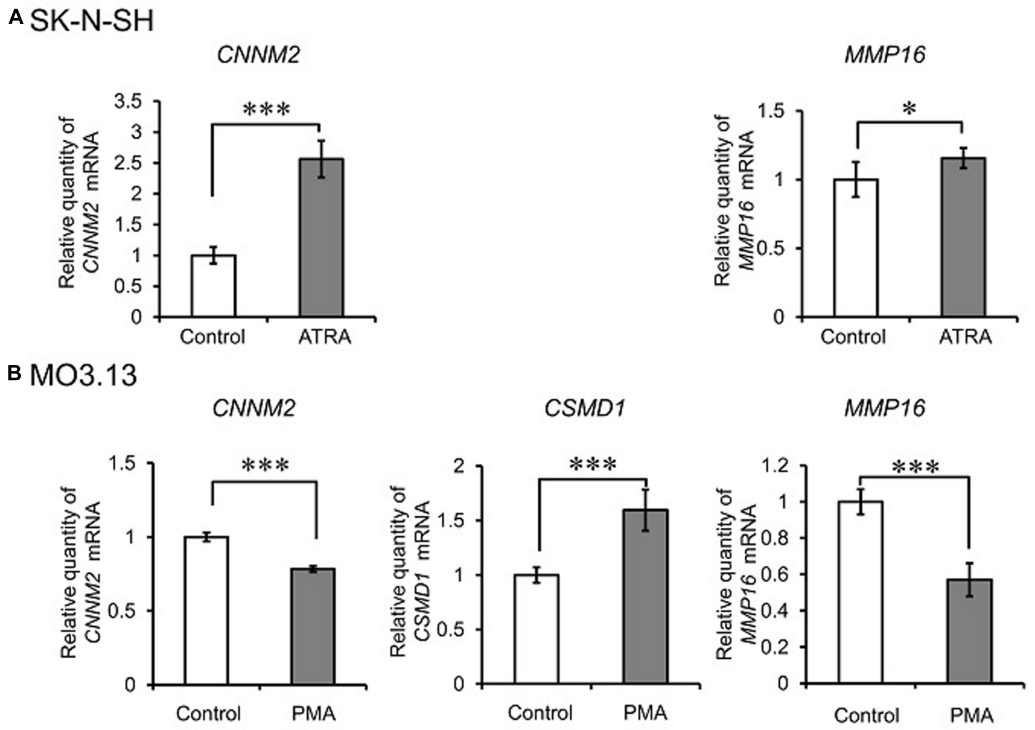

FIGURE 4 | Alteration of expression of novel schizophrenia risk genes during differentiation of neuronal and glial cell lines. qRT-PCR quantification of CNNM2, CSMD1, and MMP16 expression during neuronal differentiation of SK-N-SH cells **(A)** and oligodendrocytic maturation of MO3.13 cells **(B)**. Relative CNNM2, CSMD1, and MMP16 expression were obtained by normalizing to GAPDH from the same cDNA. Results are expressed as a ratio of control (undifferentiated cells) expression, resulting in a control ratio of 1. Error bars represent mean ± SD ($n = 3$–4 per treatment). $^{*}P < 0.05$, $^{***}P < 0.001$ between control cells and cells treated with ATRA (for SK-N-SH cells) or PMA (for MO3.13 cells).

to the asphyxia process, whereas the other two (*Csmd1* and *Mmp16*) were associated with C-section. *Cnnm2* expression was significantly downregulated in Pfc and Str under the influence of asphyxia. While such downregulation was observed only at the neonatal period in Str, it was sustained into adolescence in Pfc. In contrast, exposure to C-section was related to upregulated *Csmd1* and *Mmp16* expression. Moreover, altered *Csmd1* expression levels were conserved until adulthood in Str, but were transient in Hip. Furthermore, *Mmp16* expression was strikingly upregulated, but only immediately after C-section in Str. Using human cell lines, we provided some evidence indicative of potential involvement of *CNNM2, CSMD1*, and *MMP16* in CNS development.

Effect of Asphyxia on *Cnnm2* Expression

CNNM2 is a member of the ancient conserved domain-containing protein family (Wang et al., 2003). Members of this protein family contain a sequence motif that is present in the cyclin box, specifically, a cyclic nucleotide-monophosphate (cNMP)-binding domain (Wang et al., 2003). The *CNNM2* gene has a ubiquitous expression pattern in humans (Wang et al., 2003), and is highly expressed in the brain[1]. However, little is known about expression levels of *CNNM2* in the brain of schizophrenia patients compared with control subjects.

To our knowledge, this is the first study to show that *Cnnm2* expression is downregulated in Pfc and Str in the asphyxia model. Notably, the reduction was maintained until adulthood in Pfc, but was restricted to the perinatal period in Str. It has been reported that schizophrenia patients with obstetric complications show worsened Pfc dysfunction, such as higher intensity of negative symptoms and worse performance on Wisconsin Card Sorting Test (WCST) measures, compared to those without complications (Borkowska and Rybakowski, 2002). This suggests that the event of asphyxia may affect CNS-related gene expression, and altered gene expression continues to play a role in disturbing brain function, resulting in formation of schizophrenia symptoms.

In our human cell line experiments, we found increased *CNNM2* expression during neuronal differentiation. Additionally, because our *in vitro* experiments show that mature neurons express *CNNM2* more than undifferentiated cells, and further, that *CNNM2* expression is reduced in asphyxia-induced rats, it is possible that mature neurons may be decreased, especially in the prefrontal region in the asphyxia model. Intriguingly, in line with our findings, it has been reported that a *CNNM2* variant (Schizophrenia Psychiatric Genome-Wide Association Study [GWAS] Consortium, 2011) is associated with

[1]http://www.ebi.ac.uk/gxa/experiments/E-MTAB-513?geneQuery=ENSG00000148842

gray matter morphological vulnerability of the bilateral inferior frontal gyri (Ohi et al., 2013).

A number of brain imaging studies have demonstrated decreased myelin integrity in schizophrenia (Martins-de-Souza, 2010). However, the maturation status of oligodendrocytes in the brain of schizophrenia patients is unknown. Of note in this context, is a study showing abnormal cell cycle re-entry in postmitotic oligodendrocytes in the brains of schizophrenia patients, with mitotically active oligodendrocytes functionally deficient in schizophrenia (Katsel et al., 2008). Intriguingly, it has been suggested that cell division increases the number of mitotically active oligodendrocytes (Katsel et al., 2008). In addition, mRNA levels of myelin-associated oligodendrocyte basic protein (MOBP), an oligodendrocyte-associated gene, are increased in Pfc white matter in schizophrenia patients (Mitkus et al., 2008). We found that reduced *Cnnm2* expression levels correlated with enhanced oligodendrocytic maturation in the human SK-N-SH cell line. Thus, our finding of reduced *Cnnm2* expression in the asphyxia model suggests that an accelerated maturational process may take place in oligodendrocytes from an early developmental stage, due to asphyxia exposure during the perinatal period, resulting in abnormal regulation of the oligodendrocyte cell cycle and an unusual maturation pattern. This scenario may be relevant to disturbed myelin integrity in schizophrenia.

CNNM2 has been proposed as a Mg^{2+} transporter in Xenopus oocytes (Goytain and Quamme, 2005), whereas human CNNM2 is a principal molecular factor of intracellular Mg^{2+} homeostasis but not a Mg^{2+} transporters *per se* (Sponder et al., 2016). Intriguingly, knockdown of *CNNM2* orthologs in zebrafish resulted in impaired brain development and reduced body Mg content (Arjona et al., 2014). Further study is needed to understand the underlying molecular mechanisms of CNNM2 in the brain development and the pathophysiology of schizophrenia.

Here, we failed to find any change in *Cnnm2* expression in Hip in the asphyxia model, despite numerous studies reporting various functional and structural hippocampal changes in the asphyxia model (Boksa, 2004). There is the possibility that hippocampal abnormalities in relation to asphyxia may be accounted for by genes other than the genes examined in this study.

Effect of C-Section on CSMD1 and MMP16 Expression

Despite evidence showing that undergoing C-section at birth is itself associated with later development of schizophrenia (Byrne et al., 2000), there is inconsistency in the literature (Cannon et al., 2002). C-section delivery is also associated with other types of neurodevelopmental disorders, such as autism spectrum disorders and attention deficit hyperactivity disorder (Curran et al., 2015). We found that C-section was related to increased *Csmd1* and *Mmp16* expression.

CSMD1 encodes the CUB and Sushi multiple domains 1 protein. CSMD1 protein is highly expressed in regions of neuronal differentiation and outgrowth, and remains high in the adult in areas of increased neuronal plasticity, such as the cerebral cortex and Hip (Kraus et al., 2006). It has been suggested that CSMD1 is an important regulator of complement activation and inflammation in the developing CNS, and also that it plays a role in growth cone function (Kraus et al., 2006). Recently, a *CSMD1* variant (Schizophrenia Psychiatric Genome-Wide Association Study [GWAS] Consortium, 2011) was associated with comparatively reduced cortical activation in the middle occipital gyrus and cuneus, a posterior brain region that supports maintenance processes during performance of a spatial working memory task, and affects general cognitive ability and executive function in healthy persons (Koiliari et al., 2014). In addition to this variant (Schizophrenia Psychiatric Genome-Wide Association Study [GWAS] Consortium, 2011), other variants of this gene have been associated with increased risk for schizophrenia (Havik et al., 2011; Sakamoto et al., 2016) and associated with illness severity at end point (Sakamoto et al., 2016).

This study is the first to show increased *Csmd1* expression in Str and Hip in relation to C-section. Of particular interest is the long-lasting change in striatal expression throughout adulthood. This is in accordance with the findings of long-term changes in dopaminergic parameters, such as tyrosine hydroxylase activity and DA transporter density in Str caused by C-section in the rat (El-Khodor and Boksa, 2003; Boksa and Zhang, 2008). Further, it has been reported that genetic variation of *CSMD1* (Schizophrenia Psychiatric Genome-Wide Association Study [GWAS] Consortium, 2011) plays a role in the ratio between DA and serotonin metabolites in CSF (Luykx et al., 2014). These studies suggest that the event of C-section may intervene in gene expression, particularly *CSMD1* expression in Str, and altered gene expression continues to play a role in disturbing function of the monoaminergic systems (in particular the dopaminergic system), resulting in psychotic symptoms. In human SK-N-SH cells, we found that *CSMD1* was involved in oligodendrocytic maturation. Along with this, the fact that *Csmd1* expression is upregulated in the C-section model suggests that C-section may exert a facilitatory effect on oligodendrocyte maturation. Further, a metabolic connection has been demonstrated between myelinating oligodendrocytes and axons (Funfschilling et al., 2012), and metabolite levels in fronto-striatal-thalamic white matter pathways differ in patients with schizophrenia compared with control subjects (Beasley et al., 2009). As mentioned above, it has been suggested that abnormal regulation of the oligodendrocyte cell cycle and an unusual maturation pattern may underlie schizophrenia (Katsel et al., 2008; Mitkus et al., 2008). It is possible that myelin integrity may be disturbed by unusual oligodendrocyte developmental processes in schizophrenia. These processes may be mediated by long-lasting changes in risk gene expression.

MMP16 encodes for a matrix metalloproteinase family protein, which is involved in extracellular matrix breakdown in normal physiological processes such as embryonic development, reproduction, and tissue remodeling, as well as in physical disease processes (Andersson et al., 1996). Mmp16 mRNA is strongly expressed in the rat brain (Shofuda et al., 1997), and equally expressed in gray and white matter, suggesting possible roles for

MMP16 in CNS development and/or function (Yoshiyama et al., 1998).

We found that C-section led to upregulated *Mmmp16* expression in Str, which was observed immediately after C-section. We also found that gene expression increased during neuronal differentiation and decreased during oligodendrocytic maturation in human cell lines. Gray and white matter abnormalities are present in schizophrenia at illness onset (Douaud et al., 2007). Therefore, it is possible that changes in *MMP16* expression early in life may compromise gray and white matter construction by accelerating neural differentiation and affecting oligodendrocyte maturation.

Molecular mechanisms which mediate the influence of CSMD1 and MMP16 on brain development and the pathobiology of schizophrenia remain unresolved. Further research is needed exploring these mechanisms.

In this study, we considered both asphyxia and C-section to equally play a role in predisposition to schizophrenia phenotypes. However, aetiological engagement of C-section might be weaker than asphyxia, because epidemiological studies of the former are inconsistent (Byrne et al., 2000; Cannon et al., 2002). Rat pups born by C-section show normal or slightly reduced oxygen partial pressure levels at birth and signs of mild respiratory distress during the 1st day of life (El-Khodor and Boksa, 1997; Vaillancourt et al., 1999; Berger et al., 2000). Thus, mild hypoxia (but not the level required for asphyxia) may be induced by C-section. Of note, human neonates born by C-section also suffer from increased occurrence of mild respiratory distress during the 1st days of life (Hales et al., 1993; van den Berg et al., 2001). However, since circumstances surrounding C-section in clinical practice may be complex, animal models may not wholly represent all aspects of C-section as occurs in humans. Indeed, Cannon et al. (2002) reported significant differences between schizophrenic and comparison subjects for emergency C-sections but not for elective C-sections.

Our model of asphyxia and C-section rats showed more salient changes in those gene expressions in Str relative to Pfc and Hip. These findings are in line with the view that Str is one of the most sensitive areas to asphyxia (Loidl et al., 1994; Fabian Loidl et al., 1997).

Limitation

In this study, we examined multiple genes in three brain regions at three time points. There is a potential error in interpretation due to the nature of multiple testing. To attenuate this risk, we analyzed intergroup differences in three brain regions, instead of each brain region separately, by applying MANOVA. Based on the results, we proceeded to univariate analyses. Following Rothman's suggestion (Rothman, 1990), we did not thoroughly adjust for multiple comparisons in the analysis, which may have led to eliminating any heuristic indication of results from this study. Nevertheless, there is a potential risk of false-positives, and further study is clearly warranted.

ETHICS STATEMENT

The animal protocol used in this study was approved by an Institutional Animal Care and Use Committee at the Hamamatsu University School of Medicine. All animal experiments were performed in accordance with the Guide for Animal Experimentation at the Hamamatsu University School of Medicine.

AUTHOR CONTRIBUTIONS

AP, KI, RM, and NT designed the study. AP, KI, TW, and CI collected the data. AP, KI, RM, and NT analyzed and interpreted the data. KI and NT prepared the manuscript. All authors read and approved the final manuscript.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fnmol.2017.00341/full#supplementary-material

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frontiers in Molecular Neuroscience

REVIEW
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Molecular Neuropathology of Astrocytes and Oligodendrocytes in Alcohol Use Disorders

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Postmortem studies reveal structural and molecular alterations of astrocytes and oligodendrocytes in both the gray and white matter (GM and WM) of the prefrontal cortex (PFC) in human subjects with chronic alcohol abuse or dependence. These glial cellular changes appear to parallel and may largely explain structural and functional alterations detected using neuroimaging techniques in subjects with alcohol use disorders (AUDs). Moreover, due to the crucial roles of astrocytes and oligodendrocytes in neurotransmission and signal conduction, these cells are very likely major players in the molecular mechanisms underpinning alcoholism-related connectivity disturbances between the PFC and relevant interconnecting brain regions. The glia-mediated etiology of alcohol-related brain damage is likely multifactorial since metabolic, hormonal, hepatic and hemodynamic factors as well as direct actions of ethanol or its metabolites have the potential to disrupt distinct aspects of glial neurobiology. Studies in animal models of alcoholism and postmortem human brains have identified astrocyte markers altered in response to significant exposures to ethanol or during alcohol withdrawal, such as gap-junction proteins, glutamate transporters or enzymes related to glutamate and gamma-aminobutyric acid (GABA) metabolism. Changes in these proteins and their regulatory pathways would not only cause GM neuronal dysfunction, but also disturbances in the ability of WM axons to convey impulses. In addition, alcoholism alters the expression of astrocyte and myelin proteins and of oligodendrocyte transcription factors important for the maintenance and plasticity of myelin sheaths in WM and GM. These changes are concomitant with epigenetic DNA and histone modifications as well as alterations in regulatory microRNAs (miRNAs) that likely cause profound disturbances of gene expression and protein translation. Knowledge is also available about interactions between astrocytes and oligodendrocytes not only at the Nodes of Ranvier (NR), but also in gap junction-based astrocyte-oligodendrocyte contacts and other forms of cell-to-cell communication now understood to be critical for the maintenance and formation of myelin. Close interactions between astrocytes and oligodendrocytes also suggest that therapies for alcoholism based on a specific glial cell type pathology will require a better understanding of molecular interactions between different cell types, as well as considering the possibility of using combined molecular approaches for more effective therapies.

Keywords: glia, alcoholism, postmortem, human, animal models, molecular markers, myelin, epigenetic

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INTRODUCTION

Alcohol use disorder (AUD) is defined in the webpage of the National Institute of Alcohol Abuse and Alcoholism as a chronic relapsing brain disease involving compulsive alcohol use, loss of control over alcohol intake, and a negative emotional state when not using. This disorder results in severe behavioral, neurological and other medical pathologies that eventually depend on disturbances of cellular function and metabolism (Abrahao et al., 2017). In the nervous system, these disturbances cause abnormal exchanges of information between brain centers, including cortical and subcortical regions that control the intake of rewarding substances such as alcohol itself, or regions involved in emotional and cognitive regulation (Moselhy et al., 2001; Schulte et al., 2010). In many AUD patients, alcohol-induced brain alterations are also reflected in structural damage in the gray and white matter (GM and WM; Rosenbloom et al., 2003; Zahr and Pfefferbaum, 2017).

It is well known that a large proportion of alcoholics, despite serious behavioral and emotional pathology, show only minor or inconspicuous neurological deficits of the kind mentioned above, and thus these subjects are dubbed as "uncomplicated" alcoholics. In these subjects, AUDs do not necessarily result in catastrophic or global loss of brain tissue of neuronal or glial cell numbers (Jensen and Pakkenberg, 1993). However, application of conventional MRI imaging techniques has shown that even in these AUD subjects atrophy of the cerebral GM volume at relatively younger ages, while later in life the reduction in volume extends to the WM and cerebral ventricles (Pfefferbaum et al., 1988, 1992). This progressive decline in GM volume was first described as affecting the brain globally, but further neuroimaging investigations have demonstrated that significant age-dependent volume reduction is particularly noticeable in the prefrontal cortex (PFC; Pfefferbaum et al., 1997). Furthermore, while only macroscopic volumetric variations can be safely assessed in brain tissue with conventional MRI, diffusion tensor imaging (DTI) studies, a more recent development of MRI with increased resolution to visualize fiber bundle structure in WM, have shown that WM volume changes parallel structural alterations in specific WM axon bundles connecting PFC to brain circuits involved in reward and emotion regulation (Schulte et al., 2010) or that, even when macrostructural changes are not patent, there could be significant microstructural disturbances of axon bundles (Pfefferbaum and Sullivan, 2002). MRI- and DTI-based detection of volumetric and fiber bundle alterations most likely betray disturbed signal processing in specific brain regions such as the PFC and the hippocampus and anomalous connectivity between those brain regions. In fact, uncomplicated subjects are not entirely free of neuropathological alterations at the cellular level either because they show regionally selective degeneration of pyramidal neurons or their dendrites in some brain regions such as the PFC (Kril et al., 1997).

Role of Glial Cells in AUDs

Since neurons are the main conveyors of information between brain regions, much attention has been placed on the role of neuronal pathology in the various disorders caused by AUDs in human subjects and experimental animals (Abrahao et al., 2017). However, it is well-known that maintenance, survival and normal activity of neurons are fully dependent on the interaction with several types of glial cells (Barres, 2008). These cells assist critically in the support of neurotransmission, propagation of action potentials, survival (of neurons and other glial cells), supply of metabolites, brain injury repair, neuroprotection and synapse formation and removal. In the central nervous system, the main classes of glial cells are astrocytes, oligodendrocytes, NG2 cells and microglia. Each of these cell types identifies mainly with one or two of the support functions mentioned (for instance, astrocytes with neurotransmitter reuptake and metabolic support, oligodendrocytes with myelin formation around axons, microglia with responses to injury and repair), but some functions are performed cooperatively by two or more cell types. Thus, if prolonged alcohol exposure damages glial cells or disrupts their activity, grave disturbances of neuronal function are to be expected. Conversely, due to the existence of neuronal signals that regulate glial physiology (Haydon, 2000; Simons and Trajkovic, 2006), indirect actions of alcohol, mediated by neuronal pathology, are to be expected on the structural and functional integrity of glial cells. Many studies have shown that alcohol exposure *in vivo* and *in vitro* profoundly affects the development, morphology, physiology and gene expression of astrocytes, oligodendrocytes, microglia and NG2 cells. The effects of AUDs on oligodendrocytes were some of the first to receive attention from clinicians and investigators because alcoholism leads to severe neurological and cognitive disorders associated with myelin pathology (Sun et al., 1979; Gallucci et al., 1989; Harper, 2009). Later developments have also shown that the development, physiology, gene expression and morphology of astrocytes are profoundly affected by alcohol abuse (Kennedy and Mukerji, 1986; Renau-Piqueras et al., 1989; Cullen and Halliday, 1994; Franke, 1995).

Many reviews and original research articles have dealt with specific morphology, molecular markers and functions that characterize classes and types of astrocytes (Rajan and McKay, 1998; Laming et al., 2000; Nedergaard et al., 2003; Oberheim et al., 2006; Takano et al., 2006; Zhang and Barres, 2010; Lovatt et al., 2012; Parpura et al., 2012; Kettenmann et al., 2013), oligodendrocytes (Cahoy et al., 2008; Wegner, 2008; Emery and Lu, 2015; Fitzpatrick et al., 2015; Simons and Nave, 2015; Purger et al., 2016; Snaidero and Simons, 2017) and the other glial cell classes in the brain of vertebrates, including the human brain. In this review, I will concentrate on astrocytes and oligodendrocytes in the brain WM and cortical GM mainly because they are the primary glial cell types implicated in the integration (astrocytes) and propagation (oligodendrocytes) of neural signals originating from and arriving in the cortex and the most extensively studied regarding AUDs. I refer to the cited reviews and original articles for more detailed information on normal development, structure, molecular biology and physiology of astrocytes, oligodendrocytes and related cell subtypes. Likewise, the present review is about the glial molecular pathology in AUDs in the context of postmortem and neuroimaging studies, and does not include a detailed discussion of glial pathology

in fetal alcohol spectrum disorders (FASD), although occasional reference to FASD is made to illustrate some general points about the pathology of astrocytes or oligodendrocytes in alcohol abuse disorders.

ASTROCYTES

Diversity of Astrocytes

Since the earliest structural and developmental studies astrocytes and related cells have been classified into several subtypes according to their localization and morphology (Reichenbach and Wolburg, 2012). In fact, astrocytes that reside in specific neural niches tend to substantially differ from those in other niches. For instance, astrocytes in WM are considered to be mostly of the fibrillary type while most astrocytes in GM display a distinctive morphology and are classified as protoplasmic astrocytes (Reichenbach and Wolburg, 2012). In turn, in some WM tracts, such as the optic nerve, astrocytes are further subdivided into type 1 and type 2 astrocytes, while GM astrocytes in contact with the meningeal pia mater or those adjacent to the ventricular surfaces are also morphologically distinct from the protoplasmic astrocytes (Reichenbach and Wolburg, 2012). In the cerebellum, astrocytes take on a distinctive morphology that parallels the structure of Purkinje cell's dendrites and are identified as Bergman glia, while retinal astrocytes radially span across the retinal layers and are called Mueller cells (Kettenmann et al., 2013).

In recent years, it has become evident that the morphological variety is matched by an even richer molecular differentiation of astrocytes (Verkhratsky and Nedergaard, 2018), and that even astrocytes considered as a morphologically homogenous type (Cui et al., 2001; Oberheim et al., 2006; Khakh and Sofroniew, 2015; Hu et al., 2016), for example, cerebral cortex and WM astrocytes, can be further subdivided according to their molecular markers or their ability to divide (Zhang and Barres, 2010). These subtype-specific molecular markers ultimately betray a significant degree of physiological differentiation among astrocytes as they adapt to specific neural niches and functions.

Roles of Astrocytes in Gray and White Matter

The differential features of astrocytes in the cortical GM reflect a multitude of regulatory roles in support of cortical neuronal function (Verkhratsky and Nedergaard, 2018). These roles can be significantly disturbed either by direct actions of ethanol on receptors, transporters or metabolic enzymes of astrocytes, or through indirect actions mediated by the effects of ethanol on neurotransmission and neuronal metabolism (Verkhratsky and Parpura, 2010; Adermark and Bowers, 2016). Prominent among the critical roles of astrocytes are the exchange with blood vessels of energy-rich metabolites to support neuronal metabolism, the buffering of extracellular ions exchanged during synaptic neurotransmission/propagation of action potentials, the reuptake of synaptically-released glutamate and gamma-aminobutyric acid (GABA), the recycling of these and other transmitters for reutilization or metabolism, the release of small molecular cofactors, such as serine or glycine required for activation of the N-methyl-D-aspartate-type (NMDA-type) glutamate receptors, the release of gliotransmitters or cytokine-like molecules to communicate with other neural cells, and the expression of immune-like activities within the nervous system (Cali et al., 2008). This variety of neurophysiological roles is made possible by abundant expression of specific proteins and molecules and their associated intracellular pathways, which are in many cases exclusive to or predominantly expressed in astrocytes (Verkhratsky and Nedergaard, 2018).

Many of their characteristic intracellular pathways are found in all astrocyte subtypes. However, a division of labor also exists among astrocytes such that, for instance, some astrocytes express high levels of excitatory amino acid transporter 1 (EAAT1), other astrocytes are richer in EAAT2, or others, such as Bergmann glia express EAAT5. Most WM astrocytes and those adjacent to the pia mater express high amounts of the cytoskeletal protein glial fibrillary acidic protein (GFAP). In contrast, astrocytes in the middle layers of the cortex, while rich in glutamate transporters have very low levels of GFAP, even if dramatic increases of GFAP can occur in astrocytes after brain injury, toxicity or ischemia.

In addition to their distinctive morphology, WM astrocytes constitutionally express high levels of GFAP (levels that are significantly lower in many GM astrocytes). Even if neuron to neuron synapses are very low in WM, WM astrocytes express glutamate and glutamine transporters (Banner et al., 2002; Miguel-Hidalgo et al., 2010; Roberts et al., 2014). Unlike most GM astrocytes, WM astrocytes and astrocytes in myelinated parts of the GM, are in close contact with oligodendrocyte processes and the outermost layer of myelin, and often form gap junctions with oligodendrocyte cell membranes to allow for direct communication between the astrocyte and oligodendrocyte cytoplasms (Nualart-Marti et al., 2013). The molecular composition of these junctions is connexin subtype-specific, because connexin 43 (Cx43) and Cx30, which are contained on the astrocyte side of the gap junction, are respectively matched with Cx47 and Cx32 contained in the oligodendrocyte cell membrane to form Cx43–Cx47 and Cx30–Cx32 gap junctions (Nagy and Rash, 2000; Orthmann-Murphy et al., 2008). The importance of these astrocyte-oligodendrocyte gap junctions to establishment of myelin has been confirmed in recent studies showing that absence or downregulation of astrocytic Cx43 and Cx30 (or oligodendrocyte Cx47 and Cx32) in transgenic mice models results in major disruption of myelin formation and maintenance, as well as behavioral deficits (Lutz et al., 2009; Magnotti et al., 2011; Wasseff and Scherer, 2011). In addition, the end-feet of some processes of astrocytes reach to most of the nodes of Ranvier (NR) in the CNS. Despite the close and frequent association of these processes with NR, their function is still unknown, although some researchers have proposed that astrocytes and their perinodal processes play an important role in potassium buffering or in the stabilization and organization of myelin formation at the nodes (Black and Waxman, 1988; Kalsi et al., 2004; Serwanski et al., 2017).

End-feet of astrocytic processes also closely abut the basal lamina surrounding endothelial cells of small blood vessels.

These contacts around blood vessels are important in the regulation of water movements from the blood circulation and the maintenance and function of the brain blood barrier (Paemeleire, 2002; Simard et al., 2003; Abbott et al., 2006).

MOLECULAR PATHOLOGY OF ASTROCYTES IN ALCOHOL USE DISORDERS

Alcohol-Related Neuropathology of Astrocytes

Chronic alcohol exposure induces atrophic features in glial cells, including astrocytes and their precursors in GM (Miguel-Hidalgo and Rajkowska, 2003). Furthermore, alcoholism associates with downregulation of astrocyte specific genes, particularly in subjects with hepatic pathology (Liu et al., 2006). The atrophic changes in astrocytes appear to preferentially occur in certain brain regions, such as PFC and the hippocampus, where alcohol exposure results in reduced numbers or packing density in the general population of glial cells and in astrocytes (as identified by morphological features or GFAP labeling; Korbo, 1999; Miguel-Hidalgo et al., 2002, 2006). In addition, the expression of astrocyte-related genes is significantly downregulated (Liu et al., 2006). In some subjects, chronic AUDs are associated with persistent nutritional deficiencies or hepatic damage, resulting in serious neurological disorders such as Wernicke's encephalopathy, hepatic encephalopathy or the demyelinating disorders central pontine myelinolysis and Marchiafava-Bignami syndrome (de la Monte and Kril, 2014; Verkhratsky et al., 2014). In these cases, severe neurological symptoms correlate with macroscopically apparent, substantial degeneration of GM or WM in cerebellum, thalamus, mammillary bodies or cerebral cortex (Phillips et al., 1990; Kril and Harper, 2012). Interestingly, although neurons and oligodendrocytes are considered major targets of nutritional and metabolic disturbances, such as thiamine deficiency, astrocytes can be also critically affected by the same disturbances (Hazell, 2009). In addition, acute ethanol exposure of cultured astrocytes causes extensive gene expression changes that resemble the heat shock response (Pignataro et al., 2013).

As it could be expected, the response of astrocytes to pathogenic alcohol exposure is not limited to alterations in their number, morphology or development, but affects many of the roles played by astrocytes in the nervous system. These roles include the regulation of neuroinflammatory processes, calcium signaling, balance of excitatory and inhibitory neurotransmission, water balance/cell volume regulation, as well as the regulation of dopamine-dependent behavioral processes in brain reward circuits (Adermark and Bowers, 2016). In addition, acute or prolonged exposure of astrocytes to alcohol may substantially modify the efficacy of connections between brain areas by disturbing the maintenance of myelin (Hazell, 2009) and the buffering of ions in the proximity of nodes Ranvier. Altering ion buffering and the osmotic regulation that results from astrocyte interactions with oligodendrocytes around NR causes abnormal action potential propagation in WM and myelinated portions of GM (Gankam Kengne et al., 2011).

The alcoholism-related changes in astrocyte numbers and their markers may be due, at least partly, to direct inhibitory effects of ethanol on astrocyte proliferation or turnover. Both in astrocytes cultured from neonatal rodents (Davies and Cox, 1991; Guerri and Renau-Piqueras, 1997; Guerri, 1998) or from postmortem human brain tissue autopsies (Kane et al., 1996) ethanol exposure causes substantial inhibition of astrocyte proliferation and synthesis of DNA and protein, including reduced expression of the major astrocyte marker GFAP. After prolonged exposure, this inhibition may lead to decreased astrocyte numbers, as well as impaired ability to perform the critical functions enumerated earlier in this review. The responses of astrocytes to chronic alcohol, although mostly inhibitory, may also lead to secondary activation of gliosis-like astrocyte responses when AUDs prolong sufficiently into senescence (Miguel-Hidalgo and Rajkowska, 2003; Miguel-Hidalgo, 2009). In approaching late-age, accumulation of ethanol-related deficits in astrocyte structure and function may contribute to neuronal degeneration, and this degeneration would trigger a secondary activation of astrocyte reactivity or gliosis, which would be reflected in increased GFAP production and other gliotic changes, although it is still unclear how other markers of astrocytes respond to aging-associated neuronal degeneration.

Alcohol Effects on Glutamate Receptors and Astrocyte Components of the Cycle for Release and Reuptake of Glutamate

NMDA-Type Glutamate Receptors in AUDs

In patients with AUDs there is evidence, some of it controversial, of alcohol-related dysfunction in some aspects of glutamatergic neurotransmission such as an increase in the expression of NMDA-type glutamate receptors and a decrease in GABA receptors, particularly in chronic alcoholism (Davis and Wu, 2001). Ethanol acts antagonistically at NMDA receptors by reducing their activation by glutamate. In animal models, chronic alcohol consumption increases expression of the NR2A, NR2B and NR1 subunits of NMDA receptors in the neocortex and the hippocampus (Gass and Olive, 2008), and the increases would appear to explain the neuronal hyperexcitability found in animal models after alcohol withdrawal. Although less consistently, human studies in chronic alcoholics also show increases in ligand binding to NMDA receptors (Tsai, 1998; Tsai et al., 1998) mainly in the PFC, but not in other cortical or brain regions such as the cingulate cortex, hippocampus or cerebellar vermis (Freund and Anderson, 1996, 1999). mRNA levels for the same NMDAR subunits as above do not differ in uncomplicated alcoholics as compared to controls, but are reduced in cirrhotic alcoholics (Ridge et al., 2008).

Involvement of Astrocyte Glutamate Transporters and Glutamine Synthetase in AUDs

Regardless of how persistent glutamate receptor changes are in human chronic alcoholism, the antagonism of NMDA receptors

and the potentiation of GABA receptors by ethanol are likely to alter glutamate release and the concentrations of glutamate in the extracellular space. These neurotransmitter alterations would cause the involvement of glutamate transporters, glutamate converting enzymes and glutamate receptors, which are highly expressed by astrocytes. In the cerebral cortex, the glutamate transporters of astrocytes are crucial to the synaptic reuptake of glutamate. In addition, astrocytic GABA transporters make a very important contribution to GABA reuptake. These transporters, together with the enzyme glutamine synthetase (GS) in astrocytes, are essential components of the cycle that terminates the actions of released glutamate or GABA at many synapses, and allows for further recycling and synaptic release of glutamate and GABA. Thus, some studies have determined the cortical expression of astrocytic glutamate transporter as well as GS mRNA and protein in AUD subjects (Miguel-Hidalgo et al., 2010; Ayers-Ringler et al., 2016). Interestingly, these studies have not detected significant variations in the protein levels of either EAAT1, EAAT2 or GS in alcohol dependence, and some studies *in vitro* actually show that ethanol exposure may cause an increase in the rate of glutamate transport per astrocyte (Smith and Zsigo, 1996; Smith, 1997; Zink et al., 2004), although in some rat brain areas such the nucleus accumbens there appears to be an ethanol-induced decrease in glutamate transport that still is not linked to a reduced expression of glutamate transporters (Melendez et al., 2005).

It is possible that homeostatic mechanisms result in unaltered or increased expression of glutamate receptors or transporters in many brain regions of human alcoholics. However, it must be kept in mind that the unchanged transporter levels we found in the orbitofrontal cortex in alcohol-dependent subjects occurred only in uncomplicated alcoholism, while in subjects with comorbid major depression there were reduced levels of glutamate transporters and GS, raising the possibility that the severity of alcohol-related pathology resulting in depression involves a decrease in the astrocytic components of the glutamate cycle (Miguel-Hidalgo et al., 2010). The loss of glutamate transporters in subjects with Wernicke's encephalopathy, often associated with severe cases of chronic alcoholism (Hazell et al., 2010) may be considered further evidence for the view that the severity of pathology in some brain areas may depend on profound changes in the glutamate cycle components of astrocytes.

Animal studies have shown that different regimes of chronic alcohol intake, withdrawal, and reinstatement have diverse effects on the expression of glutamate cycle components. This diversity raises the possibility that different trajectories in the timing, length and frequency of withdrawal periods, or the comorbidity with other disorders cause ample variation in glutamate-related mRNA and protein markers in human alcoholics at the time of death. This variety would prevent finding statistically significant differences in AUD patients as compared to non-alcoholic subjects. In fact, as mentioned above, we found that among subjects with alcohol-dependence only those with a comorbid diagnosis of depression had significantly lower levels of glutamate transporters EAAT1 (and a tendency for lower EAAT2 transporters) than controls, while in alcoholics without other psychiatric diagnoses (Miguel-Hidalgo et al., 2010) there was no change, suggesting that uncomplicated AUDs may involve compensatory regulation of glutamate transport. In line with this suggestion, some animal models of alcoholism show increase in astrocytic glutamate transporter levels (Wu et al., 2011), even if alcohol itself can disrupt the function of those transporters (Mulholland et al., 2009). Interestingly, restoration of EAAT2-based glutamate transport with ceftriaxone actually reduces alcohol drinking (Lee et al., 2013), while the reduction of astrocytic EAAT1 resulting from deletion of the circadian period gene (Per2) in mice is accompanied by increased alcohol intake (Spanagel et al., 2005).

Astrocyte-Released NMDA Receptor Co-Agonists in AUDs

Regulation of glutamatergic transmission at NMDA receptors is also dependent on glycine, which acts as co-agonist at those receptors. At the same binding-site, astrocyte-released D-serine is also an active co-agonist. Both glycine and D-serine have a permissive role in NMDA receptor activation when binding to the glycine-binding site. Ethanol can compete with D-serine for the occupancy of that site, although the dependence of behavioral sensitivity on ethanol binding is related to the exact subunit composition of the NMDA receptors and thus differs across brain regions (Tsai, 1998). On the other hand, reduced affinity for glycine at the glycine site is positively associated with attenuated sensitivity to the behavioral effects of alcohol (Kiefer et al., 2003) in mice, while tolerance to partial agonists of that site appears to develop in alcohol dependent subjects (Krystal et al., 2011), pointing to an important role of astrocyte-produced D-serine in the effects of ethanol in chronic AUD patients.

Astrocyte Thrombospondin in AUD-Related Synaptic Alterations

Alcohol-related neuronal dysfunction may also depend on regressive changes at synaptic contacts that result from intermittent or prolonged alcohol exposure. Those changes may be due, at least partly, to impairments in the ability of astrocytes to provide factors involved in synapse maintenance such as thrombospondins and their receptors (Ullian et al., 2004). In animal models, alcohol exposure results in significant reduction of thrombospondin release that can persist for 24 days, in parallel with disturbed matching of presynaptic and postsynaptic structures (Risher et al., 2015). Hepatic damage caused by alcoholism in some subjects may also result in synaptic dysfunction indirectly mediated by astrocytes, because increased ammonia levels that follow liver dysfunction diminish thrombospondin secretion by astrocytes and reduce the levels of synaptic proteins (Jayakumar et al., 2014). Alcohol exposure during early or prenatal stages of development, and maybe later too, may cause persistent changes in synapse formation involving thrombospondin as well (Trindade et al., 2016). These changes are further accompanied by marked reductions in astrocyte-secreted extracellular matrix (ECM) proteins, such as laminin or heparan-sulfate proteoglycan (Lasek, 2016; Trindade et al., 2016).

Thus, repeated alcohol exposure at different stages of prenatal and postnatal development would result in abnormal regulation of astrocyte-derived factors involved in synaptogenesis.

Astrocyte Processes at the Blood-Brain Barrier and the Involvement of Aquaporins in AUD-Related Neuropathology

Astrocytes processes abut the basal lamina surrounding the endothelial cells of small blood vessels, where they contribute to blood-brain barrier (BBB) maintenance (Prat et al., 2001). In addition, those processes are essential to the exchange of metabolic substrates with the blood circulation, and to the regulation of blood flow (Koehler et al., 2009). Chronic alcoholism disturbs components of the BBB at endothelial cells (Haorah et al., 2005; Rubio-Araiz et al., 2017), impairing to varying degrees the exchange of energy and neurotrophic metabolites such as glucose, that directly impinge on neuronal and glial function (Abdul Muneer et al., 2011a). In addition, alcohol causes direct inhibitory effects on glucose uptake by astrocytes processes (Abdul Muneer et al., 2011b).

Effects of alcohol mediated by astrocytes very likely involve changes in aquaporins as well (Kong et al., 2013), in particular aquaporin 4 (AQ4), a membrane protein highly expressed in astrocytes processes at the BBB that allows effective passage of water through the cell membrane (Badaut et al., 2002; Rajkowska et al., 2013). Repeated alcohol intake in binging rat models results in significant increases in aquaporin, astrocyte swelling (linked to brain edema) and activation of neuroinflammatory cascades (Collins and Neafsey, 2012; Collins et al., 2013). Anti-inflammatory treatments can prevent the effects of the AQ4 elevation that is concomitant with increases in neuroinflammatory markers (Tajuddin et al., 2014). On the other hand, serious consequences of alcoholism, such as the loss of myelin in central pontine myelinolysis, might be associated with reduction in astrocytic aquaporins, although more research seems to be needed to fully ascertain this possibility (Popescu et al., 2013).

In summary, multifaceted actions of ethanol on astrocytic markers involved in the formation and regulation of the BBB, amplified by their reciprocal interactions with the BBB, may be important determinants of the cellular and functional pathology of alcoholism.

OLIGODENDROCYTES

Role of Oligodendrocytes in Gray and White Matter

The main function of oligodendrocytes is the formation and maintenance of myelin, which consists of tightly piled layers of oligodendrocyte cell membrane wrapped around the axons of neurons (Baumann and Pham-Dinh, 2001; Butt, 2005). The layers of myelin act as insulation against the dissipation of ionic gradients, allowing for fast, self-regenerating saltatory conduction of action potentials between consecutive NR along the axons to reach synaptic terminals, and thus for efficient exchange of neural impulses between brain centers (Nave and Werner, 2014). Despite this unity of purpose, most oligodendrocytes and their myelin sheaths are finely tuned and sensitive to the physiological and gene expression changes in the neurons whose axons they wrap and the astrocytes that surround them (Simons and Trajkovic, 2006; Nave and Werner, 2014). Conversely, oligodendrocytes produce signals and growth factors that support the axons they enwrap and the neurons and astrocytes in their vicinity (Du and Dreyfus, 2002; Nave and Trapp, 2008; Simons and Nave, 2015). Some oligodendrocytes in GM regions are intimately associated with neuronal cell bodies, but their functions remain so far unclear.

MOLECULAR PATHOLOGY OF OLIGODENDROCYTES IN ALCOHOL USE DISORDERS

Myelin Components

Postmortem cellular and *in vivo* neuroimaging studies in human subjects have revealed that prolonged and repeated alcohol intake results in various degrees of damage or adaptations in the myelin that sheaths axons in the WM and GM, as well as in the oligodendrocytes that form the myelin. In some subjects, macroscopic damage to the WM caused by alcoholism is apparent, and can be identified with loss of myelin both in neuroimaging and postmortem histochemical studies. WM and GM damage produces different neurological syndromes that can be distinguished according to the specific anatomical location and the nature of the neurological disturbances. Myelin disorders such as Marchiafava-Bignami disease, Wernicke-Korsakoff syndrome, hepatic encephalopathy, central pontine myelinolysis or alcohol cerebellar degeneration involve myelin losses in WM and GM of cortical and subcortical brain regions (Zahr and Pfefferbaum, 2017). In these disorders, BBB disruption or nutritional deficits, such as thiamine deficiency, alone or most likely in interaction with direct effects of alcohol on oligodendrocytes, are considered main culprits for myelin disturbances in chronic alcoholism (Lewohl et al., 2005; Alexander-Kaufman et al., 2007; He et al., 2007). On the other hand, given the frequent co-abuse of ethanol and tobacco, part of the deleterious effects of ethanol on myelin proteins might result from an interaction of ethanol with specific components of tobacco, such as nicotine-specific nitrosamine ketone (NNK; Tong et al., 2015; Zabala et al., 2015; Papp-Peka et al., 2017). However, even in subjects without such obvious neurological and anatomical complications (the "uncomplicated cases"), the expression of myelin and oligodendrocyte-related proteins, or their mRNAs, can be significantly altered in various brain regions, being particularly prominent in the PFC (Mayfield et al., 2002; Alexander-Kaufman et al., 2006; Liu et al., 2006), which are reflected in low levels of the main structural myelin proteins such as myelin basic protein (MBP) and possibly proteolipid protein (PLP), their companions, myelin associated-glycoprotein (MAG) and oligodendrocyte-myelin glycoprotein (Omgp; Okamoto et al., 2006), and related transcription factors

(Miguel-Hidalgo et al., 2017). Reduced expression of major myelin proteins such as MBP has been detected in models of prenatal alcohol exposure (Ozer et al., 2000; Bichenkov and Ellingson, 2001), while *in vitro* studies have shown that the effects of ethanol on the expression of myelin components could be mediated by direct regulation of PKC-like enzymes rather than by altering their expression (Bichenkov and Ellingson, 2002).

Beside disturbing the expression of myelin proteins, alcohol can induce oligodendrocyte apoptosis during prenatal development in primates such as the macaque (Creeley et al., 2013), while during early postnatal mice development (roughly equivalent to the human third gestation trimester) there is a dramatic reduction in differentiated oligodendrocytes and oligodendrocyte progenitor cells in the corpus callosum. These cell populations recover after ceasing alcohol exposure, but deficits in MBP levels or in the structure of corpus callosum fibers persist in young adult mice (Saito et al., 2016; Newville et al., 2017). In adult mice, chronic intermittent ethanol (CIE) exposure causes also significant reduction in the levels of MBP, PLP and 2′,3′-cyclic-nucleotide 3′-phosphodiesterase (CNPase) in several brain regions (Samantaray et al., 2015). These degenerative effects appear to be mediated by the calcium-activated protease calpain, because calpain inhibitors prevent the reductions of myelin-related proteins in the mice CIE model (Samantaray et al., 2015).

Gene Expression in Oligodendrocytes

In human postmortem brain, gene-expression studies of chronic alcoholism that involve the aggregate of GM and WM from frontal cerebral regions, have found significantly decreased mRNAs of myelin-related proteins in chronic alcoholics (Lewohl et al., 2000, 2001; Liu et al., 2006; Farris et al., 2015a). Low myelin-related mRNAs include those of the major structural myelin proteins MBP, PLP, myelin oligodendrocyte glycoprotein (MOG) and MAG. These reductions are particularly significant in chronic alcoholics with cirrhosis as compared to non-alcoholic controls or to non-cirrhotic alcoholics (Lewohl et al., 2005), suggesting that nutritional deficiencies or metabolic toxicity, possibly interacting with direct ethanol effects, strongly deplete the expression of myelin components in alcoholics, at least as assessed in studies that include GM in the probed tissue. In contrast, in a recent study from our laboratory, we used samples only from the WM adjacent to cortical area 47 (orbitofrontal cortex) in chronic alcoholics, and observed that mRNA levels for myelin proteins PLP, MAG and MOG and other oligodendrocyte markers were markedly lower than in controls, although the levels of MBP mRNA were not changed (Miguel-Hidalgo et al., 2017). Since cirrhosis had not been diagnosed in most subjects of our study, these results suggest that the effects of prolonged alcohol abuse in some regions of WM may occur without cirrhosis and be different from those when GM is included. However, the degree of hepatic compromise was not exactly quantified in our samples, so that it was not yet possible to separately assess indirect from direct effects of ethanol on the strong decreases in the expression of mRNAs for some myelin proteins. Despite the highly localized nature of our WM study (all samples were from WM adjacent to Brodmann's cortical area 47), it is also important to note that factors related to hepatic pathology, GM contamination of samples, or RNA quality may influence changes detected in previous studies of myelin-related markers in alcoholism (Sutherland et al., 2014) and thus replication studies with well-defined locales, or in other brain areas, appear to be necessary to draw the right conclusions regarding effects of chronic alcoholism on gene expression of glial cells in the human brain. A recent study in the hippocampus of human chronic alcoholics has in fact revealed significant decreases in several genes related to myelination in addition to alterations in specific proteins of stress-related pathways that operate in astrocytes (McClintick et al., 2013).

Studies with oligodendrocyte cultures indicate that ethanol-induced degeneration or impairment of myelin maintenance may be mediated by the ethanol metabolite acetaldehyde (Coutts and Harrison, 2015), suggesting that the ability to degrade or eliminate this metabolite may influence the effects of alcohol on myelin composition. Moreover, human subjects with alcoholism most probably differ in their drinking schedules and exposure to binge or withdrawal periods. These periods, according to the results of animal experiments, can significantly alter myelin protein expression (for example causing a recovery of MBP expression; Kipp et al., 2012; Navarro and Mandyam, 2015) and strongly influence mRNA levels at the time of death. Summarizing, despite complex interactions that probably determine the individual levels of myelin-related mRNAs and proteins at the time of death, the available animal experimentation and human postmortem evidence indicates significant effects of ethanol abuse on the expression of myelin components, and the plasticity of myelin itself. These changes would significantly affect the role of myelin maintenance in action potential propagation.

Oligodendrocyte Survival and Proliferation

In addition to the effects on myelin components and structure, ethanol exposure very likely causes damage to oligodendrocyte precursors by reducing their proliferation (Newville et al., 2017) or disrupting the expression of transcription factors, such as c-fos (Bichenkov and Ellingson, 2009), that regulate the differentiation of those precursors into mature, myelin-forming oligodendrocytes. The effects on differentiation may also include abnormal acceleration of oligodendrocyte differentiation (Aspberg and Tottmar, 1994). However, at a difference with animal models of prolonged alcohol exposure, chronic alcoholism in humans may not necessarily cause an overall reduction in neuro- or glio-genesis in well-known neurogenic niches (Sutherland et al., 2013). On the other hand, a direct role of ethanol in promoting myelin pathology and the possibility of recovery from that pathology are strongly suggested by increased MBP levels in the medial PFC of rats after prolonged periods of abstinence from ethanol (Navarro and Mandyam, 2015). Potential for recovery appears to be a consequence of the involvement of oligodendrocyte precursor cells in remyelination processes (Mi et al., 2009) during abstinence from alcohol drinking.

GLIA-RELATED EXTRACELLULAR MATRIX COMPONENTS IN AUDs

In WM, several proteoglycans and other ECM proteins are produced by astrocytes, oligodendrocytes and neurons to form adhesion complexes at the NR and axon initial segments, where they are implicated in the aggregation of voltage-gated sodium channels and other components involved in action potential generation and propagation (Zimmermann and Dours-Zimmermann, 2008; Nelson and Jenkins, 2017). Those complexes include brevican, versicans, neurocan, tenascins and neurofascins, among others, form mutual attachments involving interactions between specific protein domains. Interestingly, repetitive binge alcohol intake in adolescent rats significantly increases the levels of several of those proteins in the WM of brain areas such as the orbitofrontal cortex that participate in the pathophysiology of addictive behaviors (Coleman et al., 2014). Alcohol exposure also significantly alters the production by astrocytes and oligodendrocytes of some ECM proteins involved in the formation of perineuronal nets and synapses in the GM, and in the assembly of the basal lamina around blood vessels (Lasek, 2016), although the exact role of those changes in the mechanisms leading to behavioral and functional disturbances in AUDs is not fully understood. The importance of an involvement of ECM components in the mechanisms of alcohol addiction is suggested by the ability of ECM disturbances to modulate the seeking for alcohol and other drugs in knock-out mice lacking matrix metalloproteinase 9 (MMP-9), a protease that regulates the integrity of the ECM (Smith, 2017). These mice have reduced motivation for alcohol drinking, but rescuing MMP-9 activity in the brain's amygdala restores normal alcohol-seeking behavior (Stefaniuk et al., 2017).

EPIGENETIC CHANGES IN OLIGODENDROCYTES AND ASTROCYTES IN AUDs

Regulation of DNA transcription into mRNA is greatly dependent on epigenetic mechanisms such as DNA methylation and acetylation, and methylation of chromatin histones (Gräff et al., 2011). In addition, at the translational level, gene expression is regulated by the activity of microRNAs (miRNAs), small forms of non-coding RNA (about 22 nucleotides long) that interfere with translation into proteins by binding to specific sequences of coding mRNA (Liu and Casaccia, 2010; Li and Yao, 2012; Emery and Lu, 2015).

In recent years, several reviews have compiled studies showing that the development of astrocytes, oligodendrocytes as well as plastic changes in myelin maintenance involve complex epigenetic pathways (MacDonald and Roskams, 2009; Kim and Rosenfeld, 2010; Yu et al., 2010; Bian et al., 2013; Namihira and Nakashima, 2013; Emery and Lu, 2015). These pathways can be significantly altered by prolonged exposure to alcohol (Aspberg and Tottmar, 1994; Bichenkov and Ellingson, 2009; Alfonso-Loeches et al., 2012; Creeley et al., 2013; Coutts and Harrison, 2015; Newville et al., 2017). Methylation of DNA at specific nucleotides, and acetylation and methylation of DNA-associated histones are known to critically determine the fate and differentiation of precursors into mature astrocytes and oligodendrocytes as well as the formation of myelin (Moyon et al., 2016).

Epigenetic and miRNA-mediated mechanisms in the central nervous system play also relevant roles in the pathophysiology of neurological, neurodegenerative and psychiatric disorders (Meza-Sosa et al., 2012). The clinical relevance of increasing our knowledge about epigenetic disturbances in oligodendrocytes and astrocytes stems from the demonstration of significant epigenetic anomalies in demyelinating disorders, and the possibility of reversing them with experimental treatments targeted to epigenetic alterations (Li and Yao, 2012; Liu et al., 2016). In addition, several miRNAs have been found to regulate directly (by impairing translation) or indirectly (through other miRNAs or transcription factors suppressed by miRNAs) the production of transcription factors and proteins during development (Bian et al., 2013).

Glial Epigenetic Markers

AUDs are associated with profound brain alterations in epigenetic markers (Zhou et al., 2011; Farris et al., 2015b; Weng et al., 2015; Legastelois et al., 2017) and significant increases in miRNAs regulating the expression of many proteins (Lewohl et al., 2011). Alcohol-related epigenetic changes have been found in DNA methylation patterns and in methylation and acetylation of histones in the human PFC, hippocampus and amygdala (Ponomarev, 2013; Farris et al., 2015b) as well as in cultured astrocytes (Zhang et al., 2014). Some studies suggest that acute alcohol intake leads to histone deacetylase (HDAC) inhibition in the amygdala that would be the basis for increased histone acetylation and anxiolysis, while withdrawal, anxiety or adolescent alcohol exposure seems to be associated with increased HDAC activity (Pandey et al., 2017) and decreased acetylation (Pandey et al., 2008). This HDAC increase would lead to reduced expression of genes involved in synaptic plasticity, and, consequently, HDAC inhibitors have been suggested as a therapeutic option to reduce anxiety and alcohol intake (Pandey et al., 2017). Other researchers have demonstrated DNA methylation disturbances, mostly reductions, in humans with AUDs, although a more recent study with different methodology points to a relatively higher percentage of hypermethylated methylation sites in brain DNA of subjects with alcoholism (Tulisiak et al., 2017).

Methylation changes in the human brain appear to be accompanied by reduction in the mRNA expression of DNA methyltransferases (DNMTs), while in animal models repeated alcohol exposure results in DNMT upregulation (Tulisiak et al., 2017). Actually, the effects of alcoholism on DNA methylation patterns involve both hypo- and hypermethylation in promoters for specific genes, producing a rather complex picture of methylation effects (Tulisiak et al., 2017). However, given that alcohol exposure in animal models increases DNMTs' levels some researchers have explored DNMT inhibition as a therapeutic approach against excessive alcohol drinking, finding that 5-aza or decitabine, both DNMT inhibitors, acutely

reduce excessive alcohol drinking in rats or mice under certain conditions (Ponomarev et al., 2017; Tulisiak et al., 2017). Likewise, developmental models of alcoholism in rodents show disruption of DNA methylation and other epigenetic markers in various regions of the central nervous system (Laufer et al., 2017; Mahnke et al., 2017; Öztürk et al., 2017).

Recent studies on the effects of HDAC and DNMT inhibitors in AUD mouse models have shown that treatment with the DNMT inhibitor decitabine results in decreased ethanol drinking and upregulated expression of genes highly represented in oligodendrocytes and astrocytes in the ventral tegmental area, key region in the brain reward pathways (Ponomarev et al., 2017). In addition, exposure of cultured astrocytes to ethanol results in decreased methylation of the tissue plasminogen activator (TPA) gene promoter (Tulisiak et al., 2017). TPA is involved in ECM degradation, and has been reported to be increased in animal models of AUDs (Zhang et al., 2014). In rats, prenatal ethanol exposure leads to hypermethylation of the promoter for the astrocyte GFAP gene and to reduction in GFAP expression during early postnatal development (Vallés et al., 1997). These findings suggest that alteration of DNA or histone epigenetic markers in astrocytes and oligodendrocytes may play an important role in mediating behavioral disturbances in AUDs.

Glial miRNA Expression Changes

Some studies have also targeted putative miRNA changes in oligodendrocytes following repeated or prolonged alcohol exposure and the possibility that such alterations may bring about disturbances in myelination. In fact, alcoholism has been found associated with upregulation in the expression of miRNAs from a gene cluster in chromosome 14q32 that have for targets the mRNAs of several proteins involved in processes of oligodendrocyte proliferation and myelination (Manzardo et al., 2013). It is also unsurprising that genome-wide examination of miRNA-protein gene co-expression networks in the brain of alcohol-dependent human subjects has identified abundant epigenetic modifications in molecular networks that operate within oligodendrocytes and astrocytes (Ponomarev et al., 2012).

Studies have also shown significant increases of PFC miRNAs in subjects with AUDs (Lewohl et al., 2011), although in WM some miRNAs would be decreased (Miguel-Hidalgo et al., 2017). Increased miRNAs include some targeting major myelin proteins such as PLP1 and CNPase as well as transcription factor C11ORF9, a regulator of myelin formation. In our recent work, we found that miR-21, high in oligodendrocytes, was strongly and positively correlated with decreased PLP1 miRNA (which is not a direct target of miR-21; Miguel-Hidalgo et al., 2017). The networks and pathways regulated by differentially expressed miRNAs in human alcoholics and mice are very highly enriched in oligodendrocytes and astrocytes, some of them exclusively for each cell type (Nunez et al., 2013). Thus, miRNA increases are consistent with downregulated expression of myelin components and other oligodendrocyte pathology in AUD patients.

CONCLUDING REMARKS

Ethanol exposure in AUDs results in disturbances in the structure of astrocytes and oligodendrocytes as well as in the expression and function of specific astrocytes and myelin proteins. The disturbances are likely to impair diverse

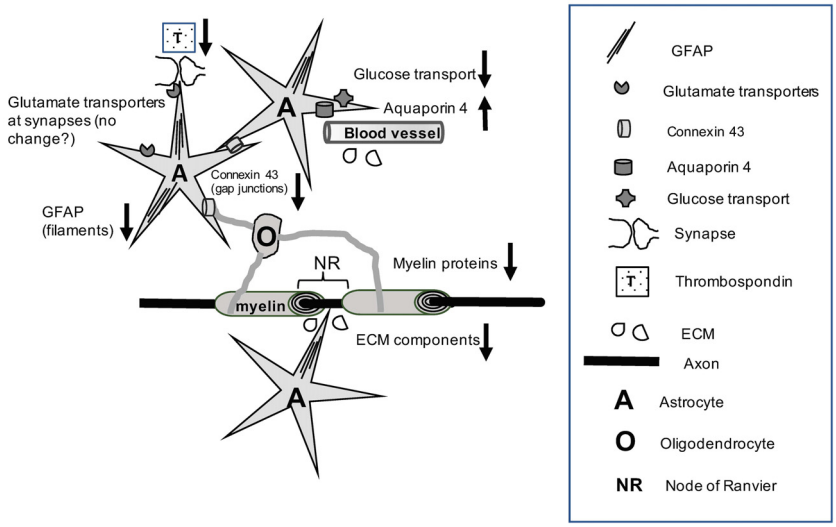

FIGURE 1 | The cartoon illustrates that chronic alcohol exposure results in a variety of molecular disturbances that affect the reciprocal interactions between astrocytes and oligodendrocytes (for example, through reduction of connexin levels), as well as between these glial cells and neurons at synapses, NR or in the wrapping of axons by myelin. Alcohol exposure also results in pathological alterations of astrocyte-derived components at the blood brain barrier (BBB) and the ECM. More recent research work has revealed epigenetic abnormalities or changed levels of specific non-coding RNAs in alcohol use disorders (AUDs). Nevertheless, further research is needed to understand the mechanisms by which alcohol-related functional pathology at the cell and protein expression levels is linked to epigenetic and non-coding RNA markers. Abbreviations: A, astrocytes; ECM, Extracellular matrix; GFAP, glial fibrillary acidic protein; NR, node of Ranvier; O, Oligodendrocyte; T, Thrombospondin; ↓, Down-regulation; ↑, Up-regulation.

aspects of neuronal function including regulation of synaptic transmission, synapse formation, metabolism, interactions with the brain blood supply, propagation of action potentials and neuroprotection. Moreover, the complex interaction between astrocytes and oligodendrocytes involves proteins that are affected by ethanol exposure, such as specific connexins at gap junctions, glutamate transporters, or ECM proteins produced by astrocytes, oligodendrocytes and neurons that are crucial for saltatory conduction at NR (**Figure 1**). However, much more research is needed to determine the mechanisms by which AUDs acting on the components that support the interactions between astrocytes and oligodendrocytes lead to failures in connectivity between brain regions, either by affecting myelin structure or the ability to regenerate action potentials between NR. In addition, chronic alcoholism causes disturbances in the expression of miRNAs and other epigenetic markers that directly influence protein expression. These regulatory changes very likely underpin alterations of proteins and functional pathways in astrocytes and oligodendrocytes observed in earlier studies. However, with a few exceptions, it is still unclear how protein expression changes and the functional pathways they serve in astrocytes and oligodendrocytes depend on non-coding RNA and epigenetic alterations and what is the contribution of these glial processes to the neuronal pathophysiology of alcoholism. In conclusion, much additional work is needed to understand at molecular and neurophysiological levels the mechanisms of alcohol-related neural damage that depend on the molecular pathology of astrocytes, oligodendrocytes and their interactions.

AUTHOR CONTRIBUTIONS

JJM-H conceived and wrote the review.

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MINI REVIEW
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Microglial Phagocytosis and Its Regulation: A Therapeutic Target in Parkinson's Disease?

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The role of phagocytosis in the neuroprotective function of microglia has been appreciated for a long time, but only more recently a dysregulation of this process has been recognized in Parkinson's disease (PD). Indeed, microglia play several critical roles in central nervous system (CNS), such as clearance of dying neurons and pathogens as well as immunomodulation, and to fulfill these complex tasks they engage distinct phenotypes. Regulation of phenotypic plasticity and phagocytosis in microglia can be impaired by defects in molecular machinery regulating critical homeostatic mechanisms, including autophagy. Here, we briefly summarize current knowledge on molecular mechanisms of microglia phagocytosis, and the neuro-pathological role of microglia in PD. Then we focus more in detail on the possible functional role of microglial phagocytosis in the pathogenesis and progression of PD. Evidence in support of either a beneficial or deleterious role of phagocytosis in dopaminergic degeneration is reported. Altered expression of target-recognizing receptors and lysosomal receptor CD68, as well as the emerging determinant role of α-synuclein (α-SYN) in phagocytic function is discussed. We finally discuss the rationale to consider phagocytic processes as a therapeutic target to prevent or slow down dopaminergic degeneration.

Keywords: microglia, phagocytosis, cytokines, alpha-synuclein, parkinson

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INTRODUCTION

Microglia are brain professional phagocytes mainly finalized to clearance of apoptotic or necrotic cells (Green et al., 2016) and removal of unfolded proteins such as amyloid beta (Aβ) or neuromelanin. Moreover, microglia participate in remodeling of neuronal connectivity by engulfment of synapses, axonal and myelin debris (Paolicelli et al., 2011) and combat central infections by direct phagocytosis of bacteria and viruses (Nau et al., 2014). These functions are carried by both unchallenged microglia in the developing brain and reactive microglia in pathological conditions (Sierra et al., 2010, 2013). Phagocytosis is part of the innate immune response of microglia, but also it mediates the adaptive responses by contributing to antigen presentation (Litman et al., 2005).

Abbreviations: α-SYN, α-synuclein; 6-OHDA, 6-hydroxydopamine; Aβ, amyloid beta; AD, Alzheimer disease; CNS, central nervous system; CPu, *Caudate putamen*; CX3CL, fracktaline receptor ligand; CX3CR1, fracktaline receptor; HSP60, Heat shock protein 60; MHC, Major Histocompatibily Complex; MPTP, 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine; PD, Parkinson disease; PI3P, phosphatidylinositol 3-phosphate; PPARγ, peroxisome proliferator activated receptor gamma; RNS, reactive nitrogen species; ROS, reactive oxygen species; SN, *Substantia Nigra*; TH-IR, tyrosine hydroxilase-immunoreactive; TREM-2, microglial triggering receptor expressed on myeloid cells-2; Wt, wild type.

Phagocytosis is traditionally regarded as beneficial for tissue homeostasis by rapidly clearing dying cells, preventing the spillover of proinflammatory and neurotoxic molecules (Green et al., 2016; Wolf et al., 2017). In this context, an increased phagocytic activity was correlated with enhanced production of anti-inflammatory and decreased production of pro-inflammatory cytokines in microglia (Fadok et al., 1998; Wolf et al., 2017). However, the current view is that different targets and related receptors finely tune microglia responses, which appear as a continuum of multiple activation states (Hanisch and Kettenmann, 2007; Sierra et al., 2013; Wolf et al., 2017). For instance, phagocytosis of apoptotic neurons mediated by microglial triggering receptor expressed on myeloid cells-2 (TREM-2) was associated with decreased production of pro-inflammatory cytokines (Takahashi et al., 2005), while myelin debris phagocytosis enhanced the pro-inflammatory and dampened the anti-inflammatory profile in microglia (Siddiqui et al., 2016).

Microglia phagocytosis is still poorly explored in terms of functional consequences and intracellular machinery involved, but recent findings indicate that phagocytosis is defective in Alzheimer's disease (AD; Lucin et al., 2013; Han et al., 2017; Krasemann et al., 2017) and might be dysregulated in other neurodegenerative disorders by genetic defects. Accordingly, p.R47H variant of TREM-2 is associated with Parkinson's disease (PD; Rayaprolu et al., 2013).

This mini-review will focus on current understanding of the role of phagocytosis in PD, and how it is regulated at the physiological and molecular level and it will discuss whether phagocytotic activity might be considered a target for therapeutic intervention in PD.

MOLECULAR MECHANISMS OF PHAGOCYTOSIS

The most important functional similarity between microglia and macrophages is their ability to perform phagocytosis, involving the three main steps "find-me", "eat-me" and "digest-me" (Sierra et al., 2013; Wolf et al., 2017). The process is initiated by the activation of several membrane receptors, which directly recognize the target to engulf. Target-recognizing receptors show a certain degree of specificity toward signaling molecules exposed on the surface of their targets (pathogens, dead cells or protein aggregates) such as phosphatidylserine, oligosaccharides or heat-shock proteins (HSPs). Accordingly, the toll-like receptors (TLRs) in complex with scavenger receptors such as CD14, have been related to pathogen recognition, but are also involved in α-synuclein (α-SYN) uptake (Stefanova et al., 2011; Venezia et al., 2017). TAM (Tyro3, Axl and Mer) receptors recognize mainly apoptotic cells and virus-infected cells exposing phosphatidylserine (Fourgeaud et al., 2016; Tufail et al., 2017). TREM-2 signals the internalization of both dead cells and protein aggregates such as Aβ (Cho et al., 2014; Han et al., 2017; Krasemann et al., 2017). In addition, many other known and unknown receptors participate in target internalization and help to elaborate both effector and immunomodulatory responses (Litman et al., 2005). Different receptors trigger different signaling pathways that stimulate F-actin polymerization and phagosome formation (Arcuri et al., 2017).

The mechanistic features of macrophage phagocytosis have been extensively studied in past years (Green et al., 2016), but the molecular machinery that coordinate engulfment and digestion of dead cells and protein aggregates by microglia, relevant for neurodegenerative diseases, only recently have become an area of growing interest (Plaza-Zabala et al., 2017). Due to poor understanding of molecular mechanisms of microglial phagocytosis, it is assumed that they are similar, if not identical among phagocytes of myeloid linage (Plaza-Zabala et al., 2017). Based on how the phagosomes are formed, we can distinguish three main types of phagocytosis: LC3 (microtubule-associated protein 1A/1B-light chain 3)-dependent (LAP), LC3-independent phagocytosis and xenophagy, a specialized type of autophagy.

LAP is triggered when a pathogen or dead cell, engaged by target recognizing receptors during phagocytosis, induces the translocation of autophagy machinery to the cargo-containing single-membrane phagosome (Martinez et al., 2015; Green et al., 2016). Three major signaling complexes are activated during LAP (see **Figure 1**). The aim of the first pathway is to ensure the production of lipidated-LC3 family proteins, which can embed in phagosomes, allowing their fusion with lysosomes (Martinez et al., 2015). The second pathway is Beclin-1 (BECN1) complex operating in association with Rubicon, Vps34 (Phosphatidylinositol 3-*kinase* class III), UV resistance-associated gene (UVRAG) and other enzymes, which are involved in the production of phosphatidylinositol 3-phosphate (PI3P), required for phagosome maturation (Wong et al., 2017). The third well-described protein complex activated by target-recognizing receptors is NADPH-oxidase type 2 (NOX2) module ensuring the superoxide production, required both for the cargo digestion and for stimulation of phagocytosis/autophagy machinery (Dodson et al., 2013; Martinez et al., 2015).

So far, LAP has not been characterized in microglia as such, but increasing evidence suggest that it may play a role in microglial phagocytosis. First, LC3 and autophagy have been recently implicated in α-SYN uptake and degradation, together with DJ-1, which is a product of *PARK7* gene and an autophagy regulator (Janda et al., 2012; Nash et al., 2017). Processing and lipidation of LC3 into LC3-II is mediated by ATGs (AuTophagy-related Gene products) proteins, which is negatively regulated presumably by mammalian target of rapamycin complex 1 (mTORC1) in microglia. Recently, mTORC1 has been shown to play a role in the regulation of autophagy (and possibly phagocytosis) mediated by TREM-2 (Ulland et al., 2017). Second, BECN1 was shown to be required for efficient microglial phagocytosis *in vitro* and in mouse brains and to be downregulated in brains of AD patients (Lucin et al., 2013). Finally, NOX2 is expressed in microglia and plays an established role in phagocytosis (Roepstorff et al., 2008; Rocha et al., 2016).

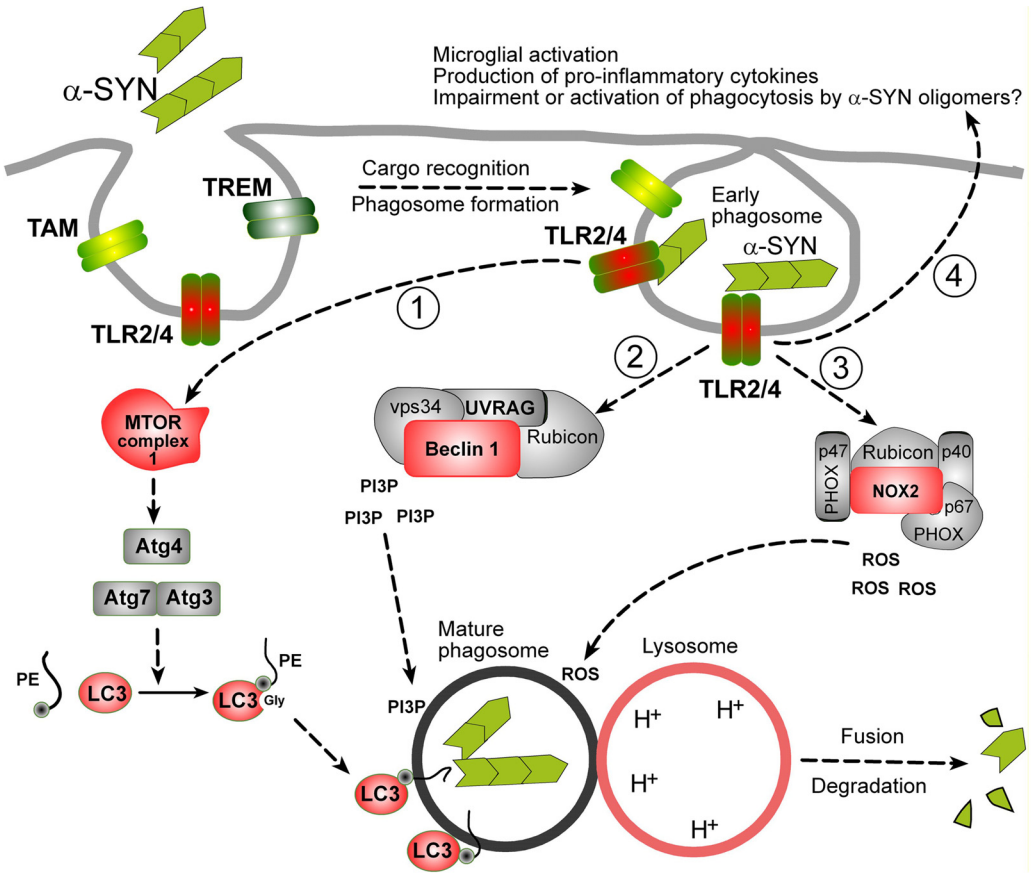

FIGURE 1 | Possible mechanisms of α-synuclein (α-SYN) oligomers phagocytosis in microglia. It is assumed that microglial phagocytosis is run by the same molecular machinery as in macrophages, partially overlapping with autophagy machinery. Proteins in red: documented evidence for an involvement in microglial phagocytosis, proteins in gray: possible role, not yet documented. Phagocytosis is initiated by the recognition of a cargo by specific phagocytosis receptors (TLRs, TREMs, TAMs or others). In case of α-SYN oligomers TLR2 and TLR4 are engaged. These receptors trigger at least three distinct molecular pathways leading to the production of: (1) lipidated-LC3 family proteins; (2) phospholipids (Phosphatidylinositol 3-phosphate, PI3P); and (3) second messengers (ROS), necessary for the delivery and fusion of phagosomes with lysosomes and degradation of the cargo. Lipidated-LC3 family proteins are produced by a cascade of events starting from unknown upstream events (likely mammalian target of rapamycin complex 1 (mTORC1) inhibition, not shown), leading to the activation of Atg3/Atg7 complex and Atg4 involved in the cleavage and lipidation of LC3 family precursors. BECN-1/Beclin-1 complex, in association with Rubicon is involved in PI3P production. ROS are produced by the activation of NOX complex, composed of PHOX subunits (p40, p47 and p67) and NOX2 and Rubicon. Beside activation of phagocytosis, TLR2/4 receptors lead to the activation to other biological responses, like indicated at the end of arrow 4. See text for details.

The alternative mechanisms of phagocytosis, either independent of LC3 and ATGs 5 and 7 (Cemma et al., 2016) or totally autophagy-dependent (xenophagy; Plaza-Zabala et al., 2017), have been described so far only in macrophages.

MICROGLIA: EMERGING ROLES IN PD

After the first report of persistent microgliosis in post-mortem PD brain, a large amount of literature was produced in the attempt to elucidate the phenotype acquired by chronically reactive microglia (Gerhard, 2016; Joers et al., 2017). Microglia in PD maintain an uncontrolled pro-inflammatory phenotype, responsible for the progression of neurodegeneration. Pro-inflammatory cytokines together with iNOS induction, reactive oxygen species (ROS) and reactive nitrogen species (RNS) production, have been found in brain, cerebrospinal fluid and blood of PD patients and in experimental PD (Sawada et al., 2006; Mogi et al., 2007; López González et al., 2016; Joers et al., 2017). In addition, microglia in PD brains and rat *Substantia Nigra* (SN) overexpressing α-SYN showed a significant increase of IgG immunostaining (He et al., 2002; Orr et al., 2005; Theodore et al., 2008).

Furthermore, several studies have suggested that microglia may dynamically change phenotype in PD depending on disease-stage, which may account for the coexistence of pro- and anti-inflammatory molecules described in PD (Sawada et al., 2006; Mogi et al., 2007; Pisanu et al., 2014; Joers et al., 2017). Moreover, gene expression of cytokines and mediators of the immune response are region and stage-dependent in PD (López González et al., 2016). In the 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP)-induced

progressive model of PD, pro-inflammatory microglia gradually increase and prevail over anti-inflammatory microglia in presence of massive dopaminergic degeneration (Pisanu et al., 2014). Therefore, in the early PD stage both pro- and anti-inflammatory microglia may coexist, while in late stages they lose their capability to assume repair functions and unremitting pro-inflammatory microglia prevail.

Finally, the interaction of α-SYN with microglia represent a key event that leads to the unremitting shift of microglia to pro-inflammatory phenotypes (Austin et al., 2006; Theodore et al., 2008; Roodveldt et al., 2010; Ingelsson, 2016). α-SYN is prevalently expressed physiologically as a monomeric form, while in PD it aggregates in oligomers which are converted into mature amyloid fibrils, main components of Lewy bodies and neuritis (Ingelsson, 2016). α-SYN aggregates are also present in the extracellular biological fluids in PD patients (Spillantini et al., 1997; Tokuda et al., 2010; Majbour et al., 2016; Vivacqua et al., 2016; Visanji et al., 2017). Importantly, exposure to human α-SYN directly activates microglia, and the PD-relevant mutations of α-SYN A30P, E46K and A53T are more potent than wild type (Wt) α-SYN in triggering inflammatory responses (Klegeris et al., 2008). Transgenic mice overexpressing human A53T α-SYN develop chronic neuroinflammation and progressive degeneration together with microglia-derived oxidative stress (Gao et al., 2011). Thus, extracellular α-SYN is clearly involved in microglia activation and it has a profound impact on phagocytosis as discussed below.

A determining role in shaping microglia in PD is played by TREM-2, selectively expressed by microglia and involved in modulating inflammatory responses and in the phagocytosis of apoptotic neurons (Jay et al., 2017). Stimulation or overexpression of TREM-2 increases, while knockdown inhibits phagocytosis of apoptotic neurons and increases pro-inflammatory gene transcription (Takahashi et al., 2005). Overexpression of TREM-2 is neuroprotective and reduces inflammation in MPTP-intoxicated mice through inhibition of the TLR4-mediated activation of nuclear factor (NF)-κB signaling (Ren et al., 2018). The fractalkine receptor CX3CR1, specifically expressed in microglia, is also involved in PD neuropathology. Through binding to neuronal CX3CL, CX3CR1 plays a fundamental role in the microglia-neurons communication (Harrison et al., 1998), being involved in homeostatic maintenance of microglia in the quiescent state, regulation of chemo-attraction and synaptic pruning/maturation (Paolicelli et al., 2011; Mecca et al., 2018). Therefore, CXCL-CX3CR1 profoundly affects microglial-mediated inflammatory responses and neurotoxicity (Sheridan and Murphy, 2013). In PD models, MPTP-intoxicated mice with CX3CR1 deficiency displayed aggravated pathology and greater loss of tyrosine hydroxilase-immunoreactive (THIR) neurons in the SNc (Cardona et al., 2006). Moreover, in the intrastriatal 6-hydroxydopamine (6-OHDA) rat model of PD, the continuous delivery of recombinant CX3CL1 suppressed microglia activation and reduced neuronal loss (Pabon et al., 2011).

MICROGLIA PHAGOCYTIC FUNCTION IN PD

Several studies described an altered phagocytic function of microglia in PD (**Table 1**). Pro-inflammatory and phagocytic microglia with increased Major Histocompatibily Complex (MHC) II expression was described in MPTP-treated monkeys and mice (Barcia et al., 2011, 2013; Depboylu et al., 2012). Upon MPTP administration, mouse microglia polarize to contact and phagocytose damaged dopaminergic neurons (Barcia et al., 2012). The increased engulfment and phagocytosis were suggested to contribute to degenerative processes (Barcia et al., 2013). Accordingly, blocking phagocytosis preserved live neurons from inflammation-induced cell death (Fricker et al., 2012). In contrast, we recently found that MPTP-induced neurodegeneration in mice was associated with decreased expression of scavenger receptor Mannose Receptor C-Type 1 (MRC1), while peroxisome proliferator activated receptor gamma (PPARγ)-mediated neuroprotection was associated with increased MRC1 expression and phagocytosis, suggesting a beneficial role of phagocytosis (Lecca et al., 2018).

Few studies focused on the immunohistochemical evaluation of CD68, a macrophagic protein and suggested marker of phagocytosis. Increased CD68 expression was described in the parkinsonian SN (Croisier et al., 2005; Doorn et al., 2014), and confirmed in the α-SYN overexpressing rat model (**Table 1**). In one study CD68 increased early prior to neurodegeneration (Theodore et al., 2008), while in another study correlated with dopamine neurons death (Sanchez-Guajardo et al., 2010). The upregulation of Axl TAM phagocytic receptor was reported in the spinal cord microglia of A53T α-SYN mouse, and loss of this receptor slightly extended survival (Fourgeaud et al., 2016), suggesting that microglia phagocytosis of motor neurons may hasten death of mice.

Different conclusions were reached by studies addressing microglial phagocytic function via functional assays, such as phagocytosis of beads or apoptotic cells (**Table 1**). Microglial phagocytosis but not inflammation was induced by rotenone and rotenone-induced neurotoxicity was prevented by phagocytosis inhibitors (Emmrich et al., 2013). Similarly, anti-inflammatory drug ibuprofen inhibited phagocytosis of dead neurons and NO production by microglia (Scheiblich and Bicker, 2017). However, a significant defect in phagocytic function was observed in fibroblasts and in monocytes of PD patients (Salman et al., 1999; Gardai et al., 2013).

Since phagocytosis has been traditionally regarded as a beneficial event associated with the anti-inflammatory phenotype of microglia, this evidence queries how relevant this assumption is in neurodegenerative diseases, where microglia display an abnormal inflammatory profile.

Studies dissecting the interaction of different α-SYN forms with microglia strongly implicate α-SYN in altered phagocytosis. These studies have highlighted the role of α-SYN variants on the induction of microglial phenotypes with abnormal phagocytic function. Microglia incubated with A53T α-SYN displayed a pro-inflammatory profile and impaired phagocytosis (Rojanathammanee et al., 2011). In contrast, Roodveldt et al.

TABLE 1 | Current evidence of altered phagocytosis in PD and experimental PD models.

Model	Alterations of phagocytosis	Reference
Human studies		
Post-mortem brain tissue	Increased expression of microglial CD68 in SN	Croisier et al. (2005) and Doorn et al. (2014)
Peripheral immune cells	Defective phagocytosis of beads in monocytes and fibroblasts	Salman et al. (1999) and Gardai et al. (2013)
Animal models		
Microglia from α-SYN knock-out mice	Increased expression of CD68, impaired phagocytic function	Austin et al. (2006)
MPTP-treated monkeys and mice	Microglia with phagocytic features in SN	Barcia et al. (2011, 2013) and Depboylu et al. (2012)
A53T α-SYN overexpressing mice	Increased expression of Ax1 TAM in spinal cord microglia	Fourgeaud et al. (2016)
MPTP-treated mice	Increased expression of MRC1	Lecca et al. (2018)
BV-2 cells, rat primary microglia	Monomeric α-SYN increases phagocytosis of microspheres Oligomeric α-SYN decreases phagocytosis of microspheres	Park et al. (2008)
Primary microglia	WT and A53T α-SYN increase phagocytosis of microspheres A30P and E46K α-SYN decrease phagocytosis of microspheres	Roodveldt et al. (2010)
BV-2 cells	A53T α-SYN decreases phagocytosis of bioparticles	Rojanathammanee et al. (2011)
Primary microglia	Soluble or fibrillar α-SYN increases phagocytosis of microspheres	Fellner et al. (2013)
Primary microglia	Adult microglia phagocytoses oligomeric α-SYN less efficiently than young microglia	Bliederhaeuser et al. (2016)
Primary microglia	Rotenone increases phagocytosis of microspheres	Emmrich et al. (2013)
LPS-treated MMGT12 cells	PPAR-γ agonist increases the expression of CD68 and the phagocytosis of beads or 6-OHDA-necrotic SH-SY5Y cells	Lecca et al. (2018)

(2010) showed that both Wt and A53T α-SYN promoted phagocytosis in microglial cells, while the A30P and E46K α-SYN induced opposite effect. Interestingly, Wt α-SYN was also associated with moderate inflammatory response, indicating the coexistence of pro-inflammatory and phagocytic profiles, and suggesting that a combination of alternative and classical activation states may occur in microglia (Roodveldt et al., 2010). However, microglia from α-SYN knock-out mice displayed increased basal and LPS-stimulated production of pro-inflammatory cytokines and expression of CD68, but impaired phagocytosis, suggesting that physiological levels of α-SYN prevent inflammation and promote phagocytosis (Austin et al., 2006). α-SYN conformation impacts microglial phagocytosis, with monomeric α-SYN stimulating, while oligomeric α-SYN inhibiting both basal and LPS-stimulated phagocytosis (Park et al., 2008). In addition, microglia phagocytosis was augmented, together with production of ROS and pro-inflammatory cytokines after treatment with soluble or fibrillar α-SYN, confirming the occurrence of mixed phenotypes in pathological conditions (Fellner et al., 2013). Finally, nitrated α-SYN increased both pro-inflammatory cytokines and the anti-inflammatory cytokine IL-10 in primary microglia (Reynolds et al., 2009). Importantly, age is a crucial factor for microglial phagocytosis, since microglia from adult mice was less efficient to engulf oligomeric α-SYN than young mice, while responding with higher TNFα release (Bliederhaeuser et al., 2016). Therefore, studies indicate a functional specificity for α-SYN conformational variants. In this regard it is important to note that in extracellular fluids of PD patients, the coexistence of multiple α-SYN conformations has been reported, with prevalence of pathological oligomeric α-SYN (Tokuda et al., 2010; Majbour et al., 2016).

The role of TLRs as mediators of α-SYN-effects on microglia is emerging, indicating a role in both inflammatory responses and phagocytosis. Aggregated but not fibrillar or monomeric α-SYN directly activated microglia through TLR2, leading to production of inflammatory mediators (Kim et al., 2013). The TLR4 was also suggested to mediate microglia phagocytosis of β-SYN (Stefanova et al., 2011). Both the TLR2 and TLR4 were elevated in peripheral immune cells and in PD brain, where TLR2 colocalized with microglia (Doorn et al., 2014; Drouin-Ouellet et al., 2014). The coexistence of α-SYN conformations and TLRs stimulation may lead to a microglia phenotype with inflammatory and phagocytic functions, which may be harmful for neurons.

A role of TREM-2 in promoting phagocytosis has been well characterized for *in vitro* clearance of Aβ and *in vivo* models of AD (Taylor et al., 2017). In contrast, the role of TREM-2 in PD-associated dysfunctional phagocytosis has not been investigated. Defective function of TREM-2 in PD may

lead to incomplete removal of apoptotic cells and debris and accumulation of toxic products that may chronically stimulate microglia to release cytotoxic species. Whether TREM-2 is affected by α-SYN accumulation is unknown.

Recent studies suggested that the CXCL-CX3CR1 axis modulates the inflammatory response induced by α-SYN overexpression. CX3CR1−/− mice displayed a reduced α-SYN-mediated inflammatory response, with reduced microglia phagocytosis (Thome et al., 2015).

Therefore, while studies point to a dysregulation (either up- or downregulation) of phagocytosis in microglia as a prominent event in the PD neuropathology, quantitative and qualitative α-SYN abnormalities emerge as the underpinning mechanisms.

CURRENT GAPS AND FUTURE PERSPECTIVES

While it is clear that the shift to pro-inflammatory phenotypes contributes to neurodegeneration, there is no consensus on the role of phagocytosis in PD and research in this field presents several gaps.

An important gap is related to its molecular mechanisms. Since microglia and macrophages share several functional and surface-receptor similarities, it has been assumed that the mechanistic features of phagocytosis should be the same in both cell types (Plaza-Zabala et al., 2017). However, beside some progress in our understanding of phagocytosis machinery involved in Aβ clearance (Krasemann et al., 2017; Sarlus and Heneka, 2017), it is unknown what types of phagocytosis can be activated in microglia, whether other microglia-specific types of phagocytosis exist, and what is the role of autophagy in this process. All these issues are heavily investigated in macrophages, but not yet in microglia, and future efforts will clarify which are the common mechanisms and targetable differences.

Most important, it is still unclear whether phagocytosis is pathologically activated or rather defective in PD. The answer to this question might be hampered by our incomplete understanding of microglial plasticity and its regulation, especially in the context of progressing PD. The current literature strongly suggests that microglia acquire mixed phenotypes in PD displaying an altered phagocytic activity, which escape from traditional classification in pro- and anti-inflammatory phenotypes. Recent innovative studies support a beneficial effect of phagocytosis stimulation in PD (Venezia et al., 2017; Lecca et al., 2018). Additional studies are needed to understand whether we can pharmacologically restore phagocytosis homeostatic levels. Considering that a prompt clearance of dead cellular bodies and protein aggregates should be beneficial in PD, we expect that this concept may prevail.

The current debate on phagocytosis in PD resembles a long-lasting debate about a role of autophagy in this pathology. Despite genetic evidence pointed toward a positive function of autophagy, experimental evidence often indicated a hyperactivation of autophagy in PD (Banerjee et al., 2010; Janda et al., 2012; Dagda et al., 2013). We understood now that conflicting results were often caused by technical limitations and misleading interpretation (Janda et al., 2012), and current view favors a beneficial role of autophagy in PD, while its pharmacological stimulation has become an achievable goal (Janda et al., 2015; Moors et al., 2017). Considering many functional and mechanistic similarities between autophagy and phagocytosis, it is safe to speculate that concomitant stimulation of both pathways in different cellular compartments, will became a therapeutic target in the future.

AUTHOR CONTRIBUTIONS

ARC addressed current knowledge of phagocytosis in PD and in PD models. EJ addressed all the molecular aspect of phagocytosis, both in general and in relation to PD. LB reviewed the literature on alpha-syn and phagocytosis and addressed this specific issue in the review.

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frontiers in Molecular Neuroscience

REVIEW
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Microglia Gone Rogue: Impacts on Psychiatric Disorders across the Lifespan

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Microglia are the predominant immune response cells and professional phagocytes of the central nervous system (CNS) that have been shown to be important for brain development and homeostasis. These cells present a broad spectrum of phenotypes across stages of the lifespan and especially in CNS diseases. Their prevalence in all neurological pathologies makes it pertinent to reexamine their distinct roles during steady-state and disease conditions. A major question in the field is determining whether the clustering and phenotypical transformation of microglial cells are leading causes of pathogenesis, or potentially neuroprotective responses to the onset of disease. The recent explosive growth in our understanding of the origin and homeostasis of microglia, uncovering their roles in shaping of the neural circuitry and synaptic plasticity, allows us to discuss their emerging functions in the contexts of cognitive control and psychiatric disorders. The distinct mesodermal origin and genetic signature of microglia in contrast to other neuroglial cells also make them an interesting target for the development of therapeutics. Here, we review the physiological roles of microglia, their contribution to the effects of environmental risk factors (e.g., maternal infection, early-life stress, dietary imbalance), and their impact on psychiatric disorders initiated during development (e.g., Nasu-Hakola disease (NHD), hereditary diffuse leukoencephaly with spheroids, Rett syndrome, autism spectrum disorders (ASDs), and obsessive-compulsive disorder (OCD)) or adulthood (e.g., alcohol and drug abuse, major depressive disorder (MDD), bipolar disorder (BD), schizophrenia, eating disorders and sleep disorders). Furthermore, we discuss the changes in microglial functions in the context of cognitive aging, and review their implication in neurodegenerative diseases of the aged adult (e.g., Alzheimer's and Parkinson's). Taking into account the recent identification of microglia-specific markers, and the availability of compounds that target these cells selectively *in vivo*, we consider the prospect of disease intervention via the microglial route.

Keywords: microglia, early-life stress, microgliopathies, autism spectrum disorder, major depressive disorder, schizophrenia, aging, neurodegenerative disease

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INTRODUCTION

The modulation of higher order cognitive functions and the dysregulation thereof that leads to neuropsychiatric diseases may commonly be attributed to brain wiring and neural connectivity. Nevertheless, mounting evidence that non-neural microglia play critical and specific roles during brain development, homeostasis and plasticity, with consequences on neurodevelopmental and neuropsychiatric disorders, should be strongly considered in this context (reviewed in Prinz and Priller, 2014; Tay et al., 2017b). Microglia are tissue resident macrophages of the central nervous system (CNS) parenchyma that share the same yolk sac origin as other long-living tissue macrophages (Gomez Perdiguero et al., 2015). Thus the myeloid identity of microglia makes this population unique within the CNS, as they could be strong candidates for therapeutic interventions, without direct impact on cell types of the neuroectodermal lineage within the brain. Previously we examined in detail the growing literature on the varied roles exerted by microglial cells in the healthy brain, across the lifespan, during which they are constant surveillants, and not simply orchestrators of immune responses (reviewed in Tremblay, 2011; Tremblay et al., 2011; Tay et al., 2017b). Here we expand the discussion and focus on the impact of defective microglial physiological roles, from prenatal to aged CNS, on the emergence of various neurodevelopmental, neuropsychiatric and neurodegenerative disorders, and discuss the potential for treatment by specifically targeting microglial cells.

ESTABLISHMENT AND MAINTENANCE OF CNS MICROGLIA

Even when considering the microglia distinct from other CNS cell types, it is important to recognize their unifying characteristics as much as their inherent differences. The mesodermal microglial network begins to establish itself at 9.0 days post conception in the murine CNS, prior to the appearance of the neuroectodermal lineage (reviewed in Tay et al., 2016, 2017b). Several studies support the notion that yolk sac-derived endogenous microglia of the brain parenchyma are a self-maintaining population that persists and functions throughout the animal's lifespan (Alliot et al., 1999; Ajami et al., 2007; Ginhoux et al., 2010; Hashimoto et al., 2013; Hoeffel et al., 2015). Yet, recent lineage tracing studies that were conducted in mouse or in human, using genetic approaches, integration of thymidine analogs (Askew et al., 2017; Tay et al., 2017a) or carbon dating (Réu et al., 2017), have provided further evidence that microglial lifespan varies across brain compartments (Lawson et al., 1992). The significance of the varied turnover kinetics of microglia on their brain microenvironment is currently unclear. While this myeloid population purportedly originates from a single erythromyeloid progenitor (Ginhoux et al., 2010; Gomez Perdiguero et al., 2015), microglial heterogeneity is reflected in their varied distribution and morphology within the CNS (Lawson et al., 1990; De Biase et al., 2017), alongside brain region-dependent differences in gene expression (Doorn et al., 2015), bioenergetics, and immunophenotype (Grabert et al., 2016). Variations in microglial density between male and female parietal cortex, amygdala, hippocampus, and preoptic area (Schwarz et al., 2012; Lenz et al., 2013), and sex differences in microglial response to neuropathic pain (Sorge et al., 2015), have been reported in mice. Groundbreaking studies also proposed that the microenvironment in which microglia evolve influences their tissue-specific identities due to a selection pressure for exclusive gene enhancers (Gosselin et al., 2014, 2017; Lavin et al., 2014). Nonetheless, we are still in a conundrum as microglia have, until now, mostly been investigated as a single entity as compared to other cells of myeloid origin (Hickman et al., 2008; Butovsky et al., 2012, 2014; Gautier et al., 2012; Chiu et al., 2013).

What are the factors required for the establishment and maintenance of microglia? We reviewed this in detail previously (Tay et al., 2017b). Here we discuss the new players reported during this past year and briefly highlight the key transcription factors and signaling pathways that are particularly significant to the associated pathologies covered below. Signaling via the microglial colony-stimulating factor 1 receptor (CSF1R; Ginhoux et al., 2010; Erblich et al., 2011; Elmore et al., 2014) in particular via the alternative CSF1R ligand interleukin (IL)-34, was reported to be necessary for the survival and proliferation of microglia throughout early to adult stages (Greter et al., 2012; Wang et al., 2012). In various contexts, the purinergic ionotropic receptor P2X7 (Rigato et al., 2012), and cytokine transforming growth factor β (TGFβ; Butovsky et al., 2014) were described to regulate microglial cell density and maturation. The recruitment of microglia into CNS compartments where they provide essential support during development requires fractalkine (CX3CL1/CX3CR1) signaling (Maggi et al., 2011; Paolicelli et al., 2011; Rogers et al., 2011; Hoshiko et al., 2012; Ueno et al., 2013; Zhan et al., 2014; Pagani et al., 2015; Hellwig et al., 2016; Milior et al., 2016) and neurogenesis-dependent CXCL12/CXCR4 signaling (Arnò et al., 2014). More recent studies also unveiled the importance of transcription factors such as MAFB (Matcovitch-Natan et al., 2016) and Sal-like 1 (SALL1; Buttgereit et al., 2016; Koso et al., 2016) for maintenance of adult microglial homeostasis and function. Besides the transmembrane protein 119 (TMEM119), a microglia-specific cell surface protein of unknown function expressed from early postnatal development until adulthood (Bennett et al., 2016), *Sall1* was proposed to constitute a microglial signature gene considering its lack of expression in other mononuclear phagocytes and CNS cell types (Buttgereit et al., 2016). Regulating the phagocytic functions of adult microglia, the TAM receptor tyrosine kinases MER and AXL were described to be necessary for the removal of apoptotic cells resulting from adult neurogenesis (Fourgeaud et al., 2016). Microglia lacking TAM were shown to be less motile *in vivo* with delayed response to brain damage, thus underscoring the importance of MER and AXL in modulating microglial physiology (Fourgeaud et al., 2016). From a systematic analysis of the transcriptional regulation and epigenetic signature of microglia from yolk sac to adult stages, three distinct temporal stages of microglial development, namely the early-microglia, pre-microglia and adult microglia, were unveiled. The authors further demonstrated that the microglial developmental

program is sensitive to environmental perturbations such as prenatal immune activation and microbiome alteration (Matcovitch-Natan et al., 2016). Indeed, it was shown earlier that reconstitution of the gut of mice raised in a germ-free facility with short-chain fatty acid by-products of bacterial fermentation was sufficient to recover a normal ramified microglial phenotype (Erny et al., 2015).

PHYSIOLOGICAL FUNCTIONS OF MICROGLIA IN THE BRAIN

Microglia fulfill their roles during development, homeostasis and plasticity mainly through their sensing and scavenging activities, and secretion of trophic factors, cytokines and chemokines. The physiological functions of microglia at steady-state, previously discussed at length (Tay et al., 2017b), are summarized below to provide a context for our main discussions on the impact of defective microglia on psychiatric disorders.

In CNS development, microglia regulate the turnover of neural precursors and neurons by phagocytosis of apoptotic cells and excess newborn neurons (Marín-Teva et al., 2004; Peri and Nüsslein-Volhard, 2008; Swinnen et al., 2013). Furthermore, microglia support neurogenesis, neuronal survival, and the maintenance and maturation of oligodendrocyte progenitor cells through their release of trophic cytokines, also in the adult brain (Sierra et al., 2010; Arnò et al., 2014; Hagemeyer et al., 2017; Wlodarczyk et al., 2017). The positioning of microglial cells along axonal tracts suggests a role in neuronal wiring during embryonic and postnatal stages (Cho et al., 2013; Squarzoni et al., 2014). From early postnatal development until normal aging, a main contribution of microglia in the healthy brain is their activity-based regulation of neuronal activity and synaptic plasticity, which is notably exerted through the refinement of synaptic connections (Wake et al., 2009; Tremblay et al., 2010; Bialas and Stevens, 2013). Real-time two-photon *in vivo* imaging has provided convincing evidence that microglia are extremely dynamic cells. Surveillant microglia continuously extend and retract highly motile processes to interact with their microenvironment, including synapses, at all stages of life (Davalos et al., 2005; Nimmerjahn et al., 2005; Wake et al., 2009; Tremblay et al., 2010; Li et al., 2012). Microglia-synapse interactions regulate the formation and elimination of synapses. As professional phagocytes of the CNS, microglia engulf axon fragments and terminals, as well as dendritic spines, thereby contributing to a crucial pruning function that is regulated by neuronal activity, learning and memory, and the ongoing experience (Watts et al., 2004; Tremblay et al., 2010; Paolicelli et al., 2011; Schafer et al., 2012; Bialas and Stevens, 2013; Squarzoni et al., 2014). Activity- or learning-based dendritic spine formation (Parkhurst et al., 2013; Miyamoto et al., 2016) is mediated through microglial release of brain-derived neurotrophic factor (BDNF; Parkhurst et al., 2013), and their elimination of axon terminals by a TGFβ-dependent cascade that involves the complement proteins C1q and C3 tagging synapses for microglial complement receptor 3 (CR3)-mediated removal (Schafer et al., 2012; Bialas and Stevens, 2013). Fractalkine signaling is also required for hippocampal-associated learning and memory, and the adaptation to a stressful or enriched environment (Maggi et al., 2011; Rogers et al., 2011; Milior et al., 2016). Taken together, functional microglia are essential for synaptic formation, maintenance and plasticity, as well as remodeling of neural networks in response to learning and environmental challenges.

CONTRIBUTION OF MICROGLIA TO THE ENVIRONMENTAL RISK FACTORS FOR PSYCHIATRIC DISEASES

Enduring fevers or maternal infections during pregnancy, and physiological injuries at birth (e.g., infection, hypoxia-ischemia and trauma) increase the risk for autism, attention deficit and hyperactivity disorder, and schizophrenia (Patterson, 2009; Brown and Derkits, 2010; Knuesel et al., 2014; Hagberg et al., 2015; Hornig et al., 2017; Instanes et al., 2017). Childhood maltreatment that comprises physical or emotional neglect and sexual abuse, is considered a major risk factor for adult psychiatric conditions that include eating disorders, alcohol and drug abuse, as well as depression (Kessler et al., 2010; Scott et al., 2012). Maternal immune activation is considered a "neurodevelopmental disease primer" that combines with genetics and other environmental cues to induce mental disorders (reviewed in Knuesel et al., 2014; Meyer, 2014) Similarly, stressful events during adulthood, or chronic post-traumatic disorder, increase the risk for depression (Kessler, 1997), accelerate aging, and may favor neurodegenerative disorders, including the sporadic, late onset, forms of Alzheimer's disease (AD) and Parkinson's disease (PD; Fidler et al., 2011; Miller and Sadeh, 2014). Such challenges could increase the vulnerability to psychiatric disorders by disrupting microglial functions. Microglial "priming" or increased sensitivity to subsequent insults is one of the proposed mechanisms. Primed microglia differ from reactive cells (frequently referred to as "activated microglia" in literature), by displaying increased expression of genes related to phagocytosis, proliferation, and vesicular release (Orre et al., 2014; Holtman et al., 2015). The response to inflammatory challenges is exacerbated in primed microglia, which release increasing amounts of cytokines (Norden et al., 2015). Priming could also prevent microglia from exerting their normal physiological functions, directly impairing neurogenesis, synaptogenesis, and the wiring of brain circuits, with severe impacts on learning, memory and other cognitive processes (**Figure 1**).

As sentinels, microglia are likely the first CNS cell type to sense psychological stress and peripheral inflammation, and mediate the effects of perinatal challenges on the developing brain. Offspring exposed to lipopolysaccharide (LPS; from gram-negative bacteria) during embryonic development show mispositioned cortical interneurons at postnatal stages (Squarzoni et al., 2014) and altered glutamatergic transmission as well as long-term potentiation (LTP) in adolescence (Roumier et al., 2008). Animals challenged by inflammation during pre- or post-natal development, or maternal separation, exhibit

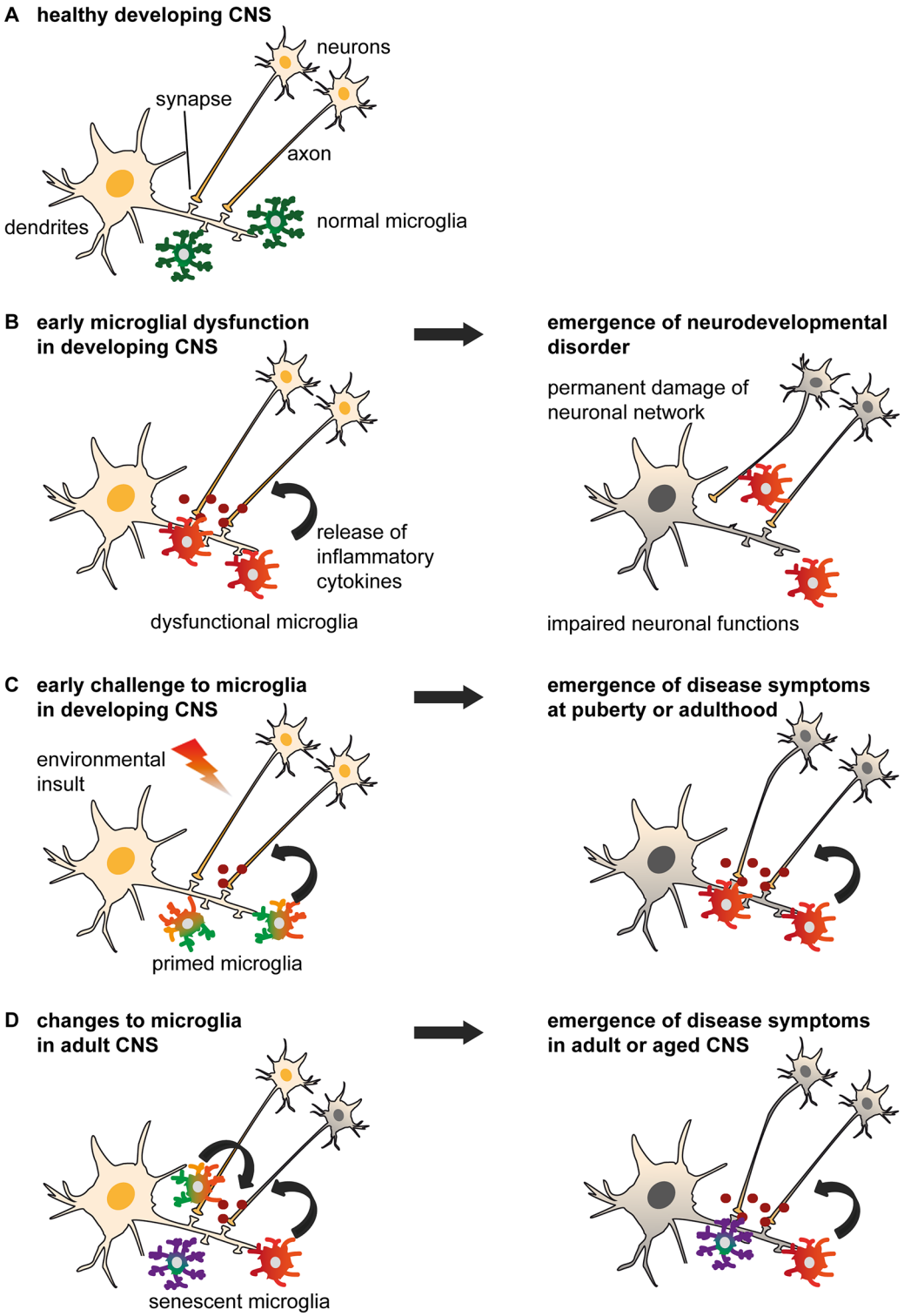

FIGURE 1 | Models of non-physiological microglia and their impacts on the onset of disease. **(A)** Normal microglia-neuron interactions in the central nervous system (CNS). **(B)** An early microglial dysfunction due to genetic or environmental (e.g., maternal or perinatal stress, inflammation, dietary deficiency) risk factors can lead to impaired neuronal functions and an early emergence of neurodevelopmental disorders. Aberrant release of cytokines, impaired pruning and phagocytic activities can affect neuronal densities, maturation and wiring, thus translating into permanent defects of the neural network. These include an imbalance of excitability to inhibition, or altered connectivity between brain regions, which are sufficient to induce the onset of psychiatric disorders during childhood (e.g., Nasu-Hakola disease (NHD),

(*Continued*)

FIGURE 1 | Continued
hereditary diffuse leukoencephaly with spheroids (HDLS), Rett syndrome (RTT), autism spectrum disorder (ASD) and obsessive-compulsive disorder (OCD)), or render an individual vulnerable to subsequent insults. **(C)** An early environmental challenge can prime microglia by altering their maturation and inflammatory states with limited immediate impacts on the neuronal network, thus resulting in asymptomatic changes. However, primed microglia are rendered more susceptible to subsequent challenges such as stress or chronic infections, and may adopt abnormal patterns of cytokine secretion or synaptic pruning later in life. These changes may progressively damage the neural system during puberty and adulthood, leading to the emergence of psychiatric disorders (e.g., alcohol and drug abuse, major depressive disorder (MDD), schizophrenia, bipolar disorder (BD), eating disorders and sleep disorders). **(D)** Changes to microglial phenotypes occurring during adulthood may be accelerated by genetic or environmental factors. Non-physiological microglia may have reduced capability to restore CNS homeostasis, or contribute to neurodegeneration and altered wiring, which result in the onset of cognitive disorders in adult [as in **(C)**] and aged patients (e.g., Alzheimer's disease (AD), dementia and Parkinson's disease (PD)).

long-lasting microglial alterations, including an increased prevalence of ameboid morphologies (reviewed in Boksa, 2010; Johnson and Kaffman, 2017). Early-life stress and prenatal inflammation also induce changes in microglial molecular signature (*C1q* and *Cx3cr1*) and phagocytic activity *ex vivo* (Delpech et al., 2016; Mattei et al., 2017), but their effects on phagocytosis are opposite, with prenatal inflammation being inhibitory (Mattei et al., 2017). Prenatal inflammation accelerates the transcriptomic maturation profile of early postnatal microglia towards an adult signature (Matcovitch-Natan et al., 2016). This shift may restrict microglial physiological functions at crucial stages of development, leading to connectivity alterations or excitatory/inhibitory synapses imbalance, and associated behavioral deficits. Maternal or perinatal stress or immune challenge in rodents, induced by cytokines or surrogates of bacteria (LPS) or viruses (viral RNA mimic polyinosinic-polycytidylic acid; Poly I:C), result in behavioral defects at adolescence or adulthood. These comprise anxiety, impairment of memory, sociability and sensorimotor gating, increased repetitive behavior and enhanced psychostimulants sensitivity (reviewed in Weinstock, 2001; Meyer and Feldon, 2009; Boksa, 2010; Careaga et al., 2017). In the offspring exposed to maternal immune challenge, abnormalities in dopaminergic and GABAergic systems, including increased dopaminergic afferences in the nucleus accumbens (NAc) and decreased inhibition of parvalbumin-positive interneurons on cortical pyramidal neurons were also reported (reviewed in Meyer and Feldon, 2009; Estes and McAllister, 2016).

In addition to stress and infections, epidemiological studies on n-3 poly-unsaturated fatty acids (PUFA), contained mainly in seafood and fishes, but not produced by humans, support the belief that a well-balanced diet is essential. An n-3 PUFA-rich maternal diet was shown to improve the intelligence quotient of children (Helland et al., 2008), whereas the absence of dietary n-3 PUFA negatively impacted on the intellectual performances (Hibbeln et al., 2007; reviewed in Luchtman et al., 2013). In mice, an n-3 PUFA-deficient diet during gestation induced the deregulation of hippocampal *Egr1*, *c-Jun*, *Bdnf* associated with neuronal plasticity in the adolescent offspring. These deleterious effects correlated with impaired microglial motility (in *Cx3cr1*-GFP reporter mice) and decreased expression of inflammation-associated genes *ex vivo*, without alteration in IBA1-positive microglial number (Madore et al., 2014). Further investigation is warranted to determine whether these effects resulted from the anti-inflammatory properties of n3-PUFA acting on microglia, or indirectly from changes in the gut microbiota (Madore et al., 2016). Microglia are affected by other nutritional factors. For instance, a high-fat and high-sucrose maternal diet that induces gestational diabetes in rats, a condition that is associated with decreased cognitive performance and psychiatric disorders in humans, increased the protein levels of pro-inflammatory cytokines and the prevalence of ameboid IBA1-positive microglia in rat neonates (Vuong et al., 2017). Such diet also impaired object memory and induced a shift of hippocampal microglia toward less ramified morphologies in the adult offspring (Vuong et al., 2017). At adulthood, high-fat diet reduces spine density in the dentate gyrus and prefrontal cortex, in addition to impairing novel object memory (Bocarsly et al., 2015; Hao et al., 2016). This diet enhanced the length of IBA1-positive microglial processes (Bocarsly et al., 2015) and the phagocytic activity of isolated microglia toward synaptosomes (Hao et al., 2016), which could mediate the induced changes in connectivity. The saturated fatty acids, which are overrepresented in high-fat, Western-type diet, and which accumulate in the hypothalamus, exert direct pro-inflammatory effects on microglia (identified by IBA1, CD11b or CD68 staining), both in culture and *in vivo* (Valdearcos et al., 2014).

ROLES OF MICROGLIA IN NEURODEVELOPMENTAL DISORDERS

Abnormal wiring of brain circuits during development is proposed to underlie mood instability, abnormal behavior, and cognitive defects that may arise and develop later in life. Here we review the evidence that certain psychiatric diseases with a neurodevelopmental origin are linked to an early microglial dysfunction (**Figures 1**, **2**).

Nasu-Hakola disease (NHD) and *hereditary diffuse leukoencephaly with spheroids* (HDLS) are human diseases associated with behavioral and cognitive alterations resulting from mutations in genes expressed by microglia. These diseases, termed "microgliopathies" (Rademakers et al., 2011), can be considered neurodevelopmental as the microglial mutations affect brain development from prenatal stages, even though disease onset occurs around the fourth decade of life.

NHD is a rare autosomal microgliopathy with a psychiatric outcome, characterized by presenile dementia and bone cysts resulting in premature death (Hakola, 1972; Nasu et al., 1973). The most characteristic abnormalities observed in the postmortem brain of NHD patients comprise a marked loss of myelin, the presence of axon spheroids, astrogliosis, and CD68-positive cells with a large soma and few processes, named "activated microglia", in the white matter of frontal and temporal lobes (Paloneva et al., 2001). NHD is caused by recessive gene

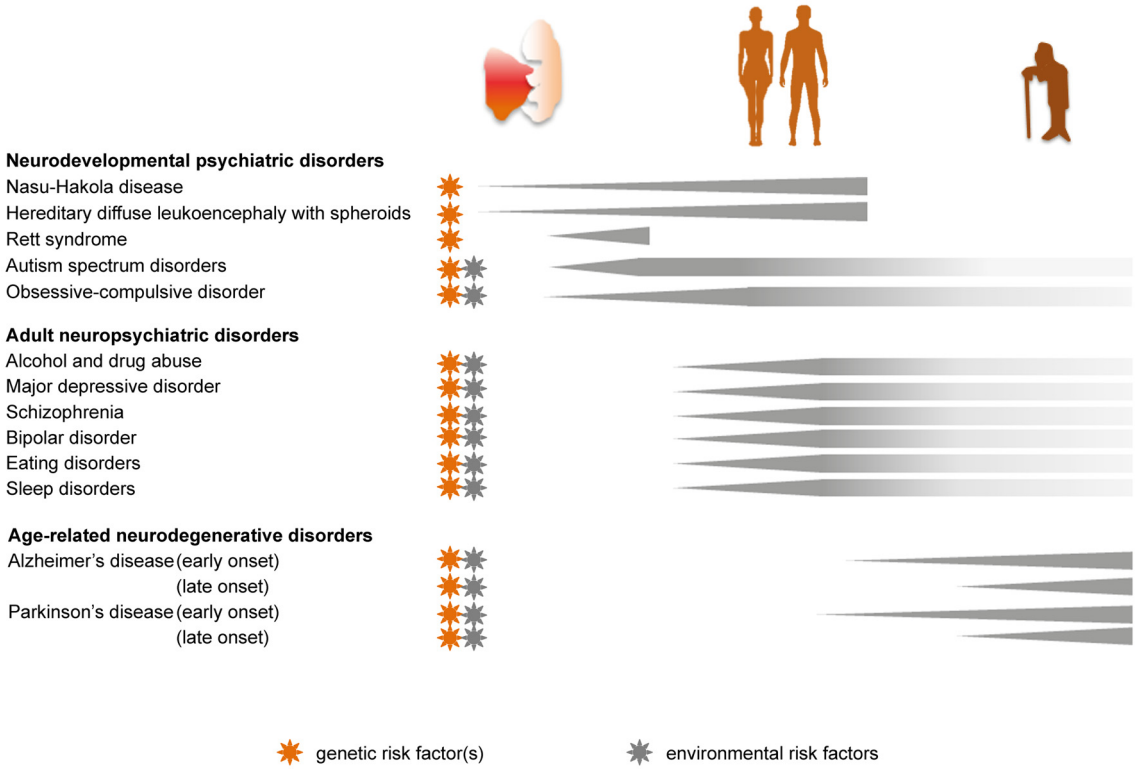

FIGURE 2 | Genetic and environmental implications for the role of microglia in the progression of psychiatric diseases across the lifespan. Pathologies caused by, or linked to, genetic risk factors are marked with an orange star. Disease-associated priming of microglia due to environmental risk factors, such as perinatal inflammation, maternal and early life stress, dietary imbalance and chronic stress during adulthood, is indicated with a gray star.

mutations of *DAP12* (*TYRO Protein Tyrosine Kinase Binding Protein*) or *TREM2* (*Triggering Receptor Expressed On Myeloid Cells 2*, Paloneva et al., 2000, 2002; Bianchin et al., 2004). DAP12 is a transmembrane protein that transduces signals from several lymphoid and myeloid receptors including TREM2. In mice, *Trem2* and *Tyrobp* (the gene encoding DAP12) are expressed by a variety of innate immune cells (Lanier and Bakker, 2000), such as microglia in the CNS (Roumier et al., 2004; Wakselman et al., 2008; Hsieh et al., 2009; Hickman et al., 2013). The molecular mechanisms linking the TREM2-DAP12 pathway to NHD remain elusive. However, analysis of DAP12-deficient mice revealed several neuronal alterations at adulthood, notably enhanced hippocampal LTP (Roumier et al., 2004) and impaired sensorimotor gating (Kaifu et al., 2003), which could account for the cognitive and behavioral symptoms of NHD patients. Transcriptional profiling of DAP12-deficient microglia at embryonic day 17.5 revealed a down-regulation of genes involved in neurite formation accompanied by defasciculation of corpus callosum axons (Pont-Lezica et al., 2014), while the lack of DAP12 impaired the outgrowth of dopaminergic axons and altered the positioning of neocortical interneurons in prenatal mice (Squarzoni et al., 2014). A role for TREM2-DAP12 pathway in clearing apoptotic neurons was also demonstrated in microglial culture (Takahashi et al., 2005; Wakselman et al., 2008) and during developmental cell death *in vivo* in mice (Takahashi et al., 2005; Wakselman et al., 2008). These data show that prenatal dysfunction of microglia due to compromised TREM2-DAP12 signaling can affect synaptic function as well as axonal outgrowth and guidance, which may trigger psychiatric defects in humans.

Another microgliopathy is HDLS, a rare autosomal dominant disease defined by progressive motor, behavioral, and cognitive alterations leading to severe dementia (Axelsson et al., 1984). Studies have shown that HDLS patients present degenerative alterations reminiscent of NHD (Axelsson et al., 1984; Rademakers et al., 2011; Sundal et al., 2012; Konno et al., 2014). HDLS is caused by mutations in the tyrosine kinase domain of *CSF1R* (Rademakers et al., 2011). In mouse brain, *Csf1r* is expressed by microglia (Geissmann et al., 2010) and, as mentioned above, is essential to their development and maintenance. The mechanisms linking CSF1R dysfunction to HDLS remain unknown. However, DAP12 regulates the ability of CSF1R to control the survival and proliferation of bone marrow-derived macrophages *in vitro* (Otero et al., 2009). This suggests that NHD and HDLS may involve a deficit of the same signaling pathway, induced through their respective mutations of *TYROBP*, *TREM2* or *CSF1R* genes.

Rett syndrome (RTT) is a X-linked mental disorder affecting mostly girls, with an onset in the first 2 years of life, in which multiple neurologic, motor, digestive and respiratory symptoms combine with an intellectual disability (Chahrour and Zoghbi, 2007). The disease is caused by mutations in

the (*MECP2*) gene encoding the transcription repressor methyl-CpG-binding protein 2 (Amir et al., 1999). As RTT patients and genetic mouse models exhibit dendritic abnormalities such as decreased spine density (reviewed in Xu et al., 2014), the disease was originally attributed to *MECP2* deficiency in the neurons. However, studies of RTT mouse models have shown that all types of glial cells including microglia ubiquitously express this gene. In mice, the loss of *Mecp2* in microglia leads to the release of high levels of glutamate, resulting in neurotoxicity and dendritic damage *in vitro* (Maezawa and Jin, 2010). Recently, microglia (in *Cx3cr1*-GFP reporter mice) were implicated in RTT through their excessive removal of axon terminals at disease end-stages in *Mecp2* null mice (Schafer et al., 2016). However, this process was independent from microglial loss of *Mecp2* expression, suggesting their contribution to the pathological "de-wiring" through the engulfment of synaptic elements rendered vulnerable by the loss of *Mecp2* in neurons or other glial cells (Schafer et al., 2016).

Autism spectrum disorders (ASDs) are characterized by impaired social communication as well as restrictive and repetitive patterns of interest and behaviors. ASDs are diagnosed at 2–3 years of age, often with clinical signs visible earlier. A common anatomical endophenotype is the transient brain overgrowth measured between 2 and 6 years of age that normalizes during adolescence or adulthood (reviewed in Courchesne et al., 2007, 2011). The cause is unknown, but may reflect abnormal axonal sprouting, cell proliferation, or deficient removal of neurons, synapses or glial cells. Impairment in the processing and integration of multiple sensory and emotional inputs, characteristic of ASD, was proposed to result from connectivity defects, notably related to dysregulated neurogenesis and neuronal migration (Packer, 2016). The connectivity hypothesis is consistent with the local variations of spine density, e.g., increased among cortical layers (Hutsler and Zhang, 2010), and altered excitation/inhibition ratio observed in sensory, social or emotional brain regions of ASD patients (Rubenstein and Merzenich, 2003). Moreover, among the hundreds of genes associated with ASD by genome-wide association studies and whole genome sequencing (Betancur, 2011; Pinto et al., 2014; Yuen et al., 2017), several were found to be involved in synapse assembly and maintenance (Peca and Feng, 2012; Pinto et al., 2014; Yuen et al., 2017). Postmortem transcriptional analyses of ASD brain samples additionally showed a down-regulation of expression modules enriched with genes related to synaptic transmission (Voineagu et al., 2011; Gupta et al., 2014).

Considering the physiological role of microglia in neurogenesis control, circuit wiring, as well as synapse stabilization and pruning, their function in ASD has been investigated (reviewed in Koyama and Ikegaya, 2015; Edmonson et al., 2016). One of the first postmortem studies of ASD patients reported an increased immunoreactivity for MHC class II (HLA-DR) within the cerebellum and cerebral cortical regions (Vargas et al., 2005). Since then, other postmortem studies confirmed that microglial alterations, mainly related to cellular density, soma volume and complexity of ramifications, were more frequent in ASD patients than age-matched controls (Morgan et al., 2010; Lee et al., 2017). However, not all ASD cases exhibited microglial abnormalities (Morgan et al., 2010), which may reflect a diversity of etiologies. Increased binding for translocator protein (TSPO), notably expressed by microglia and induced in response to inflammatory stimuli (Rupprecht et al., 2010; Karlstetter et al., 2014; Sandiego et al., 2015), was also measured by positron emission tomography (PET) imaging in several brain regions of ASD patients (Fatemi et al., 2012; Suzuki et al., 2013). Dysregulation of microglia in ASD is supported by postmortem transcriptional analyses showing an upregulation of gene expression modules enriched with microglial markers (Voineagu et al., 2011), and genes associated with an anti-inflammatory state and the anti-viral type-I interferon pathway (Gupta et al., 2014). Rare genetic variants of *CX3CR1* were also associated with an increased risk of ASD (Ishizuka et al., 2017). *Cx3cr1* knockout mice displayed phenotypes reminiscent of autism such as repetitive behavior and social interaction defects that could be caused by abnormal connectivity (Paolicelli et al., 2011; Zhan et al., 2014). In these mice, the survival of cortical neurons was impaired (Ueno et al., 2013), hippocampal excitatory synapses showed morphological and physiological features of immaturity (Paolicelli et al., 2011; Rogers et al., 2011; Zhan et al., 2014), the maturation of glutamatergic thalamocortical synapses was delayed during postnatal development (Hoshiko et al., 2012), while the positioning of neocortical interneurons was altered prenatally (Squarzoni et al., 2014). These defects could result from a delayed microglial colonization of specific brain regions including the hippocampus and cerebral cortex during development (Paolicelli et al., 2011; Hoshiko et al., 2012; reviewed in Paolicelli et al., 2014). Overall, these findings indicate that environmental risk factors, particularly perinatal infection, could impair the crucial synaptic pruning function of microglia (Knuesel et al., 2014; Delpech et al., 2016; Mattei et al., 2017). The prevalence of microglia with a primed morphology that was observed in a subset of young (6-year-old) ASD patients (Morgan et al., 2010) support this hypothesis of an active, causative role of microglia, but direct evidence is lacking. Microglial priming might alternatively reflect a secondary reaction to neuronal apoptosis and circuit rewiring that occurred to compensate for an early brain overgrowth or increase in spine density.

Obsessive-compulsive disorder (OCD) is characterized by recurrent and uncontrollable thoughts (obsessions) and actions (compulsions) leading to socially-invalidating behaviors such as stereotypy, trichotillomania and excessive cleaning. It is considered a heterogeneous disorder with distinct subtypes having different etiologies (Hirschtritt et al., 2017). Based on a knockout mouse model for the homeobox gene *Hoxb8*, expressed in the myeloid lineage, which showed compulsive grooming (Chen et al., 2010), microglial genetic deficiency was proposed to induce OCD. It was also reported that patients with frontotemporal dementia that carried mutations of the gene encoding progranulin (*GRN*), as well as mice deficient for this gene, displayed OCD and self-grooming behavior, respectively (Lui et al., 2016; Krabbe et al., 2017). While *Grn* function and expression pattern remain poorly-defined, microglia from *Grn* knockout mice, when co-cultured with neurons, more actively internalized synaptophysin-positive puncta that co-labeled with

C1qa, correlating with a selective loss of inhibitory synapses in the ventral thalamus (Lui et al., 2016; Krabbe et al., 2017). This loss could contribute to the thalamic hyperexcitability measured in these mice (Lui et al., 2016; Krabbe et al., 2017), which is reminiscent of the dysfunctional striato-thalamo-cortical circuits described in OCD patients (Burguière et al., 2015). Crossing the *Grn* knockout mice with *C1qa* knockout mice prevented the excessive synaptic pruning and rescued the OCD phenotype (Lui et al., 2016). Microglia-specific *Grn* knockouts similarly displayed an excessive grooming phenotype at adulthood (Krabbe et al., 2017), further implicating microglia in this disorder.

ROLES OF MICROGLIA IN ADULT NEUROPSYCHIATRIC DISEASES

Genome-wide studies revealed an association of immune, neuronal and synaptic pathways with several adult neuropsychiatric diseases (The Network and Pathway Analysis Subgroup of the Psychiatric Genomics Consortium, 2015), suggesting that the inflammatory CNS milieu and microglia are implicated in these disorders, either in pathogenesis or progression. In this section, we review the pre- and clinical evidence that altered microglial physiological functions may contribute to psychiatric disorders with an onset in late adolescence or adulthood (**Figures 1**, **2**).

Alcohol and drug abuse result in cognitive impairment and neurodegeneration. The proposed mechanisms, mainly derived from animal studies, include signaling through microglial Toll-like receptors (TLR), which form a subfamily of pattern recognition receptors (PRRs) allowing innate immune cells to detect changes in homeostasis through the recognition of an array of pathogen-associated molecular patterns (PAMPs; linked to microbial pathogens and cellular stress) and danger-associated molecular patterns (DAMPs; released by cellular damage). The recruitment of TLRs contributes to amplifying microglial release of pro-inflammatory mediators (Stridh et al., 2013; Yao et al., 2013).

A first binge of ethanol in rats induced microglial priming leading to increased immunoreactivity for CR3 and IBA1, and enhanced hippocampal levels of TNFα (tumor necrosis factor alpha), upon a subsequent intake (Marshall et al., 2016). Alcohol exposure in mice similarly potentiated the effects of LPS on brain levels of IL-1β and TNFα, impaired hippocampal neurogenesis, and increased immunoreactivity against IBA1 (Qin et al., 2008). After an acute binge of alcohol in mice, microglial depletion by treatment with a CSF1R inhibitor (PLX5622) increased the brain levels of anti-inflammatory genes, while reducing pro-inflammatory ones (e.g., *Tnfa*, *Ccl2*, Walter and Crews, 2017). Supporting TLRs involvement in microglial pro-inflammatory state upon alcohol exposure, administration of the TLR3 agonist Poly I:C before alcohol enhanced brain levels of TNFα, IL-1β, IL-6 and MCP-1 (monocyte chemoattractant protein-1) mRNA and protein, immunoreactivity against IBA1 and NOXgp91phox (a subunit of NADPH oxidase that generates superoxide and is expressed mainly by microglia), as well as neurodegeneration in the cerebral cortex and hippocampus of mice (Qin and Crews, 2012). These effects were blocked by treatment with minocycline, a tetracycline antibiotic with neuroprotective and anti-inflammatory properties, or the opioid receptor antagonist naltrexone, which also exerts anti-inflammatory effects notably via binding to TLRs (Qin and Crews, 2012). Knockdown of TLR4, which is triggered by LPS and recognizes DAMPs released by injured tissue, prevented alcohol-induced increase of IBA1-immunoreactivity and protected against neuronal apoptosis in cerebral cortex of mice (Alfonso-Loeches et al., 2010). Alcohol-preferring rats had high levels of TLR4 protein and MCP-1 in the central amygdala (CeA) and ventral tegmental area (VTA), while inhibition of both proteins in these areas decreased the excessive alcohol intake, suggesting that TLR4/MCP1 signaling might regulate alcohol self-administration (June et al., 2015). In human, the levels of microglia-associated MCP-1 were increased in postmortem VTA, CeA, substantia nigra, and hippocampus of alcoholics compared to healthy controls (He and Crews, 2008). An increased mRNA expression of *TLR7*, which is activated by single-stranded RNA, and *ITGAM* (encoding CD11b), was also measured in postmortem hippocampus of alcoholics (Coleman et al., 2017). These overall findings present microglial TLRs as promising therapeutic targets for alcoholism.

Similarly, TLRs have a critical contribution to opioids dependence, which is associated with cognitive deficits during both abuse and withdrawal periods, affecting attention, working and episodic memory, as well as executive functions (Dhingra et al., 2015). Chronic exposure to morphine induced apoptosis of primary fetal human microglia, which could be reversed by the opioid receptor antagonist naloxone, suggesting a prominent role of opioid receptor signaling in this process (Hu et al., 2002). Morphine also led to increased mRNA expression of *Tlr9*, a detector of unmethylated CpG dinucleotides found in bacterial and viral DNA, resulting in the apoptosis of primary mouse microglia (He et al., 2011). Morphine tolerance was postponed by blocking release of pro-inflammatory mediators. Systemic treatment with the phosphodiesterase inhibitor Ibudilast (suppressor of microglial pro-inflammatory response through TLR4 signaling) or minocycline both reduced opioids withdrawal in addition to promoting analgesia (Hutchinson et al., 2009). Microglial pannexin-1 was additionally identified as a potential clinical target for opioids withdrawal. Genetic deletion of microglial *pannexin-1* in mice dampened ATP release from spinal cord dorsal horn microglia, and blunted morphine-induced long-term facilitation, thus reducing the severity of withdrawal without affecting analgesia (Burma et al., 2017).

Methamphetamine causes neuropathology through mechanisms that comprise neurotoxicity to serotonin and dopamine neurons, as well as release of pro-inflammatory mediators, eventually resulting in cognitive impairment (Gonçalves et al., 2010; Xu et al., 2017). Microglial response to inflammatory stimuli measured by PET with TSPO ligands was most pronounced in the midbrain, striatum, thalamus, and orbitofrontal, and insular cortices of human abusers (Sekine et al., 2008). Similarly, the density of IBA1-positive

microglia increased in striatum of methamphetamine-exposed mice (Thomas et al., 2004; Lloyd et al., 2017). Acute methamphetamine enhanced mRNA levels of *Tnfa*, *Il6* and *Il1b*, in striatum and hippocampus of mice (Gonçalves et al., 2008). Co-localization of IBA1-positive microglia with the purinergic receptor P2X7R was additionally shown to increase in striatum of exposed mice, while pharmacological blockade or silencing of P2X7R in embryonic stem cell-derived microglia prevented their increased migration, reduced phagocytosis, and enhanced pro-inflammatory release induced by methamphetamine (Fernandes et al., 2016). These findings suggest that modulating microglial phenotype might help to prevent the neurological effects of chronic methamphetamine. Pharmacological treatment with minocycline indeed prevented the reduction of serotonin and dopamine levels, and the behavioral impairment of mice receiving methamphetamine (Zhang et al., 2006). In healthy humans, minocycline similarly decreased the subjective rewarding effects of dextroamphetamine (enantiomer of methamphetamine; Sofuoglu et al., 2011). Likewise, in methamphetamine abusers, Ibudilast reduced the rewarding effects of methamphetamine (Worley et al., 2016).

As with opioids and methamphetamine, the rewarding properties of cocaine are related to an increased release of dopamine in the NAc (Pontieri et al., 1995). Cocaine was shown to interact with microglial TLR4, increase *Il1b* mRNA levels in VTA, extracellular dopamine in NAc, as well as increase conditioned place preference and self-administration (Northcutt et al., 2015). These effects were all suppressed by treatment with TLR4 antagonists in mice, indicating a crucial role of TLR4 in cocaine reward and reinforcement (Northcutt et al., 2015). Increase in IL-1β mRNA and protein levels after acute cocaine administration was also measured in the cerebral cortex and NAc of rats (Cearley et al., 2011). Repeated intake of cocaine was shown to increase TNFα mRNA and protein levels in the NAc, resulting in synaptic depression and suppressed cocaine-induced behavioral sensitization in mice (Lewitus et al., 2016). In this study, microglia displayed increased IBA1-immunoreactivity, enlarged soma, and reduced process arborization in the NAc, and were identified as the cell type responsible for the release of TNFα using microglia-specific knockouts. Dopamine was additionally shown to increase microglial release of TNFα *ex vivo*, through the recruitment of D2 dopamine receptors (Lewitus et al., 2016). The weak TLR4 agonist monophosphoryl lipid A (MPLA; variant of LPS) also resulted in the suppression of behavioral sensitization, a process that required microglial TNFα (Lewitus et al., 2016), further supporting the idea that TLRs could be promising therapeutic targets.

Major depressive disorder (MDD) is characterized by anhedonia (sense of worthlessness) and cognitive impairment (Krishnan and Nestler, 2008). It affects 10%–15% of the general population worldwide. Functional magnetic resonance imaging (fMRI) and morphometric analysis indicate a consistent reduction in activity and size of the prefrontal cortex in MDD patients (Drevets et al., 1997; Rajkowska et al., 1999). Correlative fMRI analysis revealed an altered connectivity, within and between numerous brain regions relevant to resting mode, cognitive functions, and emotions (reviewed in Mulders et al., 2015). The hypothesis of a decreased connectivity in the prefrontal cortex is supported by reduced spine density and down-regulation of genes related to synaptic function (Kang et al., 2012). Despite the large number of MDD patients, we still have very limited understanding of the pathogenic mechanisms, which are obviously heterogeneous. For instance, a comprehensive comparison of transcription profiles in MDD patients and a mouse model of chronic unpredictable stress identified connectivity modules that were differentially enriched in microglia from each sex (Labonté et al., 2017).

An immunological hypothesis was proposed from the evidence that several core symptoms of MDD resemble sickness behavior (i.e., a set of adaptive behavioral changes comprising lethargy, depressed mood, reduced social exploration and loss of appetite) resulting from infectious or inflammatory conditions (Dantzer et al., 2008). According to this model, the chronicity of inflammation would induce a long-lasting depressive phenotype in subjects that are genetically predisposed or exposed to an adverse environment (Dantzer et al., 2008). This hypothesis is supported by the observation that subsets of MDD patients have elevated levels of circulating cytokines (mainly TNFα and IL-6; Dowlati et al., 2010), and increased expression of innate immunity-related genes in blood (Leday et al., 2018). In the brain, PET studies have reported increased TSPO binding in prefrontal cortex, insula and anterior cingulate cortex of MDD patients that positively correlated with their depression severity (Setiawan et al., 2015). This association of elevated inflammatory status in the CNS with depression severity was particularly significant in patients with suicidal thoughts (Holmes et al., 2018). Consistently, an increased density and enlargement of primed IBA1-positive microglia, associated with the upregulation of the genes encoding IBA1 and MCP-1, was observed in postmortem white matter of dorsal prefrontal and anterior cingulate cortex of depressed patients that had committed suicide (Steiner et al., 2008; Torres-Platas et al., 2014). Preliminary studies using minocycline as an add-on treatment (to selective serotonin reuptake inhibitors) for MDD also brought encouraging results (Dean et al., 2017; Husain et al., 2017) but require replication and further analysis regarding the inflammatory status of the patients.

Rodent models of chronic stress-induced depression revealed that connectivity (assessed by spine density or dendritic arborization) was overall decreased in prefrontal cortex, like in depressed patients, while an opposite effect was observed in NAc and amygdala (reviewed in Christoffel et al., 2011). Chronic stress also inhibited neurogenesis (reviewed in Kang et al., 2016), and affected microglial density, morphology, and gene expression, with modalities that depended on the nature and duration of the stress paradigm and examined brain region(s) (reviewed in Yirmiya et al., 2015; Calcia et al., 2016; Tian et al., 2017). Microglial hyper-ramification was reported in rat and mouse after forced swim or restraint stress (Hinwood et al., 2012, 2013; Hellwig et al., 2016), whereas repeated social defeat or chronic unpredictable stress de-ramified microglia (Kreisel et al., 2014; Wohleb et al., 2015; Milior et al., 2016; reviewed in Tian et al., 2017). These alterations could mediate vulnerability or resilience to depression. First, a number of microglial genes regulated by stress encode proteins modulating synaptic plasticity and

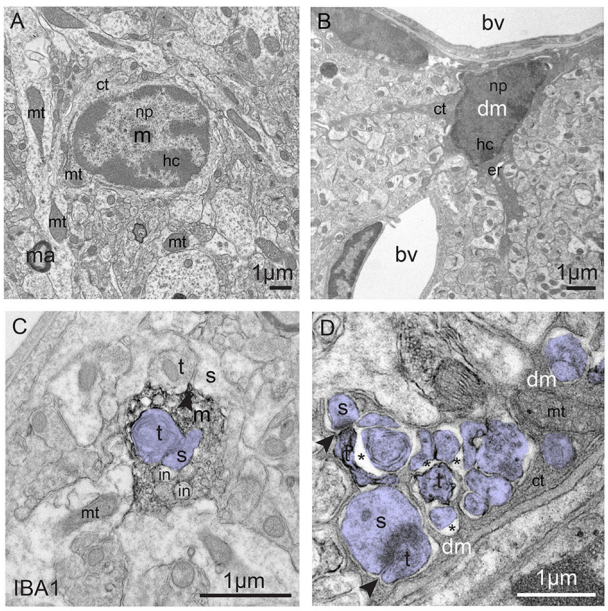

FIGURE 3 | Typical vs. dark microglia. **(A)** Typical microglia (m) that displays a light cytoplasm (ct) and nucleoplasm (np) with a clearly defined heterochromatin (hc) pattern, as well as intact organelles in the hypothalamus of a healthy adult mouse. Mitochondrion = mt; ma = myelinated axon. **(B)** Dark microglia (dm) in the hippocampus of a chronically-stressed *Cx3cr1* knockout mouse showing various signs of oxidative stress, including the darkening/condensation of its cytoplasm and nucleoplasm, making it appear as dark as mitochondria, and dilation of its endoplasmic reticulum (er) bv, blood vessel. **(C)** Processes from typical microglia are generally bulky and make focal contacts with synaptic elements. Several phagocytic inclusions (in) are shown in an IBA1-stained process, in addition to a synapse between an axon terminal (t) and a dendritic spine (s; colored in purple), within the hippocampus of a healthy adult mouse. **(D)** By contrast, dark microglia's processes extensively encircle synaptic elements (colored in purple), including shrunken terminals undergoing digestion, which are surrounded by extended extracellular space (asterisk), and entire excitatory synapses, as shown in the hippocampus of a chronically-stressed *Cx3cr1* knockout mouse. Microglial contacts with synaptic clefts are indicated by arrowheads in **(C,D)**. Blood vessels, cells and cellular elements are labeled by the large bold font. Organelles and subcellular compartments are labeled by the smaller font.

adaptive behaviors (IL-1β, TNFα; reviewed in Delpech et al., 2015). Second, phagocytosis of neuronal and synaptic material by microglia (in *Cx3cr1*-GFP reporter mice) increased in response to chronic stress (Milior et al., 2016; Wohleb et al., 2018). Repeated social defeat or chronic unpredictable stress in mice also induced a strong increase in the density of microglia exhibiting signs of oxidative stress thus appearing "dark" in electron microscopy (Bisht et al., 2016; **Figure 3**). As dark microglia were shown to interact more extensively with synapses than typical microglia, it is possible that their increased prevalence upon chronic psychological stress is related to a pathological rewiring of the brain.

Signaling between the neuronal fractalkine and its receptor CX3CR1 seems particularly relevant to the stress response (Wolf et al., 2013; Paolicelli et al., 2014; Sheridan et al., 2014). Four studies reported that *Cx3cr1*-deficient mice were resistant to chronic stress exposure (i.e., chronic unpredictable stress, forced swim, or a two-hit model combining maternal separation and chronic unpredictable stress at adulthood (Hellwig et al., 2016; Milior et al., 2016; Rimmerman et al., 2017; Winkler et al., 2017)). Altogether these murine studies suggest that the resistance of microglia to stress-induced changes in density and morphology (Hellwig et al., 2016; Milior et al., 2016) could prevent the detrimental behavioral outcomes of stress. Nevertheless, at the molecular level, microglia from *Cx3cr1* knockout mice responded to stress, albeit differently than microglia from wild-type mice. For instance, chronic stress downregulated *Ptgds* (prostaglandin D2 synthase) and *Gpr88* (a G protein coupled receptor) in microglia from wild-type mice, but upregulated these genes in *Cx3cr1* knockout mice. As treatments modulating microglial functions (e.g., minocycline, GM-CSF and M-CSF, LPS, overexpression of *Il1ra*) were shown to rescue microglial alterations, neurogenesis, and behavioral dysfunctions in stressed animals (reviewed in Hinwood et al., 2013; Kreisel et al., 2014), some of the stress-induced microglial changes might be related to resilience, instead of conferring vulnerability to depression (reviewed in Kreisel et al., 2014; Yirmiya et al., 2015).

Schizophrenia is a heterogeneous mental disorder associated with positive (e.g., hallucinations) and negative (e.g., abnormal social behavior, confused thinking) symptoms. It affects 0.3%–3% of the general population worldwide, depending on the inclusion criteria, and emerges in late adolescence or early adulthood (van Os and Kapur, 2009). Schizophrenic patients present an impaired hippocampal neurogenesis (reviewed in Kang et al., 2016) and altered neuronal connectivity, illustrated by a reproducibly low spine density in several regions of the cerebral cortex and hippocampus, which altogether contribute to gray matter loss, reduced hippocampal size, and functional hypoactivity (reviewed in Penzes et al., 2011). Moreover, a decrease of inhibitory markers (GAD67, the GABA synthetizing enzyme and parvalbumin), both at mRNA and protein levels, support the hypothesis of an excitation/inhibition imbalance (reviewed in Gonzalez-Burgos et al., 2015; Canitano and Pallagrosi, 2017).

Indirect evidence that microglia could be implicated in schizophrenia came from clinical studies reporting a significant decrease of positive and negative symptoms when minocycline was added to the antipsychotic treatment (Miyaoka et al., 2008, 2012; Levkovitz et al., 2010). Large-scale studies unraveled the risk association of schizophrenia with genetic markers across the MHC locus, particularly genes encoding complement C4, proposing an immune vulnerability that could involve microglia (Sekar et al., 2016). C4 expression was detected in subsets of neurons and astrocytes from human postmortem hippocampus and prefrontal cortex, while *C4* knockout mice displayed impaired synaptic refinement (Sekar et al., 2016). Moreover, addition of C4 increased the phagocytosis of synaptosomes by human iPSC-derived microglia *in vitro* (Sellgren et al., 2017). Whether the reduced spine density reported in postmortem schizophrenia brains resulted from an impaired synaptogenesis or an exacerbated microglial phagocytosis is still undetermined due to the lack of relevant human data. In addition, PET revealed an increased binding of TSPO ligands in the hippocampus (Doorduin et al., 2009),

total gray matter (van Berckel et al., 2008), and gray matter of frontal and temporal lobes of schizophrenic patients compared to healthy controls (Bloomfield et al., 2016). However, further investigations using more selective TSPO ligands are necessary for conclusive findings (Coughlin et al., 2016; Notter et al., 2017). In postmortem prefrontal cortex, genes of a specific inflammatory module comprising *IL6*, *IL8*, and *SERPIN3* were shown to be overexpressed, while the density of MHC class II-positive microglia was increased (Fillman et al., 2013). In these samples, dystrophy of MHC class II-positive microglia, revealed by the thinning, shortening and fragmentation of their processes, was additionally observed (Radewicz et al., 2000; Wierzba-Bobrowicz et al., 2004, 2005; Busse et al., 2012). Overall, an alteration of the genetic inflammatory profile in prefrontal cortex was fourfold more frequent in schizophrenic vs. control patients (Fillman et al., 2013). Nevertheless, changes of microglia in schizophrenia may depend on the different etiologies, disease progression, as well as history of treatment and substance abuse.

Bipolar disorder (BD) is characterized by recurrent episodes of mania followed by depression, generally beginning in adolescence or early adulthood. Few studies have investigated the roles of CNS immune regulation in BD specifically. A potential role of the immune system was suggested by the increased plasmatic levels of pro-inflammatory cytokines measured in patients during acute episodes of mania or depression, compared to recovery phases (Muneer, 2016a). Postmortem analyses reported increased mRNA and proteins levels of IL-1β, iNOS and CD11b in frontal cortex of BD patients (Rao et al., 2010). Exacerbated inflammatory response was also indicated by PET imaging showing a significant increase of TSPO binding in the right hippocampus of BD patients (Haarman et al., 2014). Although BD has a high heritability, its genetic determinants are unknown, and there is no animal model that may reliably distinguish BD from unipolar depression or schizophrenia. Nevertheless, one specific trait of BD seems to be the decrease in BDNF serum levels during manic or depressive phases. This decrease in BDNF correlated with the clinical severity, normalizing at recovery phases or in medication-induced remissions (Lin, 2009; reviewed in Muneer, 2016b). Whether microglial-derived BDNF linked to dendritic spine formation in mouse (Parkhurst et al., 2013) could underlie different synaptic plasticity levels between acute and remission episodes remains unclear. Although highly speculative, this mechanism could possibly account for the maladaptive behavior of BD patients.

Eating Disorders

Very little data exist on the pathogenesis of eating disorders, such as anorexia nervosa and bulimia nervosa, with relation to microglia. One study showed that stimulation of TLR2 by intracerebroventricular injection of the synthetic ligand Pam3CSK4 induced anorexia and increased IBA1-positive microglial density and structural contacts with proopiomelanocortin (POMC) neurons in the hypothalamic arcuate nucleus of mice (Jin et al., 2016). However, further experiments are required to determine whether this anorexia mediated by TLR2, which is likely a transient symptom of sickness behavior, shares pathogenic mechanisms with anorexia nervosa, which develops over months to years in humans. Microglia were also proposed to mediate the excessive dietary intake, by acting on the hypothalamic control of energy balance (Valdearcos et al., 2014, 2017). In particular, silencing microglial inflammatory signaling via NF-kB pathway (using microglia-specific knockouts for *Ikkβ*) or depleting microglia (with the CSF1R inhibitor PLX5622) protected high fat diet-fed mice against hyperphagia (Valdearcos et al., 2014, 2017). In contrast, microglia-specific knockouts of A20, a primary negative regulator of NF-kB activity, showed exaggerated pro-inflammatory microglial activities *ex vivo*, changes in hypothalamic densities of microglia and infiltrating myeloid cells, as well as metabolic dysfunction and obesity, independently from the diet (Valdearcos et al., 2017). These findings open the possibility to exploit microglial inhibitors in the context of human metabolic pathologies.

Sleep Disorders

Sleep is vital to maintaining health. It allows to restore synaptic homeostasis, clear the brain from toxins, and consolidate memory (Diekelmann and Born, 2010; Xie et al., 2013; Tononi and Cirelli, 2014). Sleep comprises a non-rapid-eye-movement state identified by slow EEG waves and a rapid-eye-movement sleep (REM) state associated with brain activation, as well as inhibition of muscle tone and saccadic eye movements. A PET longitudinal follow-up study showed that patients affected by idiopathic REM sleep behavior disorder were more vulnerable to subsequently developing PD and other synucleinopathies, i.e., neurodegenerative diseases characterized by an abnormal accumulation of α-synuclein in neurons and glial cells. These patients displayed increased TSPO binding in the substantia nigra, associated with a decreased dopaminergic activity in the putamen (Stokholm et al., 2017), suggesting that immune cell activation could represent a biomarker and/or therapeutic target for both idiopathic REM sleep behavior disorder and synucleinopathies. Microglia are linked to several sleep disorders, including sleep deprivation discussed below (reviewed in Nadjar et al., 2017).

Sleep deprivation induces tiredness, sleepiness, irritability and concentration difficulties, as well as more serious outcomes like cognitive impairment, anxiety and neurodegeneration when it becomes chronic (Musiek and Holtzman, 2016; Pires et al., 2016). Studies in rodent models of chronic sleep deprivation reported an increased expression of CR3 protein and hypertrophy of CR3-positive microglia in rat hippocampus (Hsu et al., 2003) and enhanced mRNA levels of pro-inflammatory cytokines in mouse brain (Wisor et al., 2011). Sleep deprived mice also presented an increase in EEG slow waves that could be reversed by the administration of minocycline (Wisor et al., 2011). An increase in IBA1-positive microglial co-localization with VGLUT1-positive glutamatergic terminals, associated with less ramified morphologies, was reported in mouse prefrontal cortex upon chronic sleep deprivation (Bellesi et al., 2017). Since the brain levels of C3 protein were concomitantly enhanced (Bellesi et al., 2017), the authors proposed that complement-mediated synaptic pruning might be exacerbated by chronic sleep deprivation. The expression of MER, regulating microglial process motility and

phagocytosis, was also increased by chronic sleep deprivation (Bellesi et al., 2017). Whether microglia help to restore the disrupted homeostasis during chronic sleep deprivation, or contribute to its detrimental consequences on synaptic loss and cognitive dysfunction remains to be investigated.

DEFECTIVE MICROGLIAL WIRING OF THE CNS IN OLD AGE

Microglia are not exempted from the natural transformation experienced by the body over time (Tay et al., 2017b). Here we discuss the impact of age-related microglial alterations and their consequences on cognitive functions (**Figures 1**, **2**).

Physiological Aging

One of the first noticeable changes with aging was the increased prevalence of dystrophic microglia primarily identified by their cytoplasmic fragmentation and appearance of short, twisted processes (Streit et al., 2004; Ritzel et al., 2015). Although seen in young age (Streit et al., 2004), their increase could imply a reduced area surveyed for harmful debris. Additionally, ameboid microglia, also encountered in early CNS development (Kaur and Ling, 1991; Leong and Ling, 1992), became more prevalent with aging (Rozovsky et al., 1998; Jyothi et al., 2015). Aged microglia, in general, showed increased oxidative stress, corroborated *ex vivo* by their increased production of reactive oxygen species (ROS; Ritzel et al., 2015) and reduced antioxidant glutathione activity (Njie et al., 2012). Dark microglia displaying various signs of oxidative stress and encircling synaptic elements with their highly-ramified processes showing strong immunoreactivity for CD11b (Bisht et al., 2016), a component of CR3 that is involved in synaptic pruning, also become numerous with aging. This suggests that dark microglia could mediate synaptic loss and ultimately lead to cognitive dysfunction (**Figure 3**).

In aging, there is also an increased presence of reactive microglia showing increased MHC class II-immunoreactivity, enlarged cell bodies, and reduced number of short and thick processes (Perry et al., 1993; Rozovsky et al., 1998; Hefendehl et al., 2014). As for the dystrophic cells described above, shorter microglial processes could be detrimental to their ability to survey the parenchyma. A reduced area surveyed by individual microglia was indeed measured in aging, but found to be compensated by a 14% increase in microglial cells in the somatosensory cortex of *Cx3cr1*-GFP reporter mice *in vivo* (Hefendehl et al., 2014). An increased microglial density is supported by previous murine studies in the retina (*Cx3cr1*-GFP mice; Damani et al., 2011), dentate gyrus and hippocampus CA1 (CR3-immnostaining; Mouton et al., 2002), as well as auditory and visual cortices (IBA1-immunostaining; Tremblay et al., 2012). Remarkably, the disparity where females had greater numbers of CR3-microglia in the dentate gyrus and CA1 than males became more pronounced with aging (Mouton et al., 2002). Another striking change in old rodents is the abundance of microglial clusters, seen in white matter (CR3-staining; Perry et al., 1993) and cerebral cortex (IBA1-staining, *Cx3cr1*-GFP mice; Tremblay et al., 2012; Hefendehl et al., 2014). The clumping of microglia could arise from their proliferation, or an increased egress from their designated territory (Hefendehl et al., 2014), resulting in parenchymal areas devoid of surveillance, which could make the brain more vulnerable to the harmful accumulation of debris. In addition, a decrease in microglial process motility during aging was detected in mouse cerebral cortex *in vivo* (Hefendehl et al., 2014) and retina *ex vivo* (Damani et al., 2011). Genes related to process motility like *Pf4*, *Itga4* and *Cxcr4* were indeed measured at lower levels in aged vs. young mouse cerebral cortex (Orre et al., 2014). These aged-related alterations of microglial density, distribution, morphology and dynamics altogether suggest a decline in their capacity to properly survey and preserve the brain milieu against threats.

Microglial release of pro- and anti-inflammatory cytokines becomes disturbed as well with age. Increased brain levels of TNFα and IL-1β mRNA or proteins were measured in aged rodents in steady-state conditions or upon immune challenge (Sierra et al., 2007; Stichel and Luebbert, 2007; Njie et al., 2012). Previous *in vitro* studies have shown that TNFα mediates neuronal loss (De Lella Ezcurra et al., 2010; Kaur et al., 2014), notably through microglial "phagoptosis" of viable neurons (Neniskyte et al., 2014). IL-1β has the capacity to alter microglial morphology from ramified to ameboid in rat hippocampal slice culture (Hailer et al., 2005). IL-1β by itself did not affect neuronal viability but its inhibition using IL-1ra, a receptor antagonist of IL-1, reduced neuronal damage after an excitotoxicity insult in rat hippocampal slice culture (Hailer et al., 2005). Pro-inflammatory IL-6 mRNA and proteins were also increased in the brain (Sierra et al., 2007) and isolated microglia (Ye and Johnson, 1999) from aged mice. Even though IL-6 is expressed by astrocytes, neurons and microglia, only the latter displayed an age-dependent increase in IL-6 production in mice (Ye and Johnson, 1999). In addition, anti-inflammatory cytokines, such as IL-10 and TGFβ1, were detected in larger quantities in the aged brain of mice (Sierra et al., 2007), leading to the hypothesis that anti-inflammatory cytokines increase during aging to dampen the detrimental effects of pro-inflammatory cytokines and prevent inflammation from impairing further microglial functions (Sierra et al., 2007).

Synapses in vulnerable regions are affected over time, as indicated by the age-related decrease in the expression of genes associated with synaptic vesicle trafficking or neuromodulation that was measured in the superior frontal gyrus and postcentral gyrus, and to a lesser degree in the hippocampus and entorhinal cortex, of human postmortem samples (Berchtold et al., 2013). Synaptic loss has been observed in multiple brain regions with aging, for instance the temporal lobe of postmortem human samples (Anderson and Rutledge, 1996), prefrontal cortex of rhesus monkeys (Peters et al., 2008) and olfactory bulb of mice (Richard et al., 2010; extensively reviewed in Petralia et al., 2014). However, in other brain regions, such as the somatosensory cortex of mice, reduced size and long-term stability of spines was instead observed *in vivo* (Mostany et al., 2013). In addition, increased brain levels of C1q, and several other components of the complement pathway, were measured in aged mice and human (Cribbs et al., 2012; Stephan et al., 2013). Mice deficient

for C3 were additionally found to be protected from synaptic loss and neuronal death during aging, in the hippocampus CA3, suggesting that microglia are implicated in synaptic loss or remodeling during normal aging by means of the complement pathway (Shi et al., 2015).

Age-Related Neurodegeneration

Age is the main risk factor for neurodegenerative diseases. The most prevalent, AD and PD, are associated to elevated CNS inflammatory milieu, synaptic dysfunction and loss, cognitive decline and dementia (Šišková and Tremblay, 2013).

AD is characterized by the appearance of neurofibrillary tangles and accumulation of amyloid β (reviewed in Perl, 2010). Seen in multiple brain regions, including frontal cortex (Scheff et al., 1990) and temporal lobes (Scheff and Price, 1993), synaptic loss is an early event in AD (Scheff et al., 2006) that correlates with the severity of cognitive dysfunction (Terry et al., 1991; Spires-Jones and Hyman, 2014). Early onset AD which typically begins around 40 years of age (Seltzer and Sherwin, 1983) has been associated with mutations in *APP*, *PSEN1* and *PSEN2* (Bekris et al., 2010), and also with polymorphisms in several microglial genes including *CD33* (Griciuc et al., 2013; Malik et al., 2013), *ABI3* (Sims et al., 2017), *PLCG2* (Sims et al., 2017), and *TREM2* (Guerreiro et al., 2013; Jonsson et al., 2013; Suárez-Calvet et al., 2016; Sims et al., 2017). Late onset AD is a sporadic form of the disease that affects a majority of patients (up to 95%) and usually develops after 65 years of age (Seltzer and Sherwin, 1983). Chronic psychological stress across the lifespan is considered a main risk factor for this late onset form of AD (Miller and Sadeh, 2014). In mice, exposure to early-life stress was shown to alter the inflammatory response to amyloid β pathology during adulthood (Hoeijmakers et al., 2017). APP/PS1 mice housed with limited bedding and nesting materials in their first postnatal week displayed reduced amyloid β deposition that was notably accompanied by increased CD68- and IBA1-immunoreactivity in the dentate gyrus at 4 months of age (Hoeijmakers et al., 2017).

While the number of microglia is similar between healthy subjects and AD patients (Griciuc et al., 2013), an increased prevalence of reactive microglia positive for MHC class II was described in the cerebral cortex of patients with senile dementia of the AD type (McGeer et al., 1987), both in gray and white matter, particularly in association with the amyloid β plaques (Mattiace et al., 1990). However, an absence of reactive IBA1-positive microglia was also reported in the cortical gray matter of postmortem AD samples (Streit et al., 2009). The authors thus suggested that microglial "activation" could be related to peripheral infections which affect the CNS over the course of AD pathology, rather than the disease itself. Additionally, as in normal aging, an increased prevalence of dystrophic microglia was discerned both in the frontal (Flanary et al., 2007) and temporal (Streit et al., 2009) lobes of postmortem AD brains, even appearing in temporal lobes before Tau pathology (Streit et al., 2009).

Upregulated in plaque-associated microglia (Frank et al., 2008; Melchior et al., 2010; Guerreiro et al., 2013), TREM2 is expressed by dark microglia (Bisht et al., 2016), disease-associated microglia (DAM; Keren-Shaul et al., 2017), and microglia dependent on the TREM2-APOE pathway (Krasemann et al., 2017), three subtypes that were described in AD mouse models. Deletion or dominant negative mutations of *Trem2* were shown to worsen AD progression in the 5xFAD and APP/PS1 mouse models of AD (Jay et al., 2015, 2017; Yuan et al., 2016; Ulland et al., 2017), but *Trem2* deletion also reduced amyloid β burden, increased neuronal loss, prevented microglial association with the plaques, and resulted in their apoptosis in the 5xFAD model (Wang Y. et al., 2015). Genetic deletion of *Trem2* in a mouse model of Tau pathology (PS19) resulted in an attenuated atrophy of the entorhinal and piriform cortices, together with increased protein levels of PSD95 in hippocampus (Leyns et al., 2017). IBA1-positive microglial density and morphology normalized in the piriform cortex and hippocampus, while the levels of genes coding for pro-inflammatory or phagocytic markers (IL-1α, IL-1β, TNFα and C1q) decreased in the piriform cortex (Leyns et al., 2017). IBA1-positive microglia expressing APOE also became less prevalent, within the piriform cortex, indicating a phenotypic shift. By contrast, Tau phosphorylation or solubility were unaltered in the piriform cortex and hippocampus (Leyns et al., 2017). These findings suggest that TREM2 could mitigate microglial response to Tau pathology, thus protecting against neurodegeneration.

Microglial capacity to phagocytose amyloid β efficiently is affected by aging and AD. As a matter of fact, microglia demonstrate an age-dependent ability to phagocytose since microglial cells isolated from 6 to 8 months old wild-type mice could not clear amyloid β fibrils with the same efficiency as microglia isolated at postnatal day 0 (Floden and Combs, 2011). Using acute hippocampal slices from 7 to 9-week old APP/PS1 mice, the phagocytic ability of microglia was also shown to be compromised in amyloid β plaque-burdened areas specifically (Krabbe et al., 2013). Similarly, an age-related decrease in mRNA expression of genes coding for amyloid β receptors (e.g., SRA, CD36, RAGE) or degrading enzymes (e.g., insulysin, neprilysin, MMP9) was detected *ex vivo* in microglia from APP/PS1 mice (Hickman et al., 2008). While amyloid β triggers synaptic loss in rat hippocampal slices (Shankar et al., 2007, 2008), microglial phagocytosis of both amyloid β and dendritic spines was shown to be mediated by microglial TDP-43, a DNA-RNA binding protein encoded by *Tardbp* gene. Microglia-specific knockouts of *Tardbp*, crossed with an APP model, displayed reduced amyloid β load, but also an exacerbated synaptic loss (Paolicelli et al., 2017). For instance, spine density was reduced, while IBA1-microglial co-localization with PSD95 puncta and expression of CD68 were increased in somatosensory and motor cortices (Paolicelli et al., 2017). This finding supports the importance of orienting microglial phagocytosis toward specific cargos in future therapies for AD and other neurodegenerative diseases.

The synaptic impairment caused by amyloid β was also associated with the complement cascade (Hong et al., 2016). Microglia-mediated synaptic pruning was shown to be abnormally activated early in AD pathology, directly affecting synaptic viability (Hong et al., 2016). In particular,

an increase of C1q-immunoreactivity was observed in regions vulnerable to synaptic alterations, defined by the loss of synapsin-PSD95 puncta, such as the hippocampus of J20 and APP/PS1 mice. Intraventricular injection of oligomeric amyloid β increased the co-localization of C1q with PSD95, and decreased microglial expression of CD68 in hippocampus. Gene deletion or pharmacological antagonism of C1q in the AD models further halted their loss of synapsin-PSD95 puncta, while rescuing LTP in acute hippocampal slices (Hong et al., 2016). This suggests that oligomeric amyloid β could drive synaptic pruning via the complement cascade or weaken synapses thus leading to their elimination. Additionally, *C3* deficient APP/PS1 mice displayed increased numbers of Vglut2-GluR1 puncta and protein levels of various synaptic markers during aging in the hippocampus, where IBA1-positive microglia recovered surveillant morphologies near plaques (Shi et al., 2017). Brain levels of pro-inflammatory TNFα, IFNγ and IL-12 proteins were also found to be reduced, and those of anti-inflammatory IL-10 to be increased (Shi et al., 2017).

The neuropathology of PD is characterized by a loss of dopaminergic neurons in the midbrain, accompanied by the presence of Lewy Bodies, which are aggregates positive for α-synuclein, and chronic elevation of brain inflammatory responses (reviewed in Wang Q. et al., 2015). Genetic and sporadic forms of the disease were both described (reviewed in Schneider and Obeso, 2015; Poewe et al., 2017). Early onset PD, which primarily affects individuals younger than 40 years of age, is caused by mutations of *SNCA*, *PINK1*, *DJ-1* and *Parkin*, or exposure to neurotoxins (reviewed in Schrag and Schott, 2006). Late onset PD that emerges between 55 and 65 years of age is instead categorized as sporadic, and associated with mutations and variants of *LRRK2* (reviewed in Volta et al., 2015) that is notably, among other cell types, expressed by microglia (reviewed in Russo et al., 2014). Polymorphisms in genes encoding pro-inflammatory cytokines such as TNF-α and IL-1β, and MHC class II were associated with a higher risk of developing PD (Wahner et al., 2007; Hamza et al., 2010). Chronic psychological stress was proposed to confer an increased susceptibility risk to late onset PD (reviewed in Hou et al., 2014; Vyas et al., 2016). In mice, chronic restraint stress that occurs before administration of the neurotoxin 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP), causing the death of midbrain dopaminergic neurons, was shown to exacerbate the motor deficits, learning impairment, further reduce the dopaminergic levels, and increase the loss of dopaminergic neurons in the substantia nigra (Lauretti et al., 2016). In wild-type mice, chronic restraint stress similarly induced a loss of dopaminergic neurons, increased the aggregation of α-synuclein, reduced proteins levels of CX3CR1 and IBA1, as well as IBA1-positive microglial density in the substantia nigra (Ong et al., 2017).

In postmortem PD brain, reactive microglia immunopositive for MHC class II, ICAM-1 (CD54), LFA-1 (CD11a), CD68, TLR2, and displaying ameboid morphologies, were encountered among several regions comprising the substantia nigra, striatum and hippocampus of postmortem PD brains (Imamura et al., 2003; Doorn et al., 2014). Microglial alterations were similarly reported in animal models of PD induced by neurotoxins. For instance, rotenone increased the density of CR3-positive microglia and altered their morphology toward enlarged cells with short, stubby processes, in the striatum and substantia nigra of rats (Sherer et al., 2003). MPTP also increased the density of MHC class II-positive microglia and their heterogeneity, especially in the substantia nigra and globus pallidus, resulting in the concomitant presence of ramified, ameboid, and multinucleated morphologies in monkeys (Hurley et al., 2003). Administration of MPTP in *Ifng* knockout mice reduced the loss of dopaminergic neurons and terminals, as well as decreased CD11b-positive microglial density in the substantia nigra (Mount et al., 2007). *In vitro*, exogenous application of aggregated α-synuclein was sufficient to transform microglia into ameboid cells, increase their phagocytosis of α-synuclein, as well as exacerbate oxidative stress (production of ROS) and the death of dopaminergic neurons in mesencephalic neuron-microglia culture (Zhang et al., 2005). The phagocytosis of α-synuclein is an age-dependent ability, with isolated microglia from older mice showing a reduced capacity to clear α-synuclein *ex vivo* (Bliederhaeuser et al., 2016). In a mouse model of PD that expresses a mutant form of human α-synuclein, IBA1-immunoreactivity was found to be dramatically increased in the spinal cord, where increased mRNA and proteins levels of AXL were also detected, in exclusive association with IBA1-positive cells (Fourgeaud et al., 2016). By contrast, upregulation of AXL was found to be minimal in the brain (Fourgeaud et al., 2016). The authors speculated that wild-type microglia might execute TAM-dependent phagoptosis of the distressed motor neurons, thus prolonging survival, considering that *Thy1-Syn*^{hA53Ttg} mice also knockout for *Mer* and *Axl* displayed a modest life extension (Fourgeaud et al., 2016). However, the roles of microglia in PD pathogenesis remain largely unknown.

PERSPECTIVES

In recent years, clinical and preclinical studies have advanced our understanding of the roles of microglia in modulating cognitive functions, and their implications in neurodevelopmental, neuropsychiatric, and neurodegenerative disorders. The evidence that CNS inflammatory response is exacerbated in several psychiatric diseases mainly came from gene expression and immunohistochemical postmortem studies, and are now supplemented by PET neuroimaging with TSPO ligands (reviewed in Mondelli et al., 2017). However, current tracers have limitations in specificity and sensitivity. Thus, the development of microglia-specific ligands is a high priority. A selective antagonist of CX_3CR1, 2-[^{18}F]FBTTP, was radiosynthetized and shown to cross the blood-brain barrier using PET imaging in mice. Its application in models of "neuroinflammation" is currently investigated (Mease et al., 2015). In parallel, clinical trials have started to assess the therapeutic potential of the tetracycline derivative, minocycline (**Figure 4**). Although its mechanisms of action remain elusive, minocycline has neuroprotective properties and is efficient at dampening pro-inflammatory responses and normalizing microglial

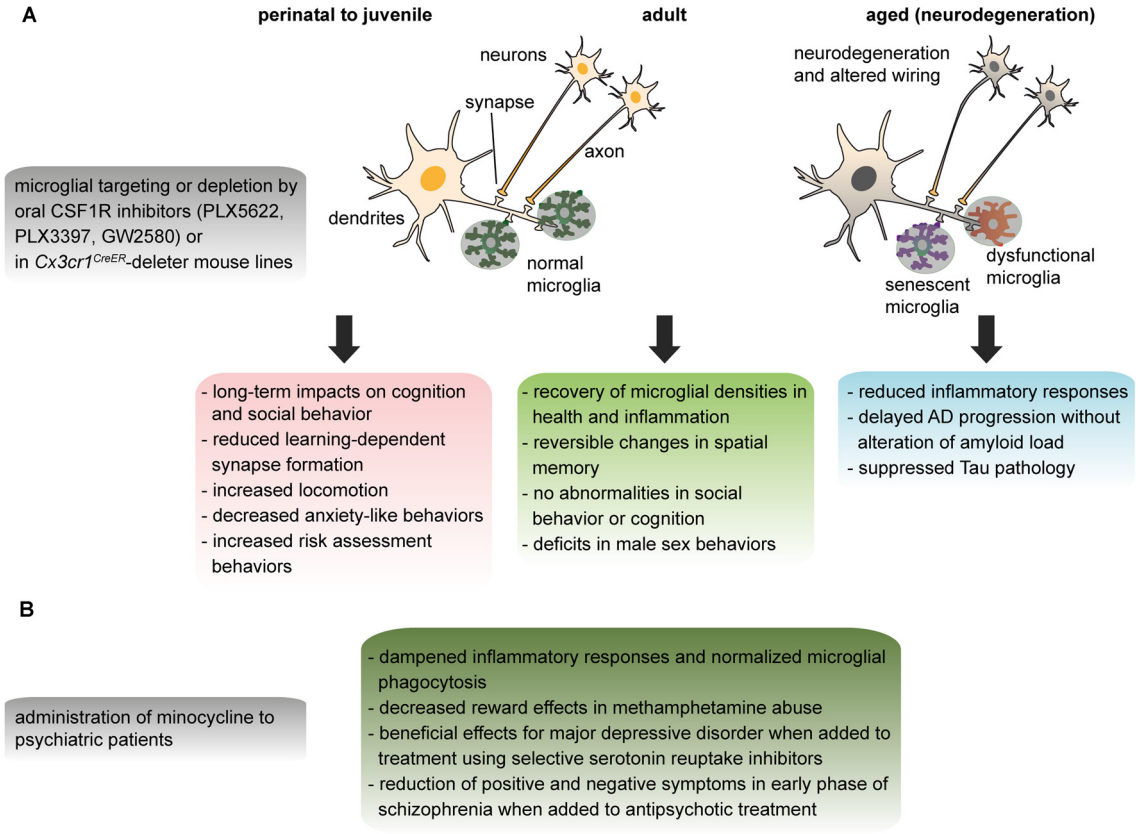

FIGURE 4 | Effects of microglial targeting in animal models and human patients discussed in this review. **(A)** Temporal effects of transient microglial depletion in rodents and nonhuman primates across the lifespan. **(B)** Potential clinical effects of minocycline on modulating the roles of microglia in adult psychiatric diseases.

phagocytosis (Plane et al., 2010; Garrido-Mesa et al., 2013; Mattei et al., 2014). Due to its excellent safety profile, it has been used alone or as an add-on treatment in numerous clinical trials for neurodegenerative diseases, and more recently, in psychiatric disorders, as we have discussed. Current clinical trials now couple the determination of brain inflammatory status (by PET imaging, combined with CSF or blood analysis), to minocycline response (e.g., clinical trial NCT02362529), in order to determine how levels of pro-inflammatory markers before the treatment could predict the clinical response. If the therapeutic effects of minocycline depend on its anti-inflammatory action, one would expect patients with an exacerbated inflammatory profile to be most responsive. Of note, even if minocycline does not target microglia specifically, it could indirectly modulate their physiological functions via astrocytes and vascular endothelial cells. Studies have demonstrated that astrocytes act under the control of reactive microglia in the context of inflammation (Pascual et al., 2012; Habbas et al., 2015; Liddelow et al., 2017).

Animal models, especially microglia-specific gene knockout strategies, have been particularly informative, considering that the readout of microglial pathophysiological alterations is not easily achieved in human subjects. For instance, several groups have taken advantage of the fractalkine receptor to specifically target microglia by crossing the conditional *Cx3cr1*^{creER} mouse lines (Goldmann et al., 2013; Parkhurst et al., 2013; Yona et al., 2013) with transgenics carrying genes of interest flanked by *loxP* sites in different disease paradigms (**Figure 4**). While the *Cx3cr1*^{creER} lines are exceptional tools for dissecting the specific roles of microglia in the brain, long-lived yolk sac-derived non-parenchymal brain macrophages may be partially targeted as collaterals, thus confounding the observations (Goldmann et al., 2016). To date a bona fide microglia-specific marker that labels all microglia, from the post-erythromyeloid progenitor stage up to old age, is still unavailable. Notably, a somatic mutation in the erythromyeloid progenitor of microglia was shown to cause neurodegenerative disease in mouse and human (Mass et al., 2017). However, as mentioned above, *Tmem119* and *Sall1* were shown to be selectively expressed by microglia across different contexts of health and disease (Bennett et al., 2016; Buttgereit et al., 2016). The impact of pharmacologically depleting microglia (Elmore et al., 2014; Torres et al., 2016; VanRyzin et al., 2016) was additionally explored in multiple preclinical animal studies (reviewed in Han et al., 2017; Lund et al., 2017; **Figure 4**). The current evidence suggest that a transient depletion during early postnatal and juvenile stages has a prolonged impact on cognition and social behavior (Parkhurst et al., 2013; VanRyzin et al., 2016), in contrast to the reversible alterations induced by adult treatment

with the selective CSF1R inhibitor PLX3397 (Elmore et al., 2014; Torres et al., 2016). Two independent investigations in AD mouse models, APP/PS1 (Olmos-Alonso et al., 2016) and 5xFAD (Spangenberg et al., 2016), further inhibited microglial proliferation or depletion using the CSF1R inhibitors GW2580 and PLX3397, respectively. Both studies concluded that the progression of AD pathology was delayed with an overall reduction of pro-inflammatory responses although amyloid β load remained unchanged. In contrast, a study that analyzed in mice the propagation of Tau protein revealed a suppression of Tau pathology when microglia were depleted using PLX3397 or intracerebroventricular infusion of clodronate liposomes, in a viral-driven neuron-specific expression of mutant human *Tau* transgene (Asai et al., 2015). Recently PLX3397 was also administered to two Rhesus macaques, in what is possibly the first attempt to deplete microglia in nonhuman primates, in order to understand the dynamics of their depletion in relation to CNS immune cell activation by PET imaging (Hillmer et al., 2017). TSPO binding suggested differential recovery of microglia after depletion in healthy vs. LPS-challenged macaques (Hillmer et al., 2017). Since these pharmacological compounds also target non-parenchymal CNS macrophages and other myeloid cells in the periphery, further characterizations of their effects based on brain compartments, gray vs. white matter, nature and stage of pathologies, and sex-dependency (VanRyzin et al., 2016; Labonté et al., 2017) are required.

CONCLUSION

Overall, the findings presented in this review indicate that microglia are increasingly implicated in the pathophysiology of various developmental and neurodegenerative psychiatric disorders, even though the exact mechanisms underlying this association are still undetermined. Critical physiological roles of microglia, including their secretion of cytokines and neurotrophins, phagocytosis, and interactions with synapses, regulate normal brain development, function and plasticity. Animal studies have revealed that abnormal neuronal and synaptic densities, impaired circuit wiring, and imbalance of excitation/inhibition, can result from altered neurogenesis or neuronal survival, impaired synaptic pruning, or altered levels of pro- and anti-inflammatory cytokines. These deficits and alterations were commonly reported in psychiatric disorders. In particular, environmental insults such as perinatal infection, early postnatal and chronic stress, were shown to compromise physiological microglial functions and induce permanent changes in the brain with direct consequences on mood and cognition. Furthermore, microglia could be primed by an early immunological challenge, rendering them more susceptible to a subsequent insult and favoring the emergence of psychiatric conditions later in life. Considering the brain region-dependent slow turnover of microglia, the accumulation of repetitive priming events such as infections and stress across a lifetime may further precipitate the age-dependent deterioration of their physiological functions, resulting in the onset of cognitive aging and neurodegenerative disorders. Due to their unique mesodermal origin, microglia are promising therapeutic targets to gain access to the CNS without directly modifying non-myeloid cell types of the CNS. To precisely characterize the microglial phenotypes implicated in different psychiatric disorders, improvement in selectivity of radiotracers for patient examinations is required. Finally, better characterization of the existing pharmacological compounds able to modulate microglial phenotypes may accelerate the implementation of effective therapies for psychiatric disorders.

AUTHOR CONTRIBUTIONS

TLT, AR and MET designed the review outline, recruited CB, ID, MKS and MSH, and supervised the overall project. All the authors contributed to the literature search and to the manuscript writing.

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MINI REVIEW
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The Blood-Brain Barrier and the EphR/Ephrin System: Perspectives on a Link Between Neurovascular and Neuropsychiatric Disorders

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Interactions among endothelial cells (EC) forming blood vessels and their surrounding cell types are essential to establish the blood-brain barrier (BBB), an integral part of the neurovascular unit (NVU). Research on the NVU has recently seen a renaissance to especially understand the neurobiology of vascular and brain pathologies and their frequently occurring comorbidities. Diverse signaling molecules activated in the near proximity of blood vessels trigger paracellular pathways which regulate the formation and stabilization of tight junctions (TJ) between EC and thereby influence BBB permeability. Among regulatory molecules, the erythropoietin-producing-hepatocellular carcinoma receptors (EphR) and their Eph receptor-interacting signals (ephrins) play a pivotal role in EC differentiation, angiogenesis and BBB integrity. Multiple EphR-ligand interactions between EC and other cell types influence different aspects of angiogenesis and BBB formation. Such interactions additionally control BBB sealing properties and thus the penetration of substances into the brain parenchyma. Thus, they play critical roles in the healthy brain and during the pathogenesis of brain disorders. In this mini-review article, we aim at integrating the constantly growing literature about the functional roles of the EphR/ephrin system for the development of the vascular system and the BBB and in the pathogenesis of neurovascular and neuropsychiatric disorders. We suggest the hypothesis that a disrupted EphR/ephrin signaling at the BBB might represent an underappreciated molecular hub of disease comorbidity. Finally, we propose the possibility that the EphR/ephrin system bears the potential of becoming a novel target for the development of alternative therapeutic treatments, focusing on such comorbidities.

Keywords: blood-brain barrier, EphR/ephrin, endothelial cells, astrocytes, neurovascular disorders, neuropsychiatric disorders

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INTRODUCTION

Structural integrity of the blood-brain barrier (BBB) is essential for the establishment and maintenance of brain homeostasis. Any disruption in its cellular or structural components may exert devastating effects on mental health. During the past couple of years, research on the neurovascular unit (NVU) has experienced a second revival. This highlighted the BBB as a potential novel target for the development of alternative treatment strategies for brain and vascular pathologies.

Structural Components and Function of the BBB

The BBB is a multicellular vascular structure separating the central nervous system from peripheral blood circulation (Obermeier et al., 2013). It is composed of cerebrovascular endothelial cells (EC) forming brain vessels, astrocytes and extracellular matrix (ECM) components providing structural support (Abbott et al., 2006). Pericytes are also relevant to form the BBB and their functional roles have been fully described elsewhere (Cabezas et al., 2014; **Figure 1**). Together, all these elements exert their functions as a selective physical (Abbott et al., 2006), transport (Begley and Brightman, 2003) and metabolic (Pardridge, 2003, 2016) barrier, tightly controlling the passage of molecules in and out of brain parenchyma and preventing the penetration of toxins or pathogens (Obermeier et al., 2013).

Endothelial Cells

Cerebral EC have a unique characteristic in comparison to peripheral EC: they are interconnected by continuous intracellular multiprotein complexes called tight junctions (TJs), which lack fenestrations and undergo extremely low rates of transcytosis (**Figure 1**). This limits paracellular passage of substances and directs molecular trafficking to take a rigorously controlled transcellular route across the BBB (Abbott et al., 2006). Such a strong physical barrier allows only small gaseous and lipophilic molecules to diffuse freely in and out of the brain, whereas bigger molecules need to be actively transferred via transporter/carrier systems, such as the glucose transporter-1 (GLUT-1) or the large neutral amino acid transporter-1 (LAT-1) located on the luminal (blood facing) or abluminal (brain facing) EC sites (Borst and Schinkel, 2013). Potentially harmful compounds like glutamate are actively cleared from the brain even against a concentration gradient requiring ATP as energy source (e.g., via excitatory amino acid transporter 1/2; EAAT1/2 (Hawkins and Viña, 2016). Generally, large hydrophilic molecules cannot be transferred across the BBB unless by specific receptor- or adsorptive-mediated transcytosis (Pardridge, 2003, 2016; Strazielle and Ghersi-Egea, 2016).

The TJ are key regulators of paracellular permeability and transendothelial electrical resistance. Major constituents of the TJ are transmembrane molecules like occludin (Yu et al., 2005), which links to the cytoskeleton via the accessory proteins zonula occludens (ZO-1/2) and claudins (Piehl et al., 2010), and junctional adhesion molecules (JAM-A, -B, -C, Mandel et al., 2012). During early embryogenesis, pre-existing vessels sprout and undergo angiogenesis (Obermeier et al., 2013). Sealing properties, including refinement of the protein complexes, establishment of efflux transporters and limitation of transcytosis, seems to only mature when sprouting vessels come in close contact with pericytes and astrocytes (Daneman et al., 2010; Obermeier et al., 2013). However, the role of astrocytes in this process is still a matter of controversy (see below). Afterwards, matured TJ are fixed and need to be maintained throughout life.

Astrocytes

Astrocytes regulate features of the BBB through the tips of their processes, called astrocytic endfeet, which surround and contact brain micro-vessels (Kettenmann and Verkhratsky, 2008). Among other functions, they regulate the ion balance around the BBB and secrete and recycle neurotrophic factors necessary to control TJ (Gee and Keller, 2005). A very elegant example of how astrocytes maintain the ionic homeostasis is represented by their synchronized spatial K^+ buffering at synaptic and BBB locations mediated by their perivascular and perisynaptic endfeet (Olsen and Sontheimer, 2008). This controls ion concentrations during normal brain activity and can thereby link and adapt responses of blood vessels to synaptic neuronal activity to guarantee the appropriate supply of oxygen and nutrients (Wolburg et al., 2011). Additionally, astrocytes secret several molecules such as the glia cell-derived neurotrophic factor (GDNF; Igarashi et al., 1999), transforming growth factor β (TGF-β; Dobolyi et al., 2012), angiopoietin 1 (ANG1; Easton, 2012), fibroblast growth factor 2 (FGF2; Reuss et al., 2003) and vascular endothelial growth factor (VEGF; Rosenstein et al., 2010) which act on EC to either promote TJ formation and/or regulate BBB permeability (**Figure 1**).

At present, it is still controversial whether astrocytes are necessary for the induction of TJ, because of the temporal shift between EC differentiation/maturation and astrocyte development. Recent work suggests that astrocytes are dispensable for the induction of TJ (Saunders et al., 2016), but are necessary for their further strengthening and maintenance throughout life (Alvarez et al., 2011, 2013). However, meningeal blood vessels which lack contacts with astrocytes display higher vascular permeability than EC-BBB, supporting indeed the necessity of astrocytes to induce BBB properties (Lécuyer et al., 2016).

Basement Membrane

The non-cellular component of the NVU is the basement membrane, which is composed of structural proteins such as collagen-IV, laminin and fibronectin, among others (Cardoso et al., 2010; **Figure 1**). The main function of the basement membrane is to provide stability to the other members of the NVU and regulate their crosstalk enabled by matrix transmembrane receptors like integrins and dystroglycans (Baeten and Akassoglou, 2011).

THE ERYTHROPOIETIN-PRODUCING-HEPATOCELLULAR CARCINOMA RECEPTORS (EphR) AND Eph RECEPTOR-INTERACTING SIGNALS (EPHRINS) SYSTEM

The erythropoietin-producing-hepatocellular carcinoma receptors (EphR)/Eph receptor-interacting signals (ephrin) system was first discovered in 1990, when ephrinA1 was characterized as a tumor necrosis factor (TNF)-inducible

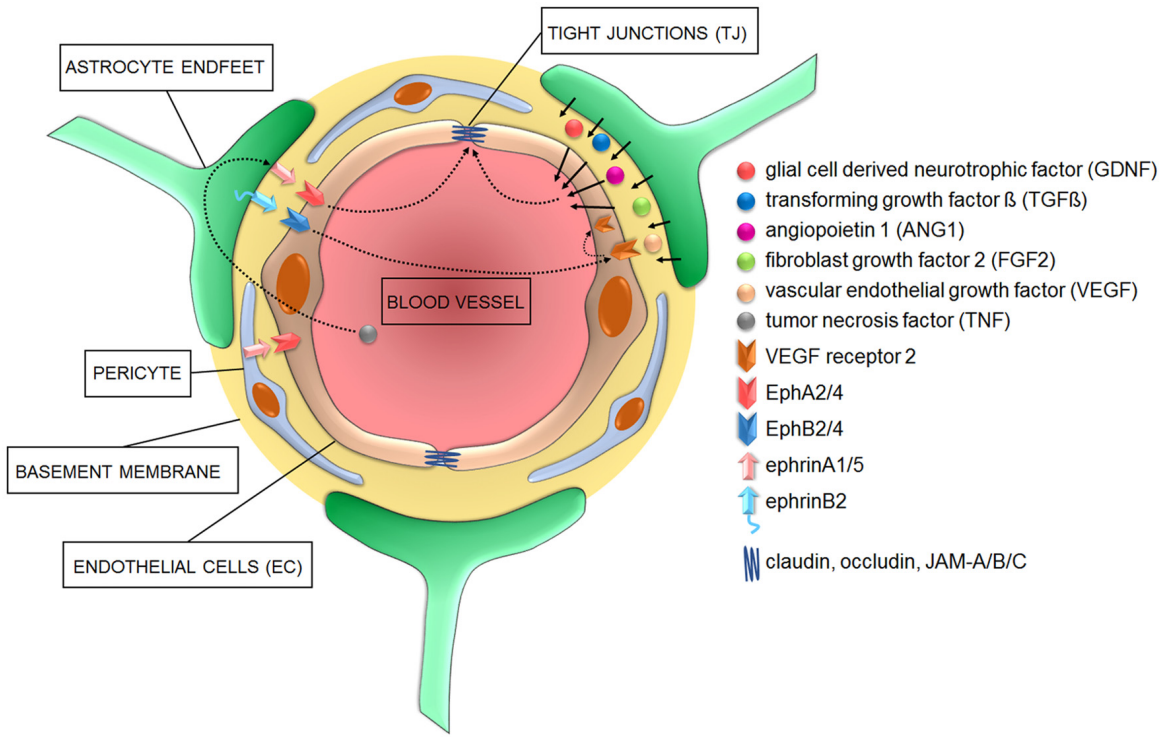

FIGURE 1 | Cellular and signaling components of the blood-brain barrier (BBB) in health conditions. Astrocyte-derived signaling molecules influence the development and/or maintenance of BBB properties. Additionally, various members of both "A" and "B" classes of the ephrin family of ligands located on either astrocyte and/or pericytes do activate EphA and EphB receptors on endothelial cells (EC) to influence EC differentiation during angiogenesis and the development of tight junctions (TJ) during barriergenesis.

protein in human umbilical vein EC (HUVEC; Holzman et al., 1990). EphRs/ephrins typically mediate contact-dependent communication between cells to control cell fates. During development, this system plays an important role in spatial organization, axon guidance, formation of synaptic connections and blood vessel remodeling. In the adulthood, it mostly regulates synaptic remodeling, epithelial differentiation, bone remodeling, immune function, insulin secretion and stem cell self-renewal (Kullander and Klein, 2002; Yamaguchi and Pasquale, 2004; Pasquale, 2005, 2008).

Eph receptors comprise the largest family of receptor tyrosine kinases (RTK). Eph receptors and ephrins can be divided into subclasses A and B. In humans, nine EphA and five EphB receptors are known. They consist of an extracellular part including a globular ligand-binding domain, a cysteine rich region two fibronectin type 3 repeats and a cytoplasmic domain comprised of a short juxtamembrane region with several conserved tyrosine residues, a sterile alpha motive protein-protein interaction domain and a C-terminal PDZ binding motif. Additionally, several alternatively spliced forms have been identified with distinct functions (Pasquale, 2010). Ephrins, on the other hand, can be further distinguished by their membrane attachment: ephrinAs are anchored via a glycosylphosphatidylinositol (GPI) linkage, whereas ephrinBs are attached via a single transmembrane domain containing a short cytoplasmic PDZ-binding motif. EphRs and ephrins can be expressed on the same cell, in mutually exclusive expression patterns or in complementary gradients, establishing a highly dynamic signaling system (Lisabeth et al., 2013).

The EphR/Ephrin System: Signaling Mechanisms

Besides the well-known bidirectional signaling activated upon cell-cell interactions, which is described in detail in some excellent reviews (Pasquale, 2008; Murai and Pasquale, 2011; Klein, 2012; Lisabeth et al., 2013), several alternative signaling mechanisms have been proposed for the EphR/ephrin system.

Upon receptor/ligand interaction, several downstream signaling cascades are activated to mediate cell adhesion or repulsion, depending on the type and abundance of ligands and receptors present on cell surfaces (Janes et al., 2012). These signaling pathways include, among others, the Src kinase family, mitogen-activated protein kinase, and integrin mediated pathways (Lackmann and Boyd, 2008; Pasquale, 2008; Pitulescu and Adams, 2010). Their activity is dependent on Rho family GTPases, including RhoA, Rac1, Cdc42 and a variety of guanine nucleotide exchange factors (GEF), like ephexins (Cowan et al., 2005; Pasquale, 2008). After the initial receptor/ligand interaction, intact

EphR/ephrin complexes together with potentially associated cytoplasmic proteins and the surrounding membrane are internalized in either cell. This Rac1-dependent mechanism is termed trans-endocytosis and provides a mechanism to switch between cell adhesion and retraction fates and to terminate receptor signaling activity (Lisabeth et al., 2013).

Besides trans-endocytosis, the activation of enzymes which initiate proteolytic cleavage represents another alternative signaling mechanism (Atapattu et al., 2014). Among such enzymes, A disintegrin and metalloproteases (ADAM) and matrix metalloproteases (MMP) are implicated in signal termination (Atapattu et al., 2014).

In mammalian tissues, members of the ADAMs family are transmembrane metalloproteases able to process and shed ectodomains of membrane bound receptors (Klein and Bischoff, 2011). They play crucial roles in pathological conditions such as inflammation or stress-mediated angiogenic responses (Weber and Saftig, 2012). Several EphRs/ephrins of both A and B subclasses can associate with ADAMs resulting in their own cleavage. Cleavage of the ligand-bound receptor leads to a breakdown of the molecular tethers between interacting cells, thereby favoring the internalization of receptor/ligand complexes, as exemplified by ADAM10 initiated cleavage of the EphA3/ephrinA2 complex during axon detachment (Hattori et al., 2000; Mancia and Shapiro, 2005) or of the EphA2/ephrinA1 complex (Salaita et al., 2010).

MMPs cleave proteins located either on membranes or in extracellular spaces (Miller et al., 2008). Their main function is to degrade structural components of the ECM to facilitate cell migration (Streuli, 1999), especially during angiogenesis and inflammatory processes (Kessenbrock et al., 2010; Palmisano and Itoh, 2010). Recently, it has been shown that MMPs cleave ephrinA1 and ephrinA2 from their GPI-anchor, leading to the release of functional soluble monomers which can act on distant Eph receptors (Beauchamp and Debinski, 2012). Followed by an initial shedding step mediated by ADAMs or MMPs, EphRs/ephrins can further be processed by intramembrane cleaving proteases such as γ-secretase (Bergmans and De Strooper, 2010) or neuropsin (Attwood et al., 2011; Morohashi and Tomita, 2013). This events generates cytoplasmic active fragments (Litterst et al., 2007; Xu and Henkemeyer, 2009) which may i.e., regulate behavioral responses such as anxiety (Attwood et al., 2011).

The signaling cluster propagation represents another noteworthy alternative signaling mechanism to be mentioned. This type of signaling, originally initiated by receptor/ligand interactions in *trans*, causes the formation of lateral clusters through receptor-receptor interactions in *cis*. These receptor clusters do no longer rely on ephrin interaction to get activated, enabling the strong amplification of an originally small signal generated by a first short cell-cell contact (e.g., EphA3/ephrinA5; Wimmer-Kleikamp et al., 2004).

Such signaling effectors of the Eph/ephrin system might become relevant in brain disorders to identify alternative targets for drug discovery.

The Role of the EphR/Ephrin System for the Development and Function of the Vasculature and the BBB

The interaction of specific cell types to properly develop the vascular system and a functional BBB is an essential process which requires the appropriate temporally- and spatially-regulated expression of distinct guidance cues. Among them, the EphR/ephrin system represents an ideal candidate to exert those functions.

During vasculogenesis, VEGF induces ephrinA1 expression which activates EphA2 on neighboring EC, thus exerting angiogenic effects—*in vitro* and *in vivo* (Cheng et al., 2002; Brantley-Sieders et al., 2004). Despite the previously mentioned controversy, astrocytes release VEGF during embryonic development and might therefore contribute to the early TJ formation. Later on in development, however, for the further differentiation of EC and formation of an efficient BBB, the inhibition of EphA2 activity in human brain micro-vessel EC (HBMEC) is instrumental to promote TJ strengthening (Zhou et al., 2011; **Figure 1**). These different functions mediated by the tightly controlled expression levels of EphA2 suggest that the regulation of EphA2 dosages may underlie the "switch" between early/angiogenic and late/barriergenic effects of EphA2 in EC. Moreover, they suggest that putative interactions between EphA2-expressing EC with ephrinA1-expressing perivascular astrocytes or pericytes may also control TJ formation in physiological conditions or their disruption during pathogenic processes (Lécuyer et al., 2016). In a different system, the pulmonary system, stimulation of arterial EC with ephrinA1 also increases their permeability (Larson et al., 2008), further supporting that the overexpression of certain EphR/ephrin interactions might influence barrier integrity, ultimately impacting brain homeostasis. Astrocytes express several other members of the EphR/ephrin system (Nestor et al., 2007) which may be relevant during both vasculogenesis and/or barriergenesis. For example, the proper interaction between EphA4/ephrinA5 located on EC and astrocyte endfeet, respectively, is necessary for the development of a normal vascular system in the hippocampus of adult mice (Hara et al., 2010). Additionally, radial glia cells provide a physical scaffold and chemical signals to support the very early stages of angiogenesis (Cheslow and Alvarez, 2016). Among such signals, EphA4 expressed on EC has been indicated to guide the invasion of the developing brain by newly forming micro-vessels in response to glial-dependent stimulation (Goldshmit et al., 2006).

With regard to the "B" types, ephrinB2 controls VEGF receptor (VEGFR)-2 internalization, which is necessary for receptor activation and VEGF-induced filopodial extension in EC during angiogenesis (Bochenek et al., 2010; Sawamiphak et al., 2010; Pitulescu and Adams, 2014). During these events, the role of the EphB2/ephrinB2 interaction is essential for blood vessel assembly (Foo et al., 2006). During cardiovascular development, EphB4/ephrinB2 signaling in EC is additionally activated to properly specify arterial vs. venous identity (Wang et al., 1998; Adams et al., 1999; Gerety et al., 1999; Gale et al., 2001; Augustin and Reiss, 2003).

DISRUPTED INTEGRITY OF THE BBB AND THE Eph/EPHRIN SYSTEM—THE "MISSING LINK" BETWEEN NEUROVASCULAR AND NEUROPSYCHIATRIC PATHOLOGIES?

BBB leakiness is a hallmark of neurovascular pathologies comorbid with neuroinflammatory processes (Lee et al., 2009; Abbott and Friedman, 2012). Recent work has also evidenced signs of BBB leakiness in neuropsychiatric disorders, which are also accompanied by elevated levels of blood-circulating pro-inflammatory cytokines and TNF (Miller et al., 2009; Janelidze et al., 2011; Liu et al., 2012; Salim et al., 2012; Najjar et al., 2013). Furthermore, preclinical and clinical studies have evidenced a wide range of comorbidity between neurovascular and neuropsychiatric disorders with concurrent neuroinflammation (Dantzer et al., 2008; Wood, 2014; Hodes et al., 2015; Patel and Frey, 2015; Seligman and Nemeroff, 2015; Miller and Raison, 2016; Barnes et al., 2017; Menard et al., 2017), thereby suggesting that common neurobiological substrates may underlie such high degrees of comorbidities. In view of the regulatory roles of the EphR/ephrin system during the development and maturation of a proper BBB sealing properties, it appears evident how this system might be considered a hub of brain disorders associated with BBB disruption.

Preclinical studies indicated that, among "A" members of the EphR/ephrin system, especially the EphA2 receptor mediates inflammation during injury, ischemia and other chronic inflammatory conditions in various murine models of neurovascular disorders (Jellinghaus et al., 2013; Thundyil et al., 2013; Ende et al., 2014). Specifically, EphA2 receptor activation occurs after brain injury and contributes to inflammation by promoting BBB permeability (Thundyil et al., 2013). Interestingly, the promoter of ephrinA1, the highest affinity ligand for EphA2, is a target of the pro-inflammatory marker TNF (Ende et al., 2014). Furthermore, whereas TNF has angiogenic properties during early embryogenesis (Cheng and Chen, 2001; Munthe and Aasheim, 2002), it triggers BBB hyperpermeability in adult epithelial tissues via the activation of both EphA2 and EphA4 in EC, with induction or exacerbation of neurovascular disorders (Jellinghaus et al., 2013; Thundyil et al., 2013; Ende et al., 2014; **Figures 1**, **2**). These evidences suggest that investigating the EphA2/ephrinA1 bidirectional signaling between interacting cellular partners at the BBB may reveal novel molecular triggers of comorbidity between inflammatory/neurovascular/neuropsychiatric disorders and indicate alternative targets of

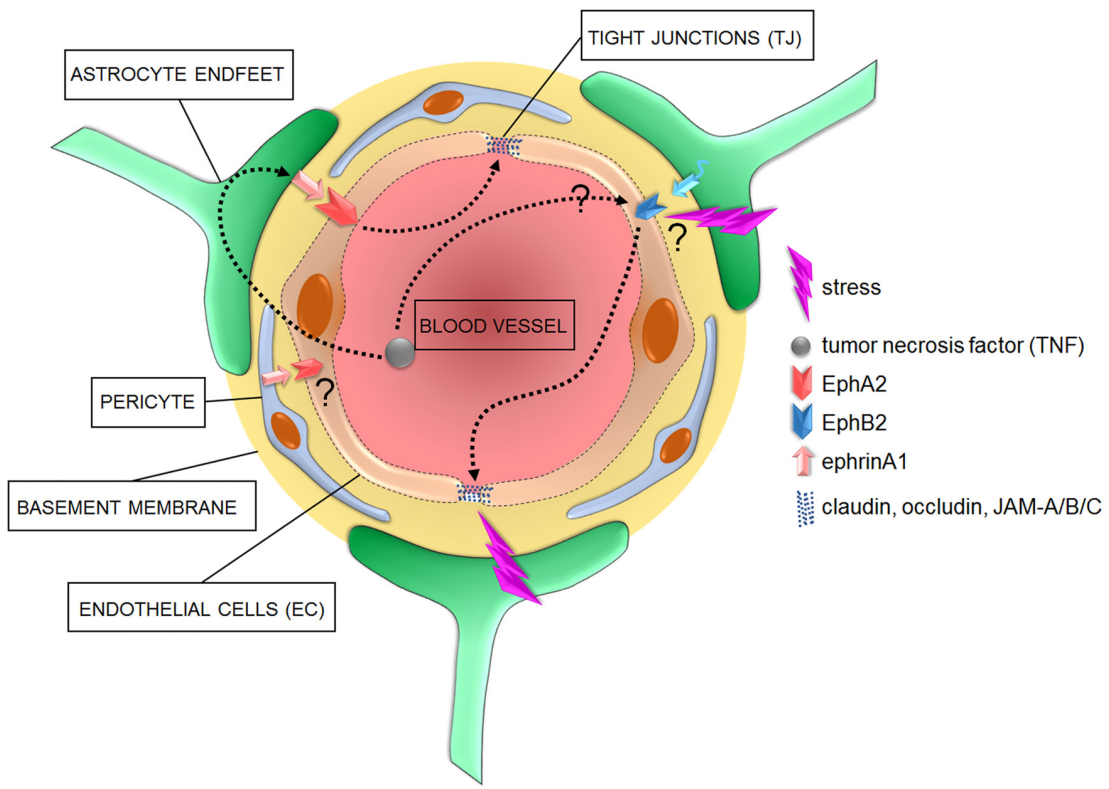

FIGURE 2 | Cellular and signaling components of the BBB in pathological conditions. In diseased brains, the overactivation of astrocyte- or pericyte-dependent ephrin signaling may affect TJ via an increased activity of Eph receptors, with consequent increased barrier permeability to blood circulating inflammatory factors such as tumor necrosis factor (TNF). Increased stress has also been shown to correlate with a compromised barrier functionality and impaired TJ which might further induce the onset of neurological and neuropsychiatric disorders. However, specific molecular mediators of such effects are yet to be identified.

therapeutic interventions. Among other candidates, although in a specific subset of neuropsychiatric disorders, it was shown that the binding between ephrinA5 expressed on astrocytes and its corresponding EphA4 receptor on EC is increased in the hippocampus of a mouse model of temporal lobe epilepsy, which shows an increased development of microvessels with detrimental consequences on brain homeostasis (Shu et al., 2016). Interestingly, the selective blockade of the EphA4/epHRin interaction is sufficient to attenuate the disease phenotype, further supporting the therapeutic relevance of a selective targeting of the EphR/ephrin system for neurovascular/psychiatric disorders.

Regarding the "B" members, a link between TNF and EphB2 has also been suggested to be relevant to induce inflammatory pathways (Pozniak et al., 2014; **Figure 2**). It has also been shown that EphB2 activity regulates cognitive functions and resilience or vulnerability to stress (Yuferov et al., 2013; Zhang et al., 2016). Among triggers of neuropsychiatric disorders, stress is one of the most detrimental (Charney and Manji, 2004). In line with this, as little as 2 days of stress provokes morphological changes in EC, accompanied by dysregulation of claudin-5 and occludin expression (**Figure 2**). These changes are paralleled by a decreased expression of GFAP, indicating an additional negative impact on astrocytes (Sántha et al., 2016). A clinical and a preclinical study also confirmed an astrocyte impaired phenotype, with reduced coverage of blood vessels by AQP-4-enriched astrocyte endfeet, in the prefrontal cortex of depressive patients and of a validated animal model of depression (Rajkowska et al., 2013; Di Benedetto et al., 2016). Moreover, a new study has clearly demonstrated the detrimental effects of stress on the permeability of BBB, with a reduction in its sealing properties (Menard et al., 2017). It would be interesting to investigate whether EphB2 might represent a molecular link between the above-mentioned stress-dependent changes in specific cellular components of the BBB, in its sealing properties and the modulatory effects on behavioral phenotypes.

A proof-of-principle that the EphR/ephrin system might represent a highly relevant therapeutic target for comorbid neurovascular and neuropsychiatric disorders has been provided by the controlled reactivation of EphB4/ephrinB2 in cardiovascular disorders, which enhanced BBB repair mechanisms (Ghori et al., 2017). This approach sounds promising to rescue BBB deficits and may putatively be beneficial to reverse comorbid maladaptive behavioral phenotypes.

CONCLUSION

Although several studies provide evidence for a compromised BBB integrity in a broad variety of psychopathologies, it is still unknown whether the BBB disruption is a cause or a consequence of the disease. Depending on the circumstances, a transient opening of the BBB might even be beneficial, e.g., during inflammation, it may allow the passage of growth factors or antibodies to hinder the inflammatory process. On the other hand, tightening of the BBB appears necessary during periods of stress or hypoxia (Abbott et al., 2006).

Investigating the expression of EphR/ephrin system in the single components of the BBB during vasculogenesis and barriergenesis as well as their interplays in health and in the pathogenesis of brain and neurovascular disorders might open new avenues to understand neurobiological underpinnings of pathological comorbidities. This may help to identify novel therapeutic targets especially beneficial for comorbid patients.

AUTHOR CONTRIBUTIONS

VAM and BDB contributed to literature screening and drafting the manuscript; both authors read and approved the final version of the manuscript.

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Intracerebroventricular Administration of ^{192}IgG-Saporin Alters Expression of Microglia-Associated Genes in the Dorsal But Not Ventral Hippocampus

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One of important aspects of development of Alzheimer's disease is degeneration of septal cholinergic neurons that innervate the hippocampus. We took advantage of widely used model of cholinergic deficit in the hippocampus, intracerebroventricular administration of ^{192}IgG-saporin (Ig-saporin), to analyze the postponed consequences of cholinergic deficit in different parts of the hippocampus. We studied effects of the immunotoxin on the behavior of rats and gene expression in the dorsal and ventral hippocampus using RNA-seq approach. We found that under normal conditions dorsal and ventral parts of the hippocampus differ in the expression of 1129 protein-coding genes and 49 non-coding RNAs (ncRNAs) and do not differ in the expression of 10 microRNAs, which were detected in both parts of the hippocampus. Ig-saporin-induced degeneration of cholinergic septal neurons did not affect rat behavior in open field, T-maze, and passive avoidance task but impaired memory retention in Morris water maze. To analyze ^{192}Ig-saporin-induced changes in the gene expression, we formed the following groups of genes: genes expressed exclusively in certain cell types (neurons, astrocytes, microglia, oligodendrocytes, and vascular cells) and, among universally expressed genes, a group of genes that encode ribosome-forming proteins. For all groups of genes, the alterations in the gene expression produced by the immunotoxin were stronger in the dorsal as compared to the ventral hippocampus. We found that, among groups of universally expressed genes, Ig-saporin increased the expression of ribosome-forming proteins in both dorsal and ventral hippocampus. Ig-saporin also strongly upregulated expression of microglia-specific genes only in the dorsal hippocampus. A subset of affected microglial genes comprised genes associated with inflammation, however, did not include genes related to acute inflammation such as interleukins-1b, -6, -15, and -18 as well as TNF. The expression of other cell-specific

genes (genes specific for neurons, astrocytes, oligodendrocytes, and vascular cells) was unaffected. The data obtained suggest that disturbance of memory-associated behavior after administration of Ig-saporin is associated with upregulation of microglia-associated genes in the dorsal but not ventral hippocampus.

Keywords: ^{192}IgG-saporin, RNA-seq, dorsal hippocampus, ventral hippocampus, microglia, rat, behavior

INTRODUCTION

Alzheimer's disease (AD) is a devastating disease which strongly reduces cognitive abilities. Hundreds of studies on the mechanisms of development of this disease revealed some critical processes that occur during AD; however, there is no clear understanding which factors are the most critical in pathology development. Currently, it is known that AD is associated with accumulation of insoluble beta-amyloid plaques in the brain; however, accumulation of these plaques does not always correlate with development of AD (Herrup, 2015). It is also known that AD is associated with accumulation of neurofibrillary tangles of hyperphosphorylated tau (Grundke-Iqbal et al., 1986; Takashima, 2013). It was also shown that AD causes strong alterations in the expression of genes (Tan et al., 2010; Berchtold et al., 2014; Wang et al., 2016) but the key factors that induce these changes are not clear.

Another important event that occurs in AD is degeneration of cholinergic neurons in the basal nuclei including septal neurons that form inputs to the hippocampus (Francis et al., 1999). Since neurons of the septum and diagonal band of Broca (DBB) are the only source of cholinergic innervation in the hippocampus, degeneration of these cells results in a strong decrease in the functioning of all acetylcholine-dependent systems. It was shown that cholinergic septal input to the hippocampus regulates excitability and oscillatory activity of hippocampal network, and its degeneration impairs hippocampal network activity [reviewed in (Teles-Grilo Ruivo and Mellor, 2013)]. However, network activity is determined not only by architecture and functioning of synapses but also by intrinsic properties of all cells that form and support network, including glial and endothelial cells. So far, there is no evidence that, in addition to its effect on hippocampal network activity, long-term cholinergic deficit may also alter the expression of genes critical for normal functioning of hippocampal cells. It was shown that activity of metabotropic acetylcholine receptors may regulate expression of some genes (Von Der Kammer et al., 1999; Albrecht, 2000); hence, it is possible to hypothesize that disruption of cholinergic innervation of the hippocampus may alter expression of genes that are critical for functioning of hippocampal cells. To analyze consequences of cholinergic deficit in the hippocampus at the mRNA level, we used a well-described model of induction of cholinergic deficit in the hippocampus – intracerebroventricular (i.c.v.) injection of immunotoxin ^{192}IgG-saporin (Ig-saporin) which causes selective degeneration of cholinergic neurons in the septum and DBB (Heckers et al., 1994).

^{192}IgG-saporin is a conjugate of antibody to NGF receptor, which is predominantly expressed on cholinergic septal neurons, and a ribosomal toxin saporin, which after penetration into a cell causes irreversible damage of ribosomes and cell death. Ig-saporin-induced degeneration of cholinergic neurons is associated with development of cognitive deficit in different behavioral tasks (Waite et al., 1995; Calza et al., 2003; Janisiewicz et al., 2004; Jeong et al., 2014; Knox and Keller, 2016).

The majority of previous studies on the effects of Ig-saporin was predominantly focused on the analysis of behavioral or electrophysiological consequences of degeneration of cholinergic neurons (Kuczewski et al., 2005; Kanju et al., 2012). Only a few studies analyzed effects of Ig-saporin on the expression of genes in the neocortex and hippocampus (Paban et al., 2010, 2011a). However, the analysis performed in these studies did not take into account specific features of different parts of the hippocampus. Here, we studied changes caused by Ig-saporin in the gene expression in the dorsal and ventral hippocampus of rats using RNA-seq approach. We analyzed ventral and dorsal parts of the hippocampus separately since these parts of hippocampus strongly differ in the expression of a large number of genes (Fanselow and Dong, 2010; Cembrowski et al., 2016) and involved in different types of learning (Moser et al., 1995; Fanselow and Dong, 2010). For instance, dorsal part mediates spatial navigation and memory formation while the ventral part is involved in anxiety and affective responses. Furthermore, these parts of the hippocampus receive cholinergic innervation from different structures (Fanselow and Dong, 2010). Using currently available databases on cell-specific expression of genes in the brain (Zhang et al., 2014; Zeisel et al., 2015), we analyzed cell-specific changes produced by i.c.v. injection of Ig-saporin in the ventral and dorsal parts of the hippocampus.

MATERIALS AND METHODS

The experiments were performed with adult male Sprague-Dawley rats (250–350 g) received from Research Center of Biomedical Technology RAMS, nursery "Pushchino." A total of 16 rats were involved in the study ($n = 8$/group). Animals were housed under standard vivarium conditions at 21 ± 1°C with a 12 h light/dark cycle, food and water were provided *ad libitum*. All experiments were performed in accordance with the ethical principles stated in the EU Directive 2010/63/EU for animal experiments and were approved by the Ethical Committee of the Institute of Higher Nervous Activity and Neurophysiology of the Russian Academy of Sciences.

Stereotaxic Surgery and Drug Administration

Rats were anesthetized with chloral hydrate (400 mg/kg, i.p.) and mounted in a Kopf stereotaxic frame. Since there is a great

variability in the Ig-saporin doses that induce substantial loss of septal cholinergic neurons, we performed pilot experiments where Ig-saporin was injected at doses 0.5, 1, 2, and 4 μg/site. In our hands, only a dose of 4 μg/site induced a strong loss of cholinergic neuron in the septum. Rats received bilateral i.c.v. infusions of saporin (4 μg/site). Hamilton syringe (Hamilton Company, United States) was lowered into the ventricle (0.8 mm posterior, 1.5 lateral to bregma). Drug (4 μl/site) was infused by a microinfusion pump (Stoelting Co., United States) at a rate of 0.2 μl/min. Rats were allowed to recover for 21 days after the surgery. The sequence of behavioral testing was as follows:

(i) days 21–31, Morris water maze; (ii) day 32, open-field test; (iii) days 33–37, T-maze; (iv) days 38–39, passive avoidance (PA) training. The samples of brain tissue were collected on day 41.

Morris Water Maze Testing (Days 21–31)

The water maze consisted of a circular pool (diameter 1 m; height 60 cm) filled with water at a temperature of 22–24°C up to a height 30 cm. It was virtually divided in four equal quadrants identified as north (N), east (E), south (S), and west (W). A platform (10 × 10 cm) was placed in the center of one of the quadrants and was 1.5–2 cm under the water surface. For each trial, a rat was placed at a starting point in the pool facing the wall. After each trial, rats were placed under a heating lamp to prevent hypothermia.

Testing procedure involved two daily trials for 60 s. For each trial, the rat was given a maximum of 60 s to reach the hidden platform, which was located in the center of the SW quadrant. The pseudorandom order of the two possible starting points was changed from day to day. When the rat climbed on the platform, it was allowed to stay there for 10 s. When a rat failed to find the platform within 60 s, it was guided to it and allowed to stay there for 10 s. Inter-trial interval was about 30 min for each rat. On day 31, the platform was removed from the pool and all the rats were given a 60-s probe trial. This delayed probe trial is used to evaluate the strength and precision of an established spatial memory by the amount of the time spent in the target quadrant. The following parameters were recorded: the latency to reach the platform, total distance moved, the swimming velocity, and tracks. Behavior was recorded automatically using Ethovision Software (Noldus, Wageningen, Netherlands).

Open-Field Test (Day 32)

Exploratory and emotional behavior was studied in a round open field. This arena (diameter of 1 m) was surrounded with a wall (height 50 cm). The arena was divided in three equal concentric zones (central, mid, and peripheral). Each rat was placed in the center of the arena and the rat's behavior was recorded automatically for 5 min under red light using Ethovision software (Noldus, Wageningen, Netherlands). The following behavioral variables were quantified: total distance moved (centimeter), velocity (centimeter/seconds), movement frequency, total movement duration (seconds), vertical activity (rearings), grooming, and number of entries to the center of the arena.

T-Maze Training (Days 33–37)

The T-maze apparatus consisted of a central arm (20 × 10 cm) and two arms (30 × 10 cm). On the first day of experiment rats were placed into the central arm and allowed to move freely for 20 min for habituation. After the habituation period, the rats were food-deprived. The next 4 days the rats were released from the starting position and the time taken to reach the goal compartment (containing a reward of several food pellets) was measured, as well as the number of wrong entries. Testing procedure involved five daily trials of 60 s. For each trial, the rat was given a maximum of 60 s to reach the goal compartment. Inter-trial interval was about 30 min for each rat. To avoid odor cues, the maze was properly cleaned with 70% ethanol between trials.

Passive Avoidance (PA) Training (Days 38–39)

The test apparatus (OpenScience, Russia) consisted of a plastic box divided into two equal compartments (30 cm × 30 cm × 30 cm): one was white-colored and brightly illuminated and the other one was black-colored and dark. The two compartments were not separated by door.

During the first trial, rat was placed into the light compartment and allowed to move freely between the two parts of the chamber for 5 min (habituation trial). After the habituation trial, the rat was placed into the same chamber (acquisition trial), behavioral conditions were similar to the habituation trial but entry into the dark compartment was paired with a 10-s electric shock (0.5 mA) provided through the metal grid covering the floor of the test camera.

After the shock, rat was immediately removed from the apparatus. In 24 h, during the retention trial, no foot shock was given and the step-through latency was recorded as a measure of retention.

Tissue Preparation for Analysis

Rats were anesthetized with chloral hydrate (400 mg/kg, i.p.) 1.5 month after the injection. They were then submitted to intracardiac perfusion with ice-cold 0.9% NaCl. The brains were removed and the frontal part of the brain, which contains septum, was dissected and post-fixed in 4% paraformaldehyde (Sigma–Aldrich, United States) solution in 0.1 M PBS (Biolot, Russia) for at least 2 days. The rest of the brain was cut along the midline and, in the left half, we isolated dorsal and ventral parts of the hippocampus (the hippocampus was cut into three equal parts, the middle part was discarded, the other parts were considered as dorsal and ventral, respectively) and took them for further analysis. Hippocampal samples were immediately transferred to vials and frozen in liquid nitrogen.

Immunohistochemistry and Cell Count

The full extent of the septum was sectioned at 50-μm-thick coronal brain sections using a vibrating blade microtome

(Leica VT1200 S, Germany). Six sections from the septum area were selected according to a random sampling scheme and stained for ChAT using a 2-day protocol. In brief, slices were incubated in 0.3% triton X-100 (SERVA, Germany) in 0.01 M PBS (PBS-T) three times for 5 min at room temperature, then for 1 h in blocking solution consisting of 5% normal goat serum (Sigma–Aldrich, United States) in PBS-T and later in blocking solution with primary antibodies (rabbit anti-Choactase 1:500, Santa Cruz Biotechnology, United States) at 4°C overnight. The next day, sections were washed in PBS-T and incubated with secondary antibodies (1:800, goat anti-rabbit IgG-biotin, Sigma–Aldrich, United States) in blocking solution at room temperature for 1 h. After additional washing in PBS, the sections were incubated with avidin–biotin–HRP complex (ABC Elite kit, Vector Labs, United States) for 1 h, and 3,3′-diaminobenzidine (SIGMA-Fast Kit, Sigma–Aldrich, United States) was used as a chromogen for development of staining. All images were acquired with a Leica DM6000B microscope (Leica, Germany) or Keyence BZ-9000 (Keyence, Japan). Imaging parameters were set to avoid signal saturations.

Delineation of brain structures was performed in images according to Paxinos and Watson atlas (Paxinos and Watson, 1998). The first section was randomly chosen at +1.2 mm from bregma and then, six sections located 150 μm from each other were chosen for analysis. All stained cell within the medial septum and DBB profiles were counted. Only cells located within one focal plane were counted in order to prevent overestimation. The mean number of cells per section was counted and was considered as a representative value for one animal.

RNA-Seq

Total RNA was isolated from the hippocampal samples using ExtractRNA Reagent (Evrogen, Russia) following manufacturer's protocol. Before preparation of libraries, total RNA in the samples was analyzed using an Agilent 2100 Bioanalyzer to confirm purity of RNA isolation and the absence of RNA degradation. In all samples, RIN > 8. For RNA-seq analysis, we took four rats from the control and Ig-saporin-treated groups of animals.

For depletion of ribosomal RNA, we used a NEBNext® rRNA Depletion Kit (Human/Mouse/Rat) in accordance with manufacturer's protocol. Then we prepared cDNA libraries using an Ion Total RNA-Seq Kit v2 for Whole Transcriptome Libraries (Ion Torrent, Life Technologies) following manufacturer's protocols. After mRNA fragmentation, mRNA was purified using magnetic beads (Magnetic Bead Cleanup Module, Ion Torrent, Life Technologies). Concentration of fragmented RNA was measured using a NanoDrop 2000.

For reverse transcription, RNA was ligated with probes complementary to the primer used for reverse transcription (Ion RT Primer v2) and, then, reverse transcribed with a SuperScript® III Enzyme Mix. The obtained cDNA was purified using a Magnetic Bead Cleanup Module. To amplify purified cDNA, we performed PCR using a Platinum® PCR SuperMix High Fidelity Mix with forward barcode-primers from a Ion Xpress™ RNA-Seq Barcode 01-16 Kit and reverse primer Ion Xpress™ RNA 3′-Barcode Primer. The number of PCR cycles was maximum shown in manufacturer' protocol. Amplified cDNA was purified using Magnetic Bead Cleanup Module. The quality of each prepared cDNA library was evaluated using NanoDrop 2000, Qubit® 2.0 Fluorometer (with Qubit® dsDNA HS Assay Kit), and Agilent 2100 Bioanalyzer (with High Sensitivity DNA Chip and Agilent High Sensitivity DNA Kit). In our samples, amount of short cDNA fragments with length of 25–160 bp did not exceed 10%.

During the next step, we performed clonal amplification using an Ion PI™ Template OT2 200 Kit v3 and an Ion OneTouch™ 2 System (Life Technologies) in accordance with manufacturer's recommendations. After amplification, the samples were centrifuged, and precipitates were resuspended in bidistilled water and kept at +4°C for 14–18 h.

Sequencing was performed using an Ion PI™ Sequencing 200 Kit v3 and an Ion PI™ Chip v2 on an Ion Proton™ sequencer. One chip contained four libraries with different barcodes.

Raw reads were mapped on transcriptome of rat genome Rnor 6.0 version by using BWA aligner (Li et al., 2009). Raw read counts were evaluated by SAMtools software (Li et al., 2009). Set of differentially expressed (DE) genes were estimated by DeSeq2 (Love et al., 2014).

List of genes that are selectively expressed in different cells types was created on the basis of data of single cell RNA-seq (Zhang et al., 2014; Zeisel et al., 2015). Genes that were not included in cell-specific sets (i.e., were expressed in more than one subpopulation of brain cells) were marked as universal. Three subgroups of genes were selected among the universal genes according to cholinergic synapse and ribosome reference pathways from KEGG database. DE genes list was analyzed for subsets of genes specific to tissues and specific to selected pathways. In this type of analysis, DE genes were selected if adjusted *P*-value of DeSeq2 test <0.05.

Raw read data were published to SRA and can be accessed by using range of accession numbers SRR5750530–SRR5750542.

Statistical Analysis

All data are presented as mean ± SEM. Across groups of behavioral data, statistical significance between means was determined using a factorial repeated measures analysis of variance (ANOVA) followed by Fisher's LSD *post hoc* test to reveal group differences on separate time intervals. The differences in behavioral activities of the rats in the round open fields, T-maze, and PA test were tested with Student's *t*-test. *P*-values < 0.05 were considered as statistically significant.

RESULTS

Effects of Intracerebroventricular Injection of Ig-Saporin on the Behavior of Rats and Expression of Choline Acetyltransferase in Cells of the Medial Septum

In order to estimate the effects of Ig-saporin on brain functioning, the control and toxin-treated animals were trained to perform spatial navigation task in the Morris water maze. Averaged

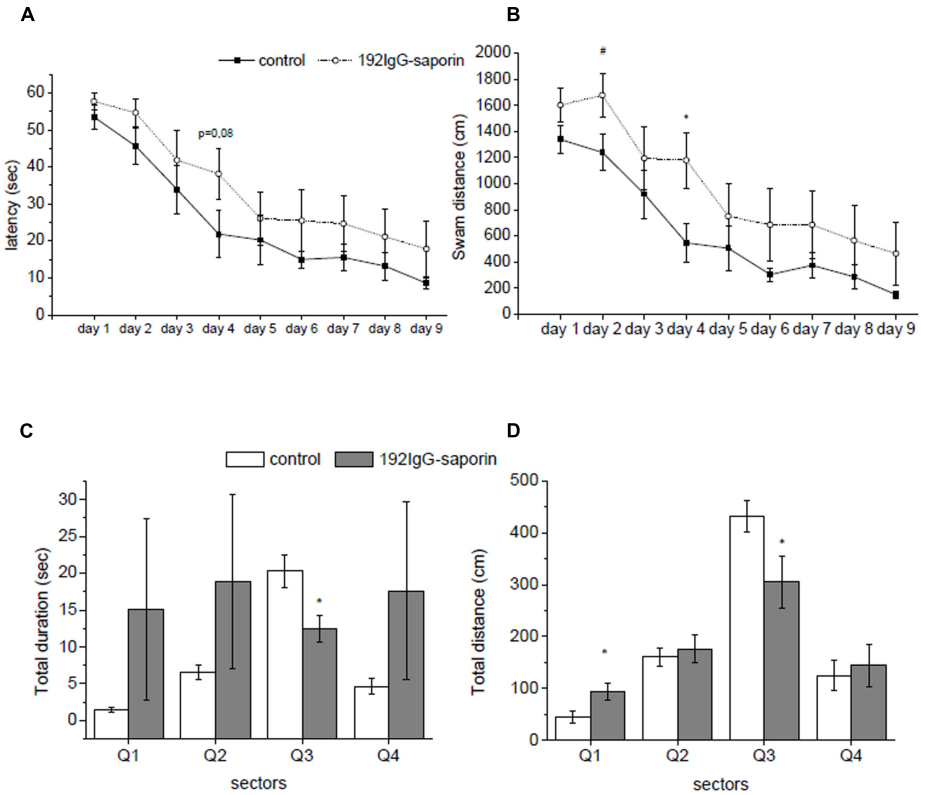

FIGURE 1 | The effects of intraventricular injection of ^{192}IgG-saporin (Ig-saporin) on spatial memory in Morris water maze task. Ig-saporin injected rats (n = 8) had longer latencies to reach the platform (seconds) **(A)** and higher distance swam (centimeter) **(B)** compared to control (n = 8) during the 9 days when the animals learned to find platform. On day 10 of testing, during probe trial saporin-treated rats (n = 8) spent significantly less time in a quadrant, where the platform was during training **(C)**, and swam shorter distance in it compared to the control (n = 8) **(D)**. Each point represents the mean ± SEM, *$P < 0.05$.

latency to find a hidden platform within the training period was analyzed. Although the animals exhibited a significant decrease in the latency to find a platform from Day 1 to Day 9 of training ($F_{8,112} = 20.35$, $P < 0.001$ according to ANOVA with repeated measures), the effect of lesion was not significant (group effect $F_{1,14} = 3.5$, $P = 0.08$) (**Figure 1A**). However, the animals from the immunotoxin-treated group exhibited a strong trend to swim longer distance to reach the platform as compared to the control group (group effect $F_{1,14} = 4.03$, $P < 0.06$), and this distance decreased during the training period in both groups studied ($F_{8,112} = 20.5$, $P < 0.001$) (**Figure 1B**). Furthermore, the immunotoxin-treated rats tended to swim more rapidly as compared to the control animals (group effect $F_{1,14} = 1.9$, $P = 0.19$) probably indicating their hyperactivity under the stressful conditions of maze training. Interestingly, we did not reveal any significant interactions between group × day factors for any of indices of learning in the water maze.

A closer look to behavioral patterns that were exhibited by rats during training revealed two time periods. During first 5 days rats learned new skill, and during the rest of training period they just reproduced known skill, and analysis of data related simultaneously to both periods may obscure effect of the immunotoxin. To evaluate whether Ig-saporin influenced learning processes, we analyzed the data for the first 5 days separately. We found that from day 1 to day 5 of training the animals exhibited a significant decrease in the latency to find a platform ($F_{4,56} = 14.7$, $P < 0.001$ according to ANOVA with repeated measures) and the effect Ig-saporin was insignificant (group effect $F_{1,14} = 3.1$, $P = 0.1$). However, in contrast to the above data on the period of 10 days, analysis of behavior during first 5 days showed that the Ig-saporin-treated animals swam longer distance to reach the platform as compared to the control group (group effect $F_{1,14} = 5.44$, $P < 0.05$). Furthermore, the immunotoxin-treated rats swam more rapidly as compared to the control animals (group effect $F_{1,14} = 4.3$, $P = 0.057$) and this may explain why the latencies were similar in these two groups.

On day 10, a probe trial without platform was performed in order to test whether the animals established long-term memory on its previous location. We found that immunotoxin-treated rats spent significantly shorter period of time in the maze quadrant, where the platform was located during training ($P < 0.01$, **Figure 1C**), and swam shorter distance within the target quadrant ($P < 0.05$, **Figure 1D**) as compared to the control animals. These data show that in spite of the minor differences in the indices of learning observed during training the rats of the Ig-saporin group could not form memory on platform location in the pool.

We also evaluated locomotor activity of rats in the "open-field" test. We found that locomotor and exploratory activity did not

differ between the experimental groups. Next, we studied learning abilities of animals in a T-maze where rats learned to find positive reinforcement. The only significant difference between lesioned and control rats was a decrease in the time spent by lesioned animals to leave the start corridor ($P < 0.05$, Mann–Whitney U-test, **Supplementary Figure S1**), which was observed during the first training day. Except this difference, analysis of animal behavior in this task did not reveal any significant differences between lesioned and control rats.

The last behavioral test that we used in our study was PA training. We found that Ig-saporin induced a trend to a decrease in the step-through latency as compared to the control animals (109.4 ± 45.3 s in Ig-saporin-treated rats vs. 160.9 ± 52.8 s in the control $P < 0.5$) (**Supplementary Figure S2**).

We found that administration of the immunotoxin caused a significant decrease in the number of ChAT-positive neurons in the septum and DBB (**Supplementary Figure S3**). Taken together, our data on behavioral and immunohistochemical experiments suggest that Ig-saporin induced mild memory impairments, which were probably associated with loss of cholinergic neurons of the medial septum/DBB complex.

Transcriptomic Analysis of Effects of Ig-Saporin on the Gene Expression in the Ventral and Dorsal Hippocampus

We performed RNA-seq followed by differential expression analysis to detect genes whose expression was altered in the dorsal and ventral parts of the hippocampus 1.5 month after i.c.v. administration of Ig-saporin and a day after the last behavioral testing. First, we compared the expression of genes between the dorsal and ventral hippocampus under normal conditions; second, we analyzed Ig-saporin-induced changes in the expression of genes in the dorsal and ventral hippocampus.

First, we analyzed differential expression of genes in the dorsal and ventral hippocampus to validate our RNA-seq approach. We found that 1178 genes are DE in the dorsal and ventral parts: 1129 protein-coding genes and 49 ncRNAs. Our results on the protein-coding genes completely correspond to the previous data on differences in the gene expression between the dorsal and ventral parts of the hippocampus (Fanselow and Dong, 2010) and recent RNAseq analysis performed in mice (Cembrowski et al., 2016). In addition, we extended the previous data by adding a set of 49 ncRNAs whose expression differ between the ventral and dorsal parts of the hippocampus. These data are summarized in Supplementary Excel Spreadsheet (**Supplementary Table S1**).

According to our RNA-seq data, only 10 of 438 currently annotated miRNAs are expressed in the hippocampus of adult rats (Mir155hg, Mir3084d, Mir770, Mir3577, Mir1949, Mir3597-2, Mir568, Mir1843b, Mir3064, and Mir664-2). We found that the expression of these miRNAs does not depend on the part of hippocampus and Ig-saporin also did not affect their expression in both parts of the hippocampus. We also analyzed changes produced by Ig-saporin in the expression of ncRNAs and found that the immunotoxin altered the expression of putative ncRNAs LOC108349525 and LOC102546946 in the dorsal hippocampus and LOC100359922 in the ventral hippocampus (**Figure 2A**). However, since functional role of these ncRNAs is unclear, currently, it is hard to interpret these results.

Next, we analyzed changes that occurred in the hippocampus 1.5 month after the induction of degeneration of cholinergic septal neurons. This time point was chosen to avoid possible effects associated with the process of degeneration of cholinergic axons in the hippocampus which may occur at early stages after injection of Ig-saporin. In general, RNA-seq analysis revealed very small changes [Log2(Fold change) < 1.5] in the expression of all genes in different parts of the hippocampus, which is similar to previously reported magnitude of changes after Ig-saporin (Paban et al., 2010). To analyze how changes in the gene expression are related to some cell subpopulations and cellular processes, we subdivided genes into several groups. According to Zhang et al. (2014) and Zeisel et al. (2015), there are some genes that are highly selectively expressed in specific cell types in the brain. For our further analysis, we took groups of genes that are selectively expressed in neurons (500), astrocytes (115), oligodendrocytes (45), microglia (249), and vascular cells [pericytes, vascular smooth muscles, and endothelium (224)]. Other genes were considered as universal genes because they are expressed in more than one cell subpopulation. Among universal genes, we considered only genes that are related to acetylcholine transmission (nicotinic and muscarinic acetylcholine receptors, acetylcholine and choline transporters) and ribosomal proteins (48).

Universal Genes

Detection of DE genes in our RNA-seq data revealed only two universal genes in the dorsal hippocampus (Mt3, RGD1359290) and one gene (Rfng) in the ventral hippocampus whose expression was significantly changed after Ig-saporin. Mt3 is a universal protein that is actively involved in Zn/Cu-exchange in cells and regulation of autophagy (Lee and Koh, 2010) and has neuroprotective role (Santos et al., 2012). The gene RGD1359290 encodes a protein that putatively has relation to ribosome functioning due to some homology to ribosomal proteins in its sequence.

Ribosome-Forming Proteins

Next, we studied Ig-saporin-induced alterations in the expression of ribosomal proteins. We found that the expression of 14 and 6 genes (out of 48), which encode ribosome-forming proteins, increased in the dorsal hippocampus and the ventral hippocampus, respectively (**Figure 2B**).

Acetylcholine-Related Genes

We found that, among both muscarinic and nicotinic acetylcholine receptors, Chrna4 was the only gene whose expression in the hippocampus was affected by Ig-saporin-induced degeneration of cholinergic fibers. Its expression significantly decreased in the dorsal but not ventral hippocampus. These data suggest that the expression of almost all acetylcholine receptors does not depend on the incoming acetylcholine activity but rather an intrinsic property of hippocampal cells. The fact that hippocampal cells preserve sensitivity to acetylcholine after immunotoxin-induced lesioning is supported by finding that

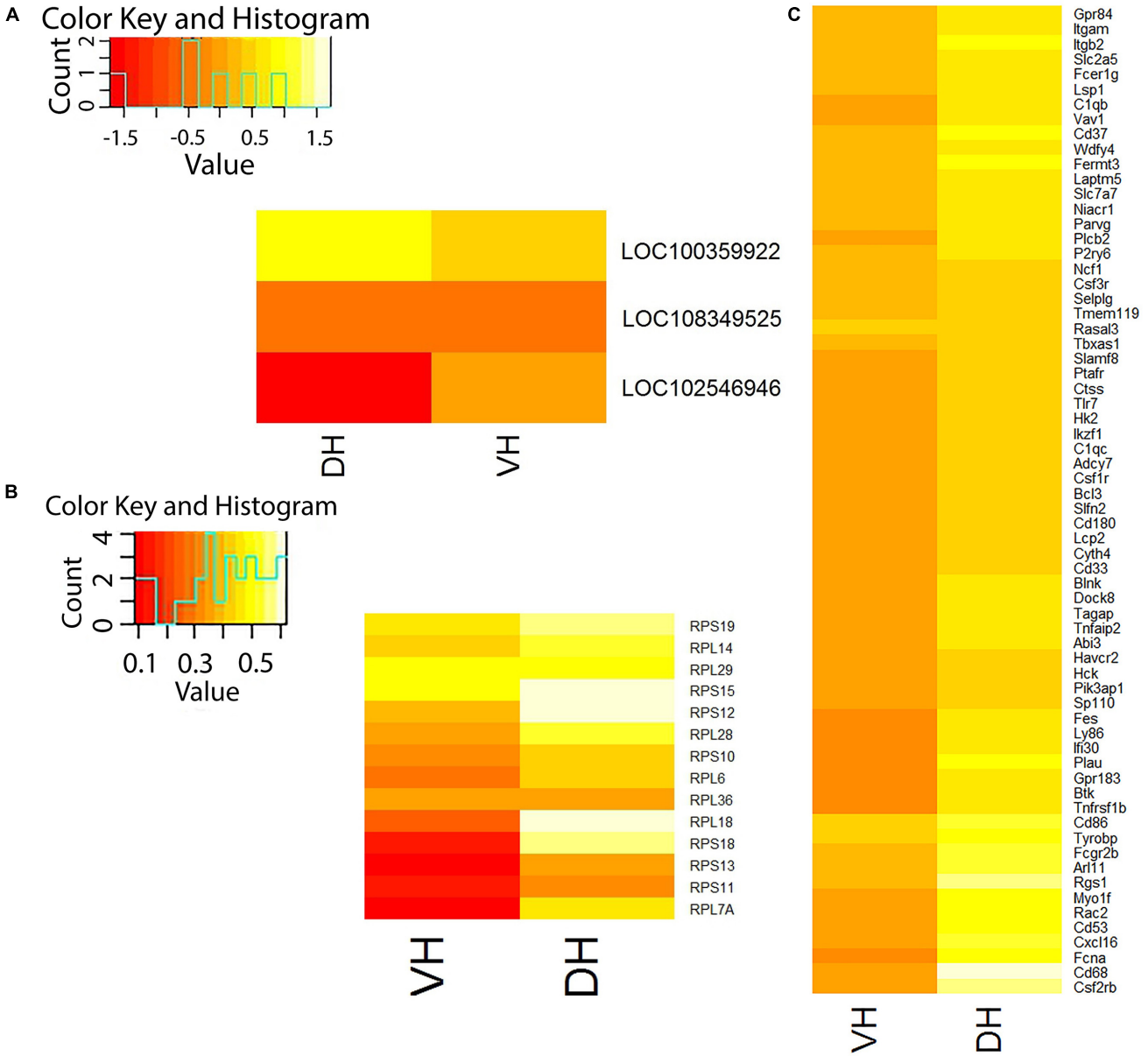

FIGURE 2 | Differentially expressed genes in the dorsal (DH) and ventral (VH) hippocampus after treatment with Ig-saporin. **(A)** Heatmap shows differential expression of non-coding RNAs in VH and DH. **(B)** Heatmap shows differential expression of ribosome-forming genes in VH and DH. **(C)** Heatmap shows differential expression of microglia-specific genes in VH and DH. It may be seen that DH is stronger affected by Ig-saporin than VH.

non-selective muscarinic agonist oxotremorine can reverse impairments caused by Ig-saporin (Van Kampen and Eckman, 2010).

Cell-Specific Genes

Analysis of differential expression of neuron- and oligodendrocyte-specific genes as well as genes that are specifically expressed in the vascular cells did not reveal any changes neither in dorsal nor in ventral part of the hippocampus.

Treatment with Ig-saporin resulted in an increase in the expression of two of astrocyte-specific genes in the hippocampus: complement protein C4b in the dorsal hippocampus and mitochondrial thiosulfate sulfurtransferase Tst in the ventral hippocampus.

Microglia-Specific Genes

Analysis of differential expression of microglia-specific genes showed that Ig-saporin altered the expression of a large number (66) of these genes only in the dorsal hippocampus whereas the ventral hippocampus remained practically unaffected (expression of only one gene increased) (**Figure 2C**). Note that the level of expression of all responded genes increased and there were

no genes whose expression decreased after Ig-saporin in both parts of the hippocampus. Out of 66 genes, 7 genes are related, according to PANTHER database (Mi et al., 2017), to formation of inflammatory response: Itgb2, Plcb2, Bcl3, Rgs1, Ptafr, Rac2, and Vav1. Note, however, we did not detect any signs of acute inflammation, like increased expression of IL1b, TNF, and IL6.

DISCUSSION

In this study, we analyzed consequences of Ig-saporin-induced degeneration of cholinergic inputs to the hippocampus at the level of gene expression. According to our behavioral data, analysis of gene expression was performed in rats with moderate impairments of learning and memory. Alterations in animal behavior observed in the Morris water maze suggest that toxin treatment predominantly affected structures involved in spatial navigation. It has been shown that different parts of the hippocampus are involved in different types of learning. The dorsal hippocampus is critical for spatial learning (Fanselow and Dong, 2010), and lesion of this, but not ventral, part of the hippocampus by injection of ibotenic acid causes severe deficits in spatial learning in a water maze (Moser et al., 1995). Taken together, these data suggest that Ig-saporin might have impaired functioning of the dorsal hippocampus which led to disturbances in spatial navigation in Morris water maze.

The major aim of the present study was to elucidate the genes whose expression is dependent on the presence of cholinergic innervation and cell subpopulations that are strongly affected by cholinergic degeneration; the latter can give us probable targets for correction of AD-like disorders. We expected that the immunotoxin-induced degeneration will induce changes in the gene expression that, at least partly, coincide in the dorsal and ventral hippocampus; this will give us basis for making suggestions on the acetylcholine-dependent regulation of gene expression. Instead of this, we found that changes produced by i.c.v. injection of Ig-saporin are different in the dorsal and ventral parts of the hippocampus. According to analysis at the level of cell-specific genes, the strongest effect of Ig-saporin was observed in the dorsal part whereas the ventral hippocampus was practically unaffected. Furthermore, this conclusion corresponds to our above suggestion that degeneration of cholinergic input to the hippocampus mostly affects the dorsal hippocampus, which leads to the development of specific learning deficits. According to our morphological data, Ig-saporin induced degeneration of both medial septal neurons, which innervate the dorsal hippocampus, and neurons in the DBB, which send projections predominantly to the ventral part of the hippocampus. If the effects of Ig-saporin were associated only with degeneration of cholinergic fibers it will be hard to expect large difference between responses in the ventral and dorsal parts of the hippocampus. These data suggest that effects induced by Ig-saporin are related not only to degeneration of cholinergic inputs but also to some additional effects of Ig-saporin. One of possible explanations of asymmetric effect of the immunotoxin is that the dorsal hippocampus is located very close to the cerebral ventricles where we injected the immunotoxin and, as a consequence, Ig-saporin diffused predominantly into this part of the hippocampus and induced stronger effects in it.

Our data suggest that the ventral hippocampus is practically insensitive to Ig-saporin and a comparison of gene expression in the control and Ig-saporin-treated animals revealed only minor changes in this structure. This means that long-term behavioral training that was performed in our study caused similar changes in the expression in the ventral hippocampus in the control and experimental rats and a comparison of these changes revealed only subtle differences. This is not the case for the dorsal hippocampus where we found alterations in the gene expression between lesioned and control rats. Obviously, the changes in the expression of genes in the DH are related to some alterations caused by the immunotoxin. Of course, we cannot exclude possibility that behavioral procedures caused different changes in the expression of genes in DH in the control and experimental animals but, obviously, these differences occurred as a result of application of the immunotoxin.

The effect of Ig-saporin on the expression of genes in the dorsal hippocampus was quite different from the Ig-saporin effects described by Paban et al. (2010) in the entire hippocampus. In general, the sets of genes affected by Ig-saporin in our study and previous studies (Paban et al., 2010, 2011b) do not coincide. First, the mentioned studies did not include the complete list of altered genes (only the genes that were altered by learning are presented) and it is hard to be absolutely sure that the altered genes that we detected in our study are absent from their list. Second, the results obtained in Paban et al. (2011b) do not correspond to the results in Paban et al. (2010), which the authors related to limitations of different technical approaches used in their studies. Third, we administered Ig-saporin into the ventricles whereas in the mentioned studies, the immunotoxin was injected into the medial septum and nucleus basalis magnocellularis. Our data suggest that intracerebroventricularly administered Ig-saporin diffuses not only in the medial septum, where it induced degeneration of cholinergic neurons, but also, probably, in the dorsal hippocampus. Fourth, we analyzed separately effects produced by Ig-saporin in the dorsal and ventral hippocampus, which was not performed in the mentioned studies. Fifth, we analyzed subset of cells, where the changes were caused by Ig-saporin, on the basis of currently available databases (Zhang et al., 2014; Zeisel et al., 2015) of cell-specific expression of genes, which was not performed in the discussed studies.

We found that, among universal genes, Ig-saporin increased expression of a group of universal genes that encode ribosome-forming proteins. Their expression was altered in both dorsal and ventral hippocampus with largest effect observed in the dorsal part. This effect of Ig-saporin may be associated, on the one hand, with degeneration of cholinergic fibers and, on the other hand, with side effect of the toxin. Saporin *per se* inactivates ribosomes and, therefore, an increase in the expression of ribosomal proteins may be just a compensation

of this action of saporin in the cells that endocytosed the immunotoxin but did not die. The specificity of Ig-saporin action is determined by antibody conjugated with saporin, however, it is unclear whether this complex immunotoxin may be endocytosed by cells that do not express Ngfr, for example, by activated microglia, which functions as macrophages. At least, we cannot exclude this possibility. On the other hand, cholinergic signaling may be one of factors that modulate processes of ribosome synthesis in hippocampal cells, however, so far there is no data on acetylcholine-dependent regulation of ribosome synthesis.

Our major unexpected findings were that lesion of cholinergic neurons practically did not influence expression of genes that are selectively expressed in all types of hippocampal cells except microglia. Previous data suggest that Ig-saporin induces a rapid activation of microglia in the septum (Rossner et al., 1995; Seeger et al., 1997). It also was shown that minocycline, which inhibits microglial activity, has a protective effect against Ig-saporin-induced degeneration of cholinergic septal neurons (Hunter et al., 2004). The authors showed that damaging effect of Ig-saporin was associated with activation of microglia in the septal area. In our study, we did not perform morphological analysis in the hippocampus; however, our transcriptomic data suggest that Ig-saporin induced postponed elevation of a large number of microglia-specific genes in the dorsal hippocampus which may result from microglia activation and/or proliferation. Note that we studied effects of Ig-saporin 1.5 months after its injection when all processes of microglial activation (if they occurred in the hippocampus after the injection) should already cease. We did not find any signs of acute microglia activation such as overexpression of pro-inflammatory interleukins (Il1B, Il6, Il15, and Il18) or TNF-alpha. This means that an increase in the expression of microglia-related genes may be a result of microglia activation that still resided in the dorsal hippocampus after acute microglia activation induced by Ig-saporin injection. This residual upregulation of microglial genes may be not only a consequence of inflammation that occurred during degeneration of cholinergic fibers but also result from deficit of acetylcholine, which is known to suppress inflammatory processes (Carnevale et al., 2007).

Finally, we found that i.c.v. injection of Ig-saporin leads to a mild cognitive deficit and is associated with very weak changes in the expression of genes. Our transcriptomic data suggest that the deficit was associated with upregulation of a number of microglia-specific genes, including inflammation-related genes, suggesting that altered functioning of microglia is among factors that at cellular level influenced cognitive abilities of animals. It was shown that microglia may influence functioning of synapses by dendritic pruning or engulfing synapse elements, especially under pathological conditions (Hong et al., 2016b). It was shown that mild cognitive impairment in a model of AD is associated with elevated expression of proteins related to complement system by microglial cells which results in strong synaptic loss (Hong et al., 2016a). Probably, under our conditions, Ig-saporin-induced activation of microglia disturbed normal functioning of hippocampal synapses which led to development of mild cognitive deficit.

Thus, our results of RNA-seq analysis suggest that i.c.v. injection of Ig-saporin strongly upregulates expression of microglia-related genes and impairs behavior associated with functioning of the dorsal hippocampus. We believe that alterations in the expression of microglia-specific genes in the part of the hippocampus, which is responsible for spatial navigation, and impairments in spatial navigation after treatment with Ig-saporin are closely related phenomena. It appears that development of mild cognitive deficit may result not from alterations in functioning of neurons but from upregulation of microglia functioning alone. In fact, we observed a situation when microglia overexpress a number of inflammation-related genes without signs of acute inflammation and these changes are practically "ignored" by other cell types. This means that induction of cholinergic deficit in the hippocampus finally leads to the development of postponed processes in microglia and activation of specific cascades, which differ from cascades associated with acute inflammation. This is important for understanding of mechanisms of the development of pathological processes, such as AD.

AUTHOR CONTRIBUTIONS

YD contributed in intracerebroventricular injections, design and performance of behavioral experiments, analysis of behavioral data, preparation of brain slices, immunohistochemistry, and writing manuscript. AK contributed to analysis of transcriptomic data. MZ contributed in intracerebroventricular injections, design, and performance of behavioral experiments. MS contributed to analysis of behavioral experiments, analysis of immunohistochemical data, and writing manuscript. EC contributed in preparation of samples for RNA-seq, RNAseq experiments. PK contributed in design and performance of RNAseq experiments. VM contributed in general design of study and writing manuscript. AB contributed in general design of study, analysis of transcriptomic data, and writing manuscript.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fnmol.2017.00429/full#supplementary-material

FIGURE S1 | Effect on IgG-saporin on learning in T-maze. Graph shows the time spent in the central arm of the maze. Rats that received IgG-saporin ($n = 8$) spent smaller time in the central arm as compared to the saline-treated rats ($n = 8$) only during the first trial. Bars represent mean ± SEM. $^*P < 0.05$.

FIGURE S2 | Effect of IgG-saporin on passive avoidance learning. Trained IgG-saporin-treated rats ($n = 8$) tended to decrease latency to enter the dark chamber compared with trained saline rats ($n = 8$). Bars represent the mean ± SEM. $^*P < 0.05$.

FIGURE S3 | Immunocytochemical staining of cholineacetyltransferase (ChAT) in the control and Ig-saporin-treated animals **(A)**. The results of counting of ChAT-positive neurons in the septum and diagonal band of Broca **(B)**. Differences between the control and experimental animals are significant in both structures (Student's *t*-test, $P < 0.05$).

TABLE S1 | Differential expression of genes between the dorsal and ventral hippocampus of control rats. The data are presented as ratio DH/VH; "+" means higher expression in the dorsal hippocampus.

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Quercetin Declines Apoptosis, Ameliorates Mitochondrial Function and Improves Retinal Ganglion Cell Survival and Function in *In Vivo* Model of Glaucoma in Rat and Retinal Ganglion Cell Culture *In Vitro*

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Glaucoma is a progressive neuropathy characterized by the loss of retinal ganglion cells (RGCs). Strategies that delay or halt RGC loss have been recognized as potentially beneficial for rescuing vision in glaucoma patients. Quercetin (Qcn) is a natural and important dietary flavonoid compound, widely distributed in fruits and vegetables. Mounting evidence suggests that Qcn has numerous neuroprotective effects. However, whether Qcn exerts neuroprotective effects on RGC in glaucoma is poorly understood. In this study, we investigated the protective effect of Qcn against RGC damage in a rat chronic ocular hypertension (COHT) model *in vivo* and hypoxia-induced primary cultured RGC damage *in vitro*, and we further explored the underlying neuroprotective mechanisms. We found that Qcn not only improved RGC survival and function from a very early stage of COHT *in vivo*, it promoted the survival of hypoxia-treated primary cultured RGCs *in vitro* via ameliorating mitochondrial function and preventing mitochondria-mediated apoptosis. Our findings suggest that Qcn has direct protective effects on RGCs that are independent of lowering the intraocular pressure (IOP). Qcn may be a promising therapeutic agent for improving RGC survival and function in glaucomatous neurodegeneration.

Keywords: quercetin, retinal ganglion cells, glaucoma, apoptosis, mitochondria, neuroprotection

INTRODUCTION

Glaucoma is a progressive neuropathy characterized by the loss of retinal ganglion cells (RGCs), and it is a major cause of irreversible visual impairment worldwide, as damaged RGCs are incapable of repair or regeneration (Calkins, 2012). Strategies that delay or halt RGC loss have been recognized as potentially helpful for rescuing vision in glaucoma. Therefore, many studies have evaluated neuroprotection for glaucoma and many neuroprotective

agents have been identified, such as N-methyl-D-aspartate (NMDA) receptor antagonists (memantine and brimonidine), glutamate release inhibitors (bis(7)-tacrine; Fang et al., 2010), calcium channel blockers (cilnidipine and lomerizine; Fitzgerald et al., 2009), neurotrophins (brain-derived neurotrophic factor and neurotrophic factor; Pease et al., 2009), antioxidants (astaxanthin and flavonoids; Yamagishi and Aihara, 2014), and others, which can be used alone or in combination with intraocular pressure (IOP)-lowering therapy. Despite intensive efforts and good laboratory evidence, these agents did not show significant efficacy in the clinic. Thus far, no neuroprotective drugs are available for glaucoma treatment (Van de Velde et al., 2015). It is imperative to identify more efficacious neuroprotective agents with potential clinical value for preventing or slowing down RGC loss as well as for preserving RGC function for multiple or, ideally, all mechanisms of glaucoma (Levin and Danesh-Meyer, 2010).

Quercetin (Qcn) is a natural and important dietary flavonoid compound that is widely distributed in fruits and vegetables. Mounting evidence suggests that Qcn has numerous beneficial effects, including anti-inflammatory (Periasamy et al., 2016), anti-apoptosis (Ben Salem et al., 2016), anti-ischemic (Ekinci Akdemir et al., 2016), anti-oxidation (Xu et al., 2016), anti-endoplasmic reticulum (ER) stress (Ben Salem et al., 2015), anti-mutagenic (Barcelos et al., 2011), and anti-viral (Wu W. et al., 2015) effects in addition to promoting mitochondrial biogenesis (Sharma et al., 2015). In the retina, it has been reported that Qcn has protective effects in multiple lesions, including retina ischemia-reperfusion injury (Arikan et al., 2015), oxidative damage of RPE cells (Hytti et al., 2015), diabetes-induced retinal neurodegeneration (Kumar et al., 2014), choroidal neovascularization in age-related macular degeneration (Zhuang et al., 2011), vascular endothelial growth factor-induced choroidal and retinal angiogenesis (Li et al., 2015), and ocular inflammation (Romero et al., 1989). However, whether Qcn exerts neuroprotective effects on RGC in glaucoma is poorly understood. The factors involved in RGC injury in glaucoma, such as oxidative stress, ischemia-reperfusion injury and glutamate excitotoxicity (Aihara, 2010), are all included in the mechanisms of cellular protection exerted by Qcn. Therefore, we speculated that Qcn may have a protective effect against RGC loss in glaucoma. To test our hypothesis, RGC function, viability, and apoptosis with or without Qcn treatment were investigated in a rat model of chronic glaucoma *in vivo* and hypoxia-induced primary cultured RGC damage *in vitro*.

MATERIALS AND METHODS

Animals and Ethics Statement

We used Wistar rats (150–200 g) and newborn Sprague-Dawley rats (3 days; SLAC Laboratory Animal Co., Ltd. Shanghai, China) in this study. All animals received humane care. The study protocol was reviewed and approved by the animal experimental ethics committee of Fudan University. The animal handling and experimental protocols adhered to the approved guidelines of Animal Care and Use Committee of Fudan University and the Association Research in Vision and Ophthalmology (ARVO) Statement for the Use of Animals in Ophthalmic and Vision Research.

Wistar rats were randomly allocated into the following four groups: (1) the NC group (normal control group)-normal age matched, untreated rats; (2) Qcn group (Qcn treated group)—these normal rats received intravitreal injection of 2 μl of 10 μM Qcn (Sigma-Aldrich, St. Louis, MO, USA) 2 days before the day of commencement of the experiment and then once a week for 4 weeks. The contralateral eyes were treated with the same volume of saline as a sham control; (3) COHT group (chronic ocular hypertension group)—COHT was unilaterally induced in these rats by the injection of 5 μl of paramagnetic polystyrene microbeads (FluoSpheres; Invitrogen, Carlsbad, CA, USA; 15-μm diameter) to the anterior chamber of the right eye, as previously reported (Sappington et al., 2010). At the same time, the left eye was treated with the same volume of saline as sham control; and (4) COHT+Qcn group, (COHT rats treated with Qcn)—COHT was induced in these rats as described for group-3 (injection of 5 μl of paramagnetic polystyrene microbeads into the anterior chamber of the right eye) and treated with Qcn as for group 2 (intravitreal injection of 2 μl of 10 μM Qcn). The left eye was treated with the same volume of saline as sham control. In this study, all animals were anesthetized by intraperitoneal injection of chloral hydrate (300 mg/kg body weight).

Intraocular Pressure (IOP) Elevation

IOP was measured in both eyes under general anesthesia once before paramagnetic polystyrene microbead injection, after 1 day, 3 days, at the end of 1st, 2nd 3rd and 4th week after microbead injection using a tonometer (Icare® Tonolab, TioLat, Helsinki, Finland). IOP was always measured between 9–10 am by the same operator. Mean ± standard deviation (SD) of the middle four readings out of six valid rebound measurements was taken as IOP.

Electroretinography (ERG) and Photopic Negative Response (PhNR) Recordings

The function of RGCs is impaired before death. It is crucial to identify early dysfunction of RGCs, before RGC loss. To assess the functional changes of RGCs in the early stage of COHT, we studied the function of whole retina and RGCs by electroretinography (ERG) and Photopic Negative Response (PhNR) at baseline and after 3, 7, and 14 days of IOP elevation, as previously reported (Rangaswamy et al., 2007; Porciatti, 2015). Each rat was dark-adapted for 1–2 h before recordings. After the rats were anesthetized, the pupils were dilated with phenylephrine hydrochloride and tropicamide. Light stimuli were delivered using a ColorDome unit on a green background with green light flashes. Recordings were generated using the Espion Visual Electrophysiology System (Espion E3, Diagnosys, Diagnosys UK Ltd, UK). Two such recordings were obtained for each eye at each time point and averaged. For the a-wave and b-wave of ERG and PhNR amplitudes, measurements were used as the difference between a peak and adjacent trough on the waveform.

Retrograde Labeling of RGCs and Counting

Seven days before sacrifice, the rats were deeply anesthetized. Then, 2 μl of 5% of FluoroGold (FG; Sigma-Aldrich, St. Louis, MO, USA) was injected into the superior colliculus on each side, as previously reported (Wu et al., 2013). At euthanasia, the eyeballs were enucleated and directly fixed in 4% paraformaldehyde for 2 h at room temperature. The retinas were then carefully dissected and prepared as flatmounts. RGCs were quantified and averaged per eight microscopic fields of identical size using a laser scanning confocal microscope (TCS SP8, Hamburg, Germany) at a final magnification of 200×. The RGCs were manually counted by two operators who were blinded to the study using ImageJ software (NIH, Bethesda, MD, USA). The RGC density is expressed as the number of cells per mm^2.

FG labeling indicates that the protective effect is most obvious at 2 weeks after Qcn administration, to further clarify the protective effect of Qcn on RGCs under COHT, we performed TUNEL and survivin staining on retinal cryosections at 2 weeks after COHT.

Terminal Deoxynucleotidyl Transferase dUTP Nick End-Labeling (TUNEL) Assay

The TUNEL assay was performed according to the manufacturer's protocol (*In Situ* Cell Detection Kit; Roche, Mannheim, Germany), as previously described (Wu J. H. et al., 2015). RGCs and retinal sections were fixed in 4% (w/v) paraformaldehyde at 4°C for 30 min. Subsequently, the TUNEL reaction mixture was added to the sample and maintained for 60 min at 37°C. The preparations were visualized using a confocal microscope (Leica SP8, Hamburg, Germany) and quantified using ImageJ software (NIH, Bethesda, MD, USA). Six microscope fields of view from six different wells were analyzed per treatment. The number of TUNEL-positive cells in ganglion cell layer (GCL) at a distance between 200 μm and 600 μm from the optic disc were counted. Only four sections were chosen from each eye, and each group contained three eyes.

Immunofluorescence

Immunofluorescence staining was performed as is reported elsewhere (Wu et al., 2013). Rat eyes were sectioned at 10 μm; then, the sections were incubated in 0.1% Triton X-100 and 3% (w/v) bovine serum albumin (BSA) for 30 min, sequentially, at room temperature to prevent nonspecific background signal. The cryosections were then incubated with primary rabbit anti-survivin (1:200, Abcam, Cambridge, MA, USA) antibodies at 4°C overnight. The following day, the samples were incubated with fluorescein-conjugated goat anti rabbit secondary antibody (1:400, Molecular Probes, Waltham, MA, USA) and Hoechst staining. The stained sections were visualized and captured by confocal microscopy (Leica SP8, Hamburg, Germany).

Cell Culture and Treatment

RGC isolation was performed as we described previously (Gao et al., 2016). Briefly, retinas were obtained from 1- to 4-day-old Sprague-Dawley rats and dissociated in 4.5 U/mL of papain solution (Worthington, Lakewood, NJ, USA). The cell suspensions were then sequentially incubated with a petri dish coated with rabbit anti-macrophage antibody (Cedarlane Laboratories, Ontario, ON, Canada) and mouse anti-Thy1.1 antibody (Abcam, Cambridge, MA, USA). RGCs were collected and seeded into appropriate plates coated with mouse-laminin (Trevigen Inc., Gaithersburg, MD, USA) and poly-D lysine (Sigma-Aldrich, St. Louis, MO, USA). The RGC purity was approximately 85% (Gao et al., 2016). RGCs were then incubated with 200 μM cobalt chloride ($CoCl_2$, Sigma-Aldrich, St. Louis, MO, USA) to induce hypoxia and apoptosis 48 h after seeding (Kim et al., 2013); then, 0, 1, 10, 20, 50 or 100 μM Qcn was added for 24 h, or the optimal concentration was given for 48 h.

Cell Counting Kit-8 Assay for RGC Viability

RGCs were seeded into 96-well plates and treated with $CoCl_2$ or/and Qcn for 24 h or 48 h. Then, 10 μl of CCK8 solution (Dojindo Laboratories, Kumamoto, Japan) was added to each well, and the samples were incubated at 37°C for 4 h before analysis at 450 nm with a Tecan Genios (Synergy H1, BIOtAK). All values are expressed as the mean ± SD of at least three wells and at least three separate experiments.

LDH Release

After each treatment, all supernatant media was collected to evaluate the lactate dehydrogenase (LDH) release from the cytoplasm of damaged RGCs. The assay was performed using an LDH cytotoxicity detection kit (Promega, Fitchburg, WI, USA) according to the manufacturer's instructions. Briefly, 50 μl of reconstituted substrate mix (Promega LDH kit) was added to each sample; after incubation at 25°C in the dark for 30 min, the enzymatic reaction was stopped with 50 μL of stop solution (Promega LDH kit). Absorbance was measured at 490 nm using a microplate reader (Synergy H1, BIOtAK). All experiments were performed in triplicate.

Flow Cytometric Analysis for Apoptosis

The proportion of apoptotic cells was measured using fluorescence-activated cell sorting (FACS) on a FACSCalibur according to the instructions in the Annexin V-FITC/propidium iodide (PI) flow cytometric assay kit (Becton Dickinson, San Jose, CA, USA). Briefly, after each treatment, cells were trypsinized and stained with Annexin V-FITC and PI at room temperature for 20 min in the dark according to the manufacturer's protocol. Then, stained cells were analyzed using FACS to differentiate the percentage of cells in early apoptosis (Annexin V+/PI−) and late apoptosis (Annexin V+/PI+). All experiments were performed in triplicate.

Western Blot Analysis

Cell protein extraction and Western blot analysis were performed as previously reported (Wu J. H. et al., 2015; Gao et al., 2017). Briefly, cultured RGCs were lysed, and total proteins were extracted on ice with cell lysis buffer (Cell Signaling Technology, Boston, MA, USA) and protease inhibitor cocktail (Sigma-Aldrich, St. Louis, MO, USA). The protein concentration

was quantified using a BCA protein assay kit (Thermo Fisher Scientific, Rockford, IL, USA). Equal amounts of protein were separated by SDS-polyacrylamide gel electrophoresis and then transferred to 0.22-μm PVDF membranes. After blocking with 5% non-fat milk for 1 h, the membranes were incubated overnight at 4°C with primary antibodies against B cell lymphoma 2 (Bcl-2, Abcam, Cambridge, MA, USA), cleaved caspase-3 antibody (Abcam, Cambridge, MA, USA) and β-actin (Abcam, Cambridge, MA, USA). Signals were monitored by the Kodak Imaging System (Kodak 440CF) using ECL Western blot substrate (Hyperfilm ECL, Thermo Fisher Scientific, Rockford, IL, USA). Then, they were quantified by densitometry using ImageJ software (NIH, Bethesda, MD, USA).

Measurement of the Mitochondrial Membrane Potential ($\Delta\psi$m)

The mitochondrial membrane potential ($\Delta\psi$m) was evaluated using MitoProbe JC-1 dye (Invitrogen, Carlsbad, CA, USA) as previously described (Zhang et al., 2016). In brief, RGCs with different treatments were incubated with JC-1 at 37°C for 30 min while protected from light and assessed via FACS (Becton Dickinson, San Jose, CA, USA) and a confocal fluorescence microscope (Leica; green: 488 nm excitation/530 nm emission; red: 550 nm excitation/590 nm emission). The JC-1 monomer (green) and J-aggregate (red) were separately detected in FL1 (green fluorescence, *x*-axis) and FL2 (red fluorescence, *y*-axis) channels. Quantitative analysis was performed using ImageJ software, and $\Delta\psi$m was indicated by the ratio of the mean red fluorescence to the mean green fluorescence.

Measurement of Reactive Oxygen Species (ROS)

To detect the reactive oxygen species (ROS) level, RGCs were incubated with 10 μM dihydroethidium (DHE) for 30 min in the dark. Fluorescence was observed with a confocal fluorescence microscope and quantified by FACS using the PE channel.

Statistical Analyses

All data are expressed as the mean ± SD. The distributions of the amplitudes and inter-event intervals between the events were compared using the Kolmogorov–Smirnov test. Statistical analysis was performed using one-way analysis of variance (ANOVA) with Bonferroni's multiple comparision test (using Prism 5.0 (Graph Pad Software Inc, San Diego, CA, USA). A P value < 0.05 was considered the threshold for significance.

RESULTS

IOP Elevation

IOP was measured in each rat prior to injection (basal) and every 3 days after injection with a rebound tonometer (**Figure 1**). There was no significant difference between the mean basal IOPs (10.57 ± 0.59 mm Hg) of the four groups ($p > 0.05$). As expected, microbead injection induced a sustained elevation in IOP, as previously reported (Samsel et al., 2011). The average

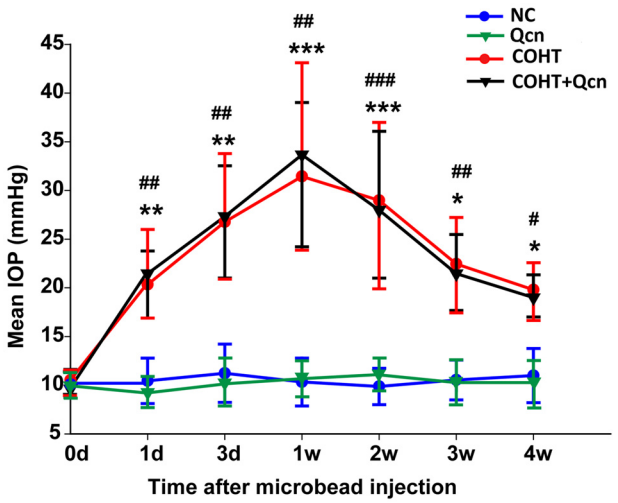

FIGURE 1 | Mean intraocular pressure (IOP) of rats in the NC, quercetin (Qcn), chronic ocular hypertension (COHT) and COHT+Qcn groups. IOP was observed from 0 weeks to 4 weeks after microbead injection. The data are given as the mean ± standard deviation (SD); $^{\#}p < 0.05$, $^{\#\#}p < 0.01$ and $^{\#\#\#}p < 0.001$ for the COHT group compared with the NC group. $^{*}p < 0.05$, $^{**}p < 0.01$ and $^{***}p < 0.001$ for the COHT+Qcn group compared with the Qcn and NC groups.

IOP of the microbead-injection eyes ranged from 18.99 ± 2.34 to 33.67 ± 9.05 mmHg at 1 day to 4 weeks after injection, which was significantly higher than that of the corresponding control eyes ($p < 0.05$). The IOP at each point after Qcn administration did not differ from the corresponding non-Qcn-treated eyes ($p > 0.05$). These results demonstrated that COHT is effectively induced by the anterior chamber injection of microbeads in rats, which is similar to human ocular hypertension and chronic glaucoma. In addition, Qcn administration had no effect on IOP.

Qcn Ameliorates RGC Dysfunction in the COHT Rat Model

The a-wave and b-wave amplitudes of the COHT group were only slightly smaller and slower than that of the NC group at an early stage of COHT (3 days, $p < 0.05$, **Figures 2A,B**), while the PhNR amplitude was significantly reduced by approximately 55% ($n = 16$, $p < 0.01$, **Figure 2C**), indicating that RGC function was impaired prior to that of other retinal cells, such as photoreceptor cells. However, the amplitudes of a-waves, b-waves and PhNR were significantly depressed by 72.17% ($p < 0.01$), 69.43% ($p < 0.01$) and 71.92%, respectively, at the 2nd week after COHT ($p < 0.01$), suggesting that retinal function, especially RGC function, is markedly impaired with the extension of COHT. Qcn administration significantly attenuated the reduction of PhNR amplitudes from a very early stage after COHT, by 54.10 ± 3.25% at the third day ($p < 0.01$) and 72.45 ± 4.56% at the 2nd week ($p < 0.01$). However, the amplitudes of a-waves and b-waves showed a delayed recovery, which was reversed by 0.97 ± 0.12- and 1.34 ± 2.35-fold at the 2nd week after COHT ($p < 0.01$).

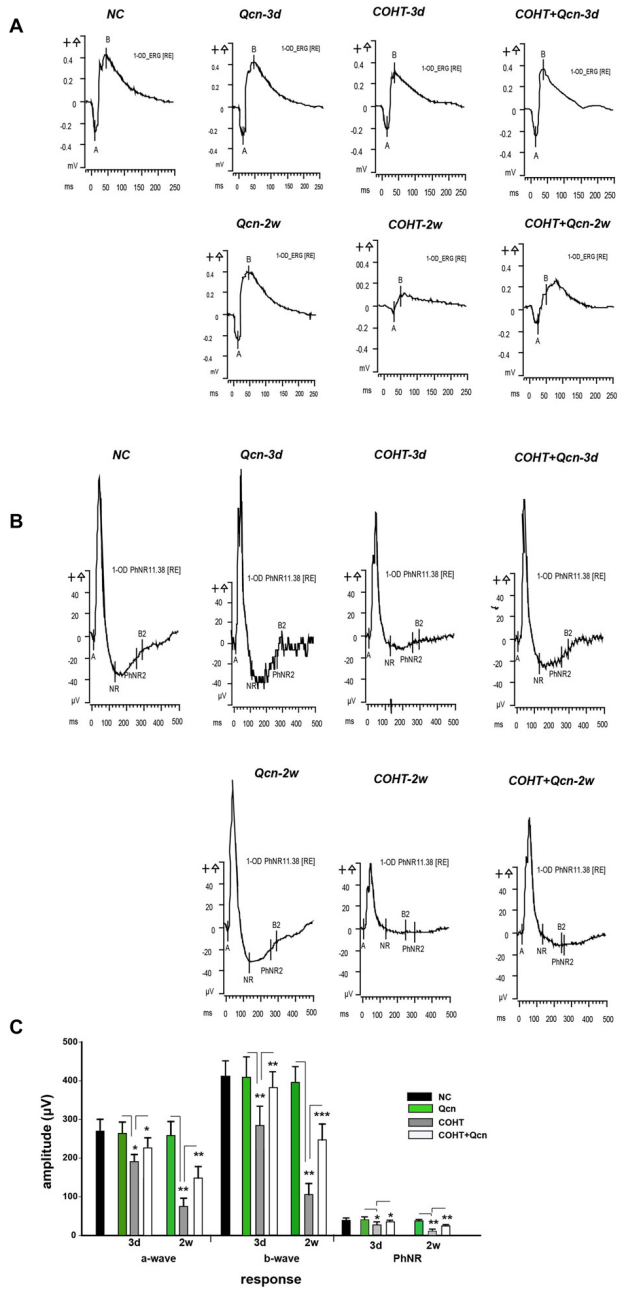

FIGURE 2 | Effects of Qcn on electroretinography (ERG) and Photopic Negative Response (PhNR) responses. **(A)** Representative ERG at baseline, 3 days and 2 weeks of COHT with or without Qcn. **(B)** Data analyses of ERG a-wave, b-wave and PhNR amplitudes for the NC, Qcn, COHT and COHT+Qcn groups after 3 days and 2 weeks of microbead injection. **(C)** PhNR amplitudes at baseline, 3 days and 2 weeks of COHT with or without Qcn. The data are expressed as the mean ± SD, $*p < 0.05$, $**p < 0.01$ and $***p < 0.001$ for the COHT group compared with the NC group and COHT+Qcn group compared with the COHT group.

Qcn injection did not affect the electroretinographic response, as no significant difference in a-waves, b-waves and PhNR amplitude were observed between Qcn group and NC group ($p > 0.05$, **Figures 2A,B**).

Qcn Promotes RGC Survival

To assess whether Qcn could increase RGC survival under COHT, RGCs were detected by retrograde FG labeling (**Figures 3A,C**). In the eyes before microbead injection and in the NC group, the mean RGC density was 2435 ± 397 cells/mm^2. After COHT, RGC somas were lost over time (**Figures 3A,B**), with a reduction of 39.4% ($p < 0.05$), 67.12% ($p < 0.01$) and 73.84% ($p < 0.001$) at the 1st, 2nd and 4th weeks, respectively. After 1, 2 and 4 weeks of Qcn treatment, the mean RGC density recovered, increasing by 19.18 ± 3.24%, 24.14 ± 5.22% and 15.89 ± 4.37%, respectively, which were significantly increases compared with the densities in the corresponding COHT group ($p < 0.05$). There was no significant difference in the RGC density between the NC and Qcn groups ($p > 0.05$). These results suggested that the intravitreal delivery of Qcn could promote RGC survival under COHT.

To further clarify the protective effect of Qcn on RGCs under COHT, we performed TUNEL and survivin staining on retinal cryosections at 2 weeks after COHT. Consistent with previous reports (Can et al., 2015), we found a significant increase in TUNEL-positive cells in the retinal GCL of retinas with COHT compared with those in the NC group (**Figures 4A,B**). However, the number of TUNEL-positive cells was significantly reduced by two-fold when Qcn was administered ($p < 0.01$). There was no significant difference in the TUNEL-positive cells between the NC and Qcn groups ($p > 0.05$). Survivin, an inhibitor of apoptosis (Zhou et al., 2016), was identified by immunofluorescence analysis (**Figure 5A**). Survivin expression in the GCL and inner nuclear layer (INL) was significantly down-regulated by 94.81% after 2 weeks of COHT compared with the expression in the NC group (**Figures 5A,B**, $p < 0.001$), while Qcn treatment remarkably reversed its expression by 11.86 ± 2.52-fold compared with that in the corresponding COHT group ($p < 0.001$).

Qcn Protects Against Hypoxia-Induced RGC Apoptosis

To further explore the underlying neuroprotective mechanism, primary RGCs were cultured under hypoxia with or without Qcn. $CoCl_2$ was used to induce hypoxia and mimic the glaucomatous micro-environment *in vitro*, as previously reported (Du et al., 2013). To achieve this goal, 1 μM, 10 μM, 20 μM, 50 μM or 100 μM Qcn was added to RGCs with 200 μM $CoCl_2$ (Wang et al., 2015), the RGC viability was analyzed using the CCK8 assay. As shown in **Figure 6A**, hypoxia significantly inhibited cell viability ($p < 0.01$), and Qcn treatment markedly increased RGC survival when the concentrations of Qcn were 10 μM ($p < 0.05$), 20 μM ($p < 0.01$) and 50 μM ($p < 0.05$) compared with the survival seen in the hypoxia group. However, the number of RGCs was significantly decreased at 100 μM Qcn ($p < 0.05$), indicating that Qcn had toxicity at high concentrations. There can be several reasons for this. First, when the drug concentration is too high, the osmotic pressure of the medium increases, which may do damage to RGCs. Second, it is reported that Qcn concentrations higher than 50 μM can led to decreased mitochondrial function of retinal pigment

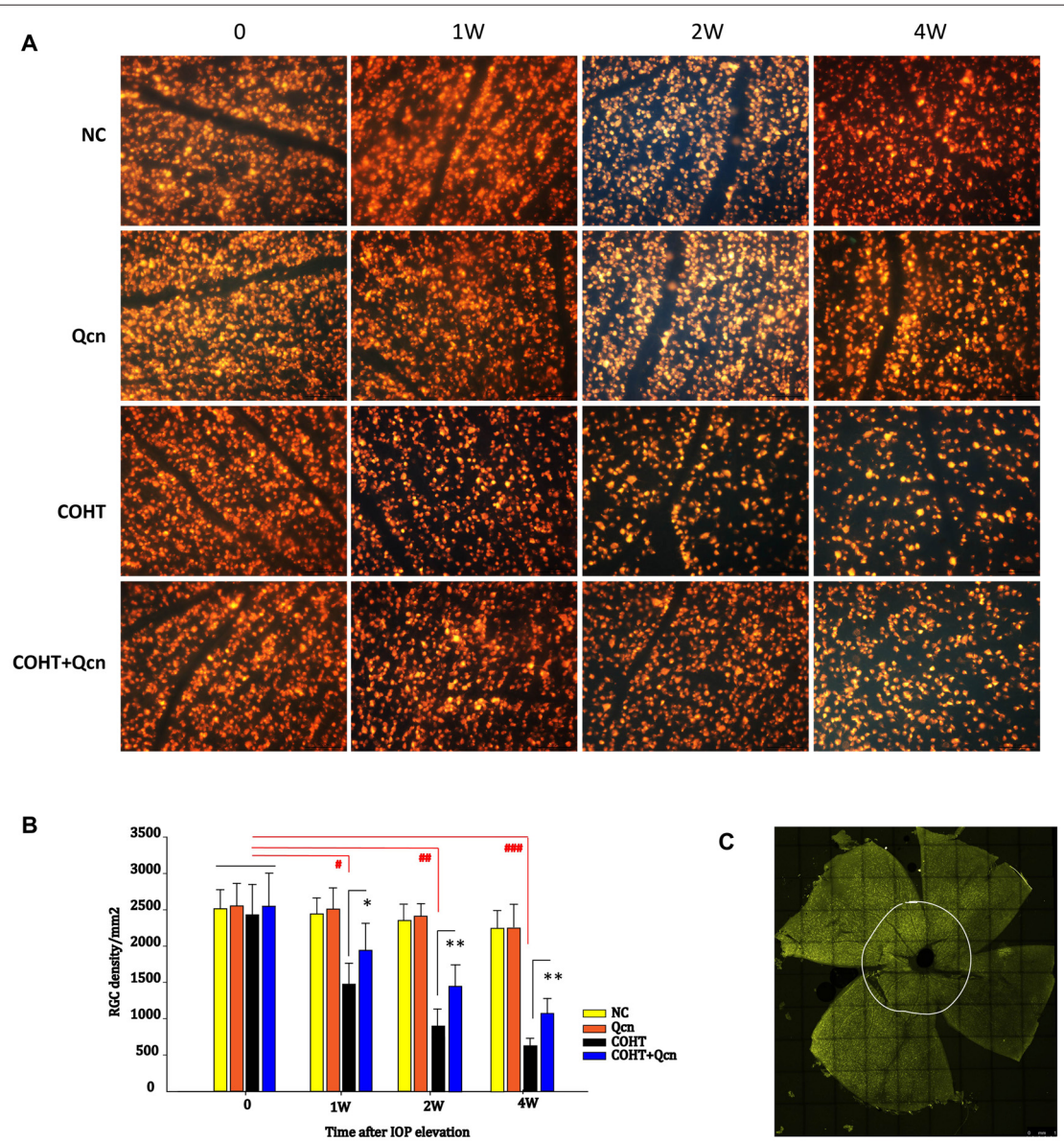

FIGURE 3 | FluoroGold (FG) labeling of surviving retinal ganglion cells (RGCs) in flat-mounted retinas in the NC, Qcn, COHT and COHT+Qcn groups after 0, 1, 2 and 4 weeks of microbead injection. All images were captured at same magnification. Scale bar, 100 μm **(A)**. **(B)** Quantification of FG-labeled RGCs. The data are given as the mean ± SD; $^{\#}p < 0.05$, $^{\#\#}p < 0.01$ and $^{\#\#\#}p < 0.001$ and $^{*}p < 0.05$, $^{**}p < 0.01$. **(C)** Whole flat-mounted retina. The point on the white circle shows the central position selected for the RGC count.

epithelial cells (Kook et al., 2008). Furthermore, too high Qcn doses may activate other molecular mechanisms or generate de novo proteins, this requires further studies. Therefore, 20 μM was used as the optimal effective concentration for subsequent studies. Then, RGCs were incubated with 20 μM Qcn under hypoxia conditions for 24 h, 48 h and 72 h. Our results showed that the RGC viability exhibited a time-dependent increase with Qcn incubation ($p < 0.05$) and reached optimum levels at 48 h ($p < 0.01$) compared with that in the hypoxia group (**Figure 6B**).

Furthermore, the protective effect of Qcn was evaluated by lactate dehydrogenase (LDH) released into culture media (**Figure 6C**). In the presence of hypoxia, LDH release from RGCs increased by 31 ± 4.36% compared with that in the NC group ($p < 0.01$); however, the effect was reversed with 20 μM Qcn ($p < 0.01$). Then, RGC apoptosis and death were evaluated by flow cytometry-based Annexin V+PI assay and TUNEL staining. As shown in **Figures 6D,E**, **7A,B**, the numbers of Annexin V (+) and TUNEL-positive RGCs were significantly increased under hypoxia (23.33 ± 4.78-fold vs. 17.98 ± 4.34-fold, respectively; $p < 0.01$), whereas Qcn-treatment remarkably decreased the number of apoptotic RGCs (decreases of 90.02 ± 5.06% vs. 83.48 ± 4.33%, $p < 0.01$). These results collectively indicate that Qcn protected RGCs from hypoxia-induced apoptosis *in vitro*, which is similar to what we observed *in vivo*.

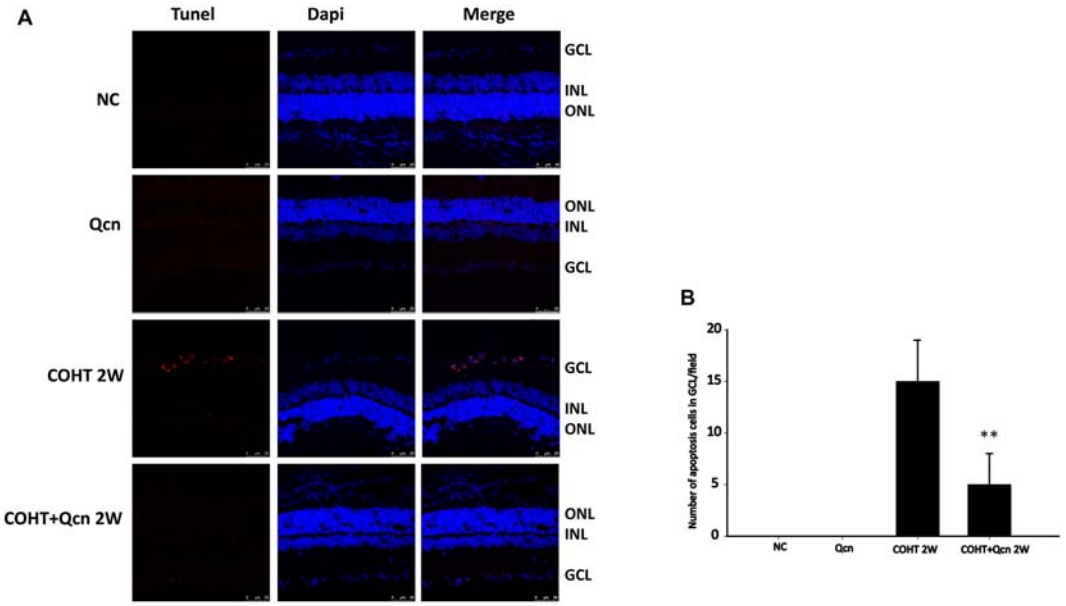

FIGURE 4 | (A) Terminal Deoxynucleotidyl Transferase dUTP Nick End-Labeling (TUNEL) analysis of retinas from four different treatment groups (NC, Qcn, COHT and COHT+Qcn groups) at 2 weeks after microbead injection. Red indicates TUNEL-positive cells, and blue indicates DAPI. GCL, retinal ganglion cell layer; INL, inner nuclear layer; and ONL, outer nuclear layer. Scale bar, 50 μm. **(B)** Quantitative analysis of TUNEL-positive cells in retinas from different treatment groups. The data are presented as the mean ± SD. $^{**}p < 0.01$.

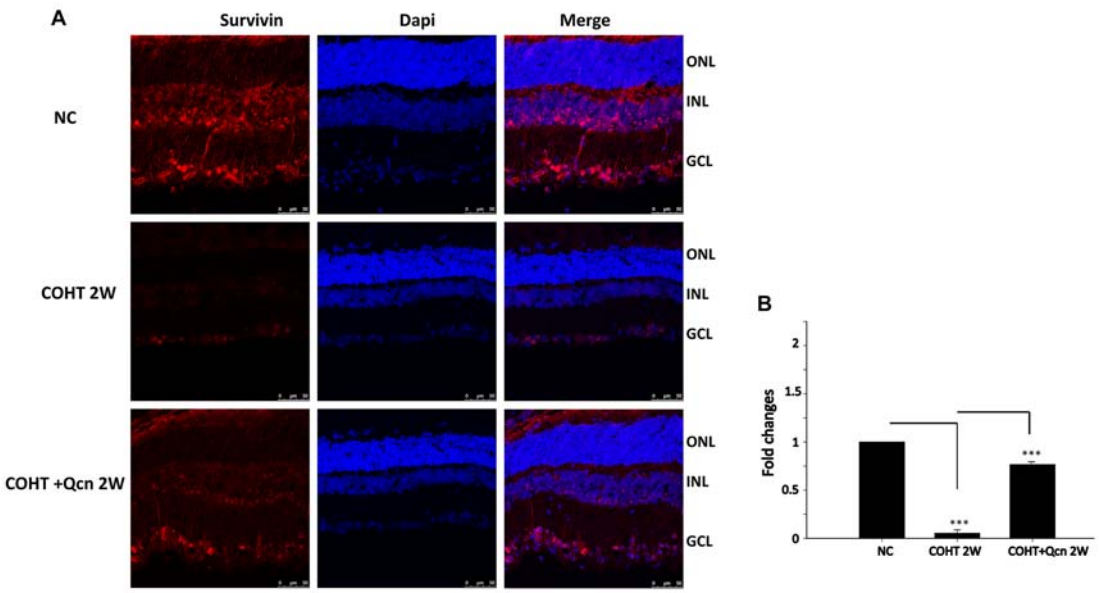

FIGURE 5 | (A) Immunofluorescence of survivin staining (red) of retinas from three different groups at 2 weeks after microbead injection. The NC group (top), COHT (middle) and COHT+Qcn group (bottom). Red indicates positive survivin staining; blue indicates DAPI; GCL, retinal ganglion cell layer; INL, inner nuclear layer; and ONL, outer nuclear layer. Scale bar, 50 μm. **(B)** Quantitative analysis of survivin-positive regions in retinas from different treatment groups. The data are presented as the mean ± SD, $^{***}p < 0.001$.

Mechanisms of the Qcn Protective Effect on RGCs

For further in-depth exploration of the mechanisms of the protective effect of Qcn on RGCs under hypoxia, the apoptotic protein levels of Bcl-2 and cleaved caspase-3, the $\Delta\Psi m$ and ROS generation were analyzed. Western blot analysis demonstrated that the expression levels of the anti-apoptotic protein Bcl-2 were reduced, while the

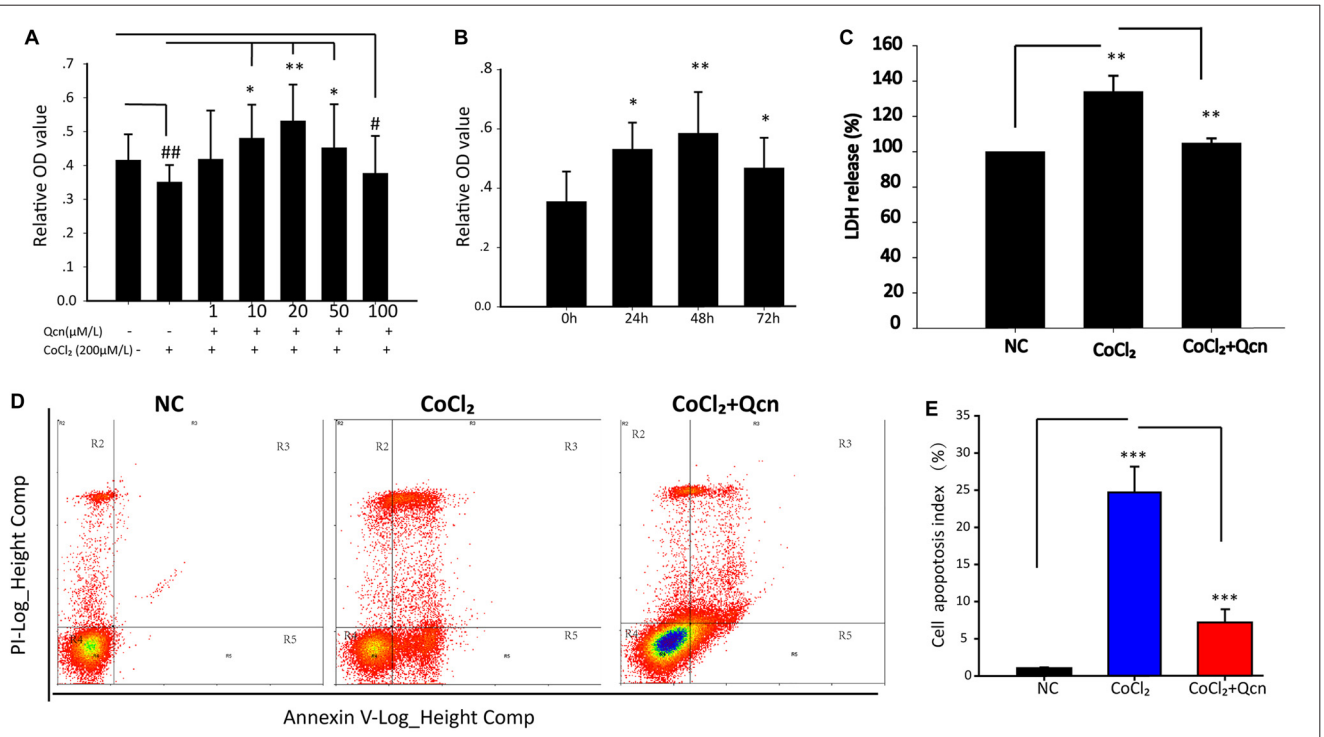

FIGURE 6 | (A) The cell viability of RGCs treated with 0–100 μM Qcn with or without $CoCl_2$ was measured by the CCK-8 assay. Bars indicate the mean ± SD; $^{\#}p < 0.05$ and $^{\#\#}p < 0.01$ compared with the NC group. $^{*}p < 0.05$ and $^{**}p < 0.01$ compared with the $CoCl_2$ without Qcn group. **(B)** RGC viability when treated with 20 μM Qcn and 200 μM $CoCl_2$ for 0, 24, 48 and 72 h. Bars indicate the mean ± SD. $^{*}p < 0.05$ and $^{**}p < 0.01$ compared with 0 h. **(C)** The impact of Qcn on lactate dehydrogenase (LDH) release of RGC treated with 20 μM Qcn and 200 μM $CoCl_2$ for 48 h. Bars indicate the mean ± SD. $^{**}p < 0.01$ when the hypoxia group was compared with the NC and $CoCl_2$+Qcn groups. **(D)** Cell apoptosis detected by AnnexinV and propidium iodide (PI) staining in RGCs treated with 20 μM Qcn and 200 μM $CoCl_2$ for 48 h. The R4 quadrant (Annexin V−/PI−), R5 quadrant (Annexin V+/PI−) and R3 quadrant (Annexin V+/PI+) indicate the percentages of viable cells, apoptotic cells and necrotic cells, respectively. **(E)** The percentage of apoptotic cells after $CoCl_2$ treatment with or without Qcn. The values represent the mean ± SD of three independent experiments. $^{***}p < 0.001$.

pro-apoptotic protein cleaved caspase-3 levels, the activated form of caspase 3, were significantly upregulated after 48 h of $CoCl_2$ treatment (**Figures 7C,D**, $p < 0.01$). However, these changes were remarkably reversed by Qcn treatment (**Figures 7C,D**, $p < 0.05$). These data indicated that Qcn could protect against hypoxia-induced RGC apoptosis by upregulating the expression of Bcl-2 and suppressing the level of cleaved caspase-3.

Qcn has been shown to play a fundamental role in modulating mitochondrial function by influencing ΔΨm (Ben Salem et al., 2016). To determine whether Qcn also has an anti-apoptotic effect on hypoxia-induced RGC by ameliorating mitochondrial function, ΔΨm and intracellular ROS levels were determined under hypoxia with or without Qcn. Quantitative data demonstrated that Qcn treatment preserved the ΔΨm by 13.58 ± 2.65% (at 12 h, $p < 0.05$) and 86.51 ± 6.86% (at 48 h, $p < 0.01$) compared with that in the control group (**Figures 8A,B**). Measurement of the fluorescence of the aggregate and monomer forms of JC-1 by flow cytometry further supported this conclusion (**Figure 8C**). Increased ROS levels are closely related to mitochondrial dysfunction. As shown in **Figures 9A,B**, a significant increase in red fluorescence representing ROS production was observed after $CoCl_2$ treatment; the increase was approximately 7.33 ± 1.58-fold ($p < 0.001$). Qcn treatment dramatically reduced the ROS generation compared with that in the $CoCl_2$-treatment group ($p < 0.01$). Flow cytometry analysis shows a leftward-shift in the log of FITC and red fluorescence in the $CoCl_2$-treatment group, while Qcn treatment effectively reduced $CoCl_2$-induced ROS production (**Figure 9C**). These findings suggest that the protective effects of Qcn are mediated, at least in part, by the direct prevention of $CoCl_2$-induced loss in ΔΨm and through an antioxidant mechanism of scavenging ROS.

DISCUSSION

Numerous studies have confirmed that Qcn has multiple biological activities and enormous potential for clinical application with safety (Sun et al., 2016). Qcn is currently undergoing clinical trials for treating cancer and is very likely to become a promising drug of choice in the near future (Madaan et al., 2016). However, as far as we know, no report is available for the use of Qcn on glaucomatous neuroprotection. Therefore, we investigated the protective effect of Qcn against RGC damage using a rat COHT model *in vivo* and explored

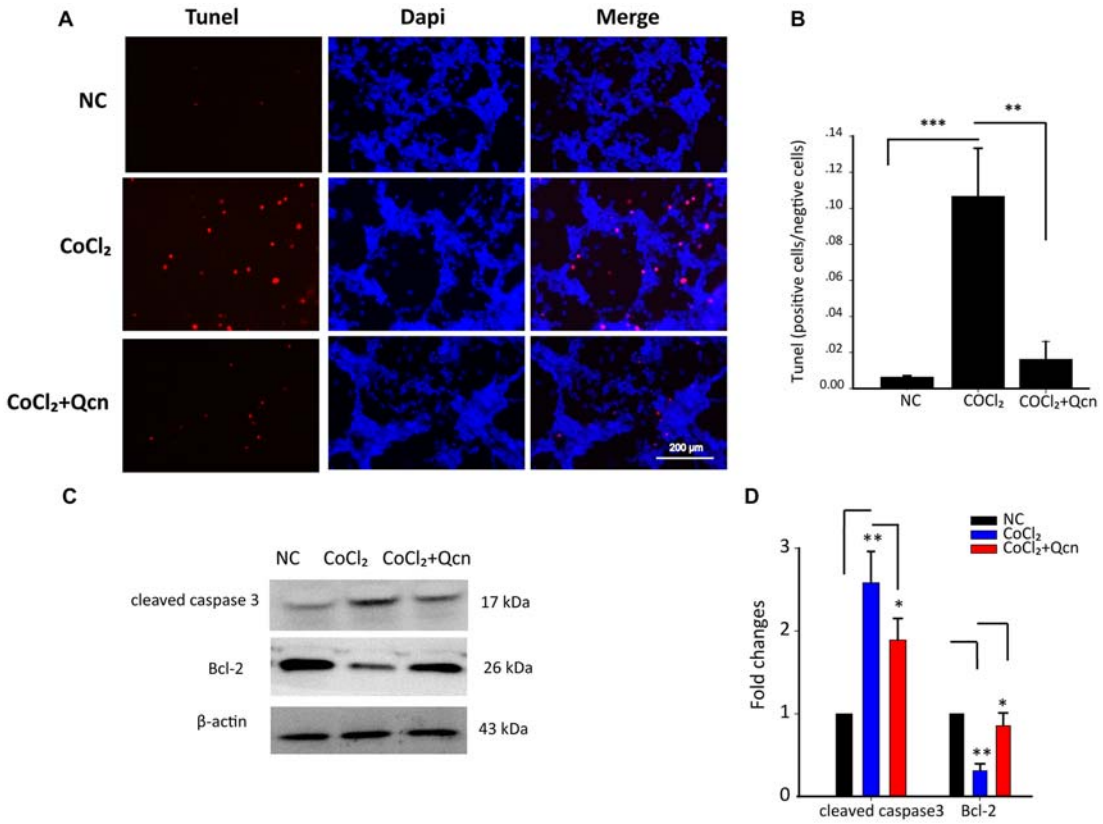

FIGURE 7 | (A) TUNEL analysis of RGCs from the following three different groups: NC group, $CoCl_2$-treatment group (48 h) and $CoCl_2$ with Qcn-treatment group (48 h). Red indicates TUNEL-positive cells, and blue indicates DAPI. All images were captured at the same magnification. Scale bar, 200 μm. **(B)** Quantitative analysis of TUNEL-positive cells in retinas from different treatment groups. The data are presented as the mean ± SD; $^{**}p < 0.01$ and $^{***}p < 0.001$. **(C,D)** Expression of cleaved caspase-3 and Bcl-2 detected by Western blot in RGCs from the following three treatment groups: NC group, $CoCl_2$-treatment group (48 h) and $CoCl_2$ with Qcn-treatment group (48 h). The bars indicate the mean ± SD. β-actin was used as an internal control. $^{*}p < 0.05$ and $^{**}p < 0.01$.

the underlying molecular mechanism by hypoxia-induced primary cultured RGC damage *in vitro*. We found that Qcn could preserve RGC function as well as prevent RGC apoptosis in a rat model of chronic glaucoma *in vivo* and hypoxia-induced RGC apoptosis *in vitro*. The mechanism does not depend on a decrease in IOP; instead, it occurs via ameliorating mitochondrial function and preventing mitochondria-mediated apoptosis.

Qcn was administered by oral or intraperitoneal injection in previous studies on the retina (Kumar et al., 2014; Arikan et al., 2015). However, to maintain an effective drug concentration and prolong the exposure of Qcn to retina after a single administration, intravitreal injection was chosen as the mode of Qcn administration in the current study. We found that this local administration was both well-tolerated and effective. Additionally, a single administration reduces animal suffering, which is supported and advocated by the Declaration of Helsinki on the care and use of animals (Villar, 1988).

There is compelling evidence that RGC dysfunction occurs early, is progressive, and precedes RGC soma loss in glaucoma (Shou et al., 2003). Therefore, early functional protection is crucial for slowing the progression of glaucomatous optic neuropathy. Although numerous neuroprotective agents have been observed to inhibit at least some RGC soma loss (surviving RGCs may not be functional) in glaucoma models (Guo et al., 2006), few studies have linked the functional improvement of these agents to RGC. PhNR is a slow negative component of the photopic full-field ERG that follows the b-wave, and it can capture RGC function throughout the entire visual field, providing a direct, objective assessment of the functional changes of RGCs (Preiser et al., 2013; Porciatti, 2015; Wilsey and Fortune, 2016). In the current study, we found that Qcn could preserve the PhNR wave early from 3 days of COHT, indicating that early RGC functional damage could be delayed or even rescued by Qcn administration with COHT. Apoptosis occurs following functional damage. FG retrograde labeling showed that Qcn effectively reduced the loss of RGCs without IOP reduction, which was further verified by TUNEL and survivin staining. Collectively, these results demonstrate that Qcn plays a protective role on RGC function and survival with COHT *in vivo*.

Apoptosis is tightly controlled by a variety of signaling pathways. Bcl-2, an anti-apoptotic protein localized to the

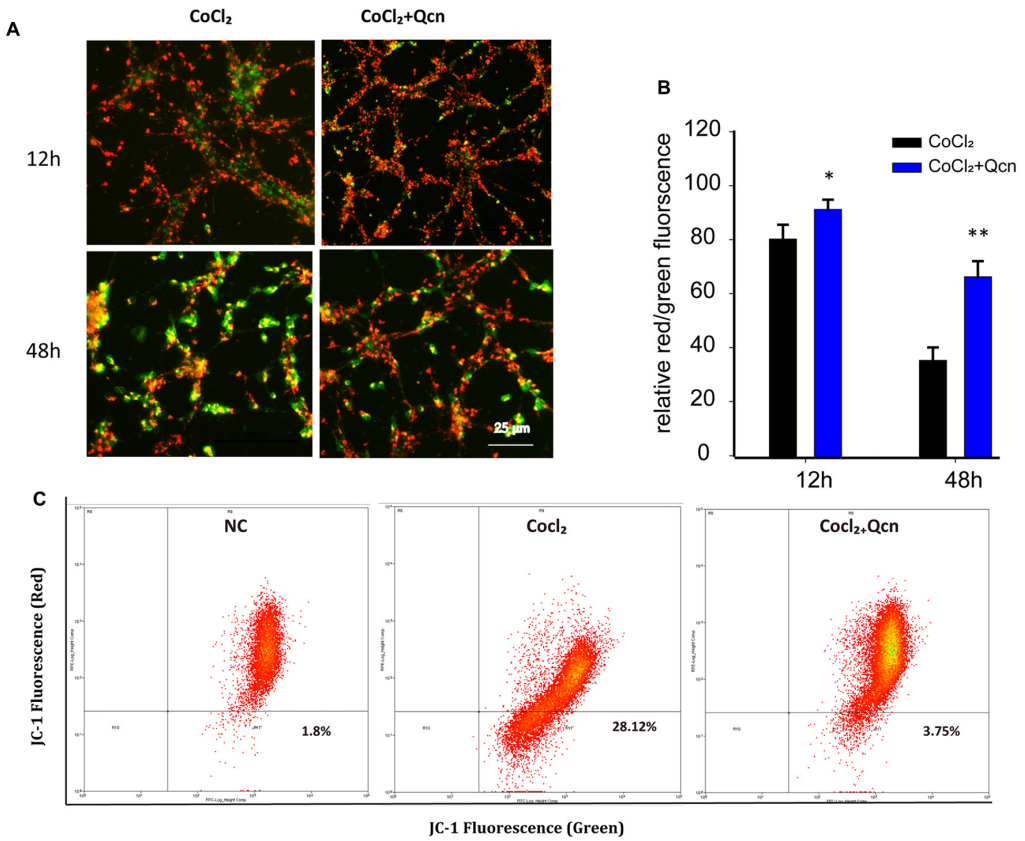

FIGURE 8 | (A) Representative images of JC-1 staining of RGCs with $CoCl_2$ and Qcn treatment for 12 h and 48 h. All images were captured at the same magnification. Scale bars, 100 μm. **(B)** Quantitative analysis of the $\Delta\psi m$ by the ratio of the red and green fluorescence. The bars indicate the mean ± SD. $^*p < 0.05$ and $^{**}p < 0.01$ for the hypoxia+Qcn group compared with the hypoxia group. **(C)** Fluorescence density of J-aggregates (*y*-axis) against JC-1 monomers (*x*-axis) displayed in a dot plot.

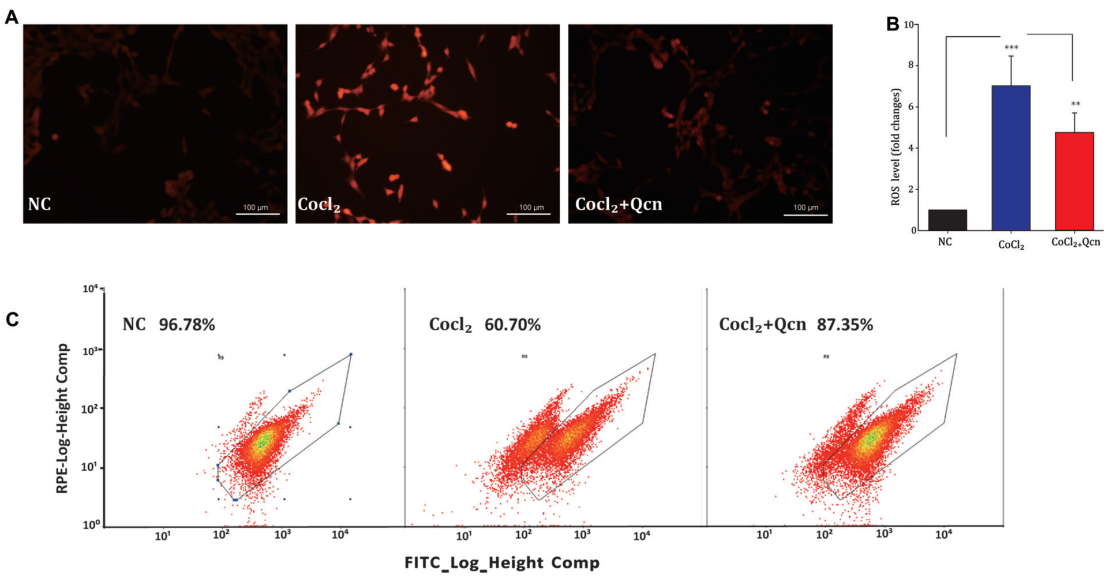

FIGURE 9 | (A) Reactive oxygen species (ROS)-induced fluorescence was visualized by confocal microscopy. Scale bar, 100 μm. **(B)** Quantitative analysis of ROS fluorescence intensities in RGCs from different treatment groups. The data are presented as the mean ± SD, $^{**}p < 0.01$ and $^{***}p < 0.001$. **(C)** Flow cytometry analysis of ROS generation shows a leftward-shift in the log of FITC in the $CoCl_2$-treatment group, whereas Qcn treatment effectively alleviates the shift.

mitochondria, is a central regulator of the intrinsic apoptotic pathway, which is also called the mitochondrial pathway (Hardwick and Soane, 2013). Bcl-2 primarily regulates cell death via its effects on mitochondrial outer membrane permeabilization (Pradelli et al., 2010), which controls the release of cytochrome C from the mitochondria to cytoplasm. The accumulation of cytochrome C in the extracellular space consequently activates caspase 3 and related downstream proteins, which eventually leads to apoptosis. Moreover, Qcn mediates the protective effects via regulating the expression of cleaved caspase-3 and Bcl-2, which has been reported by numerous studies (Kumar et al., 2014). In this context, we questioned whether these pathways were also involved in the anti-apoptotic effects of Qcn in hypoxia-induced RGCs. Our results showed that Bcl-2 expression was dramatically increased, while cleaved caspase-3 was remarkably decreased in Qcn-treated RGCs compared with the levels in the hypoxia-induced group. Bcl-2 overexpression can inhibit the accumulation of cytochrome C in the cytoplasm to inhibit cell apoptosis (Wu and Bratton, 2013). Therefore, one possible neuroprotective mechanism of Qcn is that it inhibits RGC apoptosis partially via increasing Bcl-2, while subsequently down-regulating cleaved caspase-3 expression, which is consistent with previous studies (Hu et al., 2015). $\Delta\Psi$m is a valuable indicator of mitochondrial functional status in living cells, and the lipophilic cation JC-1, a sensitive and non-invasive specific probe, is currently the gold standard for rapidly measuring $\Delta\Psi$m (Brooks et al., 2013). In this study, we found that Qcn treatment effectively recovered the $\Delta\Psi$m of RGC under hypoxia, indicating that Qcn has a protective role on mitochondrial function, which has been verified in several other types of cells and tissues, such as pancreatic β-cells (Carrasco-Pozo et al., 2016), the frontal cortex, hippocampus (Nichols et al., 2015; Gupta et al., 2017), mouse livers (Yu et al., 2016) and others. Mitochondria are the major sites of ROS production under physiologic conditions, and ROS generation is associated with mitochondrial dysfunction (Tezel, 2006). According to our data, Qcn attenuates $CoCl_2$-induced ROS formation, which further supports that Qcn modulates mitochondrial function to protect RGCs. Taken together our results demonstrated that Qcn exerts neuroprotective effects on hypoxia-induced RGC apoptosis via ameliorating mitochondrial function and preventing mitochondria-mediated apoptosis.

A variety of molecular signals—acting alone or in cooperation—have been involved in glaucomatous pathophysiology, including oxidative stress (Chen et al., 2015), glutamate excitotoxicity (Lam et al., 1999), mitochondrial dysfunction (Nickells, 1999), glia activation (Lam et al., 2009), inflammation (Levkovitch-Verbin, 2015), autophagy (Deng et al., 2013), ischemia (Almasieh et al., 2012), ER stress (Ha et al., 2015), and others, while Qcn has been reported to have many benefits and medicinal properties with regard to these pathological processes. Therefore, we propose that Qcn may offer protection to RGC with COHT via a variety of mechanisms. Further studies are needed to elucidate the exact mechanism by which Qcn protects RGCs with COHT.

In conclusion, this study provides the first direct evidence that Qcn preserved RGC function and prevented RGC apoptosis in a rat model of chronic glaucoma *in vivo* and in hypoxia-induced RGC apoptosis *in vitro*. The mechanism does not depend on a decrease in IOP but instead involves ameliorating mitochondrial function and preventing mitochondria-mediated apoptosis. Our results provide important evidence that Qcn may be a promising therapeutic strategy for ameliorating RGC damage in glaucomatous neurodegeneration.

AUTHOR CONTRIBUTIONS

J-HW and X-HS designed this work, revised it critically and finally approved the version to be published. PX, RZ, B-QY, X-JZ, J-YC, YC, W-JH and MW took part in some of the experimental studies, for example, western blotting, PCR analysis and cell culture. F-JG and S-HZ drafted, revised the manuscript and took part in a majority of the work. All authors read and approved the manuscript.

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