CEREBRAL ENDOTHELIAL AND GLIAL CELLS ARE MORE THAN BRICKS IN THE GREAT WALL OF THE BRAIN: INSIGHTS INTO THE WAY THE BLOOD-BRAIN BARRIER ACTUALLY WORKS (CELEBRATING THE CENTENARY OF GOLDMAN'S EXPERIMENTS)

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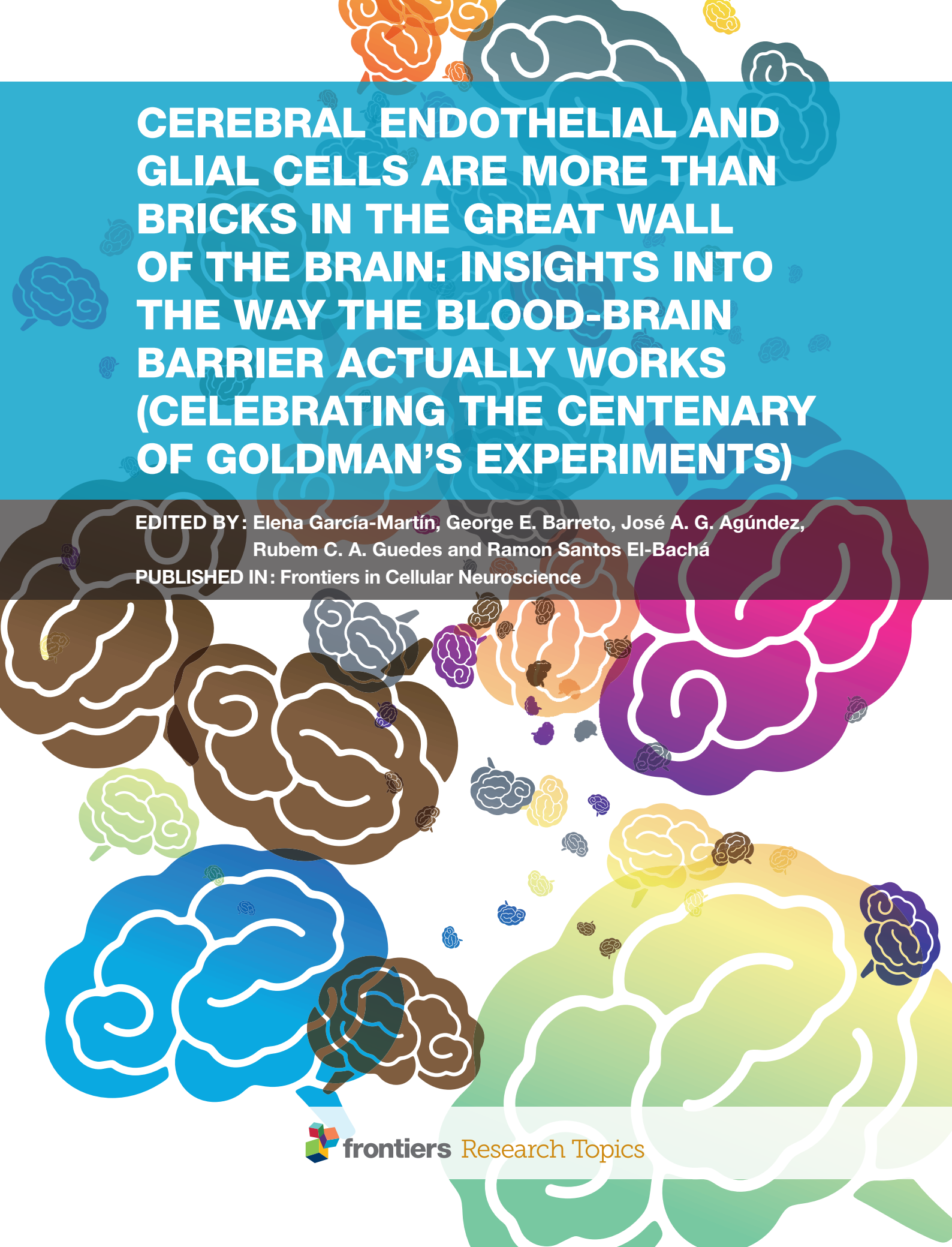

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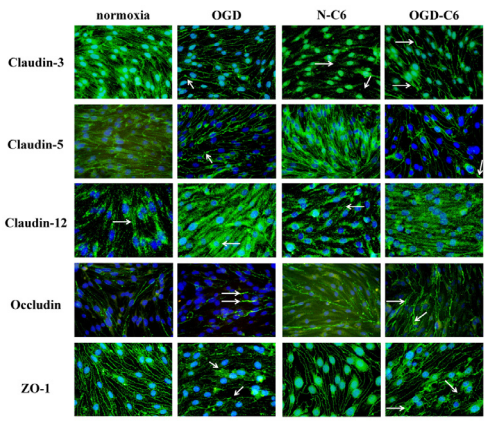

Around the year 1900 the discoveries by Paul Ehrlich, Max Lewandowsky and Edwin Goldman revealed the existence of a barrier that protects the central nervous system. Cerebral capillary endothelial cells are the major constituent of the blood-brain barrier. In contrast to peripheral endothelial cells, intercellular gaps between cerebral capillary endothelial cells are sealed by tight junctions restricting unregulated entrance of hydrophilic substances into the central nervous system. Nowadays, it is known that the communication between cerebral capillary endothelial cells with cells from the central nervous system such as astrocytes, pericytes or microglia plays a pivotal role in the regulation of the blood-brain barrier.

The currently used terms neurovascular or gliovascular units account for this important influence of the microenvironment around cerebral capillary endothelial cells. In this context, astrocytes are essential for the tightness of the blood-brain barrier by inducing tight junction protein expression under physiological conditions. On the contrary, the role of astrocytes under pathological conditions is still under investigation. The image shows the effects of astrocyte-derived factors in combination with oxygen/glucose deprivation (as a model of ischemia) on the localization of tight junction proteins claudin-3, claudin-5, claudin-12, occludin and ZO-1 of mouse brain endothelial cells.
Image taken from: Neuhaus W, Gaiser F, Mahringer A, Franz J, Riethmüller C and Förster C (2014) The pivotal role of astrocytes in an in vitro stroke model of the blood-brain barrier. Front. Cell. Neurosci. 8:352. doi: 10.3389/fncel.2014.00352

When Ehrlich discovered the first evidence of the blood-brain barrier in 1885, he probably did not perceive the Great Wall that remained hidden from consciousness inside the central nervous system. Ehrlich had observed that acidic vital dyes did not stain the brain if they were injected into the blood stream. A century ago (1913), Goldman showed that the injection of trypan blue in the cerebrospinal fluid stained only the brain, but not the other organs. For almost a century it was thought that the blood-brain barrier (BBB) consisted in a physical barrier, resulting from the restricted permeability of the cerebral endothelial cell layer, as they are joined by tight junctions. However, as scientists are always looking for news in what is already discovered, in the end of the 20th century we had evidences that cerebral endothelial and glial cells express several drug metabolizing enzymes consisting in a second protection system: a metabolic barrier. Furthermore, the drugs and their metabolites must overcome the activity of several multidrug resistance proteins that function as ATP-dependent efflux pumps, consisting in the third line of defence: the active barrier. Therefore, the way the BBB actually works should be better explained. Several endogenous compounds, as well as xenobiotics, may be activated by enzymes of the metabolic barrier, generating reactive oxygen species that could damage neurons. Therefore, endothelial and glial cells possess endogenous protecting compounds and enzymes against oxidants, consisting in an antioxidant barrier. When all these systems fail, glial cells, mainly microglia, secrete cytokines in an attempt to crosstalk with defence cells asking for help, which consists in an immune barrier. In cerebral regions that are devoid of the physical barrier, such as circumventricular organs, the metabolic, active, antioxidant and immune barriers are reinforced. It is important to understand how cells involved in the BBB interact with one another and the dynamic mechanisms of their functions. This Research Topic published in this e-Book considers recent highlights in BBB structure, cell and molecular biology, biotransformation, physiology, pathology, pharmacology, immunology and how these basic knowledges can be applied in drug discovery and clinical researches, rewriting what is already written, and paving the way that goes to the Great Wall in the Frontiers of the Brain in this new century that is just beginning.

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EDITORIAL
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Editorial on Cerebral endothelial and glial cells are more than bricks in the Great Wall of the brain: insights into the way the blood-brain barrier actually works (celebrating the centenary of Goldman's experiments)

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Keywords: blood-brain barrier, glial cells, cerebral endothelial cells, xenobiotic-metabolizing enzymes, ABC transporters, oxidative stress response, neuroinflammation

In August 23 (1913), The Lancet reported the death of Dr. Edwin Ellen Goldmann in its obituary, which had occurred on August 12th of this same year due to a malignant disease of the liver. The introduction of methylene blue as a histological reagent by Paul Ehrlich led to the discovery that all organs, but the brain, were stained after its injection into the blood stream. Goldmann showed the inverse by staining only the brain after injection of dyes into the cerebro-spinal fluid. Concerning the work of Dr. Goldmann, Dr. F. W. Mott wrote in the obituary of The Lancet (1913): "We have learnt from his work that the cerebro-spinal fluid receives from the choroid plexus important products which are carried to the nervous system tissue. Also the plexus protects the nervous tissue from the penetration of toxic substances." A century after his pioneering work, which supported the idea of the existence of a blood-brain barrier (BBB), we decided to celebrate it by proposing this Research Topic to Editors of Frontiers in Cellular Neuroscience.

We would like to thank all contributors for their valuable work helping us to present wide-ranged aspects in the field. Currently we know that a physical barrier due to the formation of tight junctions in cerebrovascular endothelial cells is the main component of the BBB. In this Research Topic, the implication of astrocyte functions in the protection of the BBB was reviewed (Cabezas et al., 2014). One year after the Goldmann's death, the First World War began in Europe leading to the death of 16 million people due to the military industry technological sophistication, which has increased since then and continues to kill several human lives. An article reviewing alterations in the brain milieu causing dysfunction or disruption of the BBB following exposure to blast shock waves was published in this Topic (Shetty et al., 2014). Since cerebrovascular diseases are prevalent worldwide, affecting the structure and functions of the BBB, cellular effectors to recover the neurovascular unity integrity were reviewed (Posada-Duque et al., 2014).

Currently we know that the BBB is more than a physical barrier. Several drug- and xenobiotic-metabolizing enzymes are expressed in endothelial and glial cells, constituting a metabolic barrier. The expression of xenobiotic metabolizing enzymes in the BBB was discussed (Agúndez et al., 2014). However, the attempt to protect neurons from xenobiotic by metabolizing them sometimes

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fails. Catechol is a compound that induces glutathione (GSH) depletion, which leads to apoptosis (Lima et al., 2008). This depletion is due to glutathione transferases (GST; EC 2.5.1.18), which catalyzes the conjugation of catechols to GSH. Therefore, the inhibition of xenobiotic metabolizing enzymes is sometimes useful to protect neurons. The inhibitory effect of 8-methoxypsoralen on GST-π activity was investigated (Oliveira et al., 2014). Although the glucuronidation of catechols was not catalyzed by brain microsomes, planar phenols could be conjugated (El-Bachá et al., 2000). The importance of UDP-glucuronosyltransferases (EC 2.4.1.17) in the BBB was reviewed (Ouzzine et al., 2014). ABC-transporters in endothelial and glial cells exert an active function in the BBB. An *in vitro* stroke model of the BBB was used to investigate how oxygen/glucose deprivation can affect tight junction proteins, as well as the expression of ABC-transporters (Neuhaus et al., 2014).

It seems that there is also an antioxidant barrier, which protects the central nervous system against the oxidative stress. The endogenous protection against reactive oxygen species can occur through the increased expression of mitochondrial enzymes in astrocytes (Cabezas et al., 2012). The expression of heme oxygenase (EC 1.14.99.3) isoform 1 (HMOX1) is modulated by pro-oxidants in neuroglia. In another study, an association between HMOX1 genetic variants and Parkinson's disease was investigated (Ayuso et al., 2014). Furthermore, biomarkers of this disease were investigated on cerebrospinal fluid of patients (Jiménez-Jiménez et al., 2014).

Cells involved in the BBB can establish immune communications. Interactions between the microglia and the vascular system (Fonseca et al., 2014), as well as the role of macrophages at the brain borders, how they enter and differentiate inside the brain, and their functions in health and neuroinflammation were reviewed (Corraliza, 2014). The effect of inflammatory stimulus on neuron/glial co-cultures infected with *N. caninum* was evaluated (Jesus et al., 2014).

The connection between the BBB and pain was reviewed, focusing on cellular and molecular mechanisms of BBB permeability induced by inflammatory or neuropathic pain and migraine (DosSantos et al., 2014). The BBB in gliomas was also reviewed (Dubois et al., 2014).

A century after the work of Goldmann, who contributed to the discovery of the BBB, several questions remains without answers. However, it is already known that BBB is far more than a physical barrier. Endothelial and glial cells also exert metabolic, antioxidant and immunological activities. It seems of great importance to understand the role of BBB in neurodegenerative diseases. Compounds that could show an ability to repair BBB injury, to suppress neuroinflammation and to provide neuroprotection remain to be discovered. The role of microglia and macrophages in the BBB needs to be better understood, if we want to design effective therapies against neuroinflammation. Efforts to discover strategies that could protect neurons in ischemic events continue to defy neuroscientists. Advances in biochemistry, molecular biology, and genetics will improve our knowledge about the BBB in the next 100 years.

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The UDP-glucuronosyltransferases of the blood-brain barrier: their role in drug metabolism and detoxication

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†Alain Minn (in memoriam) deceased January 6, 2007.

UDP-glucuronosyltransferases (UGTs) form a multigenic family of membrane-bound enzymes expressed in various tissues, including brain. They catalyze the formation of β-D-glucuronides from structurally unrelated substances (drugs, other xenobiotics, as well as endogenous compounds) by the linkage of glucuronic acid from the high energy donor, UDP-α-D-glucuronic acid. In brain, UGTs actively participate to the overall protection of the tissue against the intrusion of potentially harmful lipophilic substances that are metabolized as hydrophilic glucuronides. These metabolites are generally inactive, except for important pharmacologically glucuronides such as morphine-6-glucuronide. UGTs are mainly expressed in endothelial cells and astrocytes of the blood brain barrier (BBB). They are also associated to brain interfaces devoid of BBB, such as circumventricular organ, pineal gland, pituitary gland and neuro-olfactory tissues. Beside their key-role as a detoxication barrier, UGTs play a role in the steady-state of endogenous compounds, like steroids or dopamine (DA) that participate to the function of the brain. UGT isoforms of family 1A, 2A, 2B and 3A are expressed in brain tissues to various levels and are known to present distinct but overlapping substrate specificity. The importance of these enzyme species with regard to the formation of toxic, pharmacologically or physiologically relevant glucuronides in the brain will be discussed.

Keywords: UDP-glucuronosyltransferases, blood-brain barrier, drug glucuronidation, detoxication barrier, glucuronidation of endogenous compounds

THE UDP-GLUCURONOSYLTRANSFERASE FAMILY

UDP-glucuronosyltransferases (UGTs, EC 2.4.1.17) are a multigenic family of enzymes responsible for the glucuronidation reaction, a main process of phase II xenobiotic biotransformation (Mackenzie et al., 2005). Products of phase I reactions mediated by P450-dependent monooxygenases are major substrates of UGTs, although substances which possess a functional chemical groups (hydroxyl-, phenyl, carboxylic acid, amines, thiols) are readily glucuronidated. Some of these substances especially hydroxylated or phenolic molecules can also be sulfated by sulfotransferases (SULTs). These enzymes compete with UGTs towards the same substrates. UGTs as monooxygenases, are membrane-bound enzymes. They are predominantly associated to the endoplasmic reticulum. If cytochromes P450 are expressed on the cytosolic surface of the membranes, the UGTs are present on the luminal side. Indeed intra-membrane co-localization and interactions of cytochromes P450 and UGTs insure a topological and functional coupling for an efficient stepwise drug biotransformation (Ouzzine et al., 1999; Ishii et al., 2007).

Abbreviations: BBB, blood-brain barrier; CSF, cerebrospinal fluid; DA, dopamine; 5-HT, 5-hydroxytryptamine; OB, olfactory bulb; OM, olfactory mucosa; OS, olfactory system; UGT, Uridine-diphosphate-glucuronosyltransferase.

UGTs are glycosyltransferases that catalyze the covalent binding of glucuronic acid from the high energy donor, UDP-αD-glucuronic acid on structurally related substances with a functionalized nucleophilic group, leading to the formation of water soluble β-glucuronides and UDP (**Figure 1**). The reaction is believed to occur according to a second order nucleophilic substitution (S_N2) involving an amino acid base catalyst (Magdalou et al., 2010). The main characteristic of UGTs is their potency to glucuronidate a large array of structurally unrelated substances. They play a major role in the detoxication of xenobiotics, including drugs and environmental substances, as well as in the metabolism of endogenous compounds (bilirubin, steroid hormones, bile acids, fatty acids) (Rowland et al., 2013). As such, they efficiently contribute to the protection of the organism against hazardous chemicals, and regulate the activity of several endogenous mediators involved in cell growth and differentiation. Many therapeutic classes of drugs, some of them targeting brain tissues, such as analgesics, anticonvulsants or antipsychotics are UGT substrates (de Leon, 2003). UGTs are also involved in the glucuronidation of endogenous compounds. Particularly, the monoamine neurotransmitters, dopamine (DA) and serotonin (5-hydroxytryptamine, 5-HT) are substrates of UGTs which may play a regulating role in their physiological function and implication in brain disorders (Suominen et al., 2013).

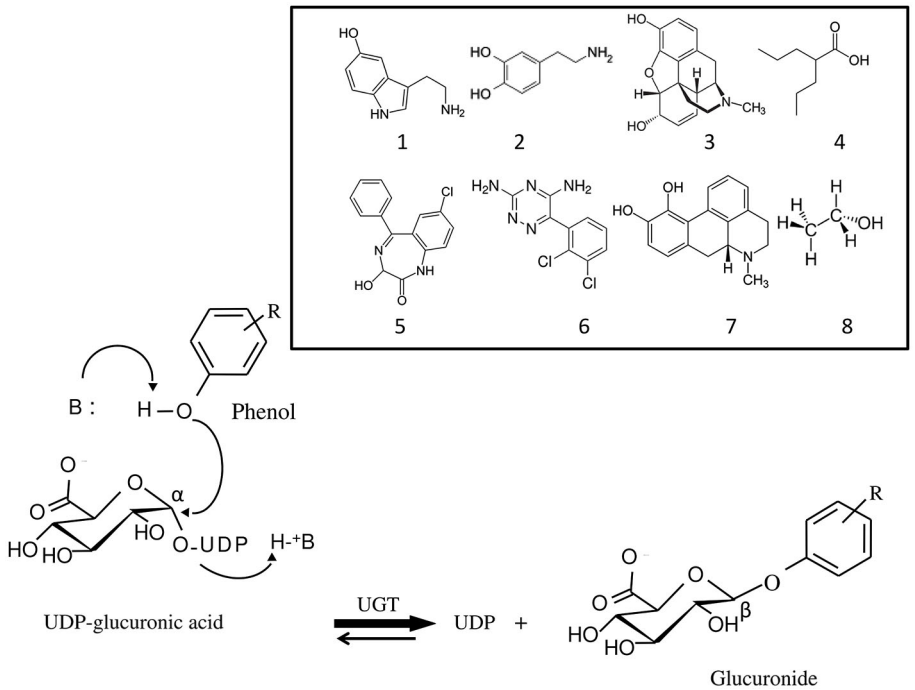

FIGURE 1 | Mechanism of the glucuronidation reaction catalyzed by UDP-glucuronosyltransferases (UGT). Glucuronidation is a bi-substrate reaction which requires an aglycone (for example, a phenol) and a high energy glucuronic acid donor, UDP-α-D-glucuronic acid, which is the common substrate to all UGT isoforms. The reaction leads to the release of UDP and formation of a β-D-glucuronide. UGT belongs to the inverting glycosyltransferase family which utilizes a direct S_N2-like mechanism involving a base (B) catalyst. Structurally unrelated substances, brain transmitters and drugs against brain disorders that are UGT substrates are shown: 1, serotonin; 2, dopamine; 3, morphine; 4, valproic acid; 5, oxazepam; 6, lamotrigine; 7, apomorphine; 8, ethanol.

This powerful detoxication barrier is due to the expression in hepatic and extrahepatic tissues of several isoforms exhibiting distinct but overlapping substrate specificity. In human up to 22 UGT isoforms have been identified to date belonging to 1A, 2A, 2B, 3A and 8A subfamilies, based on a 29 amino acids conserved signature involved in the binding site for the common substrate, UDP-glucuronic acid (Rowland et al., 2013). Among these families, UGT1A, 2A and 2B are particularly active in xenobiotic biotransformation. Interestingly, the UGT isoforms exhibit tissue specificity in terms of isoform present and level of expression. If liver contains the greatest amount and variety of UGT isoforms, other organs, such as kidney or the intestinal tract are known to express several isoforms in significant amount (Court et al., 2012; Harbourt et al., 2012).

UGT has also been found in brain and associated tissues. A glucuronidation activity has been reported upon incubation of microsomes from total brain of rat with xenobiotics and endogenous compounds known to be substrates of the enzyme (Suleman et al., 1993). This activity was much lower than that found in liver or in other extra-hepatic organs. These enzymes were progressively characterized at a protein and gene levels using immunohistochemistry (Martinasevic et al., 1998) and PCR techniques (Suleman et al., 1998), thus allowing a precise identification of the isoforms and their localization within the brain tissues.

In the brain, despite their low level of expression, UGTs participate, in cooperation with the other drug metabolizing enzymes and the various ABC efflux transporters, to a metabolic barrier which prevents efficiently the organ from the intrusion of xenobiotics. Particularly, the blood brain barrier (BBB) and the associated cells, such as the endothelial cells, play a critical role on the cerebral homeostasis. The nasal cavity which affords a direct route of entry for xenobiotics to the brain contains specific UGTs isoform that are highly expressed in the olfactory tissue. In this condition, variations of UGT expression by genetic (polymorphism), environmental (induction or repression), pathophysiological factors or during ontogenesis and perinatal development will affect the permeability and the metabolic function of this barrier prone to potential toxic effects.

This review is focused on the importance of UGT in brain function. Although if, in terms of drug metabolism, the relatively small level of expression of these enzymes in the brain areas would have a minor impact on the overall glucuronidation of xenobiotics, it surely can have a major effect when protecting locally a specific cerebral cell from the neurotoxicity of substances which enter the brain. In terms of brain homeostasis, UGTs play a subtle role in the fine tuning of the concentration of endogenous neurotransmitters, ligands of receptors, or in the modulation of the pharmacological effects of brain-directed drugs. The presence of brain specific UGT isoforms highlights the importance of this family of enzymes in this process.

A LIMITED NUMBER OF UGTs ARE EXPRESSED IN THE BLOOD-BRAIN BARRIER

The drug transporters and drug metabolizing enzymes that are present in the BBB prevent access to the brain of some lipid-soluble drugs and potentially toxic substances. It has been shown, several years ago, that rat brain microsomes exhibit high glucuronidation activity towards the reference substrate, 1-naphthol (Ghersi-Egea et al., 1987). This glucuronidation activity was found to be rather low in human brain. Interestingly, the 1-naphthol activity was also present in rat brain microvessels (Ghersi-Egea et al., 1988), indicating the capacity of endothelial cells to metabolize this compound and to participate to the protection of the brain toward this substance and related toxic compounds. The 1-naphthol activity was attributed to the UGT isoform 1A6 (UGT1A6) as it has been demonstrated that other phenolic substrates conjugated by this isoform were also metabolized in brain (Suleman et al., 1993). At cellular level, the 1-naphthol UGT1A6 activity was 10-fold higher in astrocytes, as compared with neurons and endothelial cerebrovascular cells (Suleman et al., 1998). Investigation of the expression of UGT1A6 in brain has been carried out by several authors (Martinasevic et al., 1998; Suleman et al., 1998). The expression of UGT1A6 was detected in rat astrocytes, neurone homogenates and brain microsomes by immunoblotting and by reverse transcriptase-polymerase chain reaction (RT-PCR). The presence of UGT1A6 in neuronal cells, especially the pyramidal cells of the cortex and the granular cells in the cerebellum was further confirmed by immunohistochemical localization of UGT1A6 using specific antibodies (Martinasevic et al., 1998). However, this study did not support the presence of UGT1A6 in the microvasculature of the rat brain as previously suggested. Similarly, investigation of the expression of UGTs in human brain microvessels did not reveal the presence of UGT1A6 (Shawahna et al., 2011), suggesting that this isoform was not expressed or very weakly expressed in human brain microvessels compared to rat. In line with this, it has been shown that UGT-mediated metabolism of 1-naphthol was less prominent in human brain compared to rat brain, suggesting that species differences may exist (Ghersi-Egea et al., 1993).

In addition to UGT1A6, the presence of UGT1A7, an isoform known to glucuronidate benzo(a)pyrene hydroxylated metabolites was detected by RT-PCR in rat astrocytes (Gradinaru et al., 2012). As UGT1A6, the mRNA expression of this UGT isoform is inducible by xenobiotics (Kobayashi et al., 1998). Among UGT1A isoforms, UGT1A1 was shown to be expressed in rat cerebellum at the mRNA level (Shelby et al., 2003). However glucuronidation activity towards bilirubin, the main substrate of this UGT isoform, has not been detected in rat brain, thus suggesting that UGT1A1 was not present (Suleman et al., 1993).

Recently, the expression of UGT1A4 has been investigated in brain. This isoform is known to catalyze the N-glucuronidation of primary, secondary and tertiary amines, among those are anticonvulsivants, tricyclic antidepressants and antipsychotics, such as lamotrigine, doxepin or clozapine (Li et al., 2007; Kerdpin et al., 2009). Immunobloting and glucuronidation activity using lamotrigine as a substrate indicated the presence of this UGT isoform in human brain microvascular endothelial cells (Ghosh et al., 2013). Analysis of the expression of UGT1A4 by immunohistochemistry in brain from patients with epilepsy indicated high expression in BBB endothelial cells and neurons with variable levels of UGT1A4 expression among the brain specimens analyzed. Endothelial cells from brain microvessels of these patients showed high lamotrigine glucuronidation activity compared to cells from non-pathological brain (Ghosh et al., 2013), suggesting a role in the drug-resistant epileptic brain. Although preliminary, these results emphasize the potential importance of the UGT1A4 isoform in the biotransformation of psychiatric drugs in brain.

On the other hand, the UGT2B7 isoform catalyzes the glucuronidation of morphine to 3-O- and 6-O-glucuronide, the latter metabolite presenting a higher analgesic potency than morphine (Gong et al., 1991; **Figure 2**). It has been shown that morphine-6-O-glucuronide exhibited a slow transport across the BBB compared to morphine (Bouw et al., 2001), therefore the presence of UGT2B7 in brain may lead to local formation of morphine-6-O-glucuronide that can exert its analgesic actions. Investigation of the expression of UGT2B7 and of the formation of morphine glucuronides has been conducted in rat and human brain. In rat brain, analysis of the glucuronidation activity towards morphine indicated the absence of formation of morphine glucuronides (Suleman et al., 1993), suggesting that UGT2B7 may not be expressed in rat brain at least at basal levels. In contrast, the expression of UGT2B7 has been revealed by RT-PCR in human cerebellum 1, whereas no expression of this isoform was detected in brain cortex (King et al., 1999). Analysis of the glucuronidation activity of microsomes from human brain cortex towards morphine did not show any detectable activity confirming the absence of UGT2B7 in brain cortex. Only a weak activity was present in cerebellum 1. Noteworthy, Wahlström et al. (1988) reported the presence of morphine glucuronidation activity in human brain microsomes. However, this activity was detectable in only 3 out of 19 brain samples analyzed, suggesting large inter-individual variations in brain expression of this isoform. A recent model of transgenic mouse expressing human UGT2B7 has been developed showing a differential expression

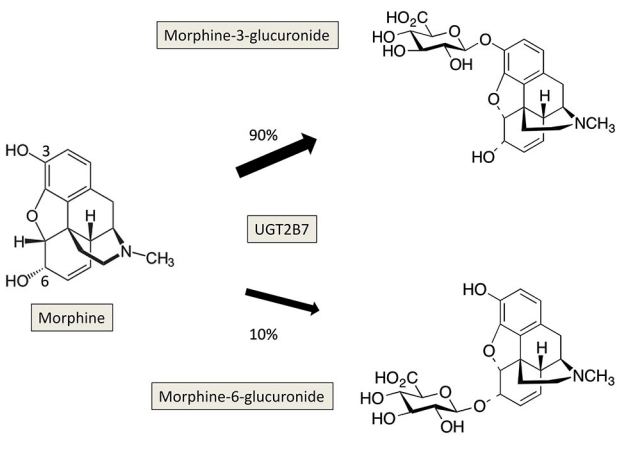

FIGURE 2 | Glucuronidation of morphine by UGT2B7 in human. The reaction occurs on the two hydroxyl groups at positions 3 and 6 of morphine, and leads to the formation of 3-, and 6-glucuronides which present different pharmacological properties.

and activity of the enzyme in several tissues including brain to a lesser extent (Yueh et al., 2011). This animal model is a powerful tool to decipher the role of UGT2B7 *in vivo* and to understand the regulation mechanisms of its expression and activity under various experimental conditions (in presence of various biologically active compounds like drugs, toxics, hormones or in response to potential UGT inhibitors).

Interestingly, Christen and Fent (2014) recently reported the transcription profile of eight UGT genes in zebrafish tissues (*Ugt1a, Ugt1b, Ugt5a1, Ugt5a3, Ugt5a4, Ugt5a5, Ugt5c2, Ugt5c3*). They found they were significantly expressed in brain, especially the UGT5a and 5c families. However their catalytic function and implication in xenobiotic metabolism are not known yet. On the other, in the antennae of the pest insect, Spodoptera littoralis, Bozzolan et al. (2014) have characterized the expression of eleven putative UGTs that were differently regulated by odorants. Moreover, the isoform UGT46A6 was up-regulated by the insecticide deltamethrin. These data suggest a protective role of this enzyme in this olfactory organ towards xenobiotics.

Altogether, among the different UGT isoforms characterized so far in various tissues, it appears that only few of them are expressed in mammal and human brain. The construction of an anatomically comprehensive atlas of the adult human brain and mouse transcriptome which is currently under way will undoubtedly bring additional information on the presence of other or novel UGT isoforms (Lein et al., 2007; Sunkin and Hohmann, 2007).

ROLE OF GLUCURONIDATION IN THE METABOLISM OF NEUROTRANSMITTERS

DOPAMINE AND SEROTONIN CONJUGATION IN BRAIN

DA and serotonin (5-hydroxytryptamine, 5-HT) are monoamine neurotransmitters. Their homeostasis in central and peripheral nervous system is important for many physiological processes as well as in pathological situations (Meiser et al., 2013). The function of DA has been linked to the regulation of motoric movements. The age-related Parkinson's disease that coincides with the degeneration of dopaminergic neurons within the *substantia nigra* is associated with typical motor symptoms such as rigidity, tremor or bradykinesia. The discovery that Parkinson's disease was associated with neostrial DA depletion (Ehringer and Hornykiewicz, 1960) led to the first treatment with L-3,4-dihydrophenelylalanine (DOPA, levodopa), which is still in use today. Schizophrenia and depression are other diseases linked to DA deregulation (Heinz and Schlagenhauf, 2010), and attention deficit hyperactivity disorder (ADHD) has been recently connected to deficient DA signaling (Tripp and Wickens, 2012). 5-HT is involved in many physiological functions such as body temperature regulation, blood pressure, perception of pain, sleep-wake cycles, and pathological processes including depression and anxiety (Berger et al., 2009).

Upon neuronal excitation, DA is released into the synaptic cleft for signal transduction. DA signaling stops by reimport to the synaptic neuron and recycling, or degradation following uptake by glial cells. DA is primarily metabolized by oxidative deamination by monoamine oxidase (MAO) and aldehyde dehydrogenase-catalyzed reactions to 3,4-dihydrophenylacetic acid (DOPAC; **Figure 3**). DOPAC can be further metabolized to homovanillic acid (HVA) by catechol-O-methyltransferases (COMT). 5-HT is metabolized by MAO and aldehyde dehydrogenase to 5-hydroxyindoleacetic acid (5-HIAA; **Figure 3**). Both DA and 5HT and their respective metabolites can undergo conjugation with glucuronic acid or sulfonate mediated by UGTs and SULTs respectively, that occurs in both central nervous system and periphery. In the brain, phase I metabolites of DA i.e., DPAC and HVA, which are linked to the functional activity of dopaminergic neurons are predominant. However, in rat, mouse and human cerebrospinal fluid (CSF) that is assumed to reflect the metabolism of neurotransmitters, DA-glucuronide and sulfate conjugates have been found (Wang et al., 1983; Tyce et al., 1986; Uutela et al., 2009a). Due to the lack of commercially available standards, most conjugates in human, and animal CSF or brain samples have been analyzed after acid or enzymatic hydrolysis (Swahn and Wiesel, 1976). In addition, several studies report the detection of conjugates but do not specify the type of conjugate (Gordon et al., 1976; Tyce et al., 1985) leading to ambiguous results. However, in rat CSF, DA glucuronide was found predominant over DA-sulfate and free DA, suggesting that glucuronidation was an important metabolic pathway for DA of central origin (Wang et al., 1983). DA-glucuronide was also detected in human CSF samples following β-glucuronidase analysis (Tyce et al., 1986). The presence of intact glucuronide as the major DA conjugate was recently confirmed in rat and mouse brain microdialysates using liquid chromatography tandem mass spectrometry (LC-MS/MS; Uutela et al., 2009a). LC-MS/MS also detected 5-HT-glucuronides at concentration 2-times than HT itself (Uutela et al., 2009b). Altogether, the results indicated that in rat, neurotransmitters are glucuronidated whereas their phase I metabolites are sulfated (Uutela et al., 2009b). In human, early studies identified sulfate conjugates of DA and 5-HT (Ratge et al., 1985; Tyce et al., 1986) and glucuronide conjugate of DA in CSF. Glucuronide conjugates of HVA, DOPAC and 5-HIAA and DOPAC sulfate were detected in a caudate nucleus human sample although HVA and 5-HIAA occurred predominantly as free metabolites (Swahn and Wiesel, 1976). Intact 5-HT and HVA-glucuronides were detected in human brain samples. However, no glucuronides were detected in CSF samples. These recent results using a direct UPLC-MS-MS method clearly indicate that sulfate conjugation of neurotransmitters predominates over glucuronidation in the human brain (Suominen et al., 2013).

UGT ISOFORMS INVOLVED IN DOPAMINE AND SEROTONIN GLUCURONIDATION

Among 22 recombinant human UGTs, only UGT1A10 was found to catalyze DA glucuronidation at substantial rate although with a low affinity, leading to the formation of both 4-O- and 3-O-glucuronide (Itäaho et al., 2009). Very low activity was detected for UGTs 1A6, 2A1, 2A3, 2B7, 2B11 and 2B17. However, no expression could be detected in human brain that could be responsible for DA-glucuronide formation (King et al., 1999; Itäaho et al., 2009). UGT1A10 was also the most active human UGT in the formation of HVA-O-glucuronide whereas UG2A1 conjugated HVA at the carboxyl-group (Suominen et al., 2013).

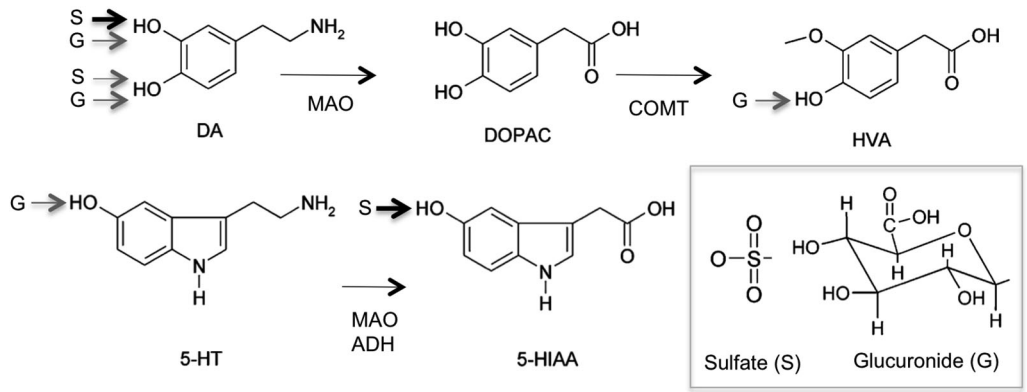

FIGURE 3 | Major metabolic pathway of serotonin (5-hydroxytryptamine) and dopamine (DA) to Phase I and Phase II metabolites (sulfate, S; glucuronide, G). Monoamine oxidase (MAO), aldehyde dehydrogenase (ADH) 3,4-dihydrophenylacetic acid (DOPAC), homovanillic acid (HVA), catechol-O-methyltransferases (COMT), 5-hydroxyindoleacetic acid (5-HIAA). Main sulfo- and glucuronoconjugate found in human brain are indicated by arrows (data from Suominen et al., 2013).

5-HT has been shown to be a substrate for UGT1A6 and was proposed as a probe for this isoform (King et al., 1999). However, we were not able to detect 5-HT glucuronidation in recombinant cells expressing UGT1A6 in our laboratory (Fournel-Gigleux et al., 1991). This may be due to the low efficiency of UGT1A6 towards its substrate compared to the reference compound, 1-naphtol (8-fold). UGT2B7 also exhibited very low activity towards this substrate (King et al., 1999). The recombinant mouse Ugt1a6a and Ugt1a6b isoforms were able to glucuronidate 5-HT with approximately same efficiencies (Uchihashi et al., 2013). Since Ugt1a6a was predominantly expressed in mouse brain, especially in the hippocampus, it was proposed that this isoform is involved in the glucuronidation 5-HT in mouse brain. Altogether, monoamine neurotransmitters and their metabolites appear as poor substrates for recombinant human UGTs.

PHYSIOLOGICAL SIGNIFICANCE OF NEUROTRANSMITTERS GLUCURONIDATION IN BRAIN

The main excretion products of DA found in human urine are HVA, DOPAC, their sulfates and glucuronides, as well as DA conjugates. In the brain, DA and 5-HT conjugation seems to play only minor roles as in microdialysates, the main metabolites are DOPAC and HVA for DA, and HIAA for 5-HT. Whether glucuronidation is more or less important than sulfoconjugation is also questionable. Reports concerning the ratio of conjugated to non-conjugated metabolites and the ratio of sulfates to glucuronides are variable. In mouse and rat, there are mainly glucuronides over sulfates, for example, the concentration of 5-HT-glucuronide was about 2.5-times higher than of free 5-HT in rat brain microdialysates (Uutela et al., 2009b). On the other hand, in human CSF, 5-HIAA-sulfate and DA-3-O-sulfate were the predominant conjugates of 5-HIAA and DA respectively, no glucuronides were detected (Suominen et al., 2013). Quantitative results of DA and 5-HT metabolism indicate that sulfation is a more important phase II metabolism in human brain. Overall, the low glucuronidation activities towards DA and 5-HT, together with the UGT expression level found in brain tissues, and the poor efficiency of recombinant UGTs are indicative of a minor role of glucuronidation in DA and serotonin metabolism. The role of neurotransmitters conjugates in the brain remains to be clarified. Whether they exert neurotoxic or neuroprotective properties or have pharmacological impact on brain function, especially in the case of sulfate conjugates is an interesting question that requires further investigation.

ROLE OF UGT IN THE GLUCURONIDATION OF XENOBIOTICS IN BRAIN

As indicated above, several lines of evidence highlight the important role of UGT in brain homeostasis and neuronal protection against the entry of drugs and other xenobiotics. In order to illustrate this part, the glucuronidation of several xenobiotics in brain is reported.

MORPHINE GLUCURONIDATION IN BRAIN

Morphine is one of the most potent and widely used opioid derivatives for acute or chronic pain relief. Once absorbed and distributed in blood flow, morphine is efficiently transferred across the BBB of rodent brain (Kalvass et al., 2007; Boström et al., 2008). The diffusion process varies according to the age or to the injected morphine dose (Bhargava et al., 1993). Interestingly, morphine can also be produced *in situ* in several tissues, including brain, from endogenous precursors such as tyramine and DA, (Laux-Biehlmann et al., 2013). *De novo* morphine synthesis from DA or L-tyrosine has been described in mammalian brain (Stefano et al., 2008) or human neuronal catecholamine-producing cell line SH-SY5Y (Poeaknapo, 2005; Muller et al., 2008). This biosynthetic pathway has been investigated by the use of potential precursors or deficient animal models such as DA-deficient mice. The results showed the requirement of DA for morphine formation (Neri et al., 2008). It has been strongly suggested that the presence of endogenous morphine in several tissues could be involved in pain modulation in response to various stimulations as stress, immune responses (Charron et al., 2011) or sepsis (Glattard et al., 2010). The recent (co)localization

of morphine and morphine-like compounds in mouse brain areas not usually known to be involved in brain responses to pain brings new questionings about the potential other(s) role(s) of morphine and its derivatives (beside antalgic activity) in mammalian brain (Laux et al., 2011).

Morphine is mostly metabolized by glucuronidation leading to the production of two metabolites: the pharmacologically inactive and major metabolite, morphine-3-β-D-glucuronide (M3G) and the highly active but minor metabolite, morphine-6-β-D glucuronide (M6G; **Figure 2**; Nagano et al., 2000; De Gregori et al., 2012). A recent work reported the formation of M3G and M6G in primary cultures of neonatal rat microglia in presence of micromolar concentrations of morphine (Togna et al., 2013). The authors suggested that morphine glucuronides found in the CSF after morphine administration was in part formed in situ. As indicated before, among the UGT isoforms expressed in brain, UGT2B7 has been reported to glucuronidate morphine in several animal species and in humans (Court et al., 2003; Stone et al., 2003; Wong et al., 2006; Abildskov et al., 2010; **Figure 2**). Although UGT2B7 supported metabolism in liver is well described, the contribution of this UGT isoform in brain is still poorly documented and remains to be investigated in terms of pharmacological effects and potential consequences on brain homeostasis.

RESVERATROL GLUCURONIDATION IN BRAIN

Resveratrol (*trans*-3, 5, 4′-trihydroxystilbene) is a natural polyphenol produced by plants more particularly present in grapes and in high concentration in red wine. This compound has been regarded as a bioactive agent with possible beneficial effects in health. Its anti-oxidant properties may have therapeutic applications for cardiovascular or brain diseases. It has also been suggested that resveratrol could maintain neuronal energy homeostasis in relation to glutamate receptors and/or ions channels (Quincozes-Santos et al., 2014). Its neuroprotective effects have been shown in ischemia prevention in brain after a brief resveratrol pretreatment (Raval et al., 2008). Dasgupta and Milbrandt (2007) showed that resveratrol could activate the AMP-activated kinase (AMPK) in mouse neuroblastoma cell lines and in rat primary cultures of neurons, promoting neurite growth. These data strongly suggest that the neuroprotective effects of resveratrol could be mediated by the activation of AMPK in neurons (Dasgupta and Milbrandt, 2007).

Bioavailability of resveratrol is limited by its extensive biotransformation via sulfo- and glucuronidation pathways in rodents and humans (Iwuchukwu et al., 2012). This metabolism mainly occurs in kidney and in liver, and is low in rat brain (Juan et al., 2010). Resveratrol biotransformation leads to the production of various monoconjugated metabolites as 3- and 4′ monosulfates and 3- and 4′-monoglucuronides (Aumont et al., 2001; Sharan et al., 2012) that are excreted in urine. Previous studies showed that resveratrol was mainly metabolized by UGTs in rat brain homogenates, astrocyte primary cultures and olfactory mucosa (OM). Interestingly, only resveratrol 3-O-glucuronide has been produced in these cellular extracts whereas neither resveratrol 4-O′-glucuronide, nor sulfated conjugates could be detected in the same experimental conditions (Sabolovic et al., 2007). However the UGT isoforms involved in these reactions have not been identified, even if UGT1A6 and UGT2B7 are known to glucuronidate resveratrol. Finally, using glioblastoma cell lines from rat and human, Sun et al. (2013) showed that mainly glucuronides were formed in rat, whereas sulfate metabolites were produced in human cellular extract.

ETHANOL GLUCURONIDATION IN BRAIN

Ethanol is primarily oxidized in the liver and also in BBB. Glucuronidation has been recently described as a novel ethanol biotransformation pathway, with the characterization of an ethylglucuronide metabolite (Walsham and Sherwood, 2012). This metabolite represents 0.02–0.06% of the ethanol intake (Janda et al., 2002). Several recombinant UGT isoforms including UGT1A6 and UGT2B7 have been shown to catalyze the formation of ethylglucuronide (Schwab and Skopp, 2014). The ethylglucuronide presents a much longer half-life than ethanol itself and its relevance in clinical diagnostics has been suggested as a marker of alcohol consumption (Rainio et al., 2014). In line, previous studies have measured high concentrations of ethylglucuronide in CSF and brain of patients who died of acute alcohol intoxication (Wurst et al., 1999). The molecular mechanism for ethylglucuronide action has not been precisely described but it has been recently suggested that it could induce the toll-like receptor 4 signaling pathway leading to pain enhancement (Lewis et al., 2013). Ethylglucuronide formation exemplifies the biological importance of glucuronidation in brain. The mechanism of formation and action requires further investigation.

POLYCHLORINATED BIPHENYL GLUCURONIDATION IN BRAIN

Brain can also be a potential target for environmental pollutants. Polychlorinated biphenyls (PCB), which are highly toxic molecules present in persistent organic pollutants such as fumes and cigarette smoke have been described to be the cause of neurological adverse effects for years. A recent work showed a delay in the neuronal migration in fetal cortex following a prenatal exposure of a PCB mixture (Naveau et al., 2014). However, the mechanism underlying PCB toxicity in brain has not been clearly described yet. It has been hypothesized that the PCB neuronal deleterious effects could be related in part with the formation of one of their phase I metabolites, the hydroxyl-PCB produced by cytochrome P450 monooxygenases (Fonnum and Mariussen, 2009). This metabolite is glucuronidated *in vitro* by the rat UGT1A6 isoform which is expressed in brain (Daidoji et al., 2005). Taken together, these results strongly suggest that this UGT could be involved in hydroxyl-PCB glucuronidation in brain which could represent a PCB detoxification pathway and prevent its neurotoxicity.

In conclusion to this part, brain tissue is able to glucuronidate various xenobiotics which can cross the BBB. This reaction mainly leads to the neutralization of their toxicity or pharmacological activity. However, the potency of the brain to hydrolyze the glucuronides once formed has not been extensively explored. This reaction which is catalyzed by β-glucuronidases, leads back to the parent compounds. The balance glucuronidation/hydrolysis reaction must be taken into account to estimate the actual concentration of xenobiotics in the brain.

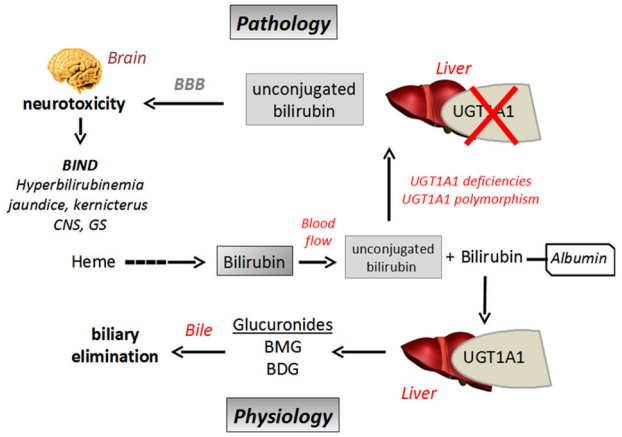

FIGURE 4 | Schematic representation of bilirubin biliary elimination pathway and neurotoxicity in a context of UGT1A1 defect. BBB, Blood Brain Barrier; BIND, Bilirubin Induced Neurological Dysfunction; BMG, bilirubin monoglucuronides; BDG, bilirubin diglucuronide; UGT, UDP-glucuronosyltransferase.

THE BRAIN TOXICITY OF BILIRUBIN IS ASSOCIATED TO A DEFECT IN BILIRUBIN GLUCURONIDATION IN THE LIVER

Bilirubin is a toxic product of heme catabolism of hemoproteins produced in high amount. Normal plasma concentration of bilirubin has been measured between 2–10 mg/l in healthy people, which results from a daily production of about 200–300 mg of bilirubin. Once produced, the main excretion/detoxication pathway of the pigment is through glucuronidation supported by the UGT1A1 isoform (Ohta et al., 2005). The reaction leads to the production of bilirubin monoglucuronides (BMG) and bilirubin diglucuronide (BDG) which are finally excreted into bile and feces (**Figure 4**).

Indeed, when bilirubin is less metabolized, because of an immature glucuronidation capacity, as in newborns for instance (neonatal icterus), or in relation to UGT polymorphism (*see below*), unbound (free) plasma bilirubin concentration increases. The hydrophobicity of bilirubin explains its ability to efficiently permeate the BBB by passive transport (diffusion), resulting in its accumulation in brain where the molecule exerts its neurotoxic effects (Gazzin et al., 2012; **Figure 4**). In this context, glucuronidation of bilirubin in tissues could be regarded as a regulation mechanism to control its diffusion through BBB, as only free/unconjugated bilirubin can reach the brain (McDonagh, 2010). It has also been shown that high free bilirubin concentrations disrupt the integrity of human brain microvascular endothelial cells in primary cultures or in monolayer cultured-cell lines in terms of permeability and secreting activity. It has been suggested that prolonged and high exposition to bilirubin could cause brain endothelial cell injury by an oxidative stress-mediated mechanism in relation to a loss in glutathione homeostasis, increase in nitrite oxide production and cytokine (IL6) release (Palmela et al., 2011).

UGT1A1 is the only UGT described to date able to glucuronidate bilirubin. Therefore glucuronidation represents a rate-limiting step for the metabolism and elimination of the pigment. UGT1A1-mediated bilirubin conjugation has never been detected in brain, meaning that the conjugation of bilirubin in the other tissues, especially in the liver can be considered as a metabolic barrier to prevent its diffusion into the brain and thus to control its neurotoxicity. Recent studies on transgenic mouse expressing the human UGT1A gene locus (encoding 9 UGT1A genes, including UGT1A1) have been conducted, showing that about 8–10% of humanized UGT1A mice died from CNS damages with neuroinflammation and reactive gliosis as a consequence of neonatal hyperbilirubinemia (Fujiwara et al., 2010). High serum bilirubin concentrations have been observed in these transgenic animals in relation to limited UGT1A expression. More precisely, a delayed UGT1A1 expression has been pointed out, which explains hyberbilirubinemia and all the associated pathological features including bilirubin-mediated neurotoxicity (Chen et al., 2012).

The UGT1A1 gene is prone to an intense genetic variability in the human population including mutations on the open reading frame or in the promoter (TATA box) region leading to a decrease or even to a complete loss of bilirubin glucuronidation. Various pathological disorders have been described, such as the frequent, although benign Gilbert's syndrome (Strassburg et al., 2008), or in contrast the rare but severe Crigler-Najjar syndromes (Bartlett and Gourley, 2011). All these diseases are characterized by moderate to high hyperbilirubinemia, respectively. A genetic defect in UGT1A1 and thus in bilirubin glucuronidation leads to high and potentially neurotoxic plasma bilirubin concentrations. When the concentration of the free fraction of the molecule exceeds the capacity of the UGT1A1 isoform, bilirubin becomes neurotoxic crossing the BBB, diffusing in specific brain regions (corpus striatum and hippocampus for instance) and inducing irreversible neuronal damages such as kernicterus (Brito et al., 2013).

In conclusion, bilirubin glucuronidation by UGT1A1 is a prerequisite and a key- step for detoxication of the pigment. Hepatic biotransformation of bilirubin by UGT1A1 controls its neurotoxicity by preventing its brain uptake and slowing down its BBB permeation, and finally by promoting its biliary excretion. On the other hand, controlling bilirubin amount in blood flow by increasing UGT1A1 expression seems to be an important strategy to prevent its neurotoxicity.

UGT AND OLFACTORY TISSUES

In the previous sections, the important role of BBB and brain UGTs in the context of brain protection against xenobiotics has been discussed. However, since the last two decades, the olfactory system (OS) has emerged as an alternate barrier structure preventing penetration of inhaled toxic molecules into the brain. This assertion mainly relies on the early identification of some tissue-specific UGTs (Lazard et al., 1990, 1991), and cytochromes P-450 enzyme subfamilies (Nef et al., 1989; Zupko et al., 1991), in bovine, rat, and also human olfactory neuroepithelium (Jedlitschky et al., 1999; Sneitz et al., 2009; Court et al., 2012). Indeed, these enzymes, which were first predicted to play important roles in chemoreception and especially in odorant signal termination (Nef et al., 1989; Lazard et al., 1991), are now ascertained as specific nasal xenobiotic metabolizing enzymes

(Thiebaud et al., 2010; Xie et al., 2013). Nowadays, UGTs, due to their potency to glucuronidate a wide range of unrelated chemicals compounds, are considered as the main enzymes that confer to OS its known role as a metabolic barrier against potentially toxic inhaled substances. In mammals, OS consists in a neurophysiologic system including the OM and the olfactory bulb (OB) whose structures respectively allow generation, processing and driving of afferent odorant signals to the olfactory cortex (Mori et al., 2006). Since both OM and OB are able to express UGTs (Leclerc et al., 2002), OS can be considered as a metabolic defense structure against toxic airborne substances, in which OM and OB respectively represent an early and a late barrier stage. In this section, we will describe the UGTs isoforms which have been clearly identified in OM and OB, and their activities and roles in terms of brain protection against airborne substances intrusion.

THE OLFACTORY MUCOSA AS AN EARLY LINE OF DEFENSE AGAINST TOXIC INHALED SUBSTANCES

The expression pattern of UGTs in the OM tissue has been investigated. Attention was first focused on the UGT2A1 isoform previously called UGTolf since Lazard et al. (1991) demonstrated that it was almost exclusively expressed in the olfactory tissue. They also detected by *in situ* immunolocalization the presence of UGT2A1 in the Bowman's glands and in the apical pole of the sustentacular cells on serial frozen sections of bovine olfactory epithelium (Lazard et al., 1991). This result was later confirmed by Jedlitschky et al. (1999) in the human olfactory epithelium. By *in situ* mRNA localization and quantitative RT-PCR assays on rat olfactory epithelium, Heydel et al. (2001) reported that UGT2A1 mRNA was detectable in Bowman's glands and sustentacular cells, and also in olfactory sensory neurons. These data were confirmed by a proteomic approach by Mayer et al. (2008). However, at a lesser extent, others UGT isoforms were also shown to be expressed in OM. Thus, Leclerc et al. (2002) described the presence of UGT1A6 mRNA in rats OM, although this latter isoform was 400 to 4,000 times less expressed than UGT2A1. Furthermore, high-throughput analysis of more than 6,000 cDNA mouse sequences indicated the expression of UGT2A2, a splice variant of UGT2A1, in neonatal OM whose glucuronidation activity towards several different endo- and xenobiotic substrates was similar to that of UGT2A1 (Strausberg et al., 2002; Sneitz et al., 2009; Court et al., 2012).

UGT2A1 is, thus, widely expressed among the cells which constitute the OM tissue. However, the expression of the UGT1A6 isoforms also contributes to the glucuronidation activity observed in the OM tissue. What does this co-localization mean? A preliminary answer would reside in the difference and complementarity of substrate specificity of UGT2A1 compared to that of UGT1A6. In one hand, UGT2A1 catalyzes mainly the glucuronidation of most odorant compounds including numerous phenols derivatives, aliphatic and monoterpenoid compounds, but also accepts numerous steroids as endogenous substrates including testosterone, 5α-androstane-17β-ol-3-one or 5α-androstane-3α-17β-diol (Jedlitschky et al., 1999). On the other hand, UGT1A6, which is expressed in various tissues (Ouzzine et al., 1994; Münzel et al., 1996), including brain (King et al., 1999) is mainly involved in the glucuronidation of planar phenols (Tukey and Strassburg, 2000), acetaminophen (Bock et al., 1987), serotonine (Krishnaswamy et al., 2003, 2004), but also glucuronidates carcinogenic arylamines and aryl hydrocarbons (Gschaidmeier et al., 1995). Moreover, UGT1A6 was recently shown to present a significant affinity toward nonsteroidal anti-inflammatory drugs and salicylate derivatives (Soikkeli et al., 2011). As expected, the chemical structure of the substrates recognized by UGT2A1 differs from that for UGT1A6 suggesting that conjugation by both enzymes allows widening the spectra of inhaled molecules that could be glucuronidated. This is consistent with the role of OM as metabolic structure preventing penetration of potentially toxic inhaled substances inside the brain. However, this assertion should be considered in the light of the recent studies regarding the complex expression regulation of these enzymes. Indeed, the expression level of UGT2A1 mRNA was shown to be significantly higher than that of UGT1A6 mRNA in rat OM but not in rat OB, indicating that olfactory UGT expression was tissue-dependent (Leclerc et al., 2002). The same authors also demonstrated that UGT2A1 and UGT1A6 expression in rat OM were age-dependent. Furthermore, Buckley and Klaassen (2007) showed that, in mice, female-predominant expression of UGT2A1 was observed in OM, while male-predominant expression of UGT1A6 was rather observed in lung, suggesting that expression of these OM UGTs could also be gender-dependent (Buckley and Klaassen, 2007). Interestingly, Thiebaud et al. (2010) reported that UGT2A1 and UGT1A6 did not share the same gene transcription inducers in rat OM. In fact, UGT2A1 mRNA expression level was up-regulated by dexamethasone (DM) whereas no modulation effect of phenobarbital, Aroclor 1254, methylcholanthrene or ethoxyquin upon the residual UGT2A1 mRNA expression level was observed. Furthermore, these authors also showed that none of these compounds could up-regulate the UGT1A6 mRNA residual expression. Several xenobiotic-response transcription factors (XRTF) involved in the modulation of some UGTs expression in rat OM have been identified. These XRTFs, including aryl hydrocarbon receptor, nuclear factor E2-related factor 2, peroxisome proliferator-activated receptor, pregnane X receptor, and glucocorticoid receptor, are known to mediate UGTs mRNAs expression upon addition of specific inducers in rat OM (Thiebaud et al., 2010), but also in mouse liver and intestine (Buckley and Klaassen, 2009). Thus, such an approach would allow: (i) to decipher the molecular basis of the enzymatic response of OM towards inhaled substances (ii) to highlight transcription factors or metabolic partners which would be targeted in therapeutic aims (Rowland et al., 2013). Besides its known role as a neurosensory structure, OM can also be considered as a dynamic structure capable to adapt the expression of its xenobiotic metabolizing enzymes to terminate olfaction signal but also to eliminate toxic inhaled compounds in the context of brain protection.

THE OLFACTORY BULB AS A LATE LINE OF DEFENSE AGAINST TOXIC INHALED SUBSTANCES

OB is a second line of defense of OS since this structure was also shown to express drug metabolizing enzymes, including UGTs (Leininger et al., 1991). OB main role is to process the olfactory signals received from olfactory sensory neurons to then ensure

their transfer towards the olfactory cortex and towards other brain areas including the amygdala, the hippocampus, the piriform cortex, and the entorhinal cortex to mainly generate olfactory information and odor memory (Johnson et al., 2000; Buck, 2005; Mori et al., 2006; Zelano et al., 2009). Few groups attempted to identify the UGTs that were specifically expressed in OB tissue, and demonstrated the expression of the UGT2A1 and UGT1A6 isoforms. The expression was heterogeneously distributed among the different cells of OB. By *in situ* hybridization analysis, Heydel et al. (2001) successfully localized UGT2A1 mRNA expression inside the granule cells and in a lesser extent in some mitral cells whereas no signal was observed in the other layers of OB. Unfortunately, no such exploration was made for UGT1A6.

UGT1A6 was identified as the main enzyme responsible for the glucuronidation of 1-naphthol in rat OB (Gradinaru et al., 1999). The activity was age-dependent and a significant increase of UGT1A6 mRNA expression level was observed in OB for rats older than 3 months (Leclerc et al., 2002). Interestingly, UGT2A1 was expressed in rat OB, although at a less amount than in OM. mRNA expression level increased for rats from 1-day up to 3-months-old, then decreased thereafter (Leclerc et al., 2002). These data are important as they suggest that OB possesses the same enzymatic potency as OM against toxic inhaled substances. These data also suggest that, as a function of age, UGT1A6 likely plays a greater role in the glucuronidation activity when compared to UGT2A1. Interestingly measurement of glucuronidation activity toward a series of structurally unrelated hydroxylated substances including odorants and phenols suggested that other UGT isoforms may be expressed in rat OB. To better understand how OB exerts its detoxification role to ensure brain protection, further exploration regarding the identification of such hypothetical isoforms should be undertaken.

The results acquired during these last two decades undoubtedly indicates that, more than a complex signal processing structure, OB acts as an additional line of defense against toxic substances that would target the cerebral tissue thanks to the presence of UGT2A1 and UGT1A6 enzymes. Moreover, this substructure of the OS has to be considered as a "late" line of defense, because OB is already recognized as a part of the forebrain and thus belongs to the cerebral tissue.

CONCLUSIONS

Compared to other organs, brain tissues are characterized by a qualitatively and quantitatively low expression of UGTs. Only few isoforms are present which are heterogeneously distributed among the several cells constituting the different brain areas. Beside their significant participation to the overall protection of the brain against the entry of xenobiotics, including pollutants present in our environment, these enzyme species are specialized toward the biotransformation of brain-directed substances, including metabolism of neurotransmitters chemically related to DA, or antipsychotic drugs. However, the contribution of UGTs expressed in liver or in gastro-intestinal tract in the protection of the brain against drugs and other xenobiotics has to be taken into account. By actively eliminating these substances from the body, they decrease the risk of their entry into the brain. On the other hand, a better knowledge on the UGTs, and other drug metabolizing enzymes and transporters in the BBB and olfactory tissues is a prerequisite to design and target pharmacological compounds toward brain able to bypass these physiological barriers.

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8-Methoxypsoralen is a competitive inhibitor of glutathione S-transferase P1-1

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The blood-brain barrier (BBB) is known to protect healthy brain cells from potentially dangerous chemical agents, but there are many evidences supporting the idea that this protective action is extended to tumor cells. Since the process of angiogenesis in brain tumors leads to BBB breakdown, biochemical characteristics of the BBB seem to be more relevant than physical barriers to protect tumor cells from chemotherapy. In fact, a number of resistance related factors were already demonstrated to be component of both BBB and tumor cells. The enzyme glutathione S-transferases (GST) detoxify electrophilic xenobiotics and endogenous secondary metabolites formed during oxidative stress. A role has been attributed to GST in the resistance of cancer cells to chemotherapeutic agents. This study characterized 8-methoxypsoralen (8-MOP) as a human GST P1-1 (hGST P1-1) inhibitor. To identify and characterize the potential inhibitory activity of 8-MOP, we studied the enzyme kinetics of the conjugation of 1-chloro-2,4-dinitrobenzene (CDNB) with GSH catalyzed by hGST P1-1. We report here that 8-MOP competitively inhibited hGST P1-1 relative to CDNB, but there was an uncompetitive inhibition relative to GSH. Chromatographic analyses suggest that 8-MOP is not a substrate. Molecular docking simulations suggest that 8-MOP binds to the active site, but its position prevents the GSH conjugation. Thus, we conclude that 8-MOP is a promising prototype for new GST inhibitors pharmacologically useful in the treatment of neurodegenerative disorders and the resistance of cancer to chemotherapy.

Keywords: GST, 8-MOP, glioblastoma

INTRODUCTION

The Brain Blood Barrier (BBB) protects central nervous system (CNS) against chemical and biological insults. It was described as composed mainly of highly specialized endothelial cells with tight junctions, and astrocyte endfeets with anchoring transmembrane proteins (Bentivoglio and Kristensson, 2014). However, nowadays it is clear that a number of enzymes are also part of this barrier (Shawahna et al., 2013). The idea that the BBB has not only a physical constitution, but also a metabolic one, is not new (Minn et al., 1991; El-Bacha and Minn, 1999), drug-metabolizing enzymes continues to be identified (Decleves et al., 2011), and they represent difficulties in drug delivery to the brain (Bentivoglio and Kristensson, 2014). Therefore, BBB is a critical obstacle for the pharmacologic treatment of brain tumors (Jovćevska et al., 2013), leading to research on BBB-disrupting strategies for enhanced drug delivery in these cases (Liu et al., 2014).

Glutathione *S*-transferases (GST; EC 2.5.1.18), multifunctional enzymes which are mainly involved in phase II metabolism and antioxidant cell systems (Di Pietro et al., 2010), are among the drug-metabolizing enzymes present in BBB. It seems that these GSTs, mainly the GSTP1 (GST-π) isoform, which is the most abundant at the BBB, protect cells against oxidative stress and are involved in the phenomenon of drug resistance (Shawahna et al., 2013). In fact, GSTs promote the conjugation between drugs and the tripeptide glutathione (GSH), and special attention has been given to these enzymes since they are strongly associated with resistance, remarkable in cancer (Sau et al., 2010). The most highly expressed GST isoenzyme in various human cancerous and precancerous tissues is also GST-π (Sau et al., 2010). Overexpression of this class of GST was associated with drug resistance or poor prognosis in many kinds of tumors (Wang et al., 2007, 2011; Pasello et al., 2008; Geng et al., 2013), including gliomas (Okcu et al., 2004; Calatozzolo et al., 2005), the most common form of primary brain tumors, and glioblastoma (the highest malignant glioma) (Lo et al., 2004), the most frequent brain tumor in adults (Brennan, 2011). Furthermore, it was documented that GST-π polymorphisms are associated with survival in anaplastic glioma patients; an explanation is that lower activity GST genotypes will allow more prolonged

exposure of tumor cells to chemotherapeutic agents (Kilburn et al., 2010).

There are experimental observations suggesting that inhibition of GST increases the response of glioblastoma cells to alkylating agents better than the inhibition of the enzyme O^6-methylguanine-DNA methyltransferase (MGMT) (Juillerat-Jeanneret et al., 2008), the most frequently associated factor to temozolomide resistance in glioblastomas (Mrugala and Chamberlain, 2008). Therefore, inhibition of Pi-class GST activity or inhibition of its expression has been shown to increase the tumor sensitivity to many drugs (Sau et al., 2010). Indeed, many inhibitors and pro-drugs targeted to GSTs have been developed for a long time, but the clinical effectiveness of these molecules is poor and do not justify therapeutic use (Mahajan and Atkins, 2005). The search for effective GST inhibitors for use in cancer therapy is not recent. Almost two decades ago, clinical studies indicated ethacrynic acid as a candidate for modulation of drug resistance (Lacreta et al., 1994). At the same time, the selective GST-π inhibitor TER-117 was synthesized (Lyttle et al., 1994 include on references), but there are no recent studies with this. Other GST-π specific inhibitors were able to revert multiple drug resistance in cholangiocarcinoma (Nakajima et al., 2003).

In this work we presented the 8-MOP, a well-known drug that has been used for decades in the treatment of skin disease (Tzaneva et al., 2009), as a novel promising molecule to develop new GST-π inhibitors, detailing its mechanism of action, and showing the potential for cancer therapy by using an *in vitro* model of glioblastoma.

MATERIALS AND METHODS

ASSAY OF GLUTATHIONE *S*-TRANSFERASE ACTIVITY

GST activity was measured as previously described (van Haaften et al., 2001) with modifications. Briefly, the reaction of 1 mM GSH with 1 mM 1-chloro-2,4-dinitrobenzene (CDNB) catalyzed by GST (GSTP1-1 at 0.1 U/ml or 0.15 mL of human glioma cells lysate in 100 mM potassium phosphate buffer, pH 6.5, at 25°C) was monitored spectrophotometrically by recording the increase in absorbance at 340 nm. Absorbance units were converted to concentration of DNP-SG (the conjugate of GSH and CDNB) as previously described (Mannervik and Guthenberg, 1981). A correction for the spontaneous reaction was made by monitoring formation of DNP-SG in the absence of enzyme. For inhibitory effect analysis, increasing concentrations of 8-MOP (Sigma®) were used. In the test with lysates of glioma cells, GST activity was expressed as percentage of the control group. 8-MOP was dissolved in DMSO, which was present in all groups and did not affect the GST activity. The effect of 8-MOP in spontaneous formation of DNP-SG was also discounted by monitoring the reaction in the presence of the drug and absence of enzyme. To study the inhibitory mechanism, substrate concentrations (CDNB or GSH) were varied (when GSH concentration was varied, the CDNB concentration was kept at 1 mM and vice

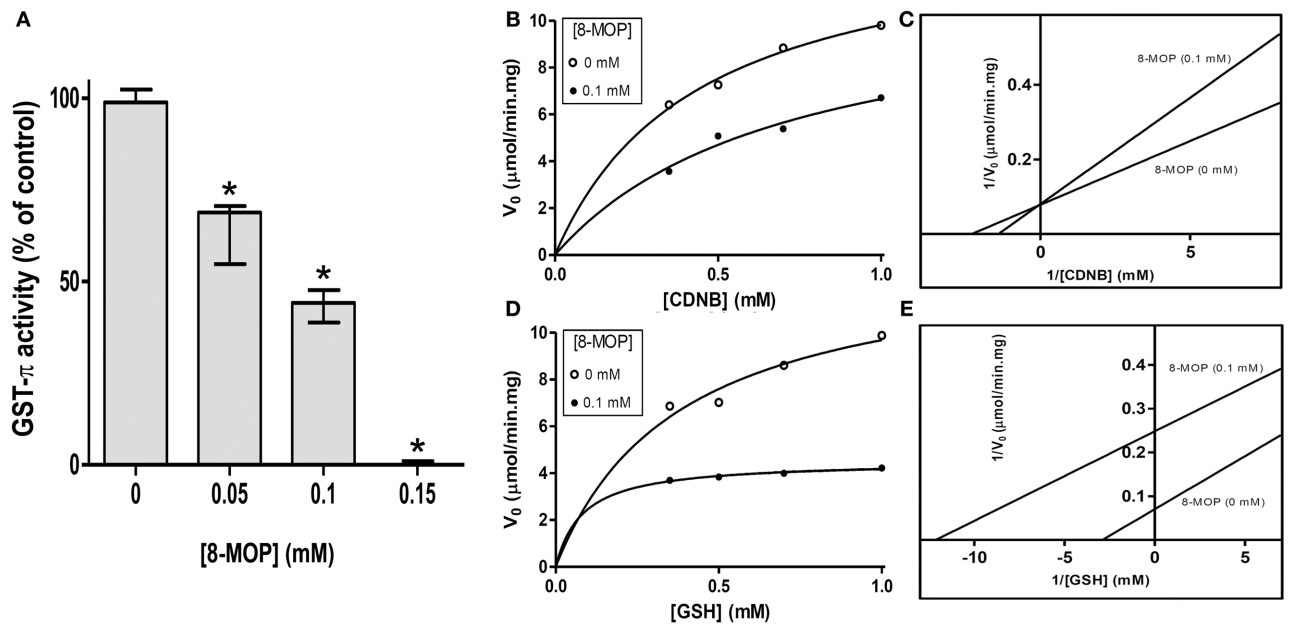

FIGURE 1 | (A) Concentration-dependent inhibition of GST-π activity by 8-MOP. Data did not present normal distribution and were analyzed by Kruskal-Wallis non-parametric test followed by Dunn's Multiple Comparison test; $^*p < 0.05$ compared to the control group. **(B)** Michaelis-Mentem ($R^2 = 0.9874$ without 8-MOP and 0.9545 with 8-MOP) and **(C)** Lineweaver-Burk plots showing competitive inhibition of human GST-π toward CDNB by 8-MOP. The K_m and V_{max} of the enzyme for CDNB were 0.30 mM ($SD = 0.05$) and 11.58 μmol/min.mg ($SD = 0.77$), respectively, but these same values in the presence of 0.1 mM 8-MOP were 0.68 mM ($SD = 0.06$) and 11.71 μmol/min.mg ($SD = 3.10$). **(D)** Michaelis-Mentem ($R^2 = 0.9733$ without 8-MOP and 0.9736 with 8-MOP) and **(E)** Lineweaver-Burk plots showing an uncompetitive inhibition of human GST-π toward GSH by 8-MOP. The K_m and V_{max} for GSH were 0.25 mM ($SD = 0.08$) and 11.36 μmol/min.mg ($SD = 1.31$), respectively. These values in the presence of 0.1 mM 8-MOP were 0.09 mM ($SD = 0.02$) and 4.70 μmol/min.mg ($SD = 0.21$). The enzyme was used at 0.1 U/mL. The graphs are representative of five independent experiments.

versa). Five independent experiments were performed with and without 8-MOP at a concentration near the IC_{50}. V_{max} and K_m values were calculated by non-linear regression following the Michaelis-Mentem mode and the mechanism was visualized by Lineweaver-Burk plot.

UV-VIS SPECTROPHOTOMETRY AND HIGH PERFORMANCE LIQUID CHROMATOGRAPHY (HPLC) ANALYSIS

Electronic absorption spectra of solutions containing CDNB with GSH or 8-MOP with GSH in the presence or absence of GST-π were recorded using a Hewlett-Packard model 8452 A spectrophotometer in 1 cm quartz cuvettes and compared to CDNB alone and 8-MOP alone spectra. Chromatographic analyses of CDNB/CDNB-conjugate and 8-MOP/8-MOP-conjugate were performed according to previous description in literature (Wang and Jiang, 2006; Vaidya and Gerk, 2007) with modifications (Supplementary Table S1). A dual wavelength ultraviolet absorbance detector was used.

IN SILICO APPROACH

A docking study of the 8-MOP in the GST structure (Protein Data Bank code 3IE3) was conducted. In this structure the enzyme is complexed with 6-(7-nitro-2,1,3-benzoxadiazol-4-ylthio)hexanol (NBDHEX) (Federici et al., 2009). The space for molecular docking was defined based on the location of the NBDHEX, as well as the amino acids with important interactions. This space consisted of a 14.0 Å sided box, spaced 1 Å grid. The AutoDock Vina 1.1.2 was used in standard configuration for molecular docking with 8-MOP. The resulting geometry of each enzyme-ligand complex was submitted to a molecular dynamic simulation (MD) using AMBER 10.0 (Case DA, AMBER 10—University of California, San Francisco, 2008). It was performed a two nanoseconds simulation, at 300 K, and with the implicit solvent model described by Hawkins et al. (1996). Separately, protein, 8-MOP, and the NBDHEX structures were submitted to the same MD calculation. The binding energies (E_{bind}) were obtained from the average potential energies of the structures resulting from the MD

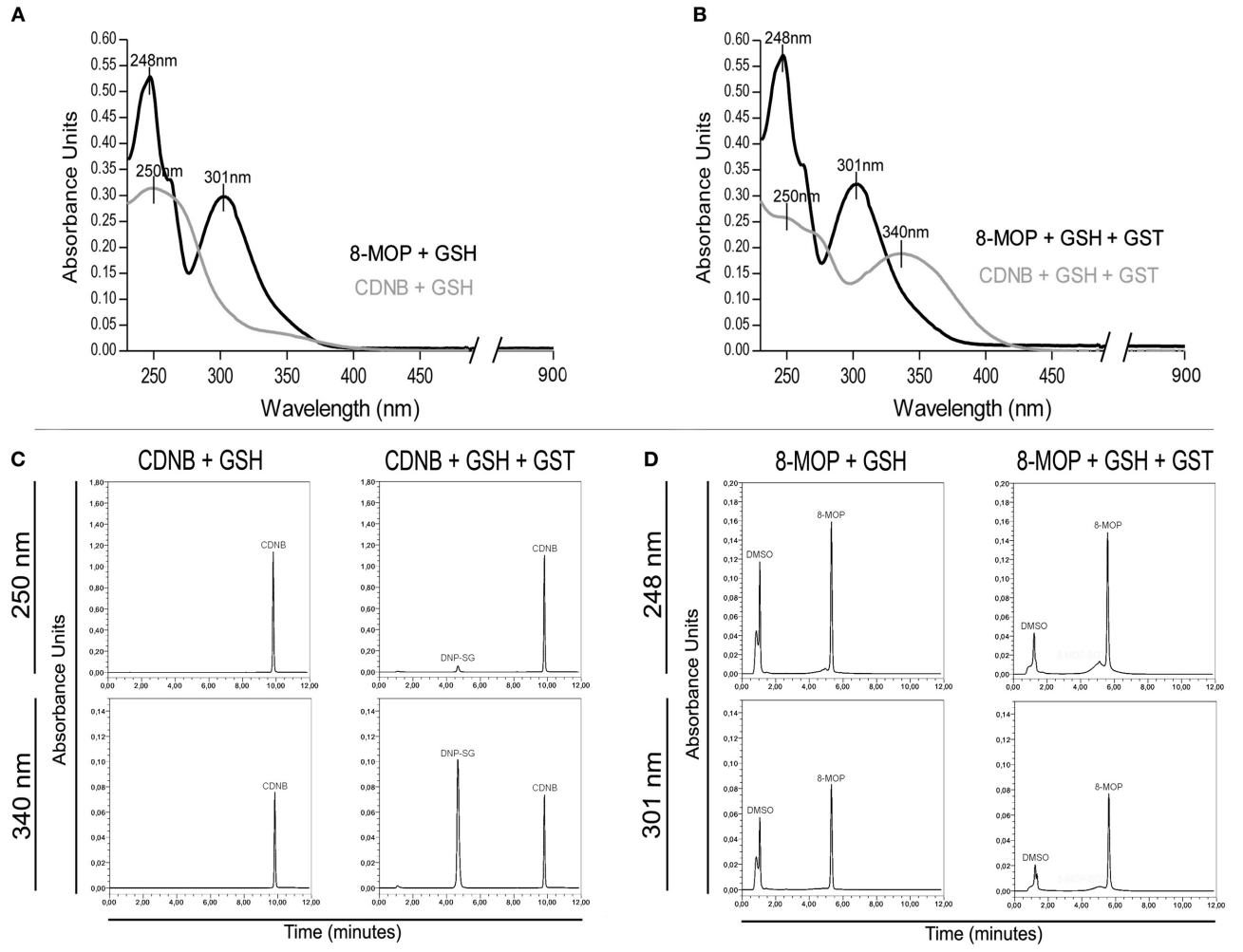

FIGURE 2 | (A) Absorption spectra of CDNB and 8-MOP in the presence of GSH. **(B)** Spectrophotometric evidences of DNP-SG, but not "8-MOP-SG," in spectra after addition of GST-π. The maximum absorbance values for the solutions containing CDNB/GSH/enzyme (250, 340 nm) and 8-MOP/GSH/enzyme (248, 301 nm) were used for HPLC analysis. **(C)** Chromatographic detection of DNP-SG (RT = 4.6 min.), and **(D)** no evidences of "8-MOP-SG."

through the equation Ebind = Ecomplex − (EGST + Eligand), in which $E_{complex}$, E_{GST} and E_{ligand} correspond to the average potential energies of the complex ligand-GST, the enzyme alone and the ligand alone respectively. PyMOL (The Molecular Graphics System, 2002—DeLano Scientific, San Carlos, CA, USA) was used for visual interpretation of the results.

CELL CULTURES AND PREPARATION OF CYTOSOLIC PROTEIN EXTRACT

Human glioblastoma GL-15 cells (Bocchini et al., 1991) were cultured at 37°C in Dulbecco modified Eagle's medium (DMEM), supplemented with 1 mM pyruvic acid, 2 mM L-(+)-glutamine, 44 mM $NaHCO_3$, 10% fetal bovine serum, 100 IU/mL penicillin and 100 μg/mL streptomycin in a humidified atmosphere of 5% CO_2 and 95% air. The culture medium was changed every 2 days. Primary cultures of astrocytes from Wistar rats were performed according to previous descriptions (Silva et al., 2008) and maintained under the same conditions described above. The study was conducted according to guidelines of the institutional animal ethics committee (Federal University of Bahia—Brazil). In order to evaluate the activity of GST from GL-15 cells, confluent cultures in 10 mm dishes were lysated with 1 mL of distilled water under vigorous shaking for 30 min. The extract was centrifuged at 5000 g (10 min) and the supernatant stored at −70°C until use in GST activity assays.

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GST-π expression in GL-15 cells was attested by immunostaining with anti-GSTP1-1 antibody. The cells were permeabilized in methanol at −20°C for 10 min and incubated with the primary antibody (rabbit polyclonal IgG anti GSTP1/2—Santa Cruz®, 1:500) for 1 h. Subsequently, cells were rinsed three times with PBS, incubated with the secondary antibody (Conjugated Alexa Fluor® 546 goat anti-rabbit IgG—Invitrogen®, 1:400) and finally observed by fluorescence microscopy (Olympus AX70 microscope—green filter). Nuclei were stained by the dye Hoechst 33258 (ex/em 340/510 nm) (Oliveira et al., 2010). For negative control, cells were incubated with only secondary antibody under the same conditions described above.

EVALUATION OF INTRACELLULAR REDUCED GLUTATHIONE CONTENT

Monochlorobimane (MCB) assay (Ublacker et al., 1991) was used to evaluate GSH depletion. After 30 min exposure to 8-MOP (0.05 or 0.4 mM) and CDNB (0.05 mM), GL-15 cells were washed three times with PBS, incubated with 1 mM MCB in medium with 1% ethanol for 40 min, washed again and observed by fluorescence microscopy (Olympus BX 51—URA2, San Jose, USA). The fluorescence mirror unit Olympus U-MWU2 was selected to observe cells (ex/em 330–385/420 nm). The exposure time of 60 ms was used in micrographs for all samples.

CELL VIABILITY MEASUREMENT AND MICROSCOPIC ANALYSIS

In order to evaluate the chemosensitizer potential of 8-MOP, cells were seeded in 96-well plates at a density of 3.1×10^4 cells/cm^2, and cultured for 24 h prior to treatments with increasing concentrations of chemotherapeutic drugs for 48 h in the presence or absence of 0.05 mM 8-MOP, which was added 2 h before treatments. Both drugs and 8-MOP were dissolved in DMSO (final concentration 0.5% v/v). Cell viability was measured by the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) method (Mosmann, 1983). In short, after treatment the MTT reagent was added to each well (1 mg/mL). Following additional 2 h incubation, 100 μL of 20% SDS was added. The absorbance was then measured at 595 nm using a microplate reader (THERMO PLATE, model TP-reader—type B). Wells without cells were used as blanks. To access cytotoxicity of 8-MOP, GL-15 cells were treated for 72 h under the same described conditions. Cell growth after long-term (10 days) exposure to a low dose 8-MOP (0.02 mM), added during each medium change, was evaluated by Trypan blue exclusion assay (Louis and Siegel, 2011) in cultures at low cell density (3.86×10^3), and expressed as percentage of cells in the first day. Changes in cell morphology were observed by contrast phase microscopy and nuclear morphology was assessed by Hoechst 33258 staining.

STATISTICS ANALYSES

Data were showed as mean with SEM or median with range according to their distribution, analyzed by Shapiro-Wilk normality test and Skewness (normal: < 1 or > -1) and Kurtosis (normal: < 2 or > -2) calculation. Parametric or non-parametric statistic tests were also chosen according to the distribution. The most appropriate test for each experiment was used and this information is in the respective figure legends. At least three independent experiments were done for each assay.

RESULTS

8-MOP INHIBITS GST-π ACTIVITY

GST activity was concentration dependently inhibited by 8-MOP (**Figure 1A**), with the IC_{50}value of 0.092 mM (Supplementary Figure S1A). The enzyme shows a characteristic Michaelis Menten behavior toward both substrates, but the inhibitor presented a double behavior. A competitive inhibition pattern was observed when 0.1 mM 8-MOP was incubated with GST- π and varying concentrations of CDNB (**Figures 1B,C**). The presence of 8-MOP

Table 1 | Energies from the docking of 8-MOP and NBDHEX.

Complexes	AMBER				Autodock Vina (Kcal/mol)
	Average potential energies (Kcal/mol)			Binding energy (Kcal/mol)	
	Complex	Ligand	GST		
NBDHEX/GST	−6131.88	41.73	−6136.48	**−37.13**	**−5.4**
8-MOP/GST	−6697.87	15.87	−6136.48	**−577.26**	**−6.4**

increased the calculated K_m value, but it did not change significantly the calculated V_{max} value, indicating that both, inhibitor and substrate (CDNB) bind to the same region of the active site. However, when 0.1 mM 8-MOP was tested varying GSH concentrations both K_m and V_{max} values decreased, which can be interpreted as an uncompetitive inhibition (**Figures 1D,E**), indicating that inhibitor and substrate (GSH) bind to different sites in the enzyme or different regions of the active site.

8-MOP IS NOT A GST-π SUBSTRATE

Since 8-MOP inhibits competitively GST-π activity, we hypothesized that this drug could be a substrate, just as CDNB. If it was true, a new compound "8-MOP-SG" (Supplementary Figure S1B) would be formed. UV-Vis spectrophotometric analysis clearly showed the generation of DNP-SG that has a different absorption spectrum when compared to CDNB, by addition of GSH in the presence of GST-π. In contrast, the addition of enzyme in the solution containing 8-MOP/GSH did not change its absorption profile, suggesting no alteration in the 8-MOP structure (**Figures 2A,B**). To certify that 8-MOP-SG was not present in the solution, HPLC was carried out using maximal absorbance values for each solution for detection. Again, DNP-SG was identified with a retention time (RT) lower than CDNB (**Figure 2C**), but a single peak was present in the chromatogram for 8-MOP/GSH plus GST-π (**Figure 2D**). The theoretical log *P*-value for CDNB and log D value for DNP-SG are 2.46 and −3.14, respectively (Supplementary Figure S2), which justifies the lower RT of DNP-SG. On the other hand, the log *P*-value for 8-MOP is 1.78 and the theoretical log D value for the proposed 8-MOP-SG is −2.58, but no peak in a very low RT

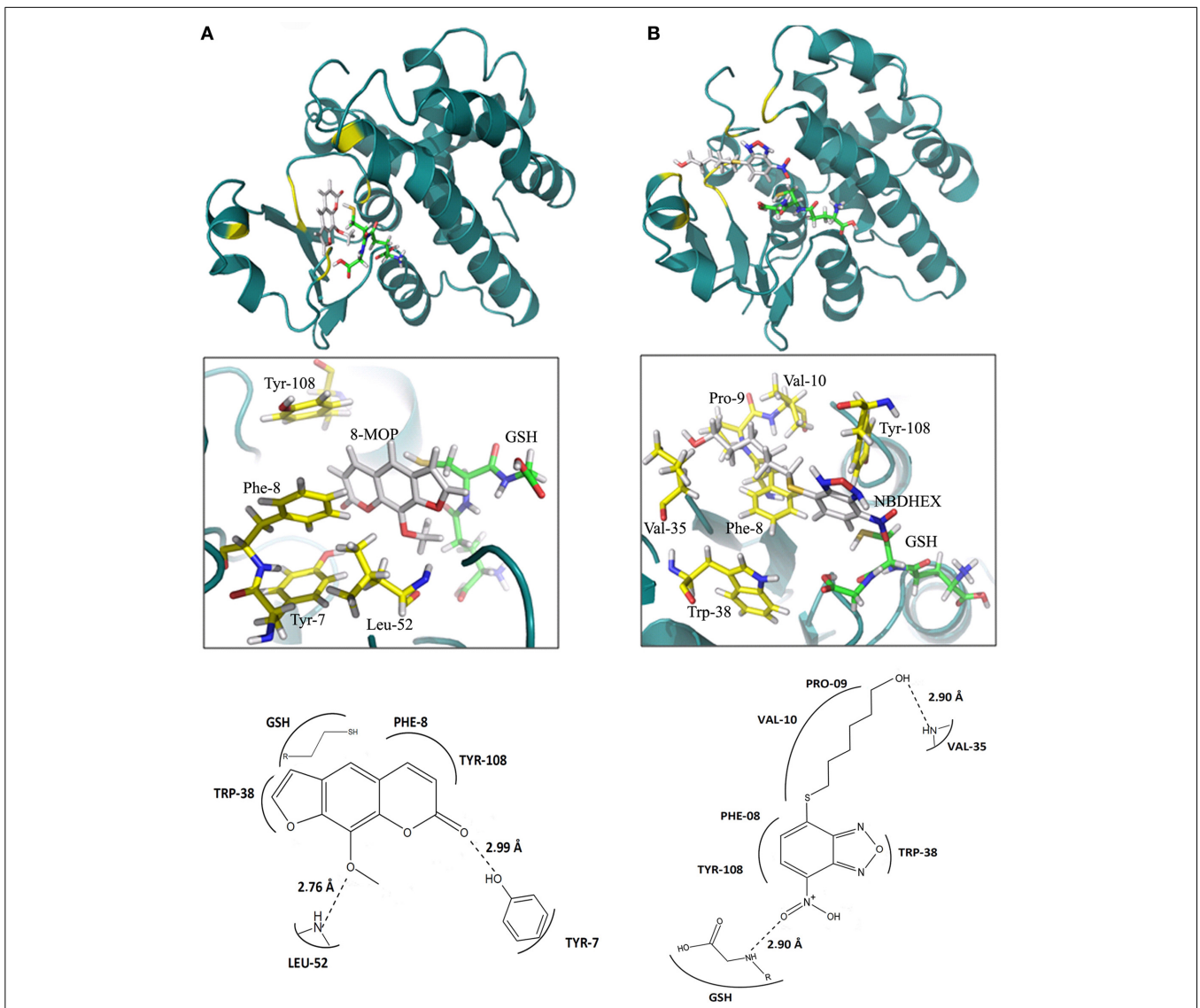

FIGURE 3 | (A) Interactions between 8-MOP (white) and GST after 2 ns simulation. The regions of interactions are in yellow. It is also showed details (interacting residues in yellow) and scheme of interactions between residues in the active site of GST and the 8-MOP. GSH is in Green. **(B)** The same for the inhibitor NBDHEX.

was visualized in the chromatogram. The absorption spectrum and chromatographic profiles were the same even after 30 days incubation (data not shown). Therefore, these data support the idea that 8-MOP-SG is not formed or it is formed in a very low extent.

8-MOP EFFICIENTLY INTERACT WITH THE ACTIVE SITE OF THE ENZYME

In silico data strongly suggest an efficient GST-π activity inhibition by 8-MOP that binds to the active site of the enzyme. The score obtained with Auto Dock Vina as well as the calculations of binding energy from the MD showed good stability of 8-MOP when compared with NBDHEX (**Table 1**). Hydrophobic interactions are made by 8-MOP coumarin core with residues Phe-8 and Tyr-108. Moreover, it is clear that the geometric position of 8-MOP inside the active site prevents the 8-MOP-SG formation (**Figure 3A**). In addition, 8-MOP makes another important interaction with Trp-38 and forms hydrogen bonds with Tyr-7 and Leu-52. These two last residues apparently do not directly interact with the NBDHEX. However, the benzoxadiazole ring of this inhibitor makes the same hydrophobic interactions with residues Phe-8 and Tyr-108 observed in 8-MOP/GST interactions (**Figure 3B**). The redocking of the inhibitor NBDHEX in the GSTP1-1 by AutoDock Vina presented a Root Mean Square Deviation (RMSD) of 1.99 Å from the respective crystal structure (Supplementary Figure S3A), which is an acceptable deviation docking value. Furthermore, RMSD vs. time graphics (Supplementary Figures S3B,C) showed less pronounced variation for the 8-MOP complex, which could indicate an effective stabilization of the system by 8-MOP.

8-MOP INHIBITS GST FROM TUMOR CELLS AND IS NOT SUBSTRATE FOR OTHER ISOFORMS OF GST

GST activity in GST-π positive tumor cells (**Figure 4A**) was investigated. K_m and V_{max} calculation could not be performed since there was not only one isoform of GST in the lysate. Then, data were analyzed by non-linear regression ($R^2 = 0.9770$) (**Figure 4B**). Substrate concentrations greater than 0.5 mM saturated the amount of enzyme present in the volume of lysate used (0.15 mL), and saturating conditions (substrate at 1 mM) were used to investigate GST activity inhibition by 8-MOP, which showed a concentration-dependent pattern (**Figure 4C**). Additionally, treatment with 0.05 mM CDNB for 15 min depleted intracellular reduced GSH, as expected, but 8-MOP did not promote GSH depletion (**Figure 4D**), even at 0.4 mM (data not shown), giving support to our hypothesis that 8-MOP does not conjugate with GSH. The addition of protein extract from tumor cells did not also change the spectrum of 8-MOP/GSH solution (data not shown).

8-MOP SENSITIZES GLIOBLASTOMA CELLS TO DRUGS AND PRESENTS INTRINSIC ANTITUMOR EFFECT

To investigate evidences of the chemosensitizer action of 8-MOP, cells from human glioblastoma (GL-15) pre-treated with a sub-toxic concentration of 8-MOP or vehicle (**Figure 5A**) were exposed to chemotherapeutic agents. High concentrations of temozolomide lead to decreases of cell viability greater than 15% (**Figure 5B**) in these cells those are totally resistant to this agent (Supplementary Figure S4). 8-MOP also increased susceptibility to etoposide (**Figure 5C**), which is a recognized substrate for GST-π. The effects, accessed by MTT assay, were clearly visualized

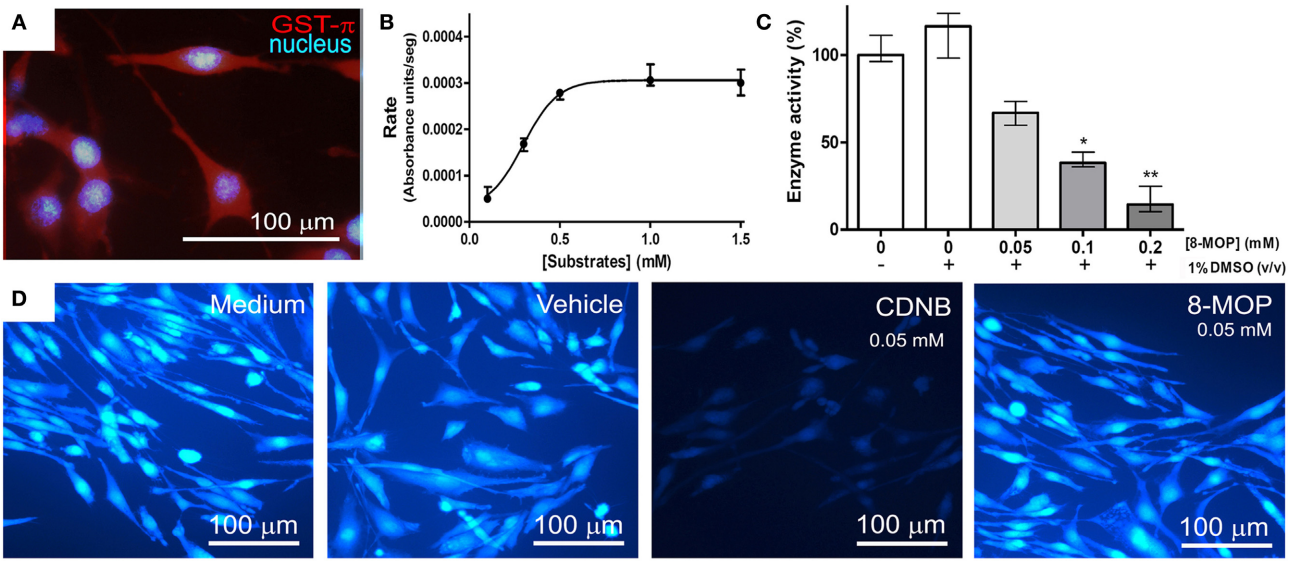

FIGURE 4 | (A) GL-15 cells labeled positively to GST-π. **(B)** Conjugation reaction between CDNB and GSH under increasing concentrations catalyzed by GST in the cytosolic protein extract of GL-15 cells, followed until the maximum rate was attained. Line represents non-linear regression ($R^2 = 0.9770$) of the data. **(C)** Concentration-dependent inhibition of GST by 8-MOP under maximum rate conditions (1 mM CDNB). Data did not present normal distribution and were analyzed by Kruskal-Wallis non-parametric test (excluding the first group without DMSO) followed by Dunn's Multiple Comparison test $^*p < 0.05$ and $^{**}p < 0.01$ compared to the control group (1% DMSO, without 8-MOP). **(D)** Intracellular GSH assessed by MCB assay. GSH was present in control conditions and was depleted by CDNB after 30 min exposure, but not by 8-MOP.

by phase contrast microscopy that shows reduction of cellularity and morphological changes promoted by the associations (**Figure 5D**). Besides chemosensitizing activity, 8-MOP presented direct and selective *in vitro* antitumor effect. It promoted significant reduction of cell viability in tumor cells, but rat astrocytes used as normal cells control were not affected to the same extent (**Figure 6A**). Microscopic findings suggest that 8-MOP promotes apoptosis in GL-15 cells (**Figures 6B,C**). A low concentration of this drug was also effective for reducing cell proliferation accessed by trypan blue assay after 10 days exposition (**Figure 6D**). No apoptotic signals were found in this experimental design.

DISCUSSION

The overexpression of GSTs in many kinds of tumors encourages studies with agents able to promote down regulation of these enzimes (Zhao et al., 2011), GST-actived pro-drugs aiming selective action (Pezzola et al., 2010; Johansson et al., 2011; Kogias et al., 2011) and GSTs inhibitors aiming overpass drug resistance (Cui et al., 2008; Tentori et al., 2011). However, nowadays, there is no GST-based approach consistent enough to be adopted as clinical adjuvant therapy. Ethacrynic acid was the first GST inhibitor, acting as substrate of some isoenzymes of GST, it was utilized to sensitize cancer cells to the cytotoxic effect of alkylating agents. However, a number of substantial side effects such as a marked diuresis have discouraged the use of this molecule in clinical practice (Quesada-Soriano et al., 2011). In this work, we presented 8-methoxypsoralen (8-MOP) as a promising GST-π inhibitor; it is a known drug that has been orally and topically used for decades in the treatment of skin disease like psoriasis and eczema (Tzaneva et al., 2009).

8-MOP concentration-dependently reduced human GST-π activity *in vitro* and also decreased total GST activity in cytosolic extract of human glioblastoma cells. To access the mechanism of action, enzyme kinetic assays were performed and showed a competitive pattern of inhibition with 8-MOP occupying the hydrophobic binding site (H-site) of the enzyme, keeping

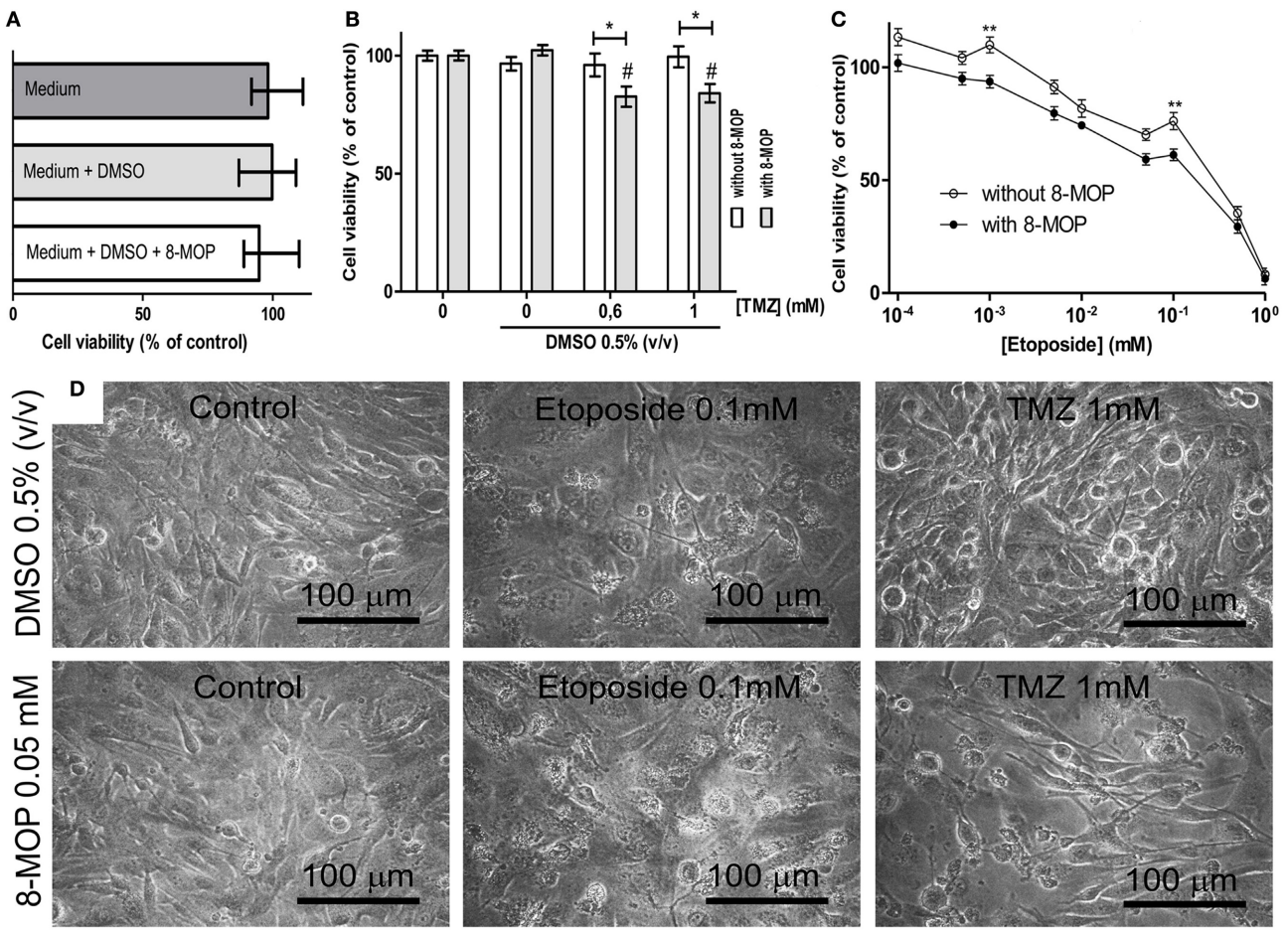

FIGURE 5 | (A) MTT assay data showing that treatment with vehicle and 8-MOP did not change cell viability. **(B)** GL-15 cells become more sensible to high concentrations of temozolomide (TMZ) after treatment with 8-MOP, that significantly reduced viability (MTT assay) of 0.6 and 1 mM TZM treated cells in 17.4% and 16%, respectively. **(C)** 8-MOP also acted as chemosensitizer for etoposide, what was statistically significant at 0.001 and 0.1 mM. DMSO was kept at 0.05% and 8-MOP at 0.05 mM in these experiments. $^{*}p < 0.05$ and $^{**}p < 0.01$. $^{\#}p < 0.01$ compared to group treated with 8-MOP alone in the experiment with TMZ (Two-way ANOVA and Bonferroni post-test). Values are means (±s.e.m.). **(D)** The pictures show reduction of cellularity and morphological changes promoted by the associations.

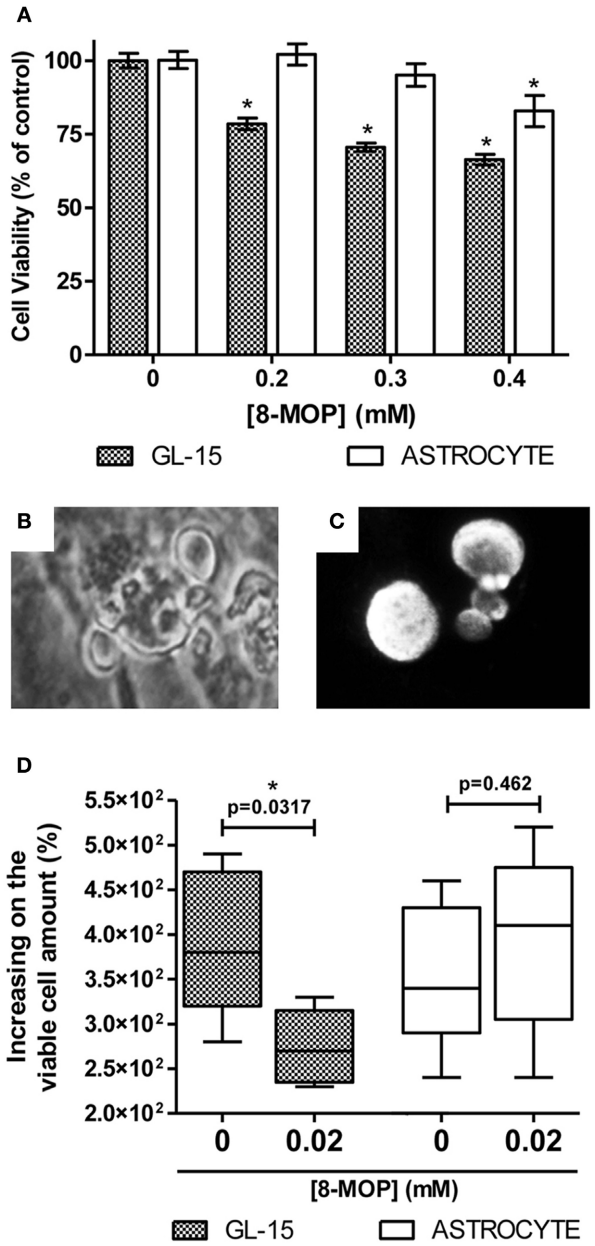

FIGURE 6 | (A) Decrease of 21.5% (± 1.9), 29.4% (± 1.4) and 33.4% (± 1.8) on GL-15 viability after 48 h treatment with 8-MOP at increasing concentrations (gray bars). Normal cells (white bars) were less affected (decrease of 17.2% (± 5.3) only at maximal concentration). Data are from MTT assay. Values are means (±s.e.m.). $*p < 0.01$ (One-way ANOVA and Dunnett's Multiple Comparison post-test, data from each cell type were statistically analyzed separately). 0.05% DMSO was present in all groups. **(B)** Cell blebbing visualized by phase contrast microscopy and **(C)** condensed/fragmented Hoechst 33258 stained nuclei of GL-15. These findings are suggestive of apoptosis. No morphological evidences of apoptosis were found among treated astrocytes. **(D)** Reduced cell proliferation (evaluated by trypan blue exclusion assay) in GL-15 cells after long-term exposure to low dose of 8-MOP (10 days, 0.02 mM). The number of cells increased 380% (320,470) in control conditions against 270% (235,315) in 8-MOP treated group. Values are median (25% percentile, 75% percentile). Astrocytes were not affected at all. $*p < 0.05$ (Mann Whitney non-parametric test).

the glutathione binding site (G-site) free. Since inhibition is competitive, 8-MOP could simply act as a substrate. However, spectrophotometric and chromatographic analyses showed no formation of the 8-MOP/GSH conjugate. Additionally, *in silico* tests showed that 8-MOP alone interacts with the active site more favorably than the inhibitor NBDHEX.

Finally, the results from experiment with monochlorobimane (MCB) suggest that 8-MOP does not act as substrate of GST from any class, since no GSH depletion occurred at all. Whereas conjugation of MCB with GSH is catalyzed by many cytosolic GSTs (Eklund et al., 2002), the MCB assay also reflects the GST activity (Ublacker et al., 1991), and then the results also confirm the reversible nature of the inhibition (since after washing it is possible to visualize fluorescence) what suggests that 8-MOP does not inhibit all cytosolic GSTs, presenting any selectivity.

The NBDHEX is a promising non-GSH peptidomimetic GST-π inhibitor (Ricci et al., 2005) that increased temozolomide efficacy in an *in vivo* model of malignant melanoma (Tentori et al., 2011) and were also effective in overcoming drug resistance in osteosarcoma cell lines (Wang et al., 2007). 8-MOP is a low molecular weight hydrophobic compound that easily crosses the cell membrane; it is also a non-peptidomimetic GST inhibitor, characteristics that made NBDHEX a promising new therapeutic possibility. Docking simulations suggested that 8-MOP interact with active site of GST-π better than NBDHEX. In fact, both, the free form of 8-MOP and the GSH conjugated form had better values of binding energy than NBDHEX. Important hydrophobic interactions made by the benzoxadiazole ring of the NBDHEX with residues Phe-08 and Tyr-108 can also be observed with the 8-MOP coumarin core. These residues are essential for the activity of two other inhibitors: clorambutil (Parker et al., 2008) and ethacrynic acid (Oakley et al., 1997). The 8-MOP also realizes important interactions with Trp-38, Tyr-7, and Leu-52. These residues apparently don't interact with the NBDHEX, as well as the two others inhibitors cited, clorambutil and ethacrynic acid. Furthermore, 8-MOP is structurally simple, what facilitates its synthesis.

Another advantage of 8-MOP is the possibility of short-term clinical using. There are a number of clinical trials with 8-MOP, that is already clinically used (Berroeta et al., 2010), and its toxicity in humans is well known (Sehgal, 1975). Also, many neurological effects of 8-MOP, as insomnia, headache and dysosmia (Vernassière et al., 2006) suggest that this substance is able to cross the blood-brain barrier. However, it is important to consider that some GST genes present polymorphism, including GSTP1 gene for which three variants (hGSTP1*A, hGSTP1*B, and hGSTP1*C) were described. The hGSTP1*C variant being expressed at a higher frequency in gliomas than in normal cells (Kilburn et al., 2010; Backos et al., 2012); this variant contains an A-to-G transition, causing an isoleucine-to-valine change and a C-to-T transition, resulting in an Ala114-Val114 (A114V) substitution. These transitions have impact in the catalytic activity of the enzyme (Lang et al., 2012), and then, 8-MOP could act differently in it. Another indication of the influence of GST variant on 8-MOP activity is that GST polymorphism influences the outcome of therapy with 8-MOP, although plasma concentrations of the drug have not been

associated with different genotypes of the enzyme (Ibbotson et al., 2012). A future clinical use of 8-MOP as GST inhibitor must take these information into account. Associations between GSTP1 polymorphism (and activity) and other pathological conditions, mainly neurodegenerative diseases, like multiple sclerosis (Alexoudi et al., 2014), Parkinson's disease (Longo et al., 2013) and essential tremor (Martínez et al., 2008), could suggest side effects of inhibition of GST activity, but, in the other hand, they also increase the potential therapeutic scope of this prototype.

Co-expression of GST-π and the efflux pump MRP1 (multidrug resistance protein 1) was associated with resistance to etoposide (Depeille et al., 2005). 8-MOP was tested as chemosensitizer in cultures of human glioblastoma cells when co-administered with this drug and the standard agent TMZ, and it was effective for both, what is probable due to its GST-π inhibitory action. 8-MOP also showed intrinsic antitumor effect against these cells. Indeed, GST-π is also involved in the regulation of apoptosis through the inhibition of the c-Jun-N-terminal kinase (JNK) signaling pathway. The enzyme binds to JNK preventing its phosphorylation and, hence, blocking its kinase activity (Tew and Townsend, 2011); the oligomerization of GST-π promotes the uncoupling and releases JNK, that can act on apoptotic pathway and amplify cell death stimuli (Laborde, 2010). That is the probable explanation for resistance toward drugs which are not substrates for GSTs in tumor cells those overexpress this enzyme (Tew and Townsend, 2011). In fact, intrinsic proapoptotic activity was also described to other GST inhibitors (Turella et al., 2005; Tregno et al., 2009). However, it is important take into account that the oligomerization of GST-π also influences (negatively) the catalytic activity of the enzyme (Bernardini et al., 2000) and several GST inhibitors, including ethacrynic acid, can promote this oligomerization (Sánchez-Gómez et al., 2013). Thus, the antitumor effect and probable apoptosis promoting activity of 8-MOP is now under investigation.

In conclusion, we have shown new application for a well-established drug. 8-MOP can represent a molecule of a novel class of GST inhibitors. The therapeutic potential of these inhibitors is not restricted to cancer treatment, they could also have application in treatment of infectious diseases since GST activity has been reported in many pathogenic parasites (Mahajan and Atkins, 2005). Ours results also have implications for current treatment using 8-MOP, like PUVA (psoralen plus ultraviolet A) therapy, and neurodegenerative diseases involving GSH depletion or any pathologic condition in which GST has an important role.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: http://www.frontiersin.org/journal/10.3389/fncel.2014.00308/abstract

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Drug and xenobiotic biotransformation in the blood–brain barrier: a neglected issue

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Drug biotransformation is a crucial mechanism for facilitating the elimination of chemicals from the organism and for decreasing their pharmacological activity. Published evidence suggests that brain drug metabolism may play a role in the development of adverse drug reactions and in the clinical response to drugs and xenobiotics. The blood–brain barrier (BBB) has been regarded mainly as a physical barrier for drugs and xenobiotics, and little attention has been paid to the BBB as a drug-metabolizing barrier. The presence of drug-metabolizing enzymes in the BBB is likely to have functional implications because local metabolism may inactivate drugs or may modify the drug's ability to cross the BBB, thus modifying drug response and the risk of developing adverse drug reactions. In this perspective paper, we discuss the expression of relevant xenobiotic metabolizing enzymes in the brain and in the BBB, and we cover current advances and future directions on the potential role of these BBB drug-metabolizing enzymes as modifiers of drug response.

Keywords: blood–brain barrier, drug metabolizing enzymes, expression profiling, CNS drug, drug response

The concept of the blood–brain barrier (BBB) was developed over a century ago, when Ehrlich observed that dyes administered intravenously do not stain the brain. Goldmann refuted the so-called "binding hypothesis" (Goldmann, 1913) and established that the central nervous system (CNS) is separated from the bloodstream by the blood–brain and cerebro-spinal fluid (CSF) barriers. At present, most of the research related to the BBB has focused on how selected molecules, drugs, metabolites, and toxic substances are able to enter and leave the brain (for a recent review, see Pardridge, 2012). In fact, the BBB has been traditionally regarded as a physical barrier that protects the CNS from non-lipophilic drugs and xenobiotics. In contrast, the putative role of the BBB as a drug-metabolizing barrier has received little attention. The presence of drug-inactivating enzymes in the BBB is likely to affect drug response, as does the presence of such enzymes in the intestine and liver (Pereira de Sousa and Bernkop-Schnurch, 2014). Local metabolism may modify the response and the risk of developing adverse drug reactions with drugs affecting the CNS, and the presence of drug-inactivating enzymes in the BBB may constitute an additional protecting mechanism against drugs and xenobiotics, which may act regardless of their lipophilicity.

With the obvious quantitative differences in enzyme expression of drug-inactivating enzymes between the BBB and the functional unit responsible for pre-systemic metabolism [intestinal enzymes (Pereira de Sousa and Bernkop-Schnurch, 2014), gut microbiota (Kang et al., 2013), and first pass in the liver], drug inactivation in the BBB may constitute a relevant quantitative or qualitative factor for CNS drugs, if these are metabolized by enzymes expressed in the BBB. In fact, a relevant problem in the study of drug response, with regard to drugs affecting the CNS, is related to large interspecies differences in drug bioavailability and distribution within the CNS, including differences between primates and humans; these differences, which would not be expected in a purely physical barrier, are likely to be related to differences in the BBB function. Regarding CNS drugs, this variability could be the consequence of variation in the expression patterns and function of drug-metabolizing enzymes and transporters in the BBB. Of great interest is the development of *in vitro* BBB models using brain vascular endothelial cell cultures which permit the characterization and quantification of genes and proteins in brain microvessels from different species (Shawahna et al., 2013). This methodological advance, together with *in vivo* studies comparing drug concentration and metabolic profiles on both sides of the BBB, will contribute in coming years to greater knowledge of the metabolic and functional implications of the BBB.

ENZYMES THAT METABOLIZE CNS DRUGS

The BBB expresses a variety of neurotransmitter-metabolizing enzymes such as monoamine oxidases (MAO), catechol *O*-methyl transferase (COMT), cholinesterases, GABA transaminase, aminopeptidases, and endopeptidases. Several drug- and xenobiotic-detoxicating enzymes are found in brain capillaries (Minn et al., 1991; de Leon, 2003; Granberg et al., 2003; Haseloff et al., 2006; Ueno, 2009; Wang et al., 2011), thus constituting an enzymatic mechanism that protects the brain from circulating neurotransmitters and from drugs and toxins. For many CNS drugs, metabolic predispositions are a crucial mechanism in determining drug effects. Among the enzymes involved in drug metabolism, two main enzyme categories (Phase I and Phase II) exist. The most relevant Phase I enzymes, in terms of

percentage of drugs that they metabolize, are the cytochrome P450 (CYP) CYP3A (including CYP3A4 and CYP3A5, which share several substrates), CYP2D6, CYP2C9 [which share several substrates with CYP2C8, particularly non-steroidal anti-inflammatory drugs (Agundez et al., 2009)], CYP1A1/1A2 and, to a lesser extent, CYP2C19, CYP2E1, CYP1B1, and other CYP enzymes (Evans and Relling, 1999). Regarding Phase II enzymes, the most relevant in terms of percentage of drugs metabolized are UDP-glucuronyltransferases (UDPs), glutathione *S*-transferases (GSTs), sulphotransferases (SULTs), and *N*-acetyltransferases (NATs) particularly NAT2 (Evans and Relling, 1999). Drugs belonging to many pharmacological groups are affected by polymorphic metabolism mediated by these enzymes, including for instance anesthetics, anti-Parkinson's disease drugs, antihistamine drugs, antipsychotics, narcotics, or antidepressants; specific recommendations for the use of these drugs in the context of variability in drug metabolism have been published (Restrepo et al., 2009; Khokhar and Tyndale, 2011; Relling and Klein, 2011; Swen et al., 2011; Agundez et al., 2013; Garcia-Martin et al., 2013).

DRUG-METABOLIZING ENZYMES IN THE BRAIN

Most available information on the expression of drug-metabolizing enzymes in the CNS corresponds to the whole brain. The expression of CYP450 in the whole brain was reported by Nishimura et al. (2003). Dutheil et al. (2009) showed the apparent selective expression in several cerebral regions in both neuronal and glial cells. The most relevant enzymes were CYP1B1, CYP2D6, CYP2E1, CYP2J2, CYP2U1, and CYP46A1, with heterogeneous distribution in different brain areas (Dutheil et al., 2009). Tissue-specific features are becoming apparent from recent studies. For instance, the dura-mater is clearly different from the other brain structures because of its particular pattern expression of CYP450, with a high level of CYP1B1 and, to a lesser extent CYP1A1, CYP2U1, CYP3A5, CYP2R1, CYP2E1, CYP2D6, and CYP46A1 (Dutheil et al., 2009). The European Bioinformatics Institute Expression Atlas[1] (available at the website), provides interesting information on the baseline expression of drug-metabolizing enzymes in the human brain. When comparing the expression in the brain and in the liver, what is striking is the high relative expression in the brain of the Phase I enzymes CYP46A1 (which is virtually absent in the liver and highly expressed in the brain), CYP1B1 and CYP2U1, which are expressed at twice the level in the brain as in the liver. CYP2R1 is expressed in a similar extent in the brain and the liver, and CYP2J2 is expressed in the brain at about 10% of the level in the liver. CYP2D6 has a marginal expression in the brain, representing about 2% of the liver levels, whereas the expression of other CYPs such as CYP1A1, CYP2C8, CYP2C9, CYP2C19, CYP3A4, CYP3A5, and CYP2E1 seems to be negligible in the human brain in basal conditions. Regarding Phase II enzymes, the expression atlas indicates that a high expression of GST enzymes is present in the human brain; specifically, a 10-fold higher expression of GSTM2, an eightfold higher expression of GSTM3, a fourfold higher expression of GSTP1 and GST4, a twofold higher expression of COMT, and about half of the expression levels of SULT1A4, as compared with the liver, respectively. No significant expression of other relevant drug-metabolizing enzymes, such as SULT1A1, UGT1A6, UGT2B7, NAT1, and NAT2 seems to occur in the human brain. Nevertheless, many of the mentioned drug-metabolizing enzymes are inducible and hence, basal values should be considered as reference values, but cannot be extrapolated to all individuals and to all situations.

DRUG-METABOLIZING ENZYMES IN THE BBB

Dauchy et al. (2008) identified CYPs in microvessels and emphasized the quantitative importance of CYP1B1 in the BBB. Shawahna et al. (2011) quantified the expression of the genes encoding Phase I and Phase II metabolizing enzymes and proteins in brain microvessels from 12 patients suffering from epilepsy or glioma. CYP1B1 and CYP2U1 transcripts were the main CYPs detected in brain microvessels whereas no other CYP proteins were detected (Decleves et al., 2011; Shawahna et al., 2011). Drug-metabolizing enzymes present in microvessels (at a protein detection level) were CYP1B1, CYP2U1, GSTP1, GSTM2, GSTM3, GSTM5, and GSTO1. In addition, detectable MRNA corresponding to CYP2D6, CYP2J2, CYP2E1, CYP2R1 were present, as well as the Phase II enzymes histamine *N*-methyltransferase (HNMT), COMT, and thiopurine *S*-methyltransferase (TPMT; Shawahna et al., 2013). Conversely, no UGTs, or NAT enzymes were identified in microvessels (Shawahna et al., 2013), which is consistent with the virtual absence of expression in the human brain according to The European Bioinformatics Institute Expression Atlas[1]. According to present knowledge, the metabolic capacity of the BBB is likely to modify drug response and, in particular, may be involved in therapeutic failure for drugs that are substrates of the enzymes present in the BBB. Not only because the drugs may be chemically inactivated, but also because drug metabolism at the BBB may modify drug polarity, making the molecules unable to cross the BBB. Because glial cells form a physical barrier in the BBB, the presence of drug-metabolizing enzymes in astrocytes and microglia constitute a line of defense that drugs cannot avoid when entering the CNS. An exhaustive list of drugs and substrates of drug-metabolizing enzymes present in the BBB falls beyond the scope of a perspective article, but some examples are the following: CYP1B1 metabolizes, among other substrates, amodiaquine, caffeine, theophylline, melatonin, and procarbazine (Shimada et al., 1997, 1999; Li et al., 2000, 2002; Spink et al., 2000; Bournique and Lemarie, 2002; Patterson and Murray, 2002; Choudhary et al., 2004; Dubey et al., 2005; Ma et al., 2005; Zhang et al., 2007), CYP46A1 participates in the metabolism of analgesics such as dextromethorphan, diclofenac, or phenacetin (Mast et al., 2003); CYP2D6 participates in the metabolism of several antidepressive agents, antipsychotics, and other CNS drugs (for a recent review, see Agúndez and García-Martín, 2014). CYP2J2 metabolizes ergocalciferol, ebastine, and astemizole (Hashizume et al., 2002; Matsumoto et al., 2002, 2003; Lee et al., 2005; Zhou et al., 2005; Aiba et al., 2006). CYP2E1 participates in the metabolism of anesthetics and ethanol (Zakhari, 2006; Restrepo et al., 2009; Martinez et al., 2010). CYP2R1 participates in the metabolism of ergocalciferol and colecalciferol (Schuster, 2011). HNMT metabolizes histamine, particularly in the CNS, and *HNMT* gene

[1]http://www.ebi.ac.uk/gxa/home

variations are relatively common and affect the enzyme activity (Garcia-Martin et al., 2009). GST enzymes in the BBB impair accumulation and cause therapeutic failure for antiepileptic drugs (Shang et al., 2008).

CLINICAL IMPLICATIONS AND FUTURE PERSPECTIVES

In contrast to the extensive investigation of drug-metabolizing enzymes in the human liver carried out in the last three decades, and compared to the present knowledge of drug transporters in the BBB, the implications of drug-metabolizing enzymes in the BBB are poorly understood. These enzymes may be a major cause of dissociation between the drug concentrations observed in the CSF and plasma, and may underlie therapeutic failure, even when plasma drug concentrations are optimal. Several issues that require further investigation include the following:

(1) Identification and quantification of all drug-metabolizing enzymes in the BBB. So far our knowledge is very limited and further studies are required to identify more enzymes, to analyze their expressions in different structures in the BBB, and to study the interindividual variability in the expression of these enzymes.

(2) Specific characteristics of the drug-metabolizing enzymes expressed in the BBB. The first exhaustive gene profiling of P450 in human brain microvessels was carried out by Dauchy et al. (2008). According to the 1000 genomes catalog[2] (available at the website), most of these enzymes show several splice variants. For instance, CYP1B1 has seven transcripts, two of which encode full-length protein, CYP2U1 has three transcripts, two of these with protein product, GSTP1 has nine transcripts, GSTM has fourteen, GSTM3 and GSTM5 have six each, and GSTO1 has seven. With the exception of CYP2D6, which has only one known transcript, the enzymes detected in the BBB at mRNA level also have several transcripts: CYP2J2 has five transcripts (although only one functional), CYP2E1 and CYP2R1 have ten transcripts each. It is crucial to know which transcripts are expressed in the BBB, both under basal conditions and in CNS or vascular disorders, as well as their characteristics (substrate specificity, Vmax, or Km).

(3) Mechanisms involved in the regulation and functional effects of drug-metabolizing enzymes in the BBB: Effects of known inducers of liver enzymes on BBB drug-metabolizing enzymes, the effect of gene variations, and factors underlying the interindividual variability in enzyme activity.

(4) Effects of known inhibitors of the liver enzymes on the BBB enzymes. This is a crucial factor that may underlie drug interactions which cannot be assessed by conventional therapeutic drug monitoring, that is, by determination of drug concentration in plasma.

In summary, besides acting as a physical barrier, the BBB constitutes a highly specialized metabolic barrier, and contains several drug-metabolizing enzymes, many of which have the ability to inactivate drugs and toxins before they enter the CNS. According to the specific pattern of enzymes, the BBB metabolic barrier has a different metabolic profile than that of pre-systemic metabolism, where CYP3A4 and CYP3A5 enzymes play a key role. These CYP3A enzymes, which show little selectivity since they are involved in the metabolism of a high percentage of clinically used drugs (Evans and Relling, 1999), are not present in the BBB (Shawahna et al., 2011). The metabolic BBB barrier seems to be selective for specific types of drugs or xenobiotics that are metabolized by the enzymes present in the BBB. Nevertheless, much additional information is necessary to gain more ground in BBB metabolism and it is expected that in the coming years we will have new information available for assessing the potential and clinical implications of local drug metabolism in the BBB, which so far have received little attention.

[2]http://browser.1000genomes.org/Homo_sapiens/Search/New?db=core

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The impact of microglial activation on blood-brain barrier in brain diseases

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The blood-brain barrier (BBB), constituted by an extensive network of endothelial cells (ECs) together with neurons and glial cells, including microglia, forms the neurovascular unit (NVU). The crosstalk between these cells guarantees a proper environment for brain function. In this context, changes in the endothelium-microglia interactions are associated with a variety of inflammation-related diseases in brain, where BBB permeability is compromised. Increasing evidences indicate that activated microglia modulate expression of tight junctions, which are essential for BBB integrity and function. On the other hand, the endothelium can regulate the state of microglial activation. Here, we review recent advances that provide insights into interactions between the microglia and the vascular system in brain diseases such as infectious/inflammatory diseases, epilepsy, ischemic stroke and neurodegenerative disorders.

Keywords: microglia, endothelium, blood-brain barrier, neuroinflammation, brain diseases, microglial activation

INTRODUCTION

The cerebrovasculature plays a crucial role in oxygen and nutrient delivery to the organ with the highest metabolic demand in the body, the brain. Moreover, it has a vital function in maintaining a stable ionic environment and protecting against neurotoxic substances critical for normal brain development and function. Brain endothelial cells (ECs) are especially suited for these functions. They act as a selective physical barrier known as the blood brain barrier (BBB), a unique specialized structure of the central nervous system (CNS) capillary bed based on EC tight junctions, with no endothelial fenestrae, and expression of specific membrane transporters, thus ensuring homeostasis and proper functioning of the brain. Brain ECs are closely associated and functionally assembled in what is termed the "neurovascular unit", composed by ECs, neurons, pericytes and glia (Iadecola, 2004). The neurovascular unit (NVU) is at the basis of neurovascular coupling, which allows cerebral blood flow to be locally regulated according to neuronal activity in specific areas of the brain, contributing to normal CNS functioning (Zlokovic, 2008).

In this review, we discuss current concepts underlying the interactions between the vascular system and glial cells, in particular, the microglia—the CNS resident macrophages—in brain diseases such as infectious/inflammatory diseases, epilepsy, ischemic stroke and neurodegenerative diseases.

MICROGLIA-ENDOTHELIUM INTERACTION DURING DEVELOPMENT

During embryogenesis, the formation of the BBB is a gradual process that starts with sprouting and invagination of newly formed branches from the perineural vascular plexus through angiogenesis (Ruhrberg and Bautch, 2013). At the distal end of the growing capillary lies a specialized EC, known as tip cell (Gerhardt et al., 2003), which guides the vascular sprout by integrating external guidance cues as well as vascular endothelial growth factor (VEGF) gradients in the extracellular matrix (Ruhrberg et al., 2002; Gerhardt et al., 2003). Vascular plexus growth is accomplished by halting tip cell migration, followed by tip cell anastomosis and subsequent lumen formation (Lenard et al., 2013). Finally, vessel stabilization and maturation depends on the association of perivascular cells on the new sprouts (Lindahl et al., 1997). The BBB properties are not intrinsic to CNS ECs, as demonstrated by transplantation experiments using the avian embryo model. Prospective abdominal vessels grafted in contact with neural tissue display functional and histochemical characteristics of the BBB (Stewart and Wiley, 1981). It has been shown that formation of EC tight junctions occurs soon

after invasion of the vessels into the developing neuroectoderm (reviewed in Wolburg and Lippoldt, 2002). The ratio of pericyte coverage is highest in the CNS microvessels, which are found to be thoroughly covered by a perivascular astroglial sheath (Mathiisen et al., 2010). Several studies have also shown that components of the NVU such as pericytes and astrocytes play a key role in regulating BBB maintenance and integrity (Janzer and Raff, 1987; Armulik et al., 2010; Daneman et al., 2010). Taken together, these results suggest that the interaction of the ECs with the neural environment is at the basis of the properties of the BBB.

Distinct immune cells within the CNS interact with the BBB. Whereas blood-borne macrophages localize between the vessel wall and the astrocytic endfeet at the meninges, choroid plexus and perivascular spaces (reviewed in Ransohoff and Engelhardt, 2012), recent *in vivo* studies present evidence that resident microglia in the brain parenchyma also interact with CNS microvessels suggesting that, besides an indisputable role in CNS development and homeostasis (reviewed in Nayak et al., 2014), those cells might be also playing a key role in regulating BBB properties during embryogenesis and disease (Fantin et al., 2010; Tammela et al., 2011). Microglia are the most abundant CNS innate immune cells, which during embryogenesis migrate from the yolk sac into the CNS parenchyma (Alliot et al., 1999). Microglia cerebral colonization precedes EC sprouting into this tissue (Cuadros et al., 1993; Checchin et al., 2006; Fantin et al., 2010; Ginhoux et al., 2010) but soon after, they localize in tight physical association with microvascular structures (Fantin et al., 2010; **Figure 1**), suggesting that microglia may play a role in angiogenesis as well as in conferring BBB properties to brain microvessels. In addition, microglia associate with endothelial tip cells, as demonstrated during embryonic brain and postnatal retinal angiogenesis (Fantin et al., 2010; Rymo et al., 2011; Tammela et al., 2011). Fantin and collaborators present *in vivo* data showing that during embryonic stages of CNS vascularization, EC stabilization and fusion are mediated by resident microglial cells (Fantin et al., 2010). Mice deficient for PU.1 (transcription regulator of CD11b and colony stimulating factor (CSF)-1) have reduced microglia, but not circulating monocytes, and present a decrease in embryonic CNS vascular connections. Because microglia appear to be physically associated with tip cell filopodia and the number of sprouts in not altered, the authors suggest that microglia play a role in CNS angiogenesis by serving as a bridge to promote tip cell fusion, vascular plexus growth following sprouting induction and tip cell migration by VEGF (Fantin et al., 2010). Similarly, specific depletion of microglia using clodronate liposomes (CL2MDP-lip) results in decreased vessel density in a mouse model of choroidal neovascularization (Espinosa-Heidmann et al., 2003). Further *in vivo* evidence for a role of microglia as a cellular chaperone controling the fusion and stabilization of vascular sprouts during CNS vascularization came from the observation that a subpopulation of F4/80/Tie-2 positive cells, specifically located near branching sites at the vascular front during vascularization stages of the retina, express VEGF-C. Despite increased vessel sprouting and filopodia bursts, VEGF-C heterozygotes present delayed retinal vascularization and decreased vessel branching density (Tammela et al., 2011). These

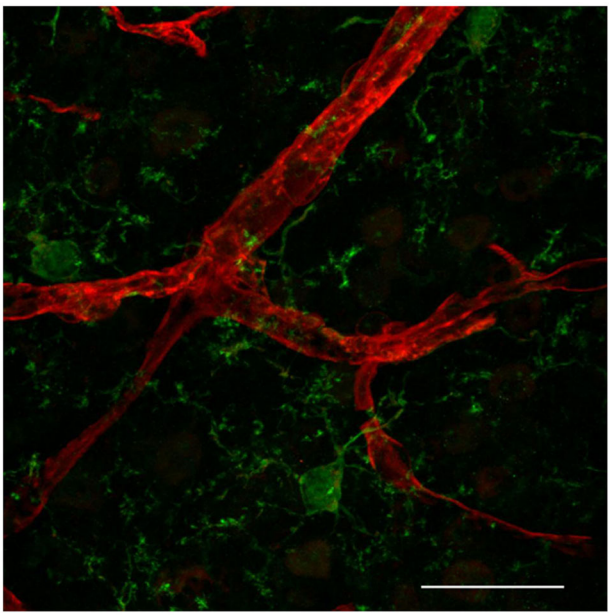

FIGURE 1 | Resident microglia associate with endothelium in the cortical microvascular bed. Representative confocal image revealing close interaction between microglia (IBA1, green) and endothelial cells (IsolectinB4, red) in a 50 micron-cryopreserved cross-section of a 1 month-old mouse cortex. Scale bar: 25 μm. This study was approved by the Ethics Committee of the Health Sciences Center at the Federal University of Rio de Janeiro (Protocol No. DAHEICB 015). The "Principles of laboratory animal care" (NIH publication No. 85–23, revised 1996) guidelines as well as The Code of Ethics of EU Directive 2010/63/EU were strictly followed for experiments.

F4/80-expressing cells present a ramified morphology, which is typical of microglial cells. The retina is part of the CNS and therefore also presents a proper blood barrier. Since the study by Tammela et al. was performed without any damage to the retinal blood barrier, and resident macrophages of the CNS are microglial cells, it thus constitute further *in vivo* evidence for microglia CNS endothelium interaction during early stages of CNS vascularization.

Although a great deal of literature has demonstrated that microglia play a role in CNS diseases where BBB breakdown is a hallmark, little is known about a possible role of these cells in inducing and/or maintaining BBB properties during CNS EC development. The fact that microglia are present in the embryonic CNS territory prior to endothelial invasion and that they participate in cerebrovasculature growth place the interactions between microglia and CNS endothelium as a possible key mechanism in BBB formation and regulation.

MICROGLIAL ACTIVATION AND THE IMPACT ON BBB

Microglia are the resident immune cells in the CNS and perform an essential role in the immune response, while they are also an important component of the NVU (Spindler and Hsu, 2012). Microglia become activated under brain injury and immunological stimuli (Kreutzberg, 1996) and undergo several alterations from a "resting state" to an active

state (Hanisch and Kettenmann, 2007; Kettenmann et al., 2011). This activation and consequent neuroinflammation are substantially involved in the progression of neurodegenerative diseases (McGeer and McGeer, 1995) and impairments of the BBB have been observed in this context (Dickstein et al., 2006; Zipser et al., 2007; Lassman et al., 2012; **Figure 2**).

Recent studies suggest that microglial activation may be related to BBB disruption. Sumi and collaborators evaluated the effects of lipopolysaccharide (LPS), a microglial activator, on BBB functions in an *in vitro* co-culture system using rat brain microvascular endothelial cells (RBEC) and microglia. Treatment with LPS on the outside of the insert (abluminal side) in both RBEC monolayer and RBEC/microglia co-culture showed no effect on transendothelial electrical resistance (TEER) in the RBEC monolayer. However, when the LPS treatment was performed on the RBEC/microglia co-culture, TEER was decreased and this was dependent on the number of microglial cells. Moreover, LPS had no effect on the permeability coefficient of sodium-fluorescein (Na-F) in RBEC monolayer, but in RBEC/microglia co-culture, LPS increased the Na-F permeability. The tight junctions and efflux transporters are the main machinery underlying the BBB function (Liebner et al., 2000; Rajasekaran et al., 2008). Immunostaining for the tight junction proteins zonula occludens-1 (ZO-1), claudin-5 and occludin exhibited a continuous distribution of these proteins along cell border in RBEC co-cultured with microglia, but when treated with LPS, this was changed to a expression pattern that was restored to a linear shape by adding DPI, a NADPH oxidase inhibitor. These results suggest that activated microglia probably produce reactive oxygen species (ROS), through NADPH oxidase, and impair BBB function (Sumi et al., 2010).

In fact, several neurodegenerative diseases include BBB dysfunction. For example, the disruption of BBB caused by oxidative stress seems to be an important step to the development of these disorders, since ROS have been shown to modulate BBB integrity by transient activation of PI3K/AKT pathway via RhoA leading to tight junction leakage (Schreibelt et al., 2007). In addition, the NADPH oxidase system of microglial cells seems to be the main producer of superoxide anion (O_2^-), causing BBB permeability (Block and Hong, 2005; Rojo et al., 2014). When microglial activation was inhibited with minocycline or superoxides produced by NADPH oxidase were blocked with apocynin, the BBB constituents were preserved *in vitro* whereas BBB disruption and hemorrhage were reduced *in vivo* (Yenari et al., 2006).

Furthermore, to clarify the involvement of tumor necrosis factor α (TNF-α) released from activated microglia on BBB integrity,

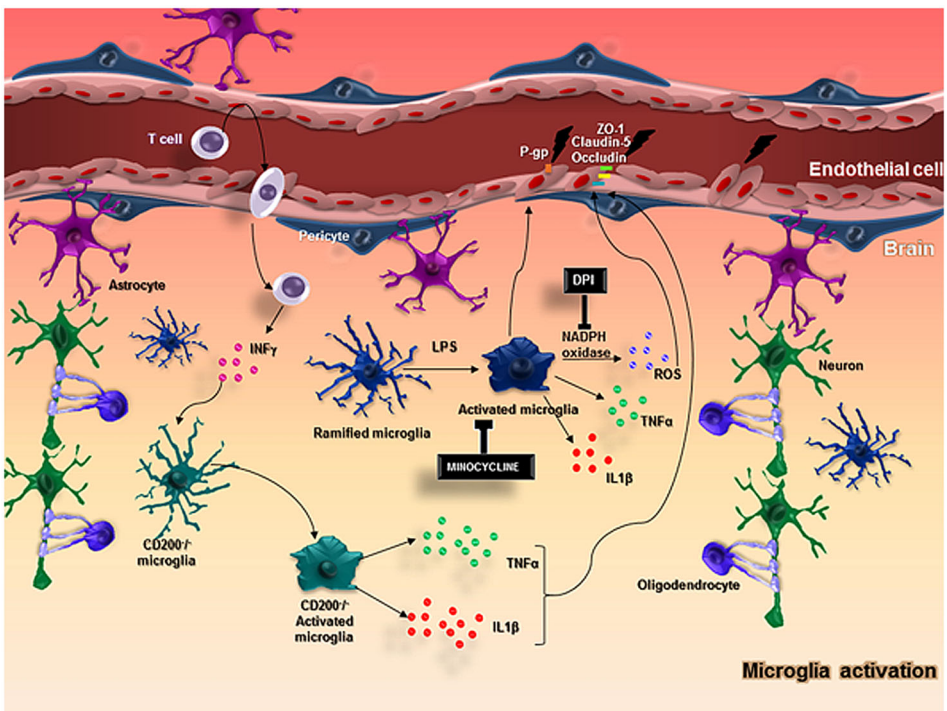

FIGURE 2 | In normal conditions, neurons and glial cells interact together to promote the homeostasis of the brain. When an injury occurs in the brain, microglia and astrocytes are capable of producing cytokines and chemokines and stimulate the adhesion molecules on ECs, allowing the migration of myeloid cells from the blood into the brain. In particular, microglial cells become activated with LPS stimulus and produce ROS through the action of NADPH oxidase, TNFα, IL-1β, that impairs BBB function altering the expression of important molecules in the BBB integrity, such as ZO-1, claudin-5, occluding and P-gp. CD200-deficient mice showed an increased BBB permeability that possibly favors T cell entrance in the brain followed by an increased IFNγ expression that activates microglial cells and enhance the release of microglial activation markers including TNFα, IL-6, contributing to keeping the BBB injury.

an *in vitro* co-culture system with mouse brain capillary ECs (MBEC4) and microglia showed that the permeability of MBEC4 cells to Na-F was increased when microglial cells were activated by LPS and it was blocked by a neutralizing antibody against TNF-α, indicating that TNF-α contributes to BBB dysfunction (Nishioku et al., 2010). On the other hand, ICAM-1 plays an important role in cell-cell adhesive interactions and its expression in cerebral microvessels is low under normal conditions. However, ICAM-1 expression increases in the presence of proinflammatory mediators, such as TNF-α, interleukin (IL)-1β, IL-4 and interferon- γ (IFNγ). The correlation between induction of ICAM-1 expression and increased BBB permeability has been shown in acute inflammation. In an effort to understand by which pathways the peripheral inflammation affects BBB function and structure, Huber and collaborators investigated the effect of λ-carrageenan-induced inflammatory pain (CIP) on the ICAM-1 expression in the BBB. The induction of ICAM-1 was region-specific and directly correlated with an increase in microglial activation (Huber et al., 2006). Moreover, the decreased expression of CD200, which occurs with ageing or in the brain of Alzheimer's Disease (AD) patients, is associated with microglial activation. CD200 is expressed on several cell types and its receptor, CD200R, is expressed on microglia. This interaction has been shown to be important in modulating inflammation and macrophage function. Denieffe and collaborators observed an increased expression of TNF-α and IFNγ in CD200 deficient mice, molecules known to promote classical activation of microglia. The increase of IFNγ levels was probably owing to the entrance of T cells and macrophages into the CNS facilitated by an increased BBB permeability, since IFNγ is normally not produced by resident cells in the brain (Denieffe et al., 2013).

Further evidence for the involvement of microglia on BBB opening was provided by the analysis of AD brain tissue, which showed overlap areas of microgliosis with fibrinogen immunoreactivity. Moreover, these representative patterns of staining were in apparent association with blood vessels. These findings incited *in vivo* experiments using intra-hippocampal injections of Aβ¬1–42 in rat brains, which caused time-dependent increase in expression of fibrinogen and microgliosis compared to the control. Moreover, IgG immunoreactivity was minimal in the control but significantly increased after Aβ¬1–42 injection. Inclusion of anti-Mac-1, a neutralizing monoclonal antibody that blocks the binding of fibrinogen to Mac-1 on microglia and inhibits microglial activation, was highly effective in decreasing IgG after Aβ¬1–42 injection. In these experiments, the permeability of BBB was determined by levels of IgG infiltration consistent with reduced microglial reactivity associated to an increased BBB preservation (Ryu and McLarnon, 2009). Therefore, microglial responses to Aβ¬1–42 can promote vascular changes that induce BBB breakdown plasma protein infiltration (Ryu and McLarnon, 2009).

Considering all the studies mentioned above, microglial activation impairs BBB function by the release of various molecules leading to a hyperpermeability condition associated with inflammation that is similar to what occurs in certain neurodegenerative disorders, including AD and Multiple Sclerosis (MS). We do not discard the entering and the action of bone marrow derived myeloid cells into the CNS in inflammatory conditions, but undoubtedly microglia certainly play an important role in these pathologies (Perry et al., 2010; Graeber et al., 2011).

An intact BBB is essential to control leukocyte trafficking either to immune response during brain infection, or after brain damage when microglial cells and macrophages must clear the debris (Shechter and Schwartz, 2013). On the other hand, the brain inflammatory response to antigen infiltration (virus, bacteria or fungus) triggered by activated microglia may result in aberrant expression of several chemokines, leading to alterations in BBB permeability (Buckner et al., 2006; Obermeier et al., 2013).

The microglial immune response to a brain infection is a complex event that involves several chemokines, particularly the monocyte chemoattractant protein-1 (MCP-1; Andjelkovic et al., 1999). MCP-1 is produced by several cell types including microglia, monocytes, macrophages, neurons, astrocytes and brain microvessel endothelial cells (BMECs; Andjelkovic et al., 1999; Andjelkovic and Pachter, 2000; Mahad and Ransohoff, 2003), and exerts biological functions by binding to its high affinity receptor CCR2, expressed by microglia, astrocytes and BMECs in the brain microenvironment (Banisadr et al., 2002; Ge et al., 2008). *In vivo* studies using MCP-1 knockout mice showed that this chemokine is important to maintenance of BBB integrity (Yao and Tsirka, 2011). On the other hand, the up-regulation of MCP-1 in the brain microenvironment during inflammatory response probably disrupts BBB integrity by redistribution of tight junction proteins, such as occludin and claudin-1, 5 and 11 and reorganization of the actin cytoskeleton in brain microvascular ECs (Stamatovic et al., 2003, 2005, 2006; Dimitrijevic et al., 2006).

Patients with bacterial/viral meningitis exhibit severe BBB impairment in the course of the disease resulting in brain edema, associated with high levels of TNF-α (Sharief et al., 1992; Glimåker et al., 1993; Leppert et al., 2000; Brivet et al., 2005; Ubenauf et al., 2007; Mook-Kanamori et al., 2011). During CNS infection by the human immunodeficiency virus (HIV), perivascular macrophages and microglia are the predominant cell types initially infected by HIV (Wiley et al., 1986). Infected monocytes are more responsive to MCP-1 than normal monocytes, which possibly increase their ability to cross the BBB through the accumulation of adhesion molecules, for instance E-selectin, in the interface between ECs from the BBB and monocytes, resulting in the infection of resident microglia and macrophages (Buckner et al., 2006; Eugenin et al., 2011). Cytokines are highly expressed in HIV-infected microglia/macrophage, leading in turn to an overexpression of IFNγ and TNF-α, inducing EC death pathway, and finally BBB dysfunction. Besides cytokine overexpression, viral proteins, mainly Tat, stimulate cyclooxygenase-2 (COX-2)—an enzyme produced in response to TNF-α during inflammation, associated with decrease of ZO-1 expression in the tight junctions, which leads to BBB rupture and various neurological disorders (Wang et al., 2008; Strazza et al., 2011).

In traumatic brain injury, the post-traumatic inflammatory response is related mainly to cytokine and metalloproteinase (MMP) expression in the lesion site, where IL-1β is associated

with ZO-1 loss and tight junction redistribution (Bolton et al., 1998; Obermeier et al., 2013). The increased levels of MMPs, particularly MMP-2, 3 and 9, have also been observed, produced mainly by microglia (Truettner et al., 2005). These proteins disrupt the basal lamina proteins and degrade the tight junction complexes, resulting in BBB breakdown and severe neurological disorders after traumatic injury (Cunningham et al., 2005; Yang et al., 2007). Using a rat model of traumatic brain injury, Readnower and collaborators showed that microglial activation-derived oxygen free radicals as well as subsequent reaction products, hydrogen peroxide and nitric oxide (NO), have the potential to harm cells, contributing to oxidative damage, neurodegeneration and BBB impairment (Readnower et al., 2010; Briones et al., 2011; Xiong et al., 2012). One of the consequences of oxidative stress is the peroxidation of membrane polyunsaturated fatty acids, giving rise to active aldehydes significantly increasing ECs permeability (Chodobski et al., 2011). The toxicity of oxygen reactive species is observed in other cerebral disorders, such as ischemia (Massberg et al., 2002). During cerebral ischemia, there is platelet accumulation in microvessels, which triggers EC activation and increases ICAM-1 expression, enhancing the neutrophil infiltration in the brain parenchyma, contributing to cerebrovascular inflammation (Thornton et al., 2010).

EPILEPSY

According to the International League Against Epilepsy (ILAE) and the International Bureau for Epilepsy (IBE) a seizure is characterized by transient abnormal excessive or hypersynchronous neuronal activity in the CNS, which leads to specific signs and symptoms. The occurrence of more than one spontaneous seizure characterizes epilepsy, a brain disorder where there is enduring predisposition to generate such seizures (Fisher et al., 2005). Despite neurons being the "effector" cells in epilepsy, the importance of cells from the NVU, specially glial cells, in the pathogenesis of this disease becomes increasingly prominent.

Many works have acknowledged the role of neuroinflammation in the pathogenesis of seizures, but little is known about the mechanisms that start the inflammatory process in the CNS. On one hand, it is known that seizures can occur in many CNS diseases where inflammation contributes to its pathophysiology such as traumatic brain injury (Kadhim et al., 2008), ischemia (Denes et al., 2010; Downes and Crack, 2010), MS (Centonze et al., 2010) and AD (Eikelenboom et al., 2011). On the other hand, there is growing evidence that peripheral inflammation causes a "mirror" inflammatory response in the CNS, characterized by microglial and endothelial cytokine production and invasion of peripheral leukocytes (Quan et al., 1999; Qin et al., 2007; Riazi et al., 2008; Pyter et al., 2009), which can in turn reduce the threshold for seizure induction.

In the epileptic brain a key neuroinflammatory mediator is IL-1β, produced by microglia and astrocytes in great amounts (Ravizza et al., 2008a). Neurons and cells from the NVU (ECs, microglia and perivascular astrocytes) express IL-1R1 receptor and therefore, mediate the effects of this cytokine (Vezzani et al., 2008). In the context of seizures, astrocytes and neurons also produce other substances that modulate microglial function, such as TGF-β, ATP, HMGB1 (a chromatin associated nuclear protein) and fractalkine (CX_3CL1; Verderio and Matteoli, 2001; Rodgers et al., 2009; Aronica et al., 2010, 2012; Dubé et al., 2010; Vezzani et al., 2011).

Microglial and astrocytic IL-1β act directly on the endothelium, altering BBB permeability and contributing to epileptogenesis. IL-1R1 activation on ECs increases BBB permeability through downregulation of tight junction proteins, mainly ZO-1, and upregulation of NO and MMPs (Ravizza et al., 2008a,b; Morin-Brureau et al., 2011; Librizzi et al., 2012). This BBB damage "from the inside" is associated with several downstream effects, all of which directly affect neuronal activity (Kofuji and Newman, 2004; Coulter and Eid, 2012). Endothelial cells activated by IL-1 β also have increased expression of adhesion molecules (ICAM-1, VCAM-1, E-Selectin and P-Selectin) that promote leukocyte adhesion, rolling and arrest in their luminal surface, releasing cytokines/proteases and damaging the BBB "from the outside". This damage "from the outside" further alters BBB's permeability to ions and proteins, contributing to sustain the mechanisms of hyperexcitability (Fujiwara and Kobayashi, 2005; Fabene et al., 2008; Kim et al., 2009).

The role of the other inflammatory mediators produced by microglia and astrocytes in the context of hyperexcitability is not as fully understood as IL-1β's. Vascular endothelial growth factor, IL-6, TNF-α, CCL-2 and prostaglandins were also shown to be important for the development of seizures: IL-6 and CCL-2, for example, are important for chemoattraction of peripheral leukocytes that interact with ECs and contribute to BBB damage "from the outside" (Obermeier et al., 2013). Vascular endothelial growth factor, contributes to BBB damage by inducing downregulation of ZO-1, besides promoting microvascular proliferation (angiogenesis) via VEGFR2 on ECs (Ravizza et al., 2008a; Morin-Brureau et al., 2011; Librizzi et al., 2012). These studies further suggest the importance of the interaction between microglia and ECs in epilepsy.

In this context, the importance of microglial and astrocytic activation is corroborated by the control of epileptic syndromes resistant to conventional anti-epileptic drugs by anti-inflammatory or immunosuppressive treatments (Najjar et al., 2011). For example, intravenous immunoglobulin (IVIG) can reduce cytokine production and astrocyte activation and, therefore, suppresses seizures. Intravenous immunoglobulin increases the circulating levels of IL-1R antagonist (IL-1Ra), blocking IL-1β signaling (Crow et al., 2007; Mikati et al., 2010; Li et al., 2012). In addition, there are also ongoing clinical trials with VX-765, a selective inhibitor of caspase-1, the enzyme that cleaves the precursor form of IL-1β into the active peptide, and therefore reduces its production (Ravizza et al., 2008b; Maroso et al., 2011; Vezzani et al., 2011). Taking all the recent data into consideration, we can suggest that the cells of the NVU, specially glial cells, have an overwhelming importance in epileptogenesis. Excitation and inflammation, traditionally considered independent pathways, are now understood as overlapping and interconnected processes; as inflammation can promote excitability, and so can excitability promote inflammation. These current findings show the increasing importance of the inflammatory pathways activated by microglial cells in inducing BBB dysfunction, which contributes

to epileptogenesis. Furthermore, this understanding is leading to the development of therapeutic strategies attempting to disrupt this self-perpetuating process of inflammation-hyperexcitability, aiming to improve the control of drug-resistant epilepsies.

STROKE

The term *stroke* comprises a heterogeneous spectrum of conditions that have in common the interruption of blood supply to the brain parenchyma. This deficit in the blood flow leads to brain damage that is divided in two regions: the rapidly, severely injured necrotic core and the surrounding *penumbra* region (Liu et al., 2010; Ronaldson and Davis, 2012). The mechanisms of brain death in the ischemic core are mainly related to direct cellular damage due to oxygen and glucose deprivation, as a collapse of ion gradients and excitotoxicity (Adibhatla et al., 2006; Arai et al., 2011; Ronaldson and Davis, 2012). The importance of the crosstalk between neurons, ECs and glial cells (in particular microglia and astrocytes) becomes more prominent in the context of the *penumbra* region, a functionally impaired, but not dead area of the ischemic brain, which is pathophysiologically characterized by hypoxia/reoxygenation stress, BBB disruption, edema and active inflammation (Lo et al., 2005).

The re-establishment of the blood flow to the *penumbra* is responsible for most of the cellular damage observed in the ischemic stroke, associated with neuronal apoptosis, increase in cellular distress secondary to ROS production, and decreased concentrations of antioxidants (GSH). In this context, it is detachable the increased expression of hypoxia-inducible factor-1 (HIF-1) and nuclear factor-κB (NF-κB), in endothelial and glial cells, especially microglial cells. The pathways up-regulated by these transcription factors are involved in disruption of the BBB and neuroinflammation (Witt et al., 2005; Lochhead et al., 2010; Yang and Rosenberg, 2011). Besides that, activated microglia itself produce ROS and NO, further contributing to endothelial and neuronal damage, microglial activation, and perpetuation of stress.

One of the key components of the cell death cascades in stroke is neuroinflammation, where the main players are microglial cells and the peripheral leukocytes, especially neutrophils and monocytes/macrophages. After the ischemic insult, the recruitment of immune cells occurs in a biphasic dynamics. In the acute phase (within minutes), microglial cells are rapidly recruited to the injury site, due to the immediate release of cytokines and chemokines. These cells are activated, enhancing the release of ROS (through NADPH oxidase and MPO), cytokines (i.e., IL-1β, IL-6 and TNF-α) and chemokines (MCP-1, CXCL-1 and MIP-1α). At this time, ECs are also stimulated by these mediators, and the expression of adhesion molecules ICAM-1, P-Selectin and VCAM is upregulated, mainly by activation of the NF-κB pathway. These molecules are also upregulated in the circulating leukocytes, thus enhancing cell migration in the late phase (Kunsch and Medford, 1999; Jin et al., 2010; Enzmann et al., 2013; Obermeier et al., 2013). In this late phase, neutrophils and peripheral macrophages become important mediators of the neuroinflammation and propagation of the acute phase events, by sustaining the production of proinflammatory mediators and ROS, besides inducing and activating MMPs (Jin et al., 2010).

In vivo models helped clarifying the kinetics of the immune cells in ischemic stroke: Denes and colleagues used cell tracking techniques and MRI imaging to describe that, despite of BBB breakdown caused by transient middle cerebral artery occlusion (tMCAo), the infiltration of neutrophils was more prominent only in longer periods of MCAo, with extensive BBB damage. Also, very few infiltrating exogenous macrophages were observed over the first 72 h period and, instead, a profound increase in proliferating resident microglia cells was observed (Denes et al., 2007). However, microglia also remains as an important effector in the late phase, as Ekdahl and colleagues reported an increased number of activated microglial cells up to 16 weeks after two hour MCAO in rats (Ekdahl et al., 2009). Therefore, these works support the concept that microglia are a crucial mediator of the neuroinflammatory response in the CNS after ischemic injury, both in early and late phase stages.

Recently, the neuroinflammatory state was shown to activate the c-Jun N-terminal kinase (JNK) pathway not only in microglia but also in ECs, leading to microglial and endothelial activation with direct and microglial-induced BBB disruption, further cytokine production and perpetuation of the neuroinflammatory process (Tu et al., 2011; Wang et al., 2012).

BBB disruption is another key event in the pathogenesis of stroke, as it can worsen the clinical picture of the patients by the formation of intracerebral vasogenic edema and hemorrhagic transformation. As it occurs in neuroinflammation, BBB disruption has a biphasic dynamics. The early opening (12–48 h) of the BBB is mainly caused by oxidative stress, being ROS the most important mediator. They activate the latent MMP-2 min after the ischemic insult, and are produced by microglia and astrocytes through the HIF-1α pathway (Yang and Rosenberg, 2011; Obermeier et al., 2013). Another phenomenon observed in this early phase is the release of cytokine pools that were trapped by BBB ECM ("molecule trapping"), that activate glial and ECs and contribute to the recruitment of peripheral immune cells (Obermeier et al., 2013). In addition, ROS and NO can also reorganize the cytoskeleton of ECs and modulate tight junction proteins claudin-5 and occludin (Yamagata et al., 2004; Schreibelt et al., 2007) activate microglial cells; and up-regulate inflammatory mediators in the first 48 h. This microglial activation by ROS enhances the production of more ROS, intensifying the early response.

A delayed secondary opening of the BBB occurs after 48 to 72 h and results from the sustained inflammatory response in the brain parenchyma. In this phase, MMPs are important mediators: microglial ROS, especially superoxide radical O_2^-, increase vascular permeability also by stimulating MMP-2/MMP-3/MMP-9 production by microglia, astrocytes and ECs (Gottschall and Deb, 1996; Asahi et al., 2001; Rosenberg et al., 2001), contributing to hemorrhagic transformation and vasogenic edema. MMPs are further stimulated by NK-κB and HIF-1α and disrupt TJ proteins (as ZO-1, claudin-5 and occludin), rendering the BBB leaky, downstream effects which were shown to be reverted by blocking NOS (Yang and Rosenberg, 2011; Gu et al., 2012; Obermeier et al., 2013).

The activation of the previously stated pathways lead microglial cells to produce a variety of molecules, such as TNF-α,

IL-1 β, IL-6, NO and insulin-like growth factor 1 (IGF-1) that interfere with BBB permeability. Apart from the effects of MMPs, microglial-induced late-phase BBB disruption is due to direct effects on ECs: IL-1β and TNF-α downregulate tight junction proteins expression in ECs (Yamagata et al., 2004; Jiao et al., 2011) and, together with IL-6, modulate the expression of adhesion molecules (ICAM-1 and VCAM; Hallenbeck, 2002). These actions not only help enhancing the influx of peripheral leukocytes, but also contribute to the formation of vasogenic edema by allowing the diffusion of sodium and water (Sandoval and Witt, 2008). These mediators also indirectly lead to BBB damage, by acting not on ECs, but on different components of the NVU: activating MMPs and acting on astrocytes and pericytes through ROS, NO and cytokines.

Blood-derived macrophages and microglia appear to have many functions in the dynamic process of CNS injury and repair, affecting BBB permeabilization, which in turn can aggravate stroke (Hallenbeck, 2002; Sandoval and Witt, 2008; Jiao et al., 2011). Preclinical and clinical studies corroborate this observation: minocycline, a member of the tetracycline antibiotic family, inhibits activation of microglia and brain infiltrating blood-derived macrophages and protects cultured neurons from excitotoxic insults by preventing microglial generation of glutamate, IL-1β and NO (Yrjänheikki et al., 1998, 1999; Tikka et al., 2001). These anti-inflammatory properties of minocycline are thought to act through inhibition of p38MAPK and MMP-9 (Tikka et al., 2001; Koistinaho et al., 2005). Inhibiting microglial activation may limit BBB disruption and reduce vasogenic edema in the context of ischemic stroke, reducing the volume of ischemic tissue and neuronal deficits, as well as preventing hemorrhagic transformation (Tikka et al., 2001; Yenari et al., 2006). So far, minocycline has been incorporated into two clinical trials involving ischemic stroke patients, demonstrating that minocycline administration (both alone and in combination with Fibrinolysis) improved neurological functional outcome following stroke (Fagan et al., 2011).

On the other hand, some groups advocate the concept that microglial cells are important for recovery from ischemic damage. Some i*n vitro* and *in vivo* studies have shown the role of IGF-1 in promoting neuroprotection and neuroregeneration (Li et al., 2010; Selvamani et al., 2012; Sohrabji and Williams, 2013; Bake et al., 2014). It has also been shown that the selective ablation of proliferating microglial cells in a transgenic mouse model exacerbates the extent of ischemic injury (Lalancette-Hébert et al., 2007). Besides that, Kitamura and colleagues demonstrated in a rat model that the intracerebroventricular injection of microglia protected the BBB and neurons against focal brain ischemia (Kitamura et al., 2004). All these data suggest that microglia has a role in the EC damage and BBB disruption in stroke, making these cells a possible target to minimize the ischemic damage in stroke patients.

NEURODEGENERATIVE DISORDERS

The BBB is seen as a protector of the brain homeostasis since it prevents the entry of several components from the blood into the brain parenchyma. In particular, during MS and AD, the mononuclear phagocytes from blood are also recruited via brain chemokines, which allows entry of cells from the bloodstream through the BBB (Britschgi and Wyss-Coray, 2007). Moreover, these inflammatory and neurodegenerative disorders cause an impairment of neural and synaptic functions due to the production of neurotoxic compounds (Zlokovic, 2008; Mizee and de Vries, 2013). MS and AD are quite distinct neurodegenerative diseases but both affect the CNS, leading to degeneration and consequently causing physical impairment and dementia, respectively.

Furthermore, several studies have shown that microglia can compromise BBB functions by the release of proinflammatory cytokines, such as TNF-α and IL-6, leading to BBB disruption (Britschgi and Wyss-Coray, 2007). Here, we will discuss the importance of microglia during the BBB breakdown in AD and MS.

ALZHEIMER'S DISEASE

Alzheimer's disease is recognized as one of the most common causes of dementia that leads to impairment of memory, thinking and behavior in humans (Zlokovic, 2008). The major pathological features of this disease are the increased production and deposition of amyloid-β (Aβ) and intracellular accumulation of neurofibrillary tangle composed of hyperphosphorylated tau protein, besides synapse and neuronal loss (Takeda et al., 2014). Also, it is commonly accompanied by BBB dysfunction (Erickson and Banks, 2013; Lyros et al., 2014). In the initial stage of AD, there is loss of BBB homeostasis, leading to the production of proinflammatory cytokines and suppressors of the cerebral blood flow by ECs, which exacerbates synapse destruction, accumulation and activation of microglia. Also, in the late phase of AD, amyloid deposits are commonly observed in larger blood vessels and smaller cerebral capillaries (Zlokovic, 2004, 2011).

Crosstalk between systemic and central innate immune systems by the release of inflammatory mediators is observed in AD (Holmes, 2013). So, another role of BBB is the control of the entrance of T lymphocytes into the brain (Town et al., 2005). In AD patients, it was observed the presence of T cells in greater numbers than in non-AD patients (Monson et al., 2014). It was proved that the migration of T cells and immune cells into the brain occurs under inflammatory conditions. In this study it was shown that T cell migration into the brain through the BBB in AD depends on the TNF-α expressed by microglia, which induce the expression of MHC class I (MHC-I) on brain ECs (Yang et al., 2013).

The microglial cells and astrocytes are the resident brain cells responsible for the immune response in the brain, and it was observed in AD brains the presence of activated microglia and astrocytes around Aβ plaques, with the release of inflammatory cytokines, such as IL-1 and IL-6, TNF-α and transforming growth factor-β (TNF-β; Wyss-Coray, 2006; Zhou et al., 2012). In AD, these cells can promote the clearance of Aβ, however, if they are not able to do that, there is an accumulation of Aβ deposits leading to neuronal death (Rogers et al., 2002). It has already been shown that the elimination of Aβ from the brain and the consequent impairment of microglial activation decrease ROS, nitrogen compounds and also inflammatory cytokines produced by microglial cells, which are correlated with the

activation of ECs (Dickstein et al., 2006). Several studies using animal models have further contributed with *in vivo* evidences of the importance of microglia during AD progression. In the study performed by El Khroury and collaborators using an AD transgenic model, a CCL2-deficient APP/PS1 mouse, which has a deficiency in the Ccr2 chemokine receptor and its ligand (CCL2) on microglia, they observed a regression in the disease (El Khoury et al., 2007; Kiyota et al., 2013). Moreover, high levels of Aβ are correlated with production of CCL2 by microglia, which leads to BBB impairment (Roberts et al., 2012).

On the other hand, it has been shown that LPS induces the production of TNF-α in the brain by microglial cells, which promotes BBB dysfunction and also causes neuronal cell death by phagocytosis (Tanaka et al., 2006; Nishioku et al., 2010; Smith et al., 2012; Neniskyte et al., 2014). Another cause of BBB breakdown is the accumulation of various molecules in the brain, such as thrombin, which is released by ECs, leading to activation of other components of the brain, like microglia and astrocytes. One study found that brain microvessels collected from AD patients produce high levels of thrombin, which directly affect neurons by the induction of cell death, and indirectly affect activation of microglia. Furthermore, it was shown that intracranial injection of thrombin activates microglia, leading to the production of NO and TNFα via JAK2-STAT3 signaling pathway, which induce BBB disruption. Thus, it appears that thrombin causes a disruption of BBB indirectly by activation of microglia (Huang et al., 2008a,b; Yin et al., 2010; Grammas, 2011).

Thus, regarding all evidences described above, we can hypothesize that microglia activation plays an important role during BBB disruption, which substantiates the need to study other factors correlated with inflammatory pathways, as potential targets of new strategies to reduce microglial-dependent inflammation in the brain of AD patients.

MULTIPLE SCLEROSIS

Multiple sclerosis is a chronic, progressive and neuroinflammatory demyelinating disease of the CNS, which involves an energy deficit, tissue remodeling, microglial activation and loss of BBB integrity (Lassmann et al., 2012).

MS is often associated with the increase of BBB permeability, and consequently inflammatory response causing the formation of lesions and demyelization. Previous studies showed that systemic inflammation contributes to the disease progression and the activation of immune cells allows the infiltration of T and B cells into the CNS, promoting the release of chemokines that in turn stimulate the migration of more immune cells, leading to BBB disruption (Holman et al., 2011; Kooij et al., 2011; Larochelle et al., 2011; Willis, 2011). Moreover, during inflammation and demyelination in MS, the activation of microglial cells lead to the release of cytotoxic factors, such as NO and ROS, provoking myelin damage, release of proinflammatory cytokines (IFNγ, TNF-α and IL-1β), as well as chemokines (MCP-1), ultimately inducing BBB disruption (Mahad and Ransohoff, 2003). Activated microglia are known to express ROS-generating enzymes, such as NADPH oxidase, which are responsible for degeneration of oligodendrocytes and increased BBB permeability by downregulating the expression of VE-cadherin, occludin and claudin-5 proteins in microvascular ECs (Fischer et al., 2012; Rochfort et al., 2014).

Hereupon, there are two different types of inflammation in MS. The first consists in the initial response to the disease, involving the infiltration of $CD4^+$ and $CD8^+$ T-cells and robust microglial activation; the second occurs due to the myelin destruction, stimulating the secondary recruitment of T cells, B cells and macrophages (Lassmann et al., 2012). There are two well-established mouse models used to study MS, which have an ineffectual immune response: Theiler's Murine Encephalomyelitis Virus-Induced Demyelinating Disease (TMEV-IDD; Rodriguez et al., 1987) and experimental autoimmune encephalomyelitis (EAE), where activation of microglia precedes infiltration of peripheral macrophages and occurs before the onset of the disease (Sriram and Steiner, 2005). *In vivo* imaging using two-photon laser scanning microscopy (2PLSM) on EAE animal showed dynamic interactions between BBB disruption and microglia activation. It was observed that microglial motility resulted from the leakage of the plasma protein fibrinogen before the onset of MS signs. In this event, fibrinogen induces the activation of microglia and consequently the release of ROS, inducing axonal damage (Davalos et al., 2012). Both in samples from patients with MS and in an animal model for MS, EAE, it was observed a high expression of MMPs. Microglia contributes to the inflammatory process through the production of MMPs, such as MMP−1, −2, −3, −9, and −19, which consequently leads to destabilization of the BBB permeability (Weaver et al., 2005; van Horssen et al., 2006).

Minocycline has been recognized as a microglia inhibitor (Li et al., 2005; Defaux et al., 2011; Kobayashi et al., 2013; Miron et al., 2013; Huang et al., 2014). Minocycline can reduce BBB breakdown by preventing microglial production of glutamate, MMPs, IL-1β and iNOS. The inhibition of microglial activation by minocycline favors the differentiation of oligodendrocyte precursors and immature oligodendrocytes. These cells are responsible for remyelination of neurons, an important event for improvement of MS condition (Li et al., 2005; Yenari et al., 2006; Defaux et al., 2011; Miron et al., 2013). Recently published data demonstrated that dipyridamole attenuates the expression of Toll-like receptor stimulation-dependent cytokines and chemokines in human microglia, reduces microglial activity in EAE mice, and consequently decreases IL-1β, TNF-α and IL-6 expression, which are responsible for increased BBB permeability (Sloka et al., 2013).

Taking into account the literature reviewed about AD and MS, we suggest that pharmacological modulation of microglial activation may control the impairment of BBB in these diseases.

CONCLUDING REMARKS

Over the last years, many studies have been performed aiming at understanding the role of BBB during ischemic injury, neurodegenerative disorders and infectious/inflammatory diseases. However, despite important advances made during the last decade, the mechanisms involved in the multifaceted interactions between the constituents of the NVU are not yet fully uncovered. In particular, how activation of microglial cells may play a key role in BBB disruption. When activated, microglia impair BBB function

through the release of several inflammatory modulators leading to a hyperpermeability condition, which is associated with several brain disorders. In addition, the consequent infiltration of peripheral immune cells in turn affects microglia function. Microglial activation may be one of the earliest phenomena involved in the progression of these diseases. In this sense, the role of the inflammatory response triggered by the activated microglia seems to be essential to understand BBB dysfunction in CNS diseases. Therefore, efforts should be directed towards the development of new approaches focusing on microglia as a potential therapeutic target.

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Recruiting specialized macrophages across the borders to restore brain functions

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Although is well accepted that the central nervous system has an immune privilege protected by the blood–brain barrier (BBB) and maintained by the glia, it is also known that in homeostatic conditions, peripheral immune cells are able to penetrate to the deepest regions of brain without altering the structural integrity of the BBB. Nearly all neurological diseases, including degenerative, autoimmune or infectious ones, compromising brain functions, develop with a common pattern of inflammation in which macrophages and microglia activation have been regarded often as the "bad guys." However, recognizing the huge heterogeneity of macrophage populations and also the different expression properties of microglia, there is increasing evidence of alternative conditions in which these cells, if primed and addressed in the correct direction, could be essential for reparative and regenerative functions. The main proposal of this review is to integrate studies about macrophage's biology at the brain borders where the ultimate challenge is to penetrate through the BBB and contribute to change or even stop the course of disease. Thanks to the efforts made in the last century, this special wall is currently recognized as a highly regulated cooperative structure, in which their components form neurovascular units. This new scenario prompted us to review the precise cross-talk between the mind and body modes of immune response.

Keywords: blood–brain barriers, recruitment, gates, macrophages, reparative functions

INTRODUCTION

The understanding of immune regulation between the body and the brain is essential to unravel the molecular basis of many etiologies affecting the central nervous system (CNS), but despite great and combined efforts of neuroscience and immunology, there are still some questions that need additional answers:

Why having the brain its own immune cells, is there still a need for the systemic immune response, not only in inflammatory or traumatic conditions but also in homeostasis? Are we starting to question the notion of the brain as an "immune privileged site" flanked by high walls? On the contrary, the intensive research made by the groups working together at these brain barriers have delimited the potential gates for leukocyte entrance as well as the spatial and temporal requirements that could allow them to pass through without compromising its integrity (Interlandi, 2013).

Each tissue of the body has its own specialized immune cells adapted to fight their common pathogens without the wise of general immunity. Nevertheless, when an important insult is threatening our integrity as a whole both, general immunity and brain innate immunity have to cooperate *in close* to restore body homeostasis. Thus, if an inflammatory process affects the body, primed T cells secrete cytokines that indeed reach the CNS. It is from there, that an innate brain immune response is generated to control the body response during the particular insult.

For example, when we have fever, symptoms as loss of appetite, arousal, blushing, or somnolence are part of this innate brain response to interleukin-1 and other T-cytokines triggered in response to the infection (Steinman, 2012). Therefore, there is increasing evidence that in both, homeostatic and inflammatory conditions, an active dialog exist between the brain and the body.

Thanks to the great efforts made by neuroimmunologists in the last 20 years now we know that this relationship is bidirectional: neurons embracing immune cells directly in peripheral tissues have been photographed and also blood-derived leukocytes are found across the barriers in healthy subjects.

This review intends to focus the attention into the routes that allow peripheral monocytes to enter and differentiate inside brain barriers and their potential function in health and neuroinflammation.

POTENTIAL GATES FOR MONOCYTE ACCESSING TO THE BRAIN

Research made in the last 10 years about the structure and cellular interplay at the blood–brain barrier (BBB) has gifted us with a new vision about the routes that leukocytes can use to cross them, even in homeostatic conditions. Indeed, experts in the field agree in considering that the concept of immune privilege has to be reinterpreted in the CNS. Ehrlich' description and demonstration of limited frontiers for molecules do not apply in the same way to cells (Wilson et al., 2010; Dyrna et al., 2013). Therefore, to better understand how monocytes can be found inside the barriers it could be helpful to briefly describe the potential gates for them to access.

To start checking the routes for entrance of blood-derived macrophages to the CNS is important to remember that meninges,

that separate the skull to the brain parenchyma, are composed of three membranes; the outer membrane – the dura mater, the middle membrane – the arachnoid – and the inner membrane – the pia matter – that surround the neuropil. All these membranes are encompassed by a BBB in which the structure of the blood vessels has huge differences on distinct and well defined sections of the vascular tree. Blood first flow through the pia mater and then is poured into the subarachnoid space where the Virchow–Robin space is formed. Blood supply to the brain enters through four arteries, the internal carotid arteries and the vertebral arteries, which merge in the circle of Willis at the base of the brain. Thereafter, they are branched in arterioles and capillaries that irrigate all the CNS parenchyma (Wilson et al., 2010).

THE BBB

The BBB comprises the capillary and postcapillary vessels at the brain and spinal cord (SC). According to its architecture but also its functionality is built up by neuro (glio) vascular units (NVU; Ballabh et al., 2003; Hawkins and Davis, 2005), and has huge differences depending on their particular location along the brain and SC. The first cellular component that separate the CNS from circulation is the brain endothelium in which cells are attached by tight junctions (TJ_s) and adherent junctions (AJ_s) (Lampron et al., 2013).

A more detailed look into the structure of the barriers have shown that recognizing the differences in the gage of vascular tree between capillary versus postcapillary vessels is key to understand why there are a limited number of portals for leukocyte recruitment:

Neuro vascular units at capillaries are composed of brain endothelial cells – with special T and J junctions – and surrounded by pericytes. There is only one basement membrane between endothelial cells and the astrocyte endfeet of the glia limitans. Therefore, the pass through this capillary barrier requires a very precise regulation. Perhaps this could be the most difficult passage, but even at capillaries is possible for blood cells to cross through two adjacent NVUs and thus avoid the endothelial junctions, as suggested by some authors (Carson et al., 2006; Dyrna et al., 2013).

Neuro vascular units in pre and post-capillary vessels, as well as in arterioles, has, however; an important difference: the astrocyte endfeets are not in intimal contact with the endothelial layer but separated from them by pericytes and smooth muscle cells forming the media and also by a key perivascular space, named in humans the Virchow–Robin space. The differences in biochemical composition of the vascular and glia basement membranes may explain why leukocytes under normal conditions could pass the former but not the latter and thus, they are found mostly trapped in the middle (Bechmann et al., 2007) but actively patrolling between frontiers, perhaps in an intermediate differentiation state.

MACROPHAGE RECRUITMENT ACROSS THE BBB

The first and key unresolved question that deserves discussion is why blood-derived macrophages are needed inside the CNS having the brain its own innate immune macrophages, the microglial cells. Therefore, many authors have tried to investigate whether macrophages and microglia have differential specific roles or rather their functions could be considered redundant. As adult microglia derive of an embryonic population of myeloid cells that migrated into the brain during mammalian embryogenesis (Cuadros and Navascués, 1998; Ginhoux et al., 2010), it is clear now that blood-derived macrophages are not the same cells as microglia, although sharing many membrane determinants and having conserved the general features of phagocytic cells. Indeed, all resident tissue macrophages have their own genetic program adapted and triggered by the innate and metabolic features of their particular tissue (Gundra et al., 2014). Thus, blood-derived macrophages must be recruited to exhibit their alternate roles as will be discussed.

Monocyte recruitment across the BBB according with the last findings would occur mostly at the level of post-capillary venules. In fact, two different steps have been proposed for them to gain access to the inner brain: first they must transmigrate from the endothelial wall to perivascular spaces and then, in a separate temporal sequence, they could progress across the glia limitans into the parenchyma (Owens et al., 2008). The demonstration that there is a population of perivascular macrophages being regularly renewed by the blood in the absence of pathology came from studies of bone marrow chimeras. Bechmann et al. (2001) used transplants of green-fluorescent-protein (GFP)-transfected bone marrow cells to clearly show that as soon as 2 weeks after transplant there was a replacement of perivascular cells by blood-borne macrophages in adult mice. These results were the first to prove that brain perivascular cells were not resident histiocytes but blood-derived macrophages. However, at this level of the BBB, even in postcapillary vessels, the migration of macrophages and T cells into the brain parenchyma have been proved to occur only under inflammatory conditions, as extensively reviewed by others (Engelhardt and Ransohoff, 2012; Lampron et al., 2013). Therefore, in healthy brains the role of perivascular macrophages could be to stay on hold living between barriers, but with a very noble function; to sense changes in immunity from inside to outside and profiling their differentiation programs accordingly.

THE BLOOD–CEREBROSPINAL FLUID (CSF) BARRIER

This barrier is present at the lateral ventricules and has two main differences with the BBB:

First, the capillaries that form the choroid plexus (CP) are fenestrated, with little holes like a *fenestra*, enough to allow the entrance of molecules bigger than 500 D, the limit estimated for molecules that can cross an intact BBB.

Second, the cellular epithelium that produce the CSF down the subarachnoid space, have lateral junctions but they are not as tight as those of the endothelial cells within the BBB. In fact, there is a consensus in assigning the CP of the ventricular system as the main route for the entrance of central immune cells into the CNS (Wilson et al., 2010). There, specialized epithelial cells (Kolmer cells) make the CSF and drop it in the subarachnoid space. Below the epithelium lay the fenestrated endothelium separated by the basal membrane of the internal meninge, the Pia matter.

Systemic monocytes within the CSF are recruited by adhesion molecules and chemokines expressed by capillary endothelial cells at these places that also allow the crossing of T cells. Otherwise,

they continue traveling, floating and circulating within the CSF from the meninges toward the CNS and then back to bath all the SC. Normally, there is from 100 to 150 ml of CSF always in circulation that is finally reabsorbed in the arachnoid villies, at the superior central sinus, by hydrostatic forces (Ransohoff and Engelhardt, 2012).

Recently, (Iliff et al., 2012) by using *in vivo* two-photon imaging techniques have demonstrated that a substantial amount of subarachnoid CSF cycles through the brain parenchyma: CSF enters the parenchyma through perivascular spaces that surround penetrating arteries, is exchanged with the brain interstitial fluid and finally cleared along perivenous drainage. They have termed this pathway as "the glymphatic system" because is supported by the water transport system of astrocytes.

The demonstration of this route suggest that immune cells of CSF could have direct contact with extracellular brain proteins and solutes and therefore participate in its clearance or alternatively start to mount an effective immune response.

MACROPHAGE RECRUITMENT ACROSS THE CHOROID PLEXUS

There is a consensus in assigning the CP as the most accessible yet selective gate for leukocyte transmigration into the brain. The cellular composition of ventricular and lumbar CSF differs from that of the blood and is dominated by CD4 + memory T cells and macrophages (Matyszak et al., 1992; Ransohoff et al., 2003).

It has been demonstrated that CP constitutively expresses adhesion molecules and chemokines which support trans-epithelial leukocyte trafficking (Steffen et al., 1996). The research to find the most suitable candidates for cell recruitment has been very intensive. Even in absence of ongoing inflammation macrophages are able to cross the CP. Therefore, adhesion molecules that could facilitate its entry to the inner brain space have been checked first between those expressed constitutively by the CP epithelium. These include P-selectine, VCAM 1 as in vascular endothelium, or the integrin receptor ICAM 1 which is localized on the apical side of the CP and suggested as a gate for "basal to apical" migration (Kunis et al., 2013). In addition, macrophages would need to be attracted by chemokines such as CCL19 and CCL20 that are constitutive in Kolmer cells (Krumbholz et al., 2007). All these results suggest that trafficking trough the CP appears to be controlled by the epithelium. Moreover, a unique feature of the CP is the presence of a large population of macrophages and dendritic cells within the stroma, implying that this compartment may provide an important source of these cells traveling along cerebral ventricles as shown by Meeker et al. (2012).

One of the first pioneering researches about the multiple roles of macrophages in CNS injury has been performed by the group of Michal Schwartz. They have demonstrated that after SC injury, blood-derived macrophages are recruited from the distant CP with a clear reparative function. The authors also show that macrophage recruitment to an injured SC came from two different places: the adjacent leptomeninges allowed the infiltration of classically activated (M1) macrophages that were involved in an effective inflammatory response, while a second remote gate through the CP allowed the entry and differentiation of macrophages that expressed a reparative (M2) phenotype. These elegant experiments suggest that in response to parenchymal damage, peripheral immune cells go through this route, which are also followed by T cells and even neutrophils in the context of sustained inflammation (Shechter et al., 2013b). Their results are supported by the evidence that in homeostasis, the phenotype of the CSF in which they patrol is immunosuppressive, having an enrichment of anti-inflammatory cytokines such as IL-13 and TGF-β, as also occurs in other immune privileged tissues (Schwartz and Baruch, 2014).

THE BLOOD–SPINAL CORD BARRIER

At this level the NVU includes endothelial cells that are surrounded by pericytes and astrocytes but the extracellular matrix has several intrinsic features. There is not CP because of the occurrence of nerve root entry zones in the transition to the peripheral nervous system. Therefore, the permeability of this BBB is higher than in other areas of the brain. On the other hand, the CSF contacts the SC at two places: the spinal subarachnoid space with leptomeninges as the interface layer and the central canal with the ependymal layer. The cellular composition of this NVU contains less pericytes surrounding endothelial cells. This has been proposed as the reason for the increased permeability of this barrier (Winkler et al., 2012).

There are many examples of leukocyte extravasation in SC injury or autoimmunity. However, there is little information about the gates of entry for them in homeostatic conditions. Schnell et al. (1999), compared the response to injury between the brain and SC in mice and found that in the SC, there was higher numbers of cells recruited at the lesion site that reached easily the parenchyma, suggesting that the BBB at this location is provided with additional gates for macrophage recruitment, perhaps due to the CSF circulation.

MACROPHAGE RECRUITMENT ACROSS THE BLOOD–SPINAL CORD BARRIER

Due to the technical difficulty of clearly differentiate resident microglia from blood-derived macrophages after a traumatic or autoimmune disorder, there is still some controversy about the role of each one during pathology. Recently, Greenhalgh and David (2014) by using a mouse model in which macrophages could be distinguished from microglia have shown that microglial cells are the first responders (within minutes) to tissue destruction and exhibit higher efficiency in removing debris than recruited macrophages. Noteworthy, this work also points to an effective and necessary recruitment of blood-derived cells to help with tissue reparation. For this reason, researches are starting thinking that the clue must be to maintain the proper balance and action time in which classic versus alternative response of macrophages are triggered.

On the other hand, during the preparation of this review, Carrillo-Salinas et al. (2014) have demonstrated that a phytocannabinoid derivative was able to switch T cell and macrophage inflammatory phenotypes *in vivo*; inhibiting Th1 and Th17 activation at the SC of experimental autoimmune encephalomyelitis (EAE) treated animals. Furthermore, they show that this compound is a potent activator of PPAR-γ receptors, which in turn are potent modulators of macrophage alternative programs

(Gallardo-Soler et al., 2008). Therefore, appears to be clear that molecules able to trigger reparative and regenerative programs to change macrophage activation could be of potential therapeutic interest for brain pathologies cursing with inflammation.

THE BLOOD–RETINA BARRIER (BRB)

The inner part of this barrier is formed by endothelial cells of retina capillaries and contains the same structural components within the NVU. The outer barrier is made by the retinal pigment epithelium. Regarding permeability properties, this barrier enables greater penetration of hydrophilic compounds.

Although much less studied, there is increasing interest in defining the potential gates for the entry of blood-derived cells into retina. Shechter et al. (2013a) have pointed to epithelial retinal cells as the interface in which takes place the intraocular migration of leukocytes, which is also favored by the fenestrated epithelium of the ciliary body (Lightman and Greenwood, 1996).

MACROPHAGE RECRUITMENT ALONG THE BRB

The permissibility of *epithelium pigmentosum* for blood cells has been demonstrated in models of uveitis and retinal lesions (Joly et al., 2009) and there is agreement in accepting that both, retinal pigment epithelium and ciliary body epithelium have immunosuppressive properties, pointing to a major role of these eye interfaces as the place for selective cell recruitment. Regulatory T cells and reparative M2 macrophages have been shown to be essential in resolving inflammation in experimental uveitis, as reviewed by Kerr et al. (2008). Being the eyes *the soul of the brain* (Perez et al., 2013) it would be desirable to gain a deeper knowledge on the role of macrophages at this important barrier.

A brief summary of macrophage location within the main brain barriers is presented in **Table 1**, in which each reference is just an example in which blood-derived macrophages have been found at these brain interfaces either in healthy or inflammatory neurological conditions. It is also important to note that blood-derived cellular transmigration, at least in the first stages of neuroinflammation, does not necessary imply the breach of the brain barriers that often while require a temporal and chronic cellular invasion as occur in non-resolving neurological diseases but also obviously, after a serious insult such are brain injury or stroke, when the brain immune surveillance is lost.

THE MANY FACES OF MACROPHAGE ACTIVATION IN BRAIN INNATE IMMUNITY

Macrophages are very heterogeneous populations of cells that have evolved epigenetically different within each tissue of the body. Therefore, although conserving its common CD markers, kupffer cells, alveolar macrophages, or microglia have its own particular program adapted to preserve the innate immune functions in both homeostatic and inflammatory conditions.

The CNS also has its own immune population, the microglia, recently shown to migrate from the Yolk Sac and colonize the brain parenchyma during embryogenesis, before the brain barriers are formed. In contrast, peripheral macrophages that enter the brain derive from circulating Ly6C^{hi} monocytes originated from bone marrow hematopoietic stem cells in a Myb-dependent manner (Schulz et al., 2012).

Moreover, these two populations remain distinct as it has been demonstrated by Ajami et al. (2011) in a model of EAE. Therefore, monocytes that cross brain borders are transformed into diverse cell populations able to acquire multiple effector functions always depending on the balance between the innate and the adaptive modes of immune response.

However, there is still some consensus in assuming that any cellular extravasation from blood to brain is or will be harmful and detrimental, especially macrophages, because they have been indeed found in all brain inflammatory diseases. The consequence of this idea has been the basis of generalized prescription of corticoids for nearly all autoimmune diseases. These protocols are still being applied.

CLASSIC VERSUS ALTERNATIVE MACROPHAGE ACTIVATION

The concept of macrophage alternative activation started 20 years ago when several groups were interested in understanding the response of antigen presenting cells (APCs) to Th2-derived cytokines (Stein et al., 1992). Independently, our group started to study the metabolic fate of L-arginine metabolism in macrophages. We found that inductors of nitric oxide synthase II (NOS II)

Table 1 | Monocyte recruitment through intacts brain barriers.

Barriers	Macrophage locations	CNS status	References
Brain endothelium(BBB)	-Perivascular macrophages at postcapillary levels	-Homeostasis	Bechmann et al. (2007)
	-Recruited and crossing through capillary endothelium	Neuroinflammation	Owens et al. (2008)
Choroid plexus(B-SCFB)	-Macrophages in choroid plexus stroma	-Homeostasis	Matyszak et al. (1992)
	-Monocyte/macrophage in CSF (25% in humans)	-Homeostasis	Meeker et al. (2012)
	-Recruitment through the fenestrated endothelium	Neuroinflammation	Ransohoff and Engelhardt (2012)
Blood-leptomeninges(B-LMB)	-Resting macrophages in leptomeningeal spaces	-Homeostasis	Bartholomäus et al. (2009)
	-M1-activated macrophages	Neuroinflamation	Shechter et al. (2013a)
Spinal cord(B-SCB)	-Macrophages recruitment from CSF	Neuroinflammation	Winkler et al. (2012)
	-M2-activated macrophages from CPt	Response to injury	Schwartz and Baruch (2014)
Retina(B-RB)	Reparative macrophages recruited by pigmented epithelium	Response to injury and inflammation	Kerr et al. (2008)

such Th1-derived cytokines, catabolized L-arginine to NO, and citrulline. Conversely, when cells were triggered with Th2-derived cytokines, such as IL-4, IL-10, or IL-13 (known to suppress iNOS expression) they switched their metabolic state to express arginase I and generated urea and ornithine (Corraliza et al., 1995). Therefore, macrophages were not *deactivated* by these *anti-inflammatory* cytokines, but acquired an *alternative pattern* of genes involved in tissue repair and remodeling (Munder et al., 1998). Subsequently, other workers introduced the terms of *M1 and M2* to mimic the models of macrophage activation with those of T cell polarization.

However, M1 and M2 cells, are only present in pathological conditions and represent two extreme opposites stages, nearly theoretical, among the plethora of patterns in which cells respond to homeostasis and inflammation, as reviewed by Gordon and Martinez (2010).

Noteworthy, the characterization of macrophage phenotypes have moved a lot further to recognize several populations on the basis of their molecular markers; the M1 classical activated phenotype is triggered by IFN-γ or LPS, or combinations of Th1-derived cytokines, but also by a great plethora of CD markers and mediators. M2-like macrophages are, in turn, a heterogeneous cell population in which different subtypes are being continuously emerging. In agreement with Martinez and Gordon (2014), these phenotypes have to be revisited, taking into account the times and places in which they are expressed.

PHENOTYPES AND ROLES OF BRAIN-RECRUITED MACROPHAGES

Brain resident macrophages do exist within the brain. They are between barriers as perivascular macrophages, meningeal and ventricular macrophages or macrophages that circulate within the CSF. Therefore, although there is still the idea of connecting cell recruitment with brain inflammation, macrophages must achieve other important roles in homeostasis such as the recognition of CNS-specific antigens as well as fagocytosis of cellular debris at places where microglia is not accessible. In fact, not only systemic macrophages go to the CNS in brain diseases but they are also found transmigrated through the CSF in basal ganglia, hippocampus and motor cortex, perivascular spaces, after liver injury (D'Mello et al., 2009), where they account for the alterations in neural transmission that occurs when the homeostasis of the body is lost.

Examples about the role of reparative macrophages in SC injury have been beautifully demonstrated and reviewed by Schwarch's group, which have made important contributions in the field of macrophage biology at brain borders. They also have highlighted the importance in discerning between the routes and phenotype markers of blood-derived monocytes for the resolution of CNS-affecting diseases (Shechter et al., 2013a) and propose the distinction between "educational gates," in which blood cells will acquire the correct phenotype, either inflammatory or reparative, and "absolute barriers," in which macrophages entrance could perhaps compromise the integrity of the BBB.

In independent experiments, it has been also shown the potential of blood monocytes in actively regenerating myelin in injured brain. Miron et al. (2013) showed that both M1 and M2-like macrophages were recruited for remyelination, but a critical switch to an M2-like state was necessary during the regenerative process. Accordingly, Jeong et al. (2013) showed that the recovery of myelin was clearly dependent on macrophage intervention and recalled the potential beneficial effects of a *protective* brain inflammation. Transient and not chronic must be the clue, because during the resolution from injury, microglia must return to a resting state and, as experimentally assessed by several authors, circulatory macrophages are "vanished" (Ajami et al., 2011). In agreement with this, Bellavance et al. (2014) have shown that recruitment of circulating PU.1-expressing cells during excitotoxicity is neuroprotective. More importantly, these macrophages that released neurotrophic factors also "disappeared" upon recovery, pointing again to a transient but essential role for macrophages in their experimental conditions. In line with this, research in a mouse model of Alzheimer disease also has shown that monocytes were crawling onto the luminal walls of Aβ venules to efficiently clear amyloid beta protein (Lampron et al., 2013).

Macrophage effector roles within the brain also affect brain tumors. Recently, Pyonteck et al. (2013), demonstrated that in a mouse model of glioblastoma, when they used a CSF-1 inhibitor to target tumor associated macrophages (TAM) surprisingly, cells were re-educated to change the tumor-induced M2 phenotype, in which arginase I was protumorigenic, toward a M1 anti-tumor phenotype maintained by GM-CSF and IFN-γ secreted by the tumor with the potential to actively kill glioma cells.

All these results strongly suggest that blood-derived macrophages can reach nearly every region within the brain, if they are driven and attracted to found the correct gates.

The impressive recent development of *in vivo* imaging techniques, together with the generation of new mouse models, open new opportunities to search for the location, properties and dynamics of these infiltrated macrophages and their participation in brain immunity.

Finally, it is worthy to mention the results shown by the Nedergaard's group (Iliff et al., 2012) which demonstrated that *a substantial portion of subarachnoid CSF cycles through the brain interstitial space.* By fluorescent-labeling of protein beta amyloid, they showed that its clearance was dependent on the expression of the water channel aquaporin-4 (AQP4) present in astrocytes, because the flux was markedly reduced in mice lacking the water channel. This transport system, CSF-dependent raises the possibility that macrophages at this location along with perivascular macrophages (Thanopoulou et al., 2010) could contribute to the clearance of Aβ protein by SR-BI, scavenger receptors.

CONCLUDING REMARKS

The huge efforts made by neuroscientists to elucidate the structural and cellular components of the different blood–CNS barriers have been essential to understand the complex interplay between the body and brain modes of immunity. In parallel, basic immunologists have changed completely the view of innate immunity recognizing that macrophages are not resting cells living within tissues to engulf foreign material; neither are they ringleaders in the process of chronic inflammation or tissue degeneration. On the contrary, macrophage plasticity and heterogeneity have made us to revise their putative functions in each tissue both, in homeostasis and in disease conditions.

The existence of a resident population of macrophages born, grown and matured along the embryonic development of mammalian brain, the microglial cells, suggest that the main roles of these macrophages are directly related with brain homeostasis, together with astrocytes. However, it is now clear that the brain have also other population of macrophages living in perivascular spaces, as well as other specialized cell populations sharing features of APCs such as pericytes at the NVUs or stromal cells integrated within the epithelial barriers. Thus, the concept of the brain as an immune privileged place could be reinterpreted as a territory with a double and cooperative immunity:

From the inside, the main role of microglial cells could be to control inflammation due to neuronal degeneration, CNS-specific antigen recognition and perhaps the most important one: to coordinate the brain response to peripheral diseases through the constant dialog with T cells and its mediators.

From the outside, naïve myeloid precursors must be necessary in the context of brain inflammation, injury, or infection in which, as in other body tissue, the general immune system must be recruited.

Inflammation is never the direct cause of a disease but the consequence of a healthy immune system able to efficiently remove cellular death, limit growth deregulation or fight against pathogen invasion and have a second reparative-healing role, essential to restore tissue homeostasis.

In conclusion, in light of the promising results obtained in animal models, it can be anticipated an increase of research focused in the characterization of human CD markers, able to distingue resident microglia and macrophage populations within barriers, a prerequisite to design new therapies adapted to human brain diseases.

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The role of the blood–brain barrier in the development and treatment of migraine and other pain disorders

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The function of the blood–brain barrier (BBB) related to chronic pain has been explored for its classical role in regulating the transcellular and paracellular transport, thus controlling the flow of drugs that act at the central nervous system, such as opioid analgesics (e.g., morphine) and non-steroidal anti-inflammatory drugs. Nonetheless, recent studies have raised the possibility that changes in the BBB permeability might be associated with chronic pain. For instance, changes in the relative amounts of occludin isoforms, resulting in significant increases in the BBB permeability, have been demonstrated after inflammatory hyperalgesia. Furthermore, inflammatory pain produces structural changes in the P-glycoprotein, the major efflux transporter at the BBB. One possible explanation for these findings is the action of substances typically released at the site of peripheral injuries that could lead to changes in the brain endothelial permeability, including substance P, calcitonin gene-related peptide, and interleukin-1 beta. Interestingly, inflammatory pain also results in microglial activation, which potentiates the BBB damage. In fact, astrocytes and microglia play a critical role in maintaining the BBB integrity and the activation of those cells is considered a key mechanism underlying chronic pain. Despite the recent advances in the understanding of BBB function in pain development as well as its interference in the efficacy of analgesic drugs, there remain unknowns regarding the molecular mechanisms involved in this process. In this review, we explore the connection between the BBB as well as the blood–spinal cord barrier and blood–nerve barrier, and pain, focusing on cellular and molecular mechanisms of BBB permeabilization induced by inflammatory or neuropathic pain and migraine.

Keywords: pain, blood–brain barrier, blood–nerve barrier, blood–spinal cord barrier, neuropathic pain, migraine, inflammatory pain and opioids

INTRODUCTION

The BBB is referred as a dynamic and functional structure that separates the systemic circulation from the CNS. The BBB has a crucial role in maintaining the proper neuronal function. It is responsible for the brain homeostasis and protects the nervous tissue from potential harmful substances, by limiting the entry of certain molecules (except the small and lipophilic) into the CNS (Rubin and Staddon, 1999). The "neurovascular unit" comprises the endothelial cells, pericytes, and astrocytes endfeet, embedded within their basal laminae. The interface between blood and CNS is represented by the space between endothelial cells/pericytes and astrocytic endfeet (Beggs et al., 2010). BBB acts as a selective barrier due to the presence of complex TJs, located between adjacent endothelial cells (Abbott et al., 2006). The TJ protein complex establishes a physical barrier and limits paracellular diffusion (Sanchez-Covarrubias et al., 2014). It is formed via an intricate communication of transmembrane, accessory, and cytoskeleton proteins. The transmembrane proteins occludin and claudins are considered the primary seal of the TJ (Fricker and Miller, 2004; Hawkins and Davis, 2005) and dynamic interactions with the accessory proteins ZO 1, 2,

Abbreviations: ABC, ATP-binding cassette; ATP, adenosine triphosphate; BBB, blood–brain barrier; BCRP (Bcrp), breast-cancer resistance protein; BNB, blood–nerve barrier; BOLD, blood oxygenation level-dependent; BSCB, blood–spinal cord barrier; CFA, complete freund's adjuvant; CGRP, calcitonin gene-related peptide; CIA, collagen-induced arthritis; CIP, lambda-carrageenan-induced inflammatory pain; CNS, central nervous system; CSD, cortical spreading depression; EBA, endothelial barrier antigen; HIV, human immunodeficiency virus; HRP, horseradish peroxidase; IASP, international association for the study of pain; ICAM-1, intercellular adhesion molecule 1; IL-1β, interleukin-1 beta; MMPs, matrix metalloproteinases; MRA, magnetic resonance angiography; MRP (Mrp), multidrug resistance protein; NMDA, *N*-methyl-D-aspartate; NSAIDS, non-steroidal anti-inflammatory drugs; OM, ophthalmoplegic migraine; P-gp, P-glycoprotein; PAG, periaqueductal gray; RA, rheumatoid arthritis; REZ, root entry zone; RM, resident macrophages; tDCS, transcranial direct current stimulation; TENS, transcutaneous electrical nerve stimulation; TJ, tight junction; TLR, toll-like receptor; TMS, transracial magnetic stimulation; TNF-α, tumor necrosis factor-alpha; VEGF, vascular endothelial growth factor; ZO, zonula occludens.

3 permit the connection between TJ and the actin cytoskeleton (Tsukamoto and Nigam, 1997). The biochemical barrier in the BBB comprises mainly influx and efflux transporters, located in the luminal and abluminal membranes of capillary endothelial cells as well as metabolizing enzymes expressed intracellularly (Hawkins and Davis, 2005; Ronaldson and Davis, 2013). ABC transporters are among the largest family of transmembrane proteins. They include P-glyprotein (P-gp), BCRP in humans and Bcrp in rodents, and MRP 1–6 in humans and Mrp 1–6 in rodents (Ronaldson and Davis, 2011; Radu et al., 2013). The main structures that compose the BBB are illustrated in the **Figure 1**.

Not all areas in the brain contain a BBB. Some areas where the BBB is absent are: hypophysis, median eminence, area postrema, preoptic recess, paraphysis, pineal gland, and endothelium of the choroid plexus (Siegel, 1999). In the spinal cord, the interface between blood and neural tissue is formed by the BSCB functionally equivalent to the BBB (Xanthos et al., 2012), while in the peripheral nerve, the perineurium, and the endothelial blood vessels form the BNB. The BNB also acts as a semipermeable membrane, regulating the microenvironment homeostasis and providing "privileged" space for peripheral axons and the corresponding supporting cells (Kanda, 2013; Lim et al., 2014).

It has been reported that the BBB morphology and function might be modulated and even disrupted in many neurological diseases, including those caused by extrinsic factors, such as meningitis (bacterial and viral) and encephalitis (e.g., herpes virus); intrinsic factors, such as ischemia/hypoxia, traumatic brain injury, small vessel diseases (e.g., hypertension, diabetes), and Alzheimer's Disease; and more recently by pain disorders, including peripheral inflammatory pain, neuropathic pain, and migraine (Rosenberg, 2012). Tissue damage can produce an intense release of signaling molecules from peripheral and central neurons as well as from blood cells. Those substances include IL-1β, TNF-α, histamine, and fractalkine. Moreover, other substances are released at the site of the injury, such as serotonin, substance P, CGRP, and ATP. These are neurotransmitters of primary sensory afferents and are not only released during tissue injury (Abbott et al., 2006; Basbaum et al., 2009; Clark and Malcangio, 2014). Many such mediators can generate significant effects in the CNS barriers (BBB, BSCB, and/or BNB). Equally important is the ability of the BBB to control the influx of pharmaceutical compounds into the CNS parenchyma, thus regulating the efficacy and side effects associated with analgesic and antiinflammatory drugs (Sanchez-Covarrubias et al., 2014).

A clear understanding of the structural and functional changes that occur in the BBB following peripheral injury/chronic pain

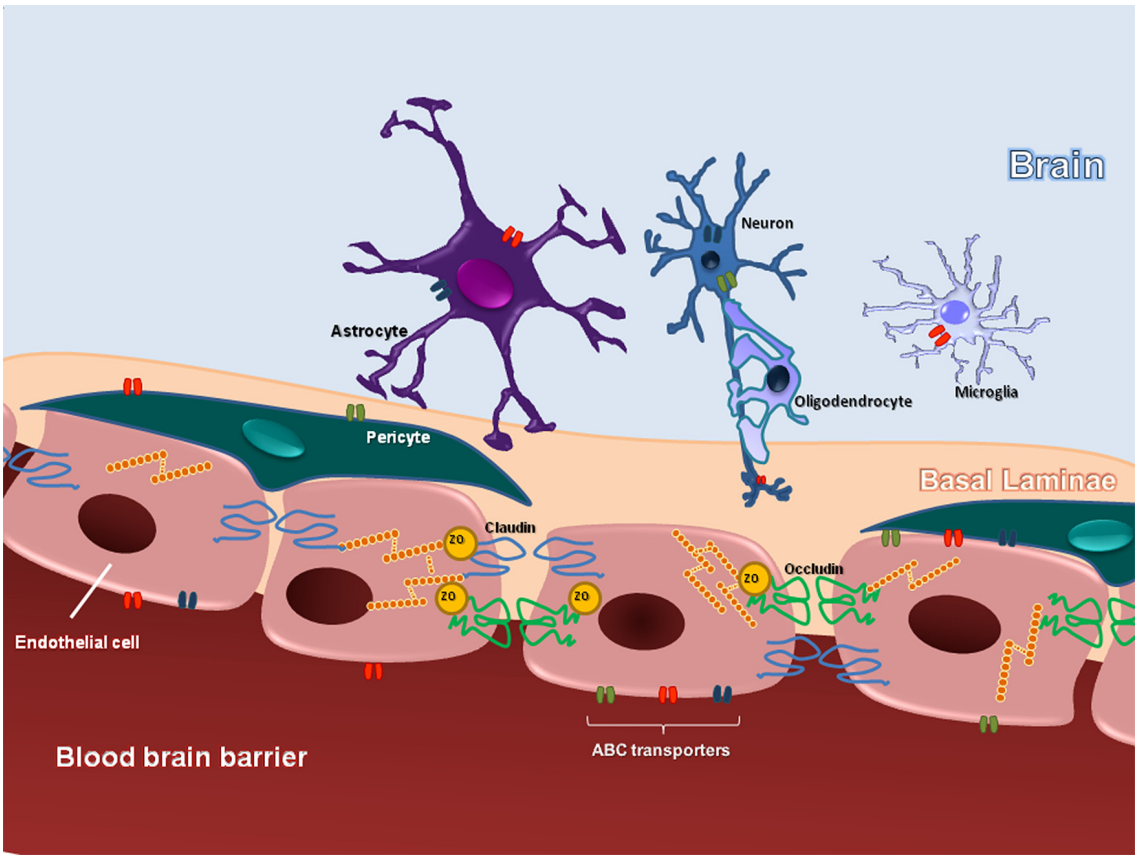

FIGURE 1 | Schematic representation, illustrating the basic structural organization of BBB.

will provide insights into the molecular mechanisms and pathophysiological profile of different clinical pain disorders, which would permit the development of more effective and perhaps safer therapeutic approaches for chronic pain management.

HOW DOES INFLAMMATORY PAIN DISRUPT THE BBB?

There is accumulating evidence that inflammatory pain states produce significant changes in the BBB permeability (**Table 1**). This may affect the delivery of therapeutic components to the brain, with great impact in the dosing regimens commonly used to treat patients with chronic pain disorders. In one of the pioneer studies investigating the effects of peripheral inflammation in the BBB function *in vivo*, three different models of subcutaneous inflammatory pain were examined (Huber et al., 2001). The results showed significantly higher distribution of sucrose, a membrane-impermeant marker, into the cerebral hemispheres after peripheral inflammation produced by subcutaneous injections of formalin, λ-carrageenan, or CFA, representing acute, short-term and long-term models of inflammatory pain, respectively. Moreover, peripheral inflammation altered the expression of TJ proteins. ZO-1 expression was significantly amplified in all models analyzed, while occludin was significantly diminished in the groups treated with λ-carrageenan or CFA. The decrease of occludin expression reported in that study was later confirmed in a model of chronic inflammatory pain, using CFA as the inducer agent (Brooks et al., 2005). The same study reported a huge increase in the expression of claudin-3 (450%) and claudin-5 (615%). Nevertheless, changes in ZO-1 could not de demonstrated. The discrepancies between both studies might be explained by differences in the methodologies adopted. In another

Table 1 | Main findings of studies investigating changes in the BBB/BSCB associated with inflammatory pain.

Barrier	Model	Main outcomes	Reference
BBB	Inflammatory pain, produced by subcutaneous injection of CFA, λ-carrageenan (CIP) or formalin, in sprague–dawley rats.	Peripheral inflammation led to an increase in the uptake of sucrose into the cerebral hemispheres, in all models studied. Western blot revealed changes in the TJ protein expression during peripheral inflammation. Occludin decreased in the groups treated with λ-carrageenan or CFA, while ZO-1 expression was increased in all inflammatory pain models. On the other hand, Claudin-1 protein expression did not change throughout the experiment.	Huber et al. (2001)
BBB	Chronic inflammatory pain, using CFA, in sprague–dawley rats.	Decrease in the expression of Occludin. Significant increase in the expression of claudin-3 (450%) and claudin-5 (615%) were also demonstrated, but the same results were not obtained with zonula occluden-1.	Brooks et al. (2005)
BBB	CIP, in sprague–dawley rats.	Increase in ICAM-1 RNA and protein expression in the thalamus, frontal, and parietal cortices; which were correlated with augmented expression of activated microglia.	Huber et al. (2006)
BBB	CIP and perineural injection of bupivacaine, in sprague–dawley rats.	Changes in the BBB integrity induced by CIP were prevented by a perineural injection of bupivacaine. This data suggests that nociceptive input is necessary to the increased BBB permeability found in λ–carrageenan models of inflammatory pain.	Campos et al. (2008)
BBB	CIP and capsaicin, in sprague–dawley rats.	Significant changes in occludin protein were observed in the lumbar spine after λ-carrageenan but not after capsaicin administration. Simultaneously, significant amounts of immunoglobulin G were seen in the lumbar and thoracic segments of the spinal cord	Xanthos et al. (2012)
BBB	CIP, in sprague–dawley rats.	Structural changes in P-gp.	McCaffrey et al. (2012)
BSCB	Perispinal inflammation induced by zymosan, in mice.	Perispinal inflammation led to changes in the reactivity of resident astrocytes and microglia within the spinal cord but maintained the integrity of the BSCB. Chronic pain did not develop.	Tenorio et al. (2013)
BBB	CIP and diclofenac treatment, in sprague-dawley rats.	Increased P-gp expression following peripheral inflammatory pain and also after diclofenac treatment. Both peripheral inflammatory pain and diclofenac treatment alone increased P-gp efflux activity, leading to a reduced morphine brain uptake. Analgesia produced by morphine was significantly reduced in animals pretreated with diclofenac, when compared to those that received diclofenac and morphine concurrently.	Sanchez-Covarrubias et al. (2014)

work, using the CIP model, paracellular permeability to [14C] sucrose was detected in the BBB, which was also paralleled by altered expression of occludin and ZO-1. However, intravenous administration of λ-carrageenan did not significantly impact the BBB permeability, indicating that the change in [14C] sucrose permeability was due to either CIP induced inflammatory or neuronal modulation of TJ (Huber et al., 2002). Furthermore, specific regional microglia activation, measured by OX42 immunoreactivity, and changes in ICAM-1 expression have been shown after CIP (Huber et al., 2006). Increased ICAM-1 expression, associated with microglia activation, has been demonstrated in central-mediated cerebral inflammation (Kyrkanides et al., 2002) and several studies have highlighted the importance of microglia to the mechanisms of neuropathic (Raghavendra et al., 2003; Tsuda et al., 2003; Coull et al., 2005; Ji and Suter, 2007; Saegusa and Tanabe, 2014) and acute inflammatory pain (Svensson et al., 2003; Ji et al., 2013). Nonetheless, it is important to mention that there is evidence that the augmented reactivity of astrocytes or microglia alone, without simultaneous changes in the BSCB or BBB, is not sufficient to generate and maintain a chronic pain state, after direct lesion, or nervous system disease. This was demonstrated in an experimental model of perispinal inflammation induced by the TLR-2 agonist zymosan (Tenorio et al., 2013). Remarkably, the previously reported regional effects of CIP in ICAM-1 expression and microglia activation occurred in brain areas that have been extensively reported to be involved in pain processing and modulation, such as the thalamus, frontal, and parietal cortices (Apkarian et al., 2004; DaSilva et al., 2007a,b, 2008, 2012; DosSantos and DaSilva, 2011; DaSilva and DosSantos, 2012; DosSantos et al., 2012a; Wager et al., 2013), leading to the hypothesis that the alterations seen in the BBB after CIP are possibly driven by a central-mediated response conducted through the spinothalamic tract. This hypothesis was further confirmed in a study showing that CIP-induced changes in the BBB integrity can be prevented by a perineural injection of bupivacaine 0.75%, implying that nociceptive input is necessary to enhance the BBB permeability in λ-carrageenan-driven inflammatory pain (Campos et al., 2008). Interestingly, the same study showed that bupivacaine nerve block also decreased the thermal allodynia and prevented variations in the expression of TJ proteins occludin, ZO-1, and claudin 5, but did not alter the paw edema formation following λ-carrageenan injection. In summary, the results indicate that the blockade of nociceptive input inhibits the functional perturbations in the BBB barrier under inflammatory pain conditions.

According to a recent study, peripheral inflammatory hyperalgesia is also responsible for a dynamic redistribution of P-gp and caveolin-1 between endothelial subcellular compartments at the BBB (McCaffrey et al., 2012). P-gp is described as the major efflux transporter at the BBB. It combines ATP hydrolysis and drug efflux to extrude drugs against concentration gradients (Miller, 2010). In addition, it has been stated that increased functional activity of P-gp during inflammatory hyperalgesia leads to a greater efflux transport of morphine, which could explain the reduced ability of this drug to gain access to the brain under inflammatory pain conditions (Seelbach et al., 2007). Hence, changes observed in the P-gp function after λ-carrageenan injection, have a potential therapeutic implication, regarding the delivery of analgesic drugs to the CNS, in particular opioids peptides, as well as other classes of pharmacological agents applied to treat peripheral inflammatory pain disorders. In addition to P-gp, it seems that MRP4, another type of ABC transporter and target of some NSADs, is also important for inflammatory pain (Lin et al., 2008).

Despite the mounting evidence linking BBB disruption and inflammatory pain, it is still controversial whether similar events take place at the level of BSCB. For example, in one paper the extravasation of Evans Blue, a dye that is classically used to measure the BBB/BSCB integrity, was reported after 48 h of carrageenan-induced inflammation (Gillardon et al., 1997) while in other studies, carrageenan- or CFA-induced inflammation apparently did not elicit Evans Blue dye leakage (Lu et al., 2009; Echeverry et al., 2011). On the other hand, it seems that morphine penetration in the spinal cord is facilitated by CFA or carrageenan administration (Lu et al., 2009). In one experiment, testing the effects of carrageenan (which produced mechanical and heat hyperalgesia that peaked at 3–24 h and lasted for 72 h) or capsaicin (which induced mechanical hyperalgesia, with peak at 2–3 h and lasting for 24 h) on the BSCB, significant alterations in endothelial cell occludin protein were seen in the lumbar spine, with a delayed onset of 72 h after intraplantar carrageenan administration. However, the same alteration was not repeated after intraplantar administration of capsaicin, which was intended to produce neurogenic inflammation. Subcutaneous injection of carrageenan did not generate significant effects on occludin protein either, illustrating that the changes observed were due to peripheral inflammation rather than a systemic inflammatory effect (Xanthos et al., 2012). The same study also tested the effects of intraplantar carrageenan using IgG extravasation in the spinal cord, another method to analyze BSCB breakdown. At the same time point that changes occurred with occludin, significant quantities of immunoglobulin G were found in the lumbar and thoracic segments of the spinal cord, probably owing to extravasation. Nonetheless, acute administration of Evans Blue dye or sodium fluorescein was not detected in the CNS parenchyma. Taken together, these findings suggest that peripheral inflammation determines transient changes in BSCB. At first glance, it would not be necessarily linked to the nociceptive signaling. However, the results also highlight the importance of using different methods to assess each particular mechanism responsible for BSCB changes after transient pathologies, and it is possible to speculate that changes induced by capsaicin in this specific study could not be detected by the methodology adopted. It is also important to emphasize that there are significant structural (Ge and Pachter, 2006) and functional (Prockop et al., 1995; Pan et al., 1997) differences between the BSCB and the BBB, including the presence of glycogen deposits in the superficial vessels of the spinal cord, higher permeability to cytokines, and tracers, and the expression of TJ proteins (Daniel et al., 1981; Prockop et al., 1995; Pan et al., 1997; Sharma, 2005; Ge and Pachter, 2006; Radu et al., 2013). All should be considered when evaluating the roles of BBB and BSCB in chronic pain. Therefore, for a more complete evaluation of all CNS barriers under inflammatory pain conditions, it would be highly recommended to compare the behavior of both the BBB and BSCB simultaneously, applying multiple procedures

to detect disruption or changes in the permeability of both barriers. Another important fact that must be considered is that the presence of Evans Blue in the brain or spinal cord parenchyma usually occurs with a considerable disruption of the BBB/BSCB. It is likely that the BBB or BSCB permeabilization mediated by inflammatory pain is a transient event, rather than an irreversible phenomenon of disruption or "breakdown" (Brooks et al., 2005; Radu et al., 2013).

There is growing evidence that acute and perhaps chronic inflammatory pain influence the functional and molecular properties of the BBB and BSCB, though probably by distinct mechanisms. The majority of the literature currently available indicates a correlation between increased BBB permeability and altered expression of some transmembrane TJ proteins that collaborate to preserve the BBB integrity. Therefore, it seems that peripheral acute or chronic inflammatory pain leads to a reorganization of the TJ proteins and altered paracellular diffusion, which may alter the delivery of therapeutic analgesic and antiinflammatory substances to the CNS. As such, the increased paracellular permeability and consequent CNS toxicity should be taken into consideration when deciding the dosing regimens for patients affected by chronic inflammatory pain.

It is important to mention that the results of the aforementioned studies linking BBB alterations and inflammatory pain must be interpreted cautiously, since they provide indirect evidence (e.g., changes in TJ protein expression or P-gp function) obtained from experimental models of inflammatory pain, generated artificially, and usually performed during relatively short periods. Hence, translational research is necessary to determine the real impact of BBB dysfunction in chronic diseases with pain of inflammatory origin, including chronic joint inflammation diseases, irritable bowel syndrome, and multiple sclerosis. For example, it has been widely recognized that a BBB pathology is present in multiple sclerosis (Zlokovic, 2008), a concept that is supported not only by experimental (Morrissey et al., 1996; Morgan et al., 2007; Kooij et al., 2009; Reijerkerk et al., 2012) but also clinical data (Plumb et al., 2002; Kirk et al., 2003; Minagar and Alexander, 2003; Leech et al., 2007; Padden et al., 2007; Cramer et al., 2014). BBB disruption has also been reported in CIA, an animal model of RA, implying that this condition could possibly be related to a dysfunctional BBB (Nishioku et al., 2010). As a matter of fact, it seems that RA increases the mortality and morbidity due to cerebrovascular diseases (Watson et al., 2003). Furthermore, it has been reported that the BBB impairment seen in CIA is potentially mediated by S100A4 (Nishioku et al., 2011). This small acidic calcium-binding protein, member of S100 family, is also upregulated in the synovial fluid and plasma of RA patients (Klingelhöfer et al., 2007), which permits a clear connection between the results obtained with the animal model of RA (CIA) and the clinical alterations seen in RA patients. In addition, despite the limited information, the decrease in the expression of TREK1, a TWIK-related potassium channel-1 that is related to pain perception (Alloui et al., 2006) and BBB regulation (Bittner et al., 2013, 2014), after colon inflammation (La and Gebhart, 2011) suggests that the involvement of the central nervous barriers in irritable bowel syndrome should be further explored. In the future, correlations between experimental outcomes and the results of controlled clinical studies will allow researchers to scrutinize the chain of events that take place in the CNS barriers in the presence of chronic inflammatory pain.

DO THE CNS BARRIERS PLAY A PIVOTAL ROLE IN THE PERIPHERAL AND CENTRAL MECHANISMS OF NEUROPATHIC PAIN?

Neuropathic pain, according to the IASP taxonomy (Merskey et al., 1994), revised in 2012 (http://www.iasp-pain.org/Education/Content.aspx?ItemNumber=1698#Neuropathicpain), is defined as "pain caused by a lesion or disease of the somatosensory nervous system." It affects approximately 2–3% of the general population (Hall et al., 2006; Bouhassira et al., 2008) with elevated costs to health systems and governments worldwide (Turk, 2002). However, this number can be even higher. Recently the prevalence of pain with neuropathic characteristics has been estimated to be between 6.9 and 10% (van Hecke et al., 2014). Neuropathic pain is considered a clinical description and not a diagnosis. It comprises several disorders, such as radiculopathies, diabetic neuropathies, trigeminal, and postherpetic neuralgia. Although the cellular and molecular mechanisms involved in neuropathic pains have not yet been totally elucidated, there is sufficient evidence that both peripheral and central mechanisms are important. Among them are the release of inflammatory mediators by activated nociceptors at the site of peripheral injury, as well as central sensitization, which encompasses several phenomena, e.g., alteration in glutamatergic neurotransmission/NMDA receptor-mediated hypersensitivity, disinhibition, and neuron-glial interactions (DaSilva et al., 2008; Basbaum et al., 2009; Gustin et al., 2011; DaSilva and DosSantos, 2012; DosSantos et al., 2012b; McMahon, 2013; Wilcox et al., 2013). There is also evidence that vascular events contribute to this process.

Notwithstanding many classical works have focused on the presence of local vascular disturbances following peripheral nerve injury in different experimental models (Myers et al., 1981, 1985; Powell et al., 1991), few studies have explored the specific cellular and molecular processes underlying the vascular events that occur in the presence of neuropathic pain (**Table 2**). To characterize the impact of vascular disturbances in the mechanisms involved in the generation of pain following neuronal damage, a recent study explored the consequences of peripheral nerve injury, produced by a partial ligation of the sciatic nerve, in the BNB functioning (Lim et al., 2014). Overall, the outcomes give rise to the hypothesis that neuropathic pain is, at least in part, associated with higher distribution of molecules that cross a defective BNB, and act at the peripheral nerve already damaged. According to the findings of that study, nerve injury triggers a "breakdown" of the BNB, which is associated with a long-lasting pain behavior. Additionally, It seems that RM play a crucial role in this process. Shortly after peripheral nerve injury, RM cells that are sparsely distributed along the nerves under basal conditions proliferate and start to express the VEGF, which in turn, initiates the "breakdown" of the BNB. This BNB breakdown permits the influx of blood borne macrophages to the endoneurial space. Those infiltrated macrophages produce

Table 2 | Summary of recent studies exploring the participation of nervous system barriers (BBB and BSCB) in the mechanisms of neuropathic pain.

Barrier	Model	Main outcomes	Reference
BSCB	Peripheral nerve injury, lidocaine administration and electrical stimulation of the sciatic nerve, in sprague–dawley rats	Peripheral nerve injury produced a transient increase in BSCB permeability. Such event did not occur when lidocaine was administrated at the site of the injury. Increases in the BSCB permeability also occurred after electrical stimulation of the sciatic nerve at intensity sufficient to activate C-fibers but not A-fibers and after application of capsaicin to the nerve. It suggests that the increase of BSCB permeability is driven by activation of TRPV1-expressing primary sensory neurons.	Beggs et al. (2010)
BNB	Neuropathic pain, produced by partial ligation of the sciatic, in mice.	Neuropathic pain related to trauma caused a significant disruption of the BNB. VEGF was expressed by RM. Intraneural injection of serum obtained from animals with nerve injury or treated with LPS generated mechanical allodynia in naive animals. Intraneural injection of fibrinogen also produced a decrease in mechanical thresholds when applied to naive nerves. Such results evidence that blood–borne molecules may contribute to neuropathic pain mechanisms.	Lim et al. (2014)

several cytokines (e.g., IL-1β, TNF-α, and fibrinogen). Fibrinogen probabaly has its effects linked to the activation of TLRs, especially TLR-4. Another interesting finding with possible clinical implications is that ProTX-II, a peptide that blocks NAV1.7 ion channel but does not pass the intact BNB, reversed the mechanical allodynia in the experimental model of neuropathic pain, an effect that is likely restricted to the site of nerve injury. Therefore, substances such ProTx-II with action restricted to peripheral nerves with compromised BNB, and that do not present a significant distribution to uninjured nerves (with preserved BNB), the brain or the spinal cord, emerge as promising therapeutic options in peripheral neuropathies, due to the limited side effects (Lim et al., 2014). Noteworthy, in the specific case of ProTX-II, significant effects would only have been reached in injured nerves, displaying altered NAV1.7 expression. In fact, changes in the NAV1.7 expression have been previously demonstrated in trigeminal neuralgia patients, indicating that such condition could be, at least in part, considered a channelopathy (Siqueira et al., 2009). Mutations in the gene encoding $Na_v1.7$ have also been linked to paroxysmal pain disorders (Fertleman et al., 2006; Han et al., 2006), illustrating its importance to the mechanisms of neuropathic pain.

A disruption of BSCB integrity, illustrated by augmented permeability along with astrocyte activation in the spinal cord, has been shown in an animal model of neuropathic pain, with chronic nerve constriction (Gordh and Sharma, 2006). In a more detailed investigation, Beggs et al. (2010) have shown that both chronic constriction injury (CCI; a model of peripheral nerve injury) and stimulation of healthy primary afferent C-fibers are capable of eliciting a surge in both the BBB and the BSCB permeability, when assessed by Evans Blue dye or HRP. Nevertheless, the most important outcome of that study was that capsaicin applied to an uninjured sciatic nerve mimicked the effects of CCI or C-fibers stimulation, supporting the concept that TRPV1-expressing C-fibers could be responsible for the upsurge in the BSCB/BBB permeability. Further clinical studies would be important to confirm if a similar process occurs in patients afflicted by neuropathic pain conditions, such as peripheral neuropathies, trigeminal, and postherpetic neuralgias. If that is the case, it could dramatically affect the penetration of analgesic agents into the CNS, determining the efficacy of those drugs and also central-mediated side effects. In the future, those findings could compose the basis to the development of therapies that purposely augment the BBB permeability by targeting the mediators involved in the afferent-induced opening of the BBB/BSCB. In addition, based on those results, it is possible to speculate that the clinical effects of novel non-pharmacological treatments that have been applied to treat neuropathic and other pain conditions, such as tDCS (Fregni et al., 2006; Antal and Paulus, 2011; DosSantos et al., 2012a) or TMS (Marlow et al., 2013; Leung et al., 2014) could be associated with transient changes in the nervous system barriers. There is recent evidence that endogenous opioids modulate the analgesia produced by those methods of non-invasive brain stimulation, through direct or indirect activation of brain areas important for opioid-mediated anti-nociception, such as the PAG (de Andrade et al., 2011; DosSantos et al., 2012a, 2014; Taylor et al., 2012). Future therapeutic protocols, combining non-pharmacological

and pharmacological agents could optimize the analgesic effects obtained with single therapies. Indeed, TENS, which reduces secondary hyperalgesia by activation of opioid receptors (Sluka et al., 1999; Kalra et al., 2001), has been successfully combined with clonidine, an a2-adrenergic agonist, to provide effective reduction of hyperalgesia in an animal model of peripheral inflammation (Sluka and Chandran, 2002). Moreover, in a pilot clinical study, prolonged pain relief was achieved by combining tDCS with a NMDA agonist (D-cycloserine) in a case of orofacial pain refractory to pharmacological treatment (Antal and Paulus, 2011), which could perhaps be linked to transient changes in the BBB or BSCB.

IS MIGRAINE PATHOPHYSIOLOGY CORRELATED TO A BBB DYSFUNCTION?

It has been estimated that approximately 11–12% of adults suffer from migraine headaches (Rasmussen, 1995; Stovner et al., 2007). The majority of patients report moderate to severe pain during the attacks, with great impact in the quality of life (Lipton et al., 2007). Migraine presents two subtypes, migraine with aura and migraine without aura (Silberstein et al., 2005). Although a large number of recent studies have tried to establish the migraine pathophysiology (Bhaskar et al., 2013; Noseda and Burstein, 2013; Sarrouilhe et al., 2014; Thissen et al., 2014), the role of the neural and vascular mechanisms in this process has been largely discussed in the literature, (Asghar et al., 2011; Grände et al., 2014). As a matter of fact, there is still a debate whether the source of the pain is in the nerves around the cranial arteries, CNS or both (Goadsby et al., 2009; Olesen et al., 2009).

It has been generally accepted that CGRP plays an important role in the migraine pathophysiology (Bell, 2014). CGRP is expressed throughout the CNS, particularly the striatum, amygdala, colliculi, and cerebellum, as well as the peripheral nervous system (Edvinsson, 2008). Recently, CGRP receptor antagonists have emerged as promising drugs to treat migraine. They could act either by blocking CGRP-induced vasodilation of meningeal blood vessels or inhibiting CGRP-mediated pain transmission in the CNS (Bell, 2014). Other approaches to block CGRP effects include the use of CGRP antibodies (Zeller et al., 2008), or specific CGRP-binding RNA-Spiegelmer (Denekas et al., 2006). The fact that CGRP receptor antagonists, such as olcegepant and telcagepant, apparently require very high doses to produce significant clinical effects in migraine patients, raises the possibility that those promising components have to cross the BBB in order to exert their effects (Tfelt-Hansen and Olesen, 2011; Bell, 2014). Thus, according to some authors, it could support the concept that CNS mechanisms are predominantly involved in the migraine pathophysiology (Tfelt-Hansen and Olesen, 2011). In fact, DaSilva et al. (2003, 2007b) have previously demonstrated specific cortical neuroplastic changes in migraine patients. Conversely, the results of a functional neuroimaging study have indicated that changes in cortical blood flow, measured by BOLD signal variations, occur during episodes of migraine with aura. In addition, dilatation of both extracranial (middle meningeal) and intracranial (middle cerebral), as demonstrated by high-resolution direct MRA, has been shown after a migraine attack induced by infusion of CGRP. Remarkably, headache and vasodilatation occurred at the same side and the administration of sumatriptan, a selective antimigraine drug, not only reduced the pain but also resulted in contraction of the middle meningeal artery (Asghar et al., 2011). Collectively, those results suggest a key role of cranial blood vessels in the migraine pathophysiology. In fact, meningeal arteries lack BBB and represent much more permeable structures, compared with cortical vessels (Edvinsson and Tfelt-Hansen, 2008; Grände et al., 2014). There have been considerable advances in the understanding of the sequence of events that lead to a migraine headache. Nevertheless, the specific structural and functional alterations that occur in the brains of patients affected by this disorder still need clarification and BBB dysfunction has emerged as a possible mechanism.

Although mild BBB opening has been previously reported in a patient suffering a severe attack of familial hemiplegic migraine type II (Dreier et al., 2005), the occurrence of BBB opening or disruption during a migraine headache is still a matter of debate (Radu et al., 2013). Migraine, as well as other neurological disorders (which are out of the scope of this study) such as epilepsy and cerebrovascular diseases, are characterized by a phenomenon known as CSD (Martins-Ferreira et al., 2000). CSD is a self-propagating wave of neuronal and glial depolarization first described by Leão (1944) in the mid-forties . Brain edema and plasma protein leakage, concomitant with altered expression of proteins that are important to maintain the BBB integrity, such as the EBA, ZO-1, and laminin (substrate protein of metalloproteinases – MMPs), were demonstrated in an animal model of CSD (Gursoy-Ozdemir et al., 2004). In addition, albumin leakage was suppressed by the injection of the matrix metalloproteinase inhibitor GM6001, but did not occur in MMP-9-null mice. It clearly indicates that the BBB disruption associated with CSD depends on the MMP-9 activity. Although those results cannot be considered exclusive of migraine, but rather related to the CSD phenomenon (that also participates in the migraine mechanisms), elevated plasma levels of MMP-9 have been reported in migraine patients (Leira et al., 2007; Imamura et al., 2008; Martins-Oliveira et al., 2012) and MMPs, especially MMP-2 and MMP-9, have been linked to BBB disruption, as well as augmented influx of inflammatory cells into the CNS (Rosenberg et al., 2001; Gurney et al., 2006; Yang et al., 2007; Bernecker et al., 2011). Furthermore, it has been suggested that MMMP-2 plasma concentrations are higher in migraine with aura than in migraine without aura (Gonçalves et al., 2013) and increased MMP-9 activity has been reported in women with migraine without aura (Martins-Oliveira et al., 2012), suggesting that distinct mechanisms are involved in each form of migraine. Nonetheless, the participation of MMP-9 in the migraine pathophysiology is not completely accepted. According to one study, plasma levels of MMP-9 should not be used as a biomarker of migraine with aura (Ashina et al., 2010). In contrast, the reduction in the plasma concentrations of MMP-3 found during the early phase of headache migraine attacks suggest that this isoform should be further investigated in migraine sufferers. However, the most important information derived from those works is that MMPs might actively contribute to the migraine pathophysiology, and perhaps other types of primary headaches, in a mechanism involving CSD and BBB disruption. Nonetheless, it is

important to state that not all primary headaches and subtypes of migraine are necessarily related to CSD, MMPs, and BBB dysfunction and that many other mechanisms can play a role in each particular condition. For example, there are studies showing that gap junctions take part in the migraine pathophysiology, being promising targets for future treatments (Sarrouilhe et al., 2014).

Finally, an ischemic and reversible "breakdown" of the BNB caused by a vasospasm of the vasa nervorum at the brainstem REZ of the oculomotor (III), trochlear (IV), or abducens (VI) nerve has been recently proposed as the pathogenic theory to explain the clinical and neuroimaging findings of OM (Ambrosetto et al., 2014). This is a rare form of episodic migraine-like headache attacks, accompanied, or followed by ophthalmoplegia related to paresis of the one or more of the following cranial nerves: III, IV, or VI. This theory seems to provide a reasonable explanation to the reversible focal thickening and enhancement of the cisternal tract at the REZ of the cranial nerve involved, usually the III, especially when occurring in children (Miglio et al., 2010; Gelfand et al., 2012). An intriguing fact is that the same alterations are not observed in the adult form of OM (Lal et al., 2009). According to this theory, the discrepancies between children and adults regarding the MRI findings in OM could reflect a differential maturation, and consequently the effectiveness of the BBB in children and adults (Ambrosetto et al., 2014).

Overall, the current literature points toward an increase in the permeability or perhaps a "breakdown" of the BBB, with vascular leakage in migraine patients, during the headache attack. This process could be triggered by CSD, in an MMP-dependent pathway (**Table 3**). Defining the pathophysiologic mechanisms that trigger a migraine attack, especially regarding the changes that occur in the BBB permeability are crucial not only to characterize the cascade of events that occur during its ictal phase, but also to provide better treatment choices, with lower side effects, for such a debilitating disorder.

HOW CAN WE MODULATE THE BBB IN ORDER TO IMPROVE THE DELIVERY OF ANALGESIC COMPOUNDS?

The majority of the substances currently available to treat moderate to severe chronic pain (e.g., opioids, anticonvulsants, and antidepressants) have their use limited due to the extensive side effects reported. In addition, tolerance and dependence can be developed over time, mainly with opioid analgesics (e.g., morphine, codeine, oxycodone, and tramadol; McMahon, 2013). Tolerance, for instance, prevents the long-term administration of opioid agonists. Notwithstanding it has been recognized that some complex phenomena, such as mu-opioid receptor desensitization, impaired recovery from desensitization, and impaired recycling after endocytosis (Williams et al., 2013) are associated with morphine tolerance, it is possible that part of the BBB components (e.g., pericytes and astrocytes) also play a role in this process (Chen et al., 2012; Luk et al., 2012). Not surprisingly, amitriptyline, a tricyclic antidepressant largely prescribed for pain control, especially in chronic neuropathic pain disorders, has been shown to attenuate astrocyte activation and consequently morphine tolerance (Huang et al., 2012). Indeed, one the most important concerns in the treatment of inflammatory as well as neuropathic pain is the deleterious drug–drug interaction when other substances (e.g., non-steroidal anti-inflammatory, NSAIDs) are combined with opioids analgesics, resulting in ineffective drug dosing. This is especially important, because chronic pain management often requires the concurrent administration of multiple pharmacological agents (Sanchez-Covarrubias et al., 2014). For instance, NSAIDs are frequently co-administrated with opioids to treat postsurgical pain (Oderda, 2012).

Particularly important in this context, is the P-gp, since it constitutes one of the most important obstacles to the delivery of pharmacological agents to the CNS in several disorders, such as epilepsy, HIV, and Alzheimer's disease (Ronaldson et al., 2008; Hartz et al., 2010; Potschka, 2012). As previously discussed in this text, a higher expression of P-gp observed in a model of inflammatory pain after λ-carrageenan injection, correlates to a lower transport of morphine in the CNS uptake, which is related to a significant reduction of its analgesic efficacy (Seelbach et al., 2007). Interestingly, not only inflammation but also diclofenac administration has been proved to cause a significant increase of P-gp expression in rat brain microvessels. Additionally, sprague-dawley rats that were pretreated with this drug revealed a lower morphine uptake (Sanchez-Covarrubias et al., 2014). One possible explanation is the drug–drug interaction between NSAIDs and opioids, with a modulatory effect of P-gp. Nevertheless, more data is needed to confirm this hypothesis.

In addition to P-gp, it has been recognized that NSAIDs also interact with other ABC transporters, mainly MRP4, and possibly MRP1 (Reid et al., 2003; Rosenbaum et al., 2005; de Groot et al., 2007). The data available supports that MRP4 has the ability to produce a cellular release of prostaglandins and that some of the most commonly prescribed NSADs (e.g., indomethacin, indoprofen and ketoprofen) act not only by inhibiting the synthesis of prostaglandin, but also by inhibiting its release, acting at the level MPR4 transporter (Reid et al., 2003).

Non-steroidal anti-inflammatory drugs are known to cross BBB. However, according to some studies indomethacin shows a greater passage through the BBB when compared to other NSAIDs (Eriksen et al., 2003; Parepally et al., 2006). As a matter of fact, this drug is the first line therapy in the treatment of some headaches, such as paroxysmal hemicrania and hemicrania continua. The efficacy of indomethacin in those disorders is so high that it is applied as a tool for differential diagnosis of those forms of primary headaches and a positive response to indomethacin is mandatory for a definitive diagnosis of hemicrania continua and paroxysmal hemicrania (Casey and Bushnell, 2000; Summ and Evers, 2013). Indomethacin is also recommended to treat other primary headaches (e.g., stabbing headache and primary cough headache; Merskey et al., 1994; Summ and Evers, 2013). The capacity to interact with MRP4 (Reid et al., 2003) and possibly MRP1 (de Groot et al., 2007), and consequently its high ability to cross the BBB, are probably crucial characteristics that determine the significant clinical efficacy of indomethacin in primary headaches (Summ and Evers, 2013). Although more information is needed regarding the interactions between NSAIDs

Table 3 | Direct and indirect evidence that migraine pathophysiology is also correlated to a BBB dysfunction.

Barrier	Model	Main outcomes	Reference
BBB	Cortical spread depression (CSD) model, in sprague–dawley rats and mice.	Direct evidence: brain edema and plasma protein leakage, associated with altered expression ZO-1, EBA, and immunoreactive laminin. Albumin leakage was suppressed by the injection of the matrix metalloproteinase inhibitor GM6001 and was not found in MMP9-null mice. Such results indicate that the BBB disruption related to CSD depends on the MMP-9 activity.	Gursoy-Ozdemir et al. (2004)
BBB	Familial hemiplegic migraine patients.	Direct evidence: quantitative analysis of gadolinium-enhanced MRI showed a mild, but significant, left-hemispheric opening of the BBB, preceding cortical edema.	Dreier et al. (2005)
BBB	Migraine patients	Indirect evidence: no differences in MMP-9 and TIMP-1 levels were found between ictal and interictal periods. However, lower plasma levels of MMP-3 were observed in the external jugular and cubital vein during migraine attacks. Such results suggest that plasma levels of MMP-9 might not be the most recommended biomarker of BBB disruption in migraine without aura. On the other hand, MMP-3 levels should be further investigated.	Ashina et al. (2010)
BBB	Migraine patients	Indirect evidence: higher MMP activity was associated with migraine, independent of aura symptoms.	Bernecker et al. (2011)
BBB	Migraine patients	Indirect evidence: patients presenting migraine without aura showed increased plasma concentrations of MMP-9 concentrations than migraine with aura patients.	Martins-Oliveira et al. (2012)
BBB	Migraine patients	Indirect evidence: patients with migraine with aura exhibited grater plasma concentrations of MMP-2 and MMP-2/TIMP-2 ratios than patients with migraine without aura and controls. CC genotype for $C^{-735}T$ polymorphism and the CC haplotype were linked to higher plasma MMP-2 concentrations in the migraine with aura group.	Gonçalves et al. (2013)

and the BSCB, it has been reported that in rats submitted to spinal cord injury, a pretreatment with NSAIDs (e.g., indomethacin or ibuprofen) not only attenuates the changes that occur in the spinal cord-evoked potentials immediately after trauma but also contributes to the reduction of edema formation and BSCB permeabilization (Sharma and Winkler, 2002).

Finally, some strategies have been applied to improve the delivery of therapeutic compounds to the CNS. For example, a conjugate of Angiopep-2 and neurotensin, called ANG2002, induced a dose–dependent analgesia, in a formalin model of persistent pain (Demeule et al., 2014). The regulated and reversible opening of the BNB has also been explored in order to develop new strategies to enhance drug delivery to the peripheral nervous system, improving the efficacy and reducing the undesirable central effects of some analgesic drugs, including opioids (Hackel et al., 2012). Though the selective blockade of nociceptive fibers at peripheral sites of injury by analgesic drugs is prevented by the BNB (Radu et al., 2013), it seems that the BNB is already disrupted in cases of peripheral nerve injures (Lim et al., 2014). Thus, the development of compounds with action limited to the peripheral nervous system would be of particular interest.

CONCLUSION AND PERSPECTIVES

There is mounting evidence that BBB/BSCB/BNB disruptions participate in the complex mechanisms that initiate or maintain inflammatory, neuropathic pain, and migraine. Regarding migraine, this process could be, at least partially, induced by MMPs. BBB and BNB also play a crucial role in the drug–drug interactions, with great impact in the efficacy as well as central-mediated side effects of analgesic agents, especially opioids peptides. Future perspectives include the complete characterization of specific changes in the nervous system barriers in order to establish the molecular mechanisms of each pain disorder. The development of novel drugs to treat neuropathic pain, with effects restricted to the peripheral nervous system would also be desirable. Finally, the contribution of polymorphisms affecting the components of the BBB and the role of epigenetics in the altered permeability of CNS barriers induced by chronic pain should be further explored.

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Protection after stroke: cellular effectors of neurovascular unit integrity

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Neurological disorders are prevalent worldwide. Cerebrovascular diseases (CVDs), which account for 55% of all neurological diseases, are the leading cause of permanent disability, cognitive and motor disorders and dementia. Stroke affects the function and structure of blood-brain barrier, the loss of cerebral blood flow regulation, oxidative stress, inflammation and the loss of neural connections. Currently, no gold standard treatments are available outside the acute therapeutic window to improve outcome in stroke patients. Some promising candidate targets have been identified for the improvement of long-term recovery after stroke, such as Rho GTPases, cell adhesion proteins, kinases, and phosphatases. Previous studies by our lab indicated that Rho GTPases (Rac and RhoA) are involved in both tissue damage and survival, as these proteins are essential for the morphology and movement of neurons, astrocytes and endothelial cells, thus playing a critical role in the balance between cell survival and death. Treatment with a pharmacological inhibitor of RhoA/ROCK blocks the activation of the neurodegeneration cascade. In addition, Rac and synaptic adhesion proteins (p120 catenin and N-catenin) play critical roles in protection against cerebral infarction and in recovery by supporting the neurovascular unit and cytoskeletal remodeling activity to maintain the integrity of the brain parenchyma. Interestingly, neuroprotective agents, such as atorvastatin, and CDK5 silencing after cerebral ischemia and in a glutamate-induced excitotoxicity model may act on the same cellular effectors to recover neurovascular unit integrity. Therefore, future efforts must focus on individually targeting the structural and functional roles of each effector of neurovascular unit and the interactions in neural and non-neural cells in the post-ischemic brain and address how to promote the recovery or prevent the loss of homeostasis in the short, medium and long term.

Keywords: stroke, NVU, BBB, CDK5, Rho GTPases, p120 catenin

INTRODUCTION

Neurological disorders are highly prevalent around the globe. In 2008, neurodegenerative disorders were responsible for 1% of disabilities worldwide (W.H.O., 2003). Strokes account for 55% of all neurological diseases and are considered the leading cause of permanent physical and mental disability (OMS, 2013). The primary risk factors of stroke include hyperlipidemia, hypertension, diabetes mellitus, and harmful habits, such as smoking and excessive alcohol consumption. The high incidence of strokes is related to an increased number of dementia cases and other emotional and cognitive disorders, such as depression and memory loss (Ovbiagele and Nguyen-Huynh, 2011). Death, physical deterioration, and altered quality of life are consequences of the natural history of strokes among patients who survive an ischemic event (Sacco, 1997, 1998; Feigin et al., 2003; Silva et al., 2006). Interestingly, the coexistence of cerebral ischemia and neurodegenerative pathologies profoundly impacts the development of dementia, suggesting a reciprocal interaction between ischemia and neurodegeneration (Nagy et al., 1997; Snowdon et al., 1997). These observations, along with the results of epidemiological studies that have indicated that Alzheimer's disease (AD) and cerebrovascular diseases share similar risk factors (Breteler, 2000), have shifted interest to vascular factors as fundamental contributors to the pathogenesis of neurodegenerative diseases (de la Torre and Mussivand, 1993; Kalaria, 2000; Iadecola and Gorelick, 2003). This hypothesis has been supported by the experimental findings that showed that amyloid-beta (Aβ) peptide, which is commonly detected in AD patients, exhibits strong cerebrovascular effects and that ischemia-induced responses to hypoxia are potent modulators of cerebral amyloidogenesis (Iadecola, 2004). Both Aβ peptide and vascular risk factors deteriorate the structure and function of the neurovascular unit (NVU, consisting of the endothelium, glia, neurons, pericytes, and the basal lamina) (Mirra and Gearing, 1997; Snowdon et al., 1997; Breteler, 2000; Kalaria, 2000; Iadecola and Gorelick, 2003; Iadecola, 2004).

The NVU acts as a guardian of cerebral homeostasis. Neurons, glia, the perivascular space, and the endothelium are closely interrelated to maintain the homeostasis of the brain microenvironment (Iadecola, 2010), regulate blood flow, modulate the exchange across the blood-brain barrier (BBB), contribute to immune vigilance and provide trophic support to the brain (Iadecola, 2010). Substantial evidence has shown that cerebrovascular dysfunction is implicated in not only cognitive impairment (such as that of cognitive origin) but also neurodegenerative diseases, such as AD (Chui et al., 1992; Alavi et al., 1998; Kalaria, 2000; Iadecola, 2004; Simpkins et al., 2005; Hachinski et al., 2006; Pendlebury et al., 2012). Ischemic stroke is exacerbated by several risk factors that affect the function and structure of blood vessels in the brain and cells associated with the NVU, reducing the ability of the brain parenchyma to repair due to the rupture of the BBB, the loss of brain blood flow regulation, oxidative stress, inflammation, and the loss of neuronal connections, ultimately increasing brain dysfunction (Deane et al., 2003; Ohab et al., 2006; Konsman et al., 2007; Weber et al., 2007; Bell et al., 2009; Wolburg et al., 2009). The study of stroke has focused on understanding the molecular and pathophysiological mechanisms of neuronal death, recovery and pharmacological intervention strategies, as well as clinical and epidemiological characteristics (Silva et al., 2006). In addition, several molecular targets associated with endothelial dysfunction and cardio-cerebrovascular risk, including CDK5, Rho GTPases, and cell adhesion proteins, are described below and presented in a hypothetical schematic in **Figure 1** to explain and propose a potential neuroprotective approach for stroke.

CEREBRAL ISCHEMIA

Cerebral ischemia is a type of stroke characterized by a transient or permanent decrease in blood flow as a result of the thrombotic or embolic occlusion of one or more cerebral arteries. Depending on the ischemic characteristics (i.e., the duration of reduced blood flow and the infarction site), this disease can result in several clinical manifestations, including paralysis or hemiplegia, aphasia, and memory and learning impairment, among others (Kemp and Mckernan, 2002).

In focal cerebral ischemia, a hypoperfusion gradient is generated, leading to the activation of diverse cell types involved in survival and cell death mechanisms (Lipton, 1999; Barreto et al., 2011, 2012). Within minutes after ischemia, the activated microglia induces cytokine and adhesion molecules; hours to days, the endothelium responds by increasing angiogenesis; in days, weeks to months, astrocytes expressing GFAP (glial-fibrillary acidic protein) generate glial scar; and the neurons trigger axonal sprouting, dendrite outgrowth, spine morphogenesis (Iadecola, 1997; Rami et al., 2008; Barreto et al., 2012). The region suffering the most severe degree of hypoperfusion, referred to as the ischemic core, progresses rapidly toward irreversible damage via necrosis. The remaining hypoperfused tissue displays altered mechanisms of blood flow autoregulation and is known as the penumbra (Baron, 2001; Moustafa and Baron, 2008). In this peri-infarct zone, neurons display functional alterations and minimal metabolic activity to preserve their structure, but these neurons ultimately advance toward apoptotic death (Mies et al., 1993; Moskowitz et al., 2010). Accordingly, the penumbra is not only a functionally affected tissue but is also potentially recoverable and, as such, represents a key target for therapeutic intervention for cerebral ischemia (Baron et al., 1995). However, unless perfusion is restored or the cells surrounding the injury site become relatively resistant, the cells in the penumbra die via apoptosis within a few hours (Lo, 2008b).

An interruption in the blood supply to the brain during ischemia results in oxygen-glucose deprivation and, consequently, reduced energy available for brain cell functions (Dingledine et al., 1999). In particular, neurons become incapable of maintaining the transmembrane ion gradients necessary for their function and homeostasis (Szydlowska and Tymianski, 2010). This event leads to excessive neuronal depolarization, an increase in the release of excitatory neurotransmitters and pro-inflammatory molecules, a reduction in the reuptake of these neurotransmitters from the extracellular space in penumbra and a GABAergic and dopaminergic misbalance in exo-focal areas (Sabogal et al., 2014). Altogether, these pathological mechanisms induce an excessive intracellular accumulation of ions such as Na^+ and Ca^{2+} and, simultaneously, the deregulation of multiple signaling pathways, activating catabolic processes mediated by proteases, lipases, and nucleases, which interrupt neuronal function and induce cell death (Dingledine et al., 1999; Szydlowska and Tymianski, 2010; Barreto et al., 2011). Increased extracellular glutamate concentrations in central nervous system (CNS) pathologies (including stroke, epilepsy and certain neurodegenerative diseases) result in the local hyperactivation of ionotropic glutamate receptors, thereby triggering neuronal cell death via excessive Na^+ and Ca^{2+} influx into neurons (Olney, 1969; Choi, 1987; Sattler and Tymianski, 2001; Greenwood and Connolly, 2007). This event is known as glutamate-mediated excitotoxicity (Lipton, 1999; Mehta et al., 2007). In the core, glutamate excitotoxicity rapidly evolves to necrosis due to ATP depletion and increased Na^+ and water influx, whilst in the penumbra, where the damage seems to be less severe, glutamate excitotoxicity produces neuronal apoptosis (Bonfoco et al., 1995; Kelly et al., 2003; Lo, 2008a; Rami et al., 2008). Glutamate-induced dendritotoxicity results in microtubule disruption and the calcium-dependent loss of MAP2 and contributes to dendritic dysfunction in acute hippocampal slices (Bindokas and Miller, 1995; Hoskison and Shuttleworth, 2006; Hoskison et al., 2007) and nearly complete dendritic loss in cortical neuron cultures (Bosel et al., 2005; Ma et al., 2009). Toxic cytoplasmic calcium concentrations during ischemia can occur due to the release of calcium from internal stores via physical damage to the mitochondria and endoplasmic reticulum (ER) or the malfunction of receptors and channels present in their membranes (Loew et al., 1994; Paschen and Doutheil, 1999). The accumulation of intramitochondrial Ca^{2+} reduces ATP synthesis, and increased ATP usage has been suggested to be a primary cause of cell death (Schinder et al., 1996). The dysregulation of Ca^{2+}-ER homeostasis following ischemia involves two phases: the accumulation of Ca^{2+} in ER stores and the subsequent release of Ca^{2+} from the ER following ischemia/reoxygenation (Chen et al., 2008). It has been suggested that Ca^{2+} released from the ER via IP3R can enter the adjacent mitochondria and trigger cytochrome c release (Rizzuto and Pozzan, 2006). The increase in the cytoplasmic calcium concentration triggers neurotoxic

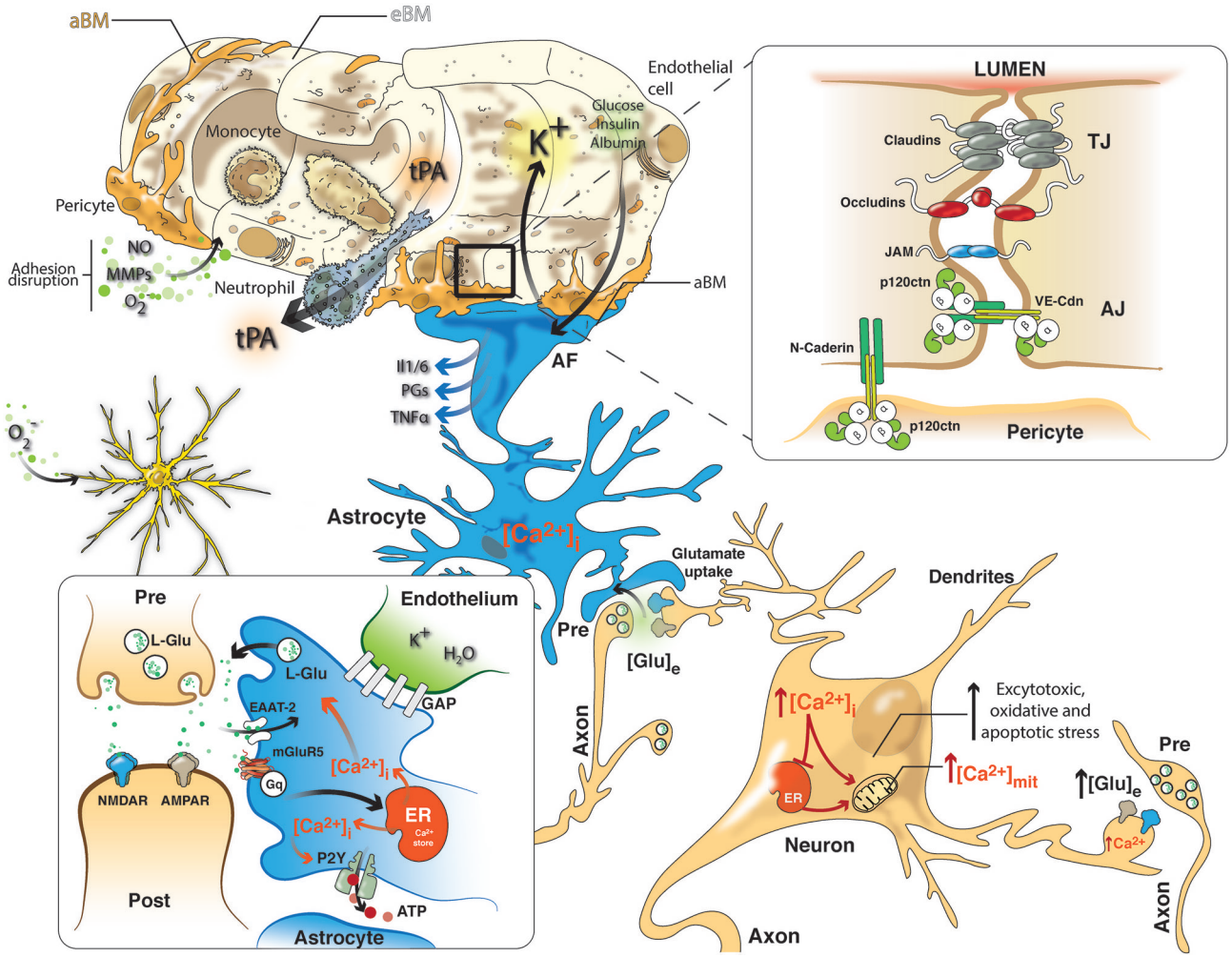

FIGURE 1 | NVU in cerebral ischemia includes a closely coupled signaling network of ECs, pericytes, astrocytes and neurons. Neurovascular uncoupling consists of the expression of various cytokines and adhesion molecules on ECs, promoting leukocyte adherence and accumulation, which thereby initiates an inflammatory response. Further, breakdown of the BBB via NO, MMPs and ROS permits neutrophil extravasation into the ischemic tissue in response to chemokines produced by astrocytes, macrophages, and microglia. Glutamate-mediated excitotoxicity via NMDA and AMPA receptors induces the hyperstimulation of neurons and subsequent calcium influx, indicating several intracellular calcium stores (mitochondria and ER), which triggers dendritotoxic and cell death pathways. Intrinsic neurovascular responses after stroke are triggered by astrocytes via the spread of calcium waves, promoting homeostatic gliotransmission. In addition, glutamate uptake by astrocytes, which requires EAATs, represents the primary neuroprotective response after excitotoxicity. Alternatively, K^+ and glucose uptake and insulin and albumin transport signal the modulation of neurovascular homeostasis. Tight junctions (TJs) consisting of claudins, occludins and JAM and adherens junctions (AJ) including members of the cadherin-catenin system maintain the integrity of the endothelium. Cell–cell adhesion may transmit intracellular signals that regulate paracellular permeability, contact-induced inhibition of cell growth and new vessel formation. After stroke, pericytes display deformed junctions and increased formation of pinocytic vesicles, which affect the polarization of astrocyte end-feet (AF), detected as the defective deposition of the astrocyte-derived basement membrane (aBM). Deposition of the endothelium-derived basement membrane (eBM) is affected by the lack of pericytes. Moreover, modifications of the molecular organization and intracellular signaling of junction proteins may exert complex effects on vascular homeostasis.

cascades, including the uncoupling of mitochondrial electron transfer from ATP synthesis and the activation and hyperstimulation of enzymes, such as calpains and other proteases, protein kinases, neuronal nitric oxide synthase (nNOS), calcineurin and endonucleases (Szydlowska and Tymianski, 2010).

In recent studies, we found that glutamate-mediated excitotoxicity decreases the number and length of dendrites and induces tau hyperphosphorylation (Posada-Duque et al., 2013). The hyperphosphorylation of tau, a neurodegeneration marker, is also increased by global and transient focal cerebral ischemia, causing alterations in spatial memory (Castro-Alvarez et al., 2011; Céspedes et al., 2013). At the cellular level, hyperactivated GSK3 protein is associated with alterations in microtubule assembly, and the activity of RhoA has been associated with the retraction of the actin cytoskeleton (Céspedes-Rubio et al., 2010). Damage to the neuronal cytoskeleton can be considered the primary cause of the loss of protein transport and neuronal stability in cerebral ischemia. Microtubule disassembly occurs after ischemia and

plays an important role in the pathophysiology of this disease (Pettigrew et al., 1996). Alterations in the cytoskeleton reflect, in part, processes of protein degradation and aggregation following ischemia (Kühn et al., 2005). For example, we found that the loss of Rac activity participates in the progression of cerebral ischemia-induced neurodegeneration and is closely associated with cognitive disorders (Gutiérrez-Vargas et al., 2010). In addition, MAP2 degradation and tau aggregation and hyperphosphorylation are considered biomarkers of ischemic damage (Pettigrew et al., 1996; Gutiérrez-Vargas et al., 2010).

NVU: THE GUARDIAN OF CEREBRAL HOMEOSTASIS

The BBB is a highly specialized endothelial brain structure composed of a specialized neurovascular system. Through pericytes, astrocytes, and microglia, the BBB separates components in circulating blood from neurons. Moreover, the BBB maintains the chemical composition of sodium, anion carbonate, glucose, lactate, essential amino acids, insulin, enkephalins, and arginine-vasopressin, which is required for the proper function of neuronal circuits in the adult brain (Zlokovic, 2008). The endothelium, the site of the anatomical BBB, neurons, and non-neuronal cells (e.g., pericytes, astrocytes, and microglia) together with the basal lamina form a functional unit, often referred to as the NVU, which participates in the establishment and function of the BBB (Lo et al., 2003; Hawkins and Davis, 2005). Cerebral endothelial cells (ECs) significantly differ from non-cerebral ECs in the following aspects: (i) the presence of intercellular tight junctions (TJs); (ii) low levels of transcytosis (pinocytosis) and paracellular diffusion of hydrophilic compounds; (iii) a high number of mitochondria, associated with increased metabolic activity; and (iv) the polarized expression of membrane receptors and transporters, which are responsible for the active passage of nutrients (Brightman and Kadota, 1992; Petty and Lo, 2002). Consistently, the permeability of plasma and ionic compounds in the cerebral endothelium is highly restricted and regulated—a phenomenon evidenced by a high trans-endothelial electric resistance (TEER; Petty and Lo, 2002; Abbott et al., 2006). Another component of the BBB, the basal lamina, is classified according to the cell source. The basal lamina of the endothelium is composed of three layers: one contains laminin-4 and -5 derived from ECs, one contains laminin-1 and -2 derived from astrocytes, and the other contains collagen IV derived from both cell types (Perlmutter and Chui, 1990).

The role of astrocytes in BBB function and integrity has been well documented; however, their molecular mechanisms of action remain unclear (Anderson et al., 2003; Giffard and Swanson, 2005; Thoren et al., 2005; Barreto et al., 2011). Astrocytes form structures called "astrocyte end-feet" that communicate with neurons, pericytes, and ECs. This cell population is very important because it modulates synaptic transmission and processes of neuronal plasticity (Ullian et al., 2001; Allen and Barres, 2005; Kucukdereli et al., 2011). These cells also maintain homeostasis by releasing sodium after action potentials, maintaining metabolic homeostasis via insulin, glucose and lactate signaling, and releasing hormones in response to various behaviors (Nedergaard et al., 2003; Newman, 2003; Abbott et al., 2006). It has been suggested that astrocytes contribute support to the BBB via the release of factors including glial cell line-derived neurotrophic factor (GDNF), angiopoietin-1, and angiotensin II (Lee et al., 2003; Hori et al., 2004; Wosik et al., 2007).

Complementarily, pericytes and the basal lamina surround ECs along the cerebral microvasculature. This cerebral microvasculature is enriched in pericytes, and the ratio of pericytes to ECs correlates to the barrier capacity of the endothelium. In addition, pericytes are actively involved in the maintenance of vessel integrity, vasoregulation, and the regulation of BBB permeability (Lindahl et al., 1997; Peppiatt et al., 2006; Armulik et al., 2010). Finally, it has been observed that neuronal projections are closely related with the cerebral endothelium (Hamel, 2006; Drake and Iadecola, 2007; Weiss et al., 2009). These perivascular neurons use perivascular astrocytes and pericytes to modulate brain blood flow and vessel dynamics (Hamel, 2006; Wang and Bordey, 2008; Sofroniew and Vinters, 2010). Recently, it has been demonstrated that the close proximity of these different cell types to neurons facilitates effective paracrine regulation, which is critical for the normal function of the CNS; however, the impaired paracrine regulation induces the development of neurological diseases (Berzin et al., 2000; Zlokovic, 2005). The normal function of the CNS requires the regulation of neurovascular coupling, microvascular hemodynamics and permeability, matrix interactions, neurotransmitter inactivation, neurotrophic support, and processes of neurogenesis and angiogenesis (Boillée et al., 2006; Deane and Zlokovic, 2007). It is currently accepted that the BBB limits the entrance of blood components into the cerebral parenchyma. Therefore, injury to any part of the neurovascular unit could permit the extravasations of vascular inflammatory cells and proteins to brain tissue (del Zoppo, 2006). Extravasation of the BBB due to cerebral ischemia, trauma, neurodegenerative processes, or vascular disorders allows these neurotoxic products to compromise synaptic and neuronal function (Hawkins and Davis, 2005; Zlokovic, 2005; Abbott et al., 2006). When the brain blood flow is interrupted, brain functions halt within seconds, and neuronal damage occurs after a few minutes (Girouard and Iadecola, 2006). An appropriate vasculature-neuron ratio is critical for normal brain function. It has been estimated that nearly every neuron in the brain has its own capillary (Zlokovic, 2005). The total length of the capillaries in the human brain is approximately 400 miles, and the surface area available for molecular transport is approximately 20 m^2 (Begley and Brightman, 2003). The length of the brain capillaries is reduced in neurodegenerative disorders, such as AD and cerebral ischemia (Bailey et al., 2004; Wu et al., 2005). These vascular reductions diminish the transport of energy substrates and nutrients across the BBB and reduce the clearance of potentially neurotoxic substances during brain injury.

BBB DYSFUNCTION IN CEREBRAL ISCHEMIA

In a variety of neurological diseases (e.g., AD, ischemia, Huntington's disease, Parkinson's disease, and ALS), BBB dysfunction has been detected not only as a late event but also at early stages of disease progression. There are two types of BBB dysfunction involving increased permeability: passive diffusion with edema formation and massive cellular infiltration across the BBB (Weiss et al., 2009). Acute obstruction of brain blood vessels due to coagulation during cerebral ischemia involves a complex series of

cellular and molecular events in the brain parenchyma (del Zoppo and Mabuchi, 2003). The sequence of events from ischemia to reperfusion begins with perivascular inflammation and increased BBB permeability, which greatly contribute to brain damage, thus supporting the notion that cerebral ischemia is primarily a vascular disorder (Benchenane et al., 2004). The release of oxidants, proteolytic enzymes, and proinflammatory cytokines alters BBB permeability, leading to brain edema (Dirnagl et al., 1999). In addition, metalloproteinases (MMPs) released by activated leukocytes affect NVU integrity, degrading the basal lamina and the proteins involved in cell-cell adhesion (Hamann et al., 1995; Rosenberg et al., 1998; Asahi et al., 2001). MMP-9 is particularly critical during this process, as previous studies have demonstrated that BBB integrity is maintained after cerebral ischemia in MMP-9-deficient mice (Asahi et al., 2001; Montaner et al., 2001). However, increased trans-endothelial migration of leukocytes is characteristic during cerebral ischemia (Lindsberg et al., 1996; Planas et al., 2006). Following ischemia, circulating leukocytes release inflammatory cytokines and activate various cell types within the NVU. For example, the increased expression of intercellular adhesion molecule-1 (ICAM-1) by ECs facilitates the trans-endothelial migration of leukocytes (Lindsberg et al., 1996). Furthermore, it has been demonstrated that treatment with anti-ICAM-1 antibodies reduces infarct size in rat models of cerebral ischemia (Zhang et al., 1995). Another process of BBB dysfunction that increases damage during cerebral ischemia is the secretion of TGFβ by astrocytes, which diminishes endothelial capillarity, fibrinolytic enzyme expression, and basal lamina proteolysis. Additionally, BBB disruption coincides with the induction of aquaporin 4 (AQP4) expression and the presence of reactive astrocytes in the perivascular glial cells (Tran et al., 1999; Lo et al., 2003; Tomás-Camardiel et al., 2005; Vakili et al., 2005). Several chemical agents circulating in the plasma or secreted from cells associated with the BBB are capable of increasing brain endothelial permeability and impairing its transport and metabolic functions (Laflamme et al., 1999; Abbott, 2000; Webb and Muir, 2000). These agents include histamine, serotonin, glutamate, purine nucleotides (ATP, ADP, and AMP), adenosine, platelet-activating factor, phospholipase A2, arachidonic acid, prostaglandins, leukotrienes, tumor necrosis factor-α (TNFα), free radicals and nitric oxide (Laflamme et al., 1999; Abbott, 2000; Webb and Muir, 2000; Tan et al., 2002; Stolp and Dziegielewska, 2009). Bradykinin, which is produced during inflammation in stroke, acts on endothelial and astroglial bradykinin B2 receptors, leading to an increase in intracellular Ca^{2+} concentrations. In astrocytes, this event can trigger the production of interleukin (IL)-6 and IL-1 via nuclear factor-κB (NF-κB) activation (Deli et al., 1995; Schwaninger et al., 1999; Didier et al., 2003; Perry et al., 2003). The role of the microglia in pathological NVU activity has been proposed to induce damage to the endothelium and the basal lamina via receptors for nucleotides, such as ATP, and opening new routes of communication between ECs, pericytes, astrocytes and microglia is important for BBB repair (Deitmer et al., 2001; Andersson et al., 2005). However, recent studies have demonstrated that enhancing the activation of microglia promotes tissue repair and remodeling after stroke by decreasing the levels of the inflammatory markers IL-1β and TNF-α and increasing IL-1ra, IL-10, and arginase 1 expression (Gelosa et al., 2014; Shin et al., 2014).

Apparently, the NVU performs a coordinated response after an ischemic insult to maintain and re-establish blood flow over time, thus reducing damage to tissue and neurons. Aside from the notion that occlusion affects vulnerable regions, this hypothesis supports the need to understand the interactions between each component of the NVU in the pathophysiology of cerebral ischemia and recovery following therapy (Nedergaard et al., 2003; Zonta et al., 2003; Iadecola, 2004; Abbott et al., 2006; Koehler et al., 2009). The strategy of protection of neuronal function in humans was not successful in preventing damage progression, indicating that there is a mechanism that is inherent to NVU coupling. Moreover, there is a relapse of the NVU function that leads to vascular dementia (del Zoppo, 2010), despite the intrinsic recovery of the tissue and of cognitive abilities. Although the use of neuroprotective agents has generally failed as a potential therapeutic strategy following cerebral ischemia in translational trials, thrombolysis using tissue plasminogen activator (tPA) continues to be the gold standard intervention during the acute phase after stroke (van der Worp and van Gijn, 2007). Importantly, neuroprotective agents should be administered early by the emergency medical system (EMS) to improve neuroprotection in the long term (Saver, 2013), which also helps to prevent the impairment of the integrity of the NVU and avoid cerebral dysfunction. Therefore, in an attempt to understand the interdependency of the components of the NVU, its integrity and how to protect it, we will focus on certain implicated tissue and cellular effectors.

INTERCELLULAR JUNCTIONS IN THE BBB

ECs are primarily connected via junction complexes, which consist of TJs and adherens junctions (AJ; Hawkins and Davis, 2005). Although gap junctions (GJ) have also been found in the BBB, their role remains unclear (Nagasawa et al., 2006). TJs are primarily the junctions that confer a low paracellular permeability and TEER to the BBB (Bazzoni et al., 2005). TJ physiology is complex. TJ proteins and their adaptor molecules, which are connected to the cytoskeleton, are often affected during acute and chronic brain disease (Wolburg and Lippoldt, 2002). Occludin was the first integral protein to be identified in TJs of ECs involved in the BBB. It has been demonstrated that the N-terminal domain of occludin plays an important role in TJ assembly and the maintenance of barrier function (Bamforth et al., 1999). In contrast, deletion of the occludin gene in mice results in a complex phenotype, including a delay in postnatal growth, due to the efficient permeability of the barrier caused by a diminished TEER and an increased paracellular flow of macromolecules (Bamforth et al., 1999; Saitou et al., 2000). Occludin is also vulnerable to degradation by MMPs (Rosenberg and Yang, 2007). Damage caused by reperfusion in ischemic models in rodents leads to a biphasic opening of the BBB—a process directed by MMP-2 activation during the acute phase that occurs for several hours after reperfusion (Zlokovic, 2006). This initial transient opening is followed by more intense damage to blood vessels from 24 to 48 h after reperfusion, which is associated with the expression of MMP-9 and MMP-3 (Suzuki et al., 2007). MMPs can also degrade basal lamina components, such as fibronectin, contributing to

BBB rupture (Cheng et al., 2006; Zlokovic, 2006). Claudins are a family consisting of more than 20 members that generate TJ chains via hemophilic claudin-claudin interactions. Claudin −5, −3, and −12 are located on the BBB, whereas the localization of claudin-1 is controversial. With regard to the function of claudins at the BBB, it has been found that each claudin isoform regulates the diffusion of a group of molecules of a particular size (Wolburg and Lippoldt, 2002; Lee et al., 2003; Nitta et al., 2003). Although claudin-5 protein expression is low in brain ECs, during BBB rupture, this protein is a target of MMP-2- and MMP-9-mediated degradation after ischemia and, together with occludin, is found near astrocytes (Yang et al., 2006). Integral TJ membrane proteins are attached to the cytoskeleton by cytoplasmic multi-domain scaffolding proteins, such as ZO-1, ZO-2, and ZO-3 (Hawkins and Davis, 2005; Hawkins et al., 2007), and these proteins are also targets of MMP-mediated degradation during CNS injury. Essentially, BBB opening during cerebral ischemia is clearly induced by the MMP-mediated degradation of TJs; however, it has recently been demonstrated that MMPs also participate in the regulation of neurogenesis and angiogenesis during the recovery phase after injury (Fujioka et al., 2012). This beneficial characteristic of MMP activity was demonstrated in a late event ischemia model (7–14 days), in which treatment with MMP inhibitors 7 days after stroke suppressed neurovascular remodeling, increased injury, and impaired cognitive function. It has been suggested that these effects are primarily due to a decrease in VEGF availability. Therefore, clarification regarding the therapeutic potential of MMP inhibition for different brain pathologies has been recommended (Zhao et al., 2006). Alternatively, AJs typically crosstalk with TJs. AJs are a type of cell-cell adhesive contact found in many tissues, and AJs use a cadherin dimer as the primary mediator of cell-cell adhesion. Endothelial permeability is regulated in part by the dynamic opening and closing of AJs (Bazzoni and Dejana, 2004). In ECs, AJs primarily consist of vascular endothelial cadherin (VE-cadherin), an endothelium-specific member of the cadherin that binds to p120 catenin, β-catenin, and plakoglobin via its intracytoplasmic domain (Dejana et al., 2008). Multiple endogenous pathways that increase vascular permeability affect the function and organization of VE-cadherin (Bazzoni and Dejana, 2004). Loss of VE-cadherin is accompanied by an increase in vascular permeability and leukocyte diapedesis, and internalization and cleavage of VE-cadherin can disassemble AJs at cell-cell junctions (Lampugnani and Dejana, 2007). Changes in AJ proteins expression contribute to increased BBB permeability and leukocyte infiltration into the CNS (Allport et al., 1997; Johnson-Léger et al., 2000). Several mechanisms by which VE-cadherin regulates endothelial function have been proposed, such as the direct activation of signaling molecules to maintain survival and the organization of the actin cytoskeleton (e.g., PI3 kinase and Rac), the regulation of gene transcription co-factors (e.g., p120 catenin, β-catenin), the formation of complexes with growth factor receptors, including the vascular endothelium growth factor receptor-2 (VEGFR-2), and the modulation of VEGFR-2 signaling (Bazzoni and Dejana, 2004). The roles of AJs in the functional and morphological changes in the BBB during cerebral ischemia have not been addressed in depth. The direct activation of oxygen-sensitive transcription factors, such as HIF-1 and reactive oxygen species (ROS), can directly activate transcription factors, such as NF-κB, which alter AJ formation. The increase in intracellular calcium levels that characterizes cerebral ischemia can lead to the transduction of signals that regulate AJ and TJ transcription, consequently inducing changes in BBB function (Brown and Davis, 2002). Based on these studies, it is necessary to develop strategies to maintain NVU structure and function after cerebral ischemia by focusing on obtaining a deeper understanding of the cell-cell junctions present in the BBB.

Rho GTPases IN ECs REGULATE BBB INTEGRITY

The importance of the cytoskeleton in BBB integrity and establishment was initially demonstrated in mice deficient in the actin-binding protein dystrophin (Nico et al., 2003). These mice displayed increased vascular permeability, as the ECs and astrocytes formed a disorganized actin cytoskeleton, as well as altered subcellular localization of junction proteins in the endothelium and aquaporin-4 at astrocyte end-feet (Nico et al., 2003). These findings demonstrated that the arrangement of actin filaments and their junctions to TJs and/or AJs are critical for normal BBB function. The actin cytoskeleton plays an essential role in maintaining the barrier function of the endothelium because it determines cell shape, facilitates cell-matrix adhesion, and participates in the regulation of junction complexes (Dejana, 2004). It has been determined that the dynamics and structure of the actin filaments are primarily regulated by Rho GTPase proteins (Ridley, 2001; Lampugnani et al., 2002; Aspenstrom et al., 2007; Aghajanian et al., 2008; Vandenbroucke et al., 2008). The Rho family of GTPases belongs to the Ras superfamily, which consists of low molecular weight, monomeric G-proteins. Rho GTPases have recently been classified into six subfamilies: RhoA, Rac1, Cdc42, Rnd, RhoBTb, and Rho/Miro (Ridley, 2001; Lampugnani et al., 2002; Aspenstrom et al., 2007; Aghajanian et al., 2008; Vandenbroucke et al., 2008). Specifically, RhoA induces the formation of actin stress fibers and focal adhesions, Rac1 stimulates lamellipodial protrusions (membrane ruffling), and Cdc42 promotes the formation of filopodia (actin microfilaments) (Ridley and Hall, 1992; Nobes and Hall, 1995). In *in vivo* experiments, multiple vasoactive agents improved endothelial barrier integrity by forming a dense F-actin band via changes in the activities of various Rho GTPases (Garcia et al., 2001; Temmesfeld-Wollbrück et al., 2007; Tauseef et al., 2008). Initially, RhoA activation was found to be important because RhoA regulates cell contraction and endothelial hyperpermeability (Sun et al., 2006). In subsequent years, attention was directed toward the involvement of Rac and Cdc42 in the assembly and stability of inter-endothelial junctions. Furthermore, a recently discovered role of RhoA and its downstream effector ROCK in barrier maintenance was identified (Broman et al., 2007; Fu and Birukov, 2009). An important role of Rho GTPases in BBB regulation is that they act as mediators of barrier damage or protection (Beckers et al., 2010). Although Rac1 primarily modulates the stability of cell-cell junctions, its activity on NADPH oxidase-mediated ROS generation participates in BBB damage after injury (Broman et al., 2007; Monaghan-Benson and Burridge, 2009). Likewise, RhoA plays a dual role in barrier regulation. For example, RhoA exerts a

protective function under basal conditions, but it is involved in BBB dysfunction after EC activation by thrombin (Broman et al., 2007; van Nieuw Amerongen et al., 2008). In particular, RhoA activity is involved in the progression of neuronal death during cerebral ischemia (Semenova et al., 2007). In our study, we found that there is a differential regulation of Rho GTPases due to global and transient focal cerebral ischemia. We demonstrated that Rho/ROCK inhibition is involved in neuroprotection (Castro-Alvarez et al., 2011). Additionally, we found that treatment with statins—a class of drugs used to reduce cholesterol—diminished RhoA activity, which is associated with decreased levels of neurodegeneration markers and neuronal death (Céspedes-Rubio et al., 2010). Some of the mechanisms associated with the neuroprotective role of statins include the downregulation of RhoA activity and the subsequent induction of endothelial nitric oxide synthase (eNOS) activity (Wang et al., 2008). Finally, we discovered that Rac1 activity is involved in long-term recovery from neurodegeneration after cerebral ischemia (Gutiérrez-Vargas et al., 2010). Because the function of Rho GTPase is essential for NVU regulation (including the endothelium, neurons, and adhesion proteins), the challenge of maintaining BBB integrity lies in determining the complex regulation of Rho GTPases, which will provide indications for new therapeutic strategies for ischemic injury.

CDK5, A TARGET FOR CEREBRAL ISCHEMIA

CDK5 is a serine-threonine kinase that participates in neuronal development and function and is involved in the regulation of various processes, such as neuritogenesis, synapse formation, and synaptic transmission (López-Tobón et al., 2011; Su and Tsai, 2011). CDK5 also plays an important role in the regulation of apoptosis during development, which is essential for the pruning and fine-tuning of neural connections, and is involved in cognitive functions, such as memory and learning. In addition, the deregulation of CDK5 activity is associated with neuronal death in neurodegenerative diseases (Dhavan and Tsai, 2001; Lai and Ip, 2009). CDK5 activity is primarily regulated by p35 and p39, and the phosphorylation of CDK5 at Ser159 and Tyr15 increases its activity (Zukerberg et al., 2000; Grant et al., 2001). Cleavage of p35 by calpains results in the formation of a CDK5/p25 complex, leading to considerable sustained abnormal CDK5 kinase activity (Cheung et al., 2006). Morphological characteristics, such as the density and morphology of dendritic filaments, and biochemical characteristics, such as the composition of postsynaptic density scaffold proteins and the number of neurotransmitter receptors, determines the properties of synaptic activity. Many synaptic proteins isolated from adult brain synaptosomes are putative CDK5 substrates, rendering CDK5 a synapse regulator (Collins et al., 2005). CDK5 is involved in cognitive deterioration in cerebral ischemia and neurodegeneration (Slevin and Krupinski, 2009; Menn et al., 2010). In addition to the many studies showing that CDK5 hyperactivation is involved in tau hyperphosphorylation and the subsequent development of dementia in neurodegenerative diseases (Ko et al., 2001; Camins et al., 2006), multiple pieces of evidence have implicated CDK5 in the progression of cerebral ischemia pathology. Increased expression levels of CDK5 and its activator p35 are detected in peri-infarct neurons following middle cerebral artery occlusion (MCAO; Green et al., 1997; Mitsios et al., 2007). In cerebral ischemia models, pharmacological inhibition of CDK5 significantly reduces the infarct size after 24 h of reperfusion (Weishaupt et al., 2003). AD and other tauopathies, such as cerebral ischemia, are characterized by the hyperphosphorylation of the protein tau (Grundke-Iqbal et al., 1986; Avila et al., 2004). Particularly, CDK5, along with its respective activators, phosphorylates tau at epitopes that are associated with neurodegenerative processes. Deregulated CDK5 activity is produced by the membrane-released CDK5/p25 complex, leading p25 to induce an atypical and sustained activation of CDK5, which leads to the hyperphosphorylation of its substrates that are associated with disease progression. Overexpression of p25 in neuronal cultures produces cytoskeletal damage, tau hyperphosphorylation, and apoptosis (Patrick et al., 1999; Wen et al., 2008). Because CDK5 inhibition protects against neuronal death and reduces tau hyperphosphorylation (Lopes et al., 2007; Piedrahita et al., 2010) and because the atypical activation of CDK5 is a fundamental component of the progression of the neurofibrillary pathology, CDK5 inhibition has been proposed as a potential therapeutic strategy for neurological diseases. During glutamate-mediated excitotoxicity, the excessive influx of Ca^{2+} can activate the Ca^{2+}-dependent protease calpain. Calpain is involved in the degradation of numerous enzymes and cytoskeletal components, thus linking its activity to a variety of intracellular events involved in CNS alterations associated with excitotoxicity, such as hypoxia, ischemia, epilepsy, and AD (Ray and Banik, 2003). Recently, it has been shown that CDK5 phosphorylates the NMDA receptor subunit NR2A, causing neuronal death in the CA1 area of the hippocampus in a transient global cerebral ischemia model. This finding suggests a possible therapeutic approach in which CDK5 inhibition targets glutamate receptors (Wang et al., 2003). Alternatively, previous studies have reported the involvement of CDK5 in the progression of neurovascular pathology. Importantly, patients suffering from cerebral ischemia display strong up-regulation of CDK5, p35, and p25 in peri-infarct blood vessels; additionally, human brain microvascular endothelial cells (HBMECs) subjected to glucose deprivation display hyperactivation of CDK5, suggesting a crucial role of CDK5 in the microvasculature in response to cerebral ischemia (Slevin and Krupinski, 2009).

CDK5 IN THE ENDOTHELIUM AND ASTROCYTES

Early investigation of CDK5 function in ECs included studies that demonstrated decreased CDK5 expression when EC proliferation was inhibited by angiotensin. In a 2008 study, Liu and colleagues demonstrated that mitogenic growth factors activated the PI3K/Akt pathway, followed by CDK5-mediated phosphorylation of PIKE-A (a factor that hyperactivates phosphatidylinositol 3-kinase (PI3K)) in glioblastoma cells. These findings suggest that CDK5 may play a significant role in the regulation of EC proliferation (Sharma et al., 2004; Liu et al., 2008). One recently emergent function of CDK5 is that it acts as a regulator of the endothelium during angiogenesis and EC migration, thus contributing to various pathological conditions. Therefore, CDK5 has been proposed as a novel target for anti-angiogenic therapy (Liebl et al., 2010). In *in vivo* and *in vitro*

models, such as neovascularization of the mouse cornea and endothelial tube formation, pharmacological inhibition of CDK5 resulted in reduced endothelial cell motility and angiogenesis, suggesting an effect independent of its previously characterized functions in neurons and a specific role of CDK5 signaling in the endothelium (Liebl et al., 2011). In contrast to its activity in neurons, the reduction in endothelial cell motility induced by CDK5 inhibition was not caused by altering the function of focal adhesions or microtubules, but rather by a reduction in lamellipodia formation (Liebl et al., 2010). CDK5 inhibition diminished Rac1 activity, leading to actin cytoskeleton disorganization, which suggests that CDK5 exerts its effects on endothelial cell migration via Rac1. Taken together, these findings indicate CDK5 as a pharmacologically accessible target for anti-angiogenic therapy and provide a basis for a new therapeutic strategy for cerebral ischemia.

One event that follows cerebral ischemia is the formation of new blood vessels. Recently, it has been shown that targeting p35/CDK5 using a specific peptide inhibitor (CIP) in a hypoxia model preserves cell motility and temporal control of actin cytoskeletal dynamics and protects and promotes angiogenesis (Bosutti et al., 2013). Although new vessels are the source of trophic factors during the early stages of brain parenchyma recovery, sustained angiogenesis becomes a physical barrier to neural circuit formation and connection refinement (Liebl et al., 2010). In addition, a previous study proposed roscovitine (an inhibitor of CDK5 and other CDKs) administration for the prevention of endothelial activation and the leukocyte-EC interaction during parenchymal leukocyte infiltration (Berberich et al., 2011). These studies could expand the use of CDK5 inhibition because of its promising anti-inflammatory effects, in addition to maintaining BBB integrity. Finally, CDK5 regulates eNOS enzyme activity. eNOS is an essential enzyme responsible for the production of endothelium-derived NO, which is a key molecule that performs multiple functions, including vascular homeostasis, angiogenesis, and cell cycle regulation (van Haperen et al., 2002; Desjardins and Balligand, 2006; Bonnin et al., 2012). CDK5 phosphorylates eNOS at Ser113 and Ser116, thus regulating nitric oxide (NO) levels (van Haperen et al., 2002; Desjardins and Balligand, 2006; Cho et al., 2010; Lee et al., 2010). eNOS deregulation contributes to the pathophysiology of multiple diseases, such as atherosclerosis, hypertension, and cancer (Desjardins and Balligand, 2006). It is well known that eNOS phosphorylation modulates its activity (Harris et al., 2001; Mount et al., 2007). Phosphorylation of eNOS by CDK5 is associated with decreased NO production (Cho et al., 2010). This mechanism may explain how NO is maintained at minimal levels at a basal state in ECs and how CDK5 contributes to the pathogenesis of diseases associated with reduced NO release from ECs. However, further studies are required to clarify these issues.

A few studies have shown that CDK5 and p35 are expressed in astrocytes in the adult brain. Additionally, it has been demonstrated that the activity of CDK5 and its activator p35 participate in the process of scratch-wound migration in a wound-healing model via the regulation of microtubules in primary astrocyte cultures (He et al., 2007). Likewise, increased CDK5 activity is involved in tau hyperphosphorylation, oxidative stress and the degeneration of astrocytes in a murine senescence model (García-Matas et al., 2008). Primarily, in GFAP/tau transgenic mice, a unique astrocytic tau pathology model, astrocytes have been identified as playing a role in the neurodegenerative pathology that is associated with CDK5 hyperphosphorylation (Forman et al., 2005). To date, there have been few findings involving CDK5 in astrocytic and endothelial function. Therefore, further studies of the possible role that this kinase might play in these cell types, as well as in other brain neurovascular cell types, namely microglia and oligodendrocytes, should be addressed.

REGULATION OF CELL ADHESION BY p120 CATENIN IN NVUs

The neural component of the NVU is partially regulated by a group of cell adhesion molecules. Specifically, the catenin/cadherin system interacts with and regulates the architecture of the actin cytoskeleton (Salinas and Price, 2005). Neuronal morphology and plasticity require a dynamic actin cytoskeleton (Dillon and Goda, 2005). p120 catenin, in particular, regulates synapse development and morphology via Rho GTPases, which control actin cytoskeleton dynamics (Elia et al., 2006). Simultaneously, CDK5, via the phosphorylation of δ-catenin (a p120 catenin family member), participates in the processes of synaptic transmission (Poore et al., 2010). Therefore, with respect to neurons, it is possible to suggest an association between CDK5 activity and modulation of p120 catenin that facilitates neural plasticity processes. However, the possible relationship between the functions of CDK5 and p120 catenin has not been explored in the endothelium. p120 catenin plays an essential role in the regulation of cadherins, and increased endothelial adhesion mediated by this protein (Anastasiadis and Reynolds, 2000) has been proposed to regulate the actin cytoskeleton via Rho GTPases (Anastasiadis, 2007). In addition, evidence indicates that the interaction between p120 catenin and cadherins promotes AJ stability (Xiao et al., 2003; Kowalczyk and Reynolds, 2004; Oas et al., 2010). Previous studies have shown that RNAi-mediated silencing of p120 catenin results in the loss of cadherin expression and subsequent destabilization of AJs in the microvasculature (Davis et al., 2003; Xiao et al., 2003). The binding of p120 catenin to VE-cadherin prevents clathrin-dependent endocytosis, as the cadherin-catenin system remains intact in the membrane. In addition, cytoplasmic p120 catenin that is dissociated from VE-cadherin acts as a regulator of Rho GTPase activity, which is involved in cell migration and morphological changes via pseudopodia and stress fiber formation (Anastasiadis and Reynolds, 2001). A recent study has suggested the fine regulation of endothelial cell adhesion by AJs, in which p120 catenin plays a critical role in regulating the distribution of cadherins in lipid rafts of endothelial membranes, which has consequences on the stabilization of cadherins and cell signaling (Gentil-Dit-Maurin et al., 2010). The finding that the deletion of p120 catenin from skin increases inflammation via RhoA-NF-κB has important implications for diseases in humans (Perez-Moreno et al., 2006). The NF-κB pathway is involved in cell survival, inflammation, and often tumor progression (Cozzolino et al., 2003; Perez-Moreno et al., 2006). Finally, these experimental findings, together with our results (Céspedes-Rubio et al., 2010), suggest p120 catenin as a critical biomarker of the recovery of the NVU after cerebral ischemia.

NEURON-TO-ASTROCYTE CONTROL OF NEUROVASCULAR COUPLING

Originally, astrocytes were considered supporting cells that play no role in neurotransmission in the CNS. However, studies have shown that astrocytes display a large conductance of potassium ions (K^+) during an action potential in presynaptic neurons. K^+ passes into the blood vessel via gap junctions for removal after a long period of neuronal activity (Massey et al., 2004; Wallraff et al., 2004; Jabs et al., 2005). Previous studies have shown that this passive role of astrocytes in the synapse changes over time, as hippocampal astrocytes in culture respond to chemical transmitters, such as glutamate (neurotransmitter), generating a Ca^{2+} wave that propagates to surrounding astrocytes (Leybaert et al., 1998; Cotrina et al., 2000). Initially, glutamate was shown as a propagator of waves in astrocytes. However, other investigations have suggested that norepinephrine, GABA, acetylcholine, histamine and adenosine induce Ca^{2+} elevation in glial cells derived from *ex vivo* preparations of adult brain slices (Duffy and Macvicar, 1995; Porter and McCarthy, 1995; Kulik et al., 1999; Shelton and McCarthy, 2000; Bowser and Khakh, 2004). Some of the signals in the neuron-to-astrocyte direction are mediated by metabotropic receptors, such as mGluR1/5. The activation of these receptors by glutamate released at synapses induces phospholipase C (PLC) activation, IP3 formation and, subsequently, IP3R activation, which facilitates the release of calcium into the cytosol from the ER (Kawabata et al., 1996; Wang et al., 2000). Calcium waves transmit signals between astrocytes, as this Ca^{2+} signal propagates to neighboring astrocytes via the diffusion or release of IP3 and the subsequent activation of purinergic P2Y receptors in neighboring astrocytes (Venance et al., 1997; Guthrie et al., 1999). Finally, the discovery of gliotransmitters (including glutamate, D-serine and ATP), chemicals released from astrocytes that affect the transmission between pre- and post-synaptic neurons, led to the concept of the tripartite synapse, which states that the activation of astrocytes associated with increased intracellular Ca^{2+} results in the direct activation of the neighboring neurons (Fiacco and Mccarthy, 2004; Gordon et al., 2005; Panatier et al., 2006). Neuronal activation by astrocytes is thought to occur via NMDA receptors that are primarily composed of NR2B subunits and is directly involved in neuronal plasticity (Fellin et al., 2004; Massey et al., 2004). Recently, studies using conditional knockout of CDK5 in the adult mouse brain showed improved performance in spatial learning tasks and enhanced hippocampal long-term potentiation via NR2B-mediated excitatory postsynaptic currents (Hawasli et al., 2007; Plattner et al., 2014). This finding, in association with the finding that gene silencing of CDK5 decreased neuropathologic characteristics of neurodegeneration, such as neurofibrillary tangles (Piedrahita et al., 2010), may suggest that the use of RNAi-mediated suppression of CDK5 expression in astrocytes promotes the tripartite synapse and recovery from cognitive impartment after stroke.

Concomitant with the function of astrocytes in the regulation of synapses, these cells directly regulate cerebral circulation, as they modulate the level of neural activity and, consequently, that of local microcirculation (Roy and Sherrington, 1890; Friedland and Iadecola, 1991; Pellerin and Magistretti, 1994). Sites of synaptic transmission that display high-energy demand during neuronal activity also display increased blood flow, which results in the rapid expansion of arterioles and capillaries (Lou et al., 1987; Magistretti et al., 1999; Kasischke et al., 2004). The propagation of calcium waves in astrocytes via glutamate receptors has been proposed to regulate blood flow during neuronal activity because these cells secrete vasoactive substances, such as NO, the product of iNOS, factors derived from the activity of cyclooxygenase (COX) and epoxygenase, such as prostaglandin E2 (PGE2), epoxyeicosatrienoic acid, and ATP, towards neurons, astrocytes, and ECs (Oomagari et al., 1991; Alkayed et al., 1997; Wiencken and Casagrande, 1999; Arcuino et al., 2002). Neuron-to-astrocyte activation via the activity of Ca^{2+}-sensitive phospholipase A2 and the accumulation of arachidonic acid can induce vasodilation or vasoconstriction through its metabolic pathways. COX-2-dependent accumulation of PGE2 leads to vasodilation, whereas diffusion of arachidonic acid into smooth muscle leads to the accumulation of 20-HETE, causing vasoconstriction (Bezzi et al., 1998; Harder et al., 2002). This phenomenon reflects neurovascular coupling and has been defined as vasomotion (Filosa et al., 2004), in which the microvascular hemodynamics are regulated by the energy and oxygen demand to an undersupplied organ, i.e., low O_2 conditions. This event triggers astrocytes to induce vasodilation in the endothelium to promote the entry of nutrients and O_2 (Ward et al., 2000; Brown et al., 2002; Lovick et al., 2005). This bifunctional role of astrocytes in neurovascular coupling and synaptic transmission implicates these cells as important targets for functional recovery of the NVU, including neurons, glia, and ECs, after cerebral ischemia.

ASTROCYTES AND MODULATION OF EXCITOTOXICITY

Astrocytes, the most abundant CNS cell population, are a type of glial cell that performs diverse functions, such as providing trophic and metabolic support to neurons (Rouach et al., 2008; Dienel, 2013), participating in differentiation, neuronal polarity, and synaptogenesis during development (new synapse formation) (Allen, 2013) and facilitating synaptic processes (Ullian et al., 2001; Allen and Barres, 2005; Kucukdereli et al., 2011). These functions render astrocytes as essential for the maintenance of brain homeostasis (Parpura and Verkhratsky, 2012) and neuronal survival (Sofroniew, 2005). In recent years, it has been determined that astrocytes participate in cerebral recovery following cell damage, particularly following damage caused by cerebrovascular diseases (CVD) or neurodegenerative disease. In such injuries, a common process that induces cell death is glutamate-mediated excitotoxicity (Matute et al., 2007; Guimarães et al., 2010). Astrocytes influence neuronal survival following brain injury via glutamate uptake, free radical removal, water transport, and cytokine and NO production. Glutamate uptake is a process in which astrocytes absorb extracellular glutamate via highly expressed glutamate transporters to maintain transmitter homeostasis (Rothstein et al., 1996; Bush et al., 1999; Swanson et al., 2004). Glutamate transporters play a major role in maintaining brain homeostasis, and the astrocytic excitatory amino acid transporters (EAATs) EAAT1 and EAAT2 are functionally dominant. Astrocytic EAATs play important roles in various neuropathologies in which astrocytes undergo cytoskeletal changes (Zagami et al., 2009; Lau et al., 2010). In addition, after brain

damage, astrocytes assist in the recovery of plasticity processes, such as long-term potentiation (LTP), via the expression of trophic and molecular factors on their surface that favor neuronal growth or regeneration and new synapse formation (Matute et al., 2007; Adelson et al., 2012; Lin et al., 2014; Wang et al., 2014).

Astrocytes respond to brain injury via a process called reactive astrogliosis (Sofroniew and Vinters, 2010), in which astrocytes change their morphology and metabolism, converting from a fibrillary or protoplasmic morphology to a hypertrophic or swollen structure, which is primarily characterized by the elevated expression of intermediate filaments and proteins related to metabolic acidity. Over time, this astroglial hyperreactivity process creates a scar that impedes NVU repair; thus, reactive astrogliosis is considered an important marker of structural injury (Di Giovanni et al., 2005; Buffo et al., 2010). In an NVU homeostasis context, astrocytes are star-shaped (fibrillar) and have many extensions irradiating from the soma towards neighboring cells, which facilitate their performance of functions involving neuronal migration, support, and maintenance (Zonta et al., 2003; Sofroniew, 2005; Wang and Bordey, 2008; Buffo et al., 2010). In contrast, in a pathological context, such as ischemia, astrocytes near the injured area change, acquiring a fibrous appearance and thick extensions (Xiong et al., 2011; Barreto et al., 2012). The morphological changes in astrocytes are primarily mediated by actin cytoskeleton remodeling (Hall, 1998; Hall and Nobes, 2000). Furthermore, this actin cytoskeleton repair system is mediated by Rho GTPases (Bustelo et al., 2007). Recently, it has been demonstrated that RhoA/ROCK activity induces a protoplasmic morphology in primary astrocyte cultures, as opposed to the function of Rac1, which favors stellation or the generation of new protrusions in astrocytes (Racchetti et al., 2012). In addition, ROCK plays a major role in determining the cell surface expression of EAAT1/2, providing evidence for an association between transporter function and astrocytic glutamate uptake. ROCK inhibitors elevate glutamate transporter function; this activity profile may contribute to their beneficial effects on neuropathologies (Lau et al., 2011). In contrast, the role of Rac1 in astrocytes is associated with not only their stellar morphology but also their survival. A study conducted using human glioma cells demonstrated that suppressing Rac1 activity induces apoptosis, in contrast to the results using primary astrocytes from humans (Senger et al., 2002). This evidence suggests that Rac1 regulates certain pro-survival pathways that are abnormally activated in this type of tumor. In addition, Rac activation by PI3K has been associated with PI3K/Akt activation, which promotes cell survival (Dey et al., 2008; Linseman and Loucks, 2008; Read and Gorman, 2009). Similarly, the Rac1 activator Tiam performs an important function in astrocyte polarization and migration during development or cell repair by affecting the organization of the microfilament and microtubule networks (Ellenbroek et al., 2012). Alternatively, cellular senescence is a tumor-suppressive process that is characterized by irreversible cell cycle exit, a unique pathway that is upregulated by CDK5 activation. The increased CDK5 activity further reduces GTPase Rac1 activity and Pak activation. The repression of GTPase Rac1 activity by CDK5 that is required for the expression of the senescent phenotype may suggest CDK5-mediated Rac1 suppression as a marker of cell death (Alexander et al., 2004).

Finally, filamentous tau aggregates have been detected in astrocytes in human disease and in animal models, and this process disturbs glutamate uptake and other astrocyte functions, leading to focal neurodegeneration (Forman et al., 2005; Dabir et al., 2006). GSK3β and Cdk5 kinase activity, which regulate tau phosphorylation, are also increased in astrocytes of the senescence-accelerated mice prone (SAMP8) model. Inhibition of GSK3β using lithium or inhibition of CDK5 using roscovitine has been shown to reduce tau phosphorylation at Ser396. Moreover, a reduced mitochondrial membrane potential in SAMP8 mouse astrocytes suppresses glutamate uptake in astrocytes, which is a critical neuroprotective mechanism (García-Matas et al., 2008).

A POSSIBLE THERAPEUTIC STRATEGY FOR NVU MODULATION

Our research group found that CDK5 gene silencing reduces histopathological markers associated with cognitive disorders (PHF-1), preventing the neurodegeneration and neuronal loss in AD mice (Piedrahita et al., 2010). We also found that Rho GTPases (Rac and RhoA) are involved in both damage and survival signaling mechanisms. For example, our recent studies show that inhibition of RhoA/ROCK using Y27632 blocks the cascade of neurodegeneration triggered by cerebral infarction, prevents the deregulation of kinases involved in neuronal cytoskeletal remodeling, such as CDK5, favors synaptic connectivity and protects against cognitive deterioration (Castro-Alvarez et al., 2011). Fasudil, Y27632, and other ROCK inhibitors have previously been proposed as potential treatments for atherosclerosis and vascular disease (Zhou et al., 2011). Complementarily, we found that Rac and synaptic adhesion proteins (p120 catenin and N-catenin) are critical for the recovery from and protection against brain infarction (Céspedes-Rubio et al., 2010; Gutiérrez-Vargas et al., 2010; Posada-Duque et al., 2013). Stroke and AD are associated with altered morphology or loss of dendritic spines (Fiala et al., 2002; Brown et al., 2008; Li and Murphy, 2008). At the postsynaptic terminal of an excitatory glutamatergic synapse in the mouse hippocampus, CDK5 phosphorylates the N-terminal domain of PSD95 to regulate the synaptic recruitment and clustering of ion channels, particularly K^+ channels and NMDA receptors (NMDARs; Fu et al., 2001; Morabito et al., 2004; Hawasli et al., 2007). The proteins identified in the postsynaptic density include cell surface receptors, cytoplasmic signaling enzymes, and cytoskeletal and scaffold proteins. These proteins include the Rho GTPases (Jordan et al., 2004; Peng et al., 2004) RhoA, Rac1, and Cdc42, which regulate the actin cytoskeleton. Actin is the primary cytoskeletal component of dendritic spines. Accordingly, the Rho family of GTPases has been implicated in the regulation of dendritic spine morphogenesis (Govek et al., 2005; Tada and Sheng, 2006). In particular, RhoA activation exerts a strong inhibitory effect on spine morphogenesis and causes a profound loss of spines, in contrast to Rac and Cdc42 activity, which promote dendritic spine morphogenesis (Govek et al., 2005). p120ctn, a member of the cadherin/catenin system, regulates Rho GTPases to modulate actin cytoskeletal remodeling during dendritic spine formation (Togashi et al., 2002; Elia et al., 2006; Kwiatkowski

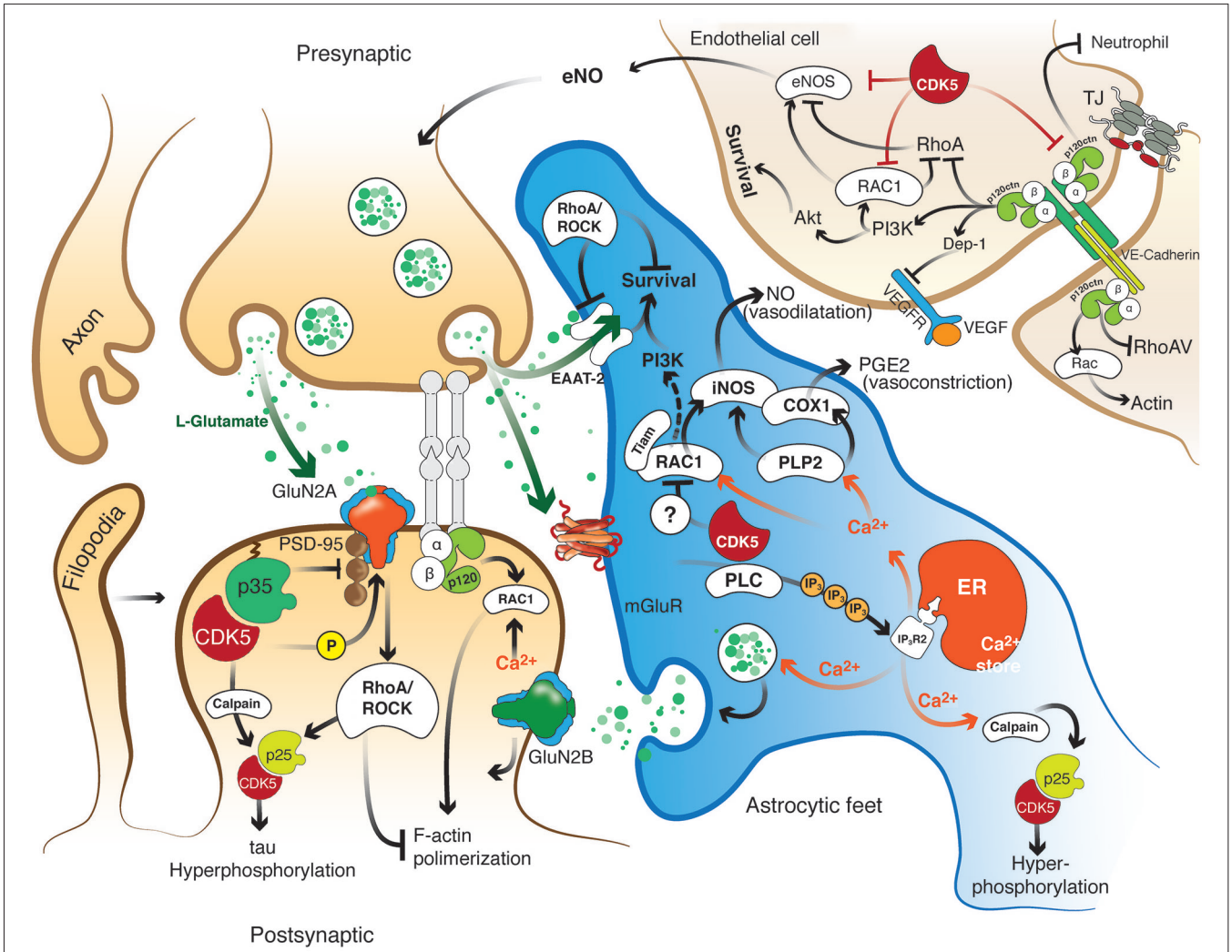

FIGURE 2 | Hypothetical model of the role of the effectors Rho GTPase, p120 catenin, and CDK5 in the recovery of the structure and function of the NVU after cerebral ischemia. CDK5 and RhoA/ROCK targeted to ECs, astrocytes and neurons may exert protection against excitotoxicity and promote neuronal plasticity and adhesion integrity. Reduction of CDK5 expression in neurons and astrocytes may facilitate synaptic plasticity, which requires NR2B for LTP and Rac1 and p120 catenin signaling for dendritic spine morphogenesis and synaptic adhesion. Decreased expression of CDK5 induces LTP via NR2B (GluN2B)-mediated excitatory postsynaptic currents, and the consequent induction or accumulation of p120ctn inhibits RhoA/ROCK, preventing F-actin retraction and inducing Rac1 activation, thus facilitating neurotransmission. In addition, CDK5 inhibition may prevent tau hyperphosphorylation and facilitate glutamate uptake in astrocytes, preserve actin remodeling and promote angiogenesis via eNOS activation. In turn, ROCK inhibition also prevents tau hyperphosphorylation and dendritic spine retraction and increases the expression of EAATs to enhance glutamate uptake and promote BBB integrity. Moreover, actin remodeling induced by Tiam/Rac1 favors stellation and survival in astrocytes. Likewise, the signaling molecules VE-cadherin and p120 catenin activate Rac/PI3 kinase and suppress RhoA to maintain the survival and organization of the actin cytoskeleton, form complexes with VEGFR-2 to promote BBB adhesion and integrity, and prevent neutrophil diapedesis. ROCK and CDK5 targeting may promote calcium wave stimulation via glutamate receptor and PLC/IP3 signaling in astrocytes to facilitate NO and PGE2 release, thus regulating endothelial vasodilatation and vasoconstriction.

et al., 2007). p120ctn also regulates spine length via Rho GTPases, whereas the control of head width requires interactions with cadherin (Elia et al., 2006; Lee et al., 2008; Ishiyama et al., 2010). In addition, the postsynaptic membrane contains a high concentration of NMDARs and associated signaling proteins, which are assembled via scaffolding proteins into the postsynaptic density. Excitotoxicity contributes to the pathogenesis of stroke, and synaptic dysfunction represents an early step in the pathology of dementia (Kamenetz et al., 2003; Walsh and Selkoe, 2004). Therefore, based on the aforementioned results and in an effort to find solutions to health issues, such as cerebral infarction, these studies suggest that Rho GTPases, p120 catenin, and CDK5 are primary effectors that function in coordination in the recovery of the structure and function of the NVU after cerebral ischemic infarction in a cell type-specific manner (**Figure 2**). These targets act in various neuroprotective contexts, such as

AD, post-ischemic and excitotoxicity murine models. Treatment with CDK5 RNAi, roscovitine or atorvastatin improves motor, cognitive and sensory function in the post-ischemic brain and triggers neuronal plasticity and survival. The intrinsic plasticity recovery after stroke, in accordance with the results using injury models in our previous studies, may suggest that the regulation of Rho GTPases are associated with actin cytoskeletal remodeling; p120ctn and synaptic adhesion proteins expression, together with β-catenin and N-catenin, at post-synapses improve neurotransmission. Therefore, future efforts must focus on individually targeting the structural and functional roles of each effector and the interactions in neural (astrocytes, microglia, and neurons) and non-neural cells (pericytes and ECs) in the post-ischemic brain and address how to promote the recovery or prevent the loss of homeostasis in the short, medium and long term.

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The pivotal role of astrocytes in an *in vitro* stroke model of the blood-brain barrier

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Stabilization of the blood-brain barrier during and after stroke can lead to less adverse outcome. For elucidation of underlying mechanisms and development of novel therapeutic strategies validated *in vitro* disease models of the blood-brain barrier could be very helpful. To mimic *in vitro* stroke conditions we have established a blood-brain barrier *in vitro* model based on mouse cell line cerebEND and applied oxygen/glucose deprivation (OGD). The role of astrocytes in this disease model was investigated by using cell line C6. Transwell studies pointed out that addition of astrocytes during OGD increased the barrier damage significantly in comparison to the endothelial monoculture shown by changes of transendothelial electrical resistance as well as fluorescein permeability data. Analysis on mRNA and protein levels by qPCR, western blotting and immunofluorescence microscopy of tight junction molecules claudin-3,-5,-12, occludin and ZO-1 revealed that their regulation and localisation is associated with the functional barrier breakdown. Furthermore, soluble factors of astrocytes, OGD and their combination were able to induce changes of functionality and expression of ABC-transporters Abcb1a (P-gp), Abcg2 (bcrp), and Abcc4 (mrp4). Moreover, the expression of proteases (matrixmetalloproteinases MMP-2, MMP-3, MMP-9, and t-PA) as well as of their endogenous inhibitors (TIMP-1, TIMP-3, PAI-1) was altered by astrocyte factors and OGD which resulted in significant changes of total MMP and t-PA activity. Morphological rearrangements induced by OGD and treatment with astrocyte factors were confirmed at a nanometer scale using atomic force microscopy. In conclusion, astrocytes play a major role in blood-brain barrier breakdown during OGD *in vitro*.

Keywords: blood-brain barrier, *in vitro*, stroke, oxygen/glucose deprivation, ischemia, traumatic brain injury, cerebEND, C6

INTRODUCTION

Impairment of the blood-brain barrier (BBB) leads to vasogenic edema and subsequent brain damage after acute cerebral ischemic insults such as stroke or traumatic brain injury. Reduced permeability of the BBB is associated with decreased harm and improved outcome after stroke (Yang and Rosenberg, 2011). Consequently, elucidation of underlying mechanisms causing BBB breakdown may lead to novel targets for future treatments to reduce adverse stroke outcomes. The BBB itself is considered as the most important interface between blood circulation and the central nervous system (CNS). Physiological roles of the BBB are to maintain the homoestasis in the CNS, to prevent the passage of bacteria, viruses and unwelcomed substances into the CNS and to efflux CNS waste products. In the 1960's Reese and Brightman found out by electron microscopy that the brain capillary endothelium is the main component of the BBB, which is sealed by intercellular tight junctions. In addition to the presence of tight junctions further major differences of the brain capillary endothelium in comparison to the peripheral endothelium is the lack of fenestrae and reduced pinocytotic activity (Brightman and Reese, 1969; Joo, 1996). During ischemia several key properties of the BBB are changed. The physical barrier is disrupted which results in increased permeability for marker molecules of the paracellular route such as albumin bound Evans blue, fluorescent labeled dextrans, fluorescein, mannitol, and sucrose. This loss of barrier function against hydrophilic compounds was associated with alterations within the tight junctional complex reflected by decreased expression or delocalisation of tight junction proteins such as occludin (András et al., 2007; Kleinschnitz et al., 2011). Furthermore, changed expression and functionality of ATP-binding cassette (ABC)-transporter proteins such as P-glycoprotein (P-gp or Abcb1) and breast cancer resistance protein (bcrp or Abcg2) were reported under ischemic or hypoxic conditions. One of the consequences would be an altered transport barrier accompanied with a loss of the protective function against a wide array of xenobiotics. In addition, the activity of proteases such as matrixmetalloproteinases (MMPs) and tissue-plasminogen activator (t-PA) is increased during stroke that was

shown to be associated with degradation of the basal lamina and integrity loss of the BBB (reviewed in Jin et al., 2010 and Patak and Hermann, 2011).

Nowadays, the BBB is understood as a complex regulated system. Terms such as neuro- or gliavascular unit (NVU, GVU) describe the strong influence of the microenvironment on the brain endothelium. Neighboring cell types such as astrocytes, pericytes, microglia or even neurons are known to influence the functionality of the BBB in health as well as in disease, which is supported by their physical proximity and consequent small diffusion distances for signaling molecules (Abbott et al., 2006; Cecchelli et al., 2007). During ischemia the undersupply with glucose and oxygen causes reduced ATP production and loss of available energy at the NVU. This contributes significantly to the breakdown of the BBB (Ronaldson and Davis, 2012). Therefore, application of oxygen/glucose deprivation (OGD) on CNS cells *in vitro* is an established model for stroke. Recent studies showed that BBB *in vitro* models based on brain endothelial cells co- or even triple-cultured with astrocytes and/or pericytes are able to reflect the physiology of the BBB in a more accurate manner (Török et al., 2003; Nakagawa et al., 2009; Ceruti et al., 2011). In this context, in case of *in vitro* stroke models of the BBB, lately reports confirmed that astrocytes aggravated the breakdown of the physical barrier (Mysiorek et al., 2009). However, underlying mechanisms were not described in detail and the need to investigate and understand them seemed to be essential for validation purposes. In previous studies, we have applied and optimized oxygen/glucose deprivation (OGD) conditions for brain endothelial mono-cultures to mimick stroke *in vitro* and to study molecular mechanisms lying behind the successful, functional stabilization of the BBB *in vivo* during stroke or traumatic brain injury (Kleinschnitz et al., 2011; Neuhaus et al., 2012a; Thal et al., 2013). The aim of the present study was to extend our stroke *in vitro* models of the BBB with astrocytes by co-cultivation of mouse BBB cell line cerebEND with rat cell line C6 and to investigate the influence of astrocytes under OGD-conditions on several BBB relevant parameters such as functionality of the physical as well as transport barrier, associated tight junction molecule and Abc-transporter expression, expression and functionality of MMPs, t-PA and of their endogenous inhibitors, and last but not least whether morphological changes were detectable via atomic force microscopy.

MATERIALS AND METHODS

MATERIALS

Collagen IV from human placenta (C5533), PBS (D8537), Triton-X 100 (T8787), DMEM (D5796), Calcein-AM (17783), DAPI (D8417), db-cAMP (D0260), MK571 (M7571), Ko143 (K2144), verapamil.HCl (V4629), β-mercaptoethanol (M6250), fluorescein sodium (F6377), albumin from bovine serum for immunofluorescence microscopy (fraction V, A9647) and for western blotting (A7906) were purchased from Sigma-Aldrich. Bodipy-FL-prazosin (B-7433), DMEM without glucose (11966-025, Gibco®) was obtained from Life technologies (USA), and fluo-cAMP (F002-01) was from Biolog (Bremen, Germany). FCS Gold EU approved was bought from PAA Laboratories (A15151, Lot A15111-2018, Linz, Austria) and was heat-inactivated in a water-bath at 56°C for 30 min. Penicillin/streptomycin (100X, 10,000 Units/mL, 10,000 µg/mL, A2213) and 0.05% Tyrpsin/0.02% EDTA-solution (L2143) were from BioChrom AG (Berlin, Germany). 6-well, 12-well and 24-well plates and 24-well Transwell® inserts (0.4 µm pore size, PET) were obtained from Becton and Dickinson (REF353046, REF353043, REF353095, REF353226, USA). Gelatine was from SERVA (22151, Heidelberg, Germany), nuclease-free water was purchased from Ambion (AM9937, USA). All other substances were of analytical grade.

CELL CULTURE

Mouse brain endothelial cell line cerebEND was produced from isolated brain microvascular endothelial cells from cerebellum by Silwedel and Förster (2006). cerebENDs were cultured in DMEM medium supplemented with 10% FCS and 1% penicillin/streptomycin in 0.5% gelatine coated cell culture tissue flasks and were subcultivated by trypsination in a ratio of 1:3 once a week as published recently (Neuhaus et al., 2012a). Rat glioma cell line C6 was obtained from ATCC and cultured with the same medium as cerebENDs in 0.5% gelatine coated cell culture tissue flasks. Subcultivation was accomplished in a ratio of 1:20 once a week. Cells were maintained in an incubator at 37°C, 95% humidity and a 5% CO2/95% air atmosphere.

TRANSWELL EXPERIMENTS

24-well plate inserts were coated with 100 µg/mL collagen IV (dissolved in acetic acid according to the manufacturer's instruction) at RT for 2 h and were washed with PBS for three times to get rid off the residual acetic acid. CerebENDs were seeded at a density of 40,000 cells/cm^2 on the 24-well inserts, and every other day growth medium was renewed. On day 7 after seeding apical growth medium was supplemented with 100 nM hydrocortisone (100 µM stock dissolved in ethanol) and C6 cells were seeded at a density of 20,000 cells/cm^2 in 0.5% gelatine coated 24-well plates. On day 9 inserts with cerebENDs were placed over C6 cell containing well-plates to form the co-culture set-up still with 100 nM hydrocortisone in the apical chamber. The Transwell insert cultivation procedure was summarized in Figure 1S. Oxygen/glucose deprivation (OGD) experiments were conducted on day 13. cerebEND cells in inserts and C6 cells in well-plates were washed with PBS twice before they were reesembled and serum-free DMEM with or without glucose was added. Transendothelial electrical resistance (TEER) was measured using chopstick electrodes from Millipore after 30 min of equibrilation at RT as previously published (Neuhaus et al., 2006). To apply OGD, cells in serum-free DMEM without glucose were incubated in a hypoxia incubator (HERACELL 150i, ThermoFisher, USA) with 1% O_2, 5% CO_2, saturated humidity atmosphere and 37°C for 4 h. As controls, blank inserts without cells, mono-cultured cerebEND cells and cerebENDs co-cultured with C6 cells were incubated in a normoxia incubator at air O_2, 5% CO_2, saturated humidity atmosphere and 37°C for the same time. After incubation TEER was measured again after a 30 min temperature equibrilation phase. To study fluorescein permeability of cell layers, apical experimental medium was exchanged with 300 µL DMEM with or without glucose containing 10 µM fluorescein and transport study was accomplished for 1 h at 37°C under normoxic conditions. DMEM

with or without glucose as blank solutions, basolateral samples and residual apical stock solutions were measured in 100 µL triplicates in black 96-well plates (GreinerBioOne, Kremsmünster, Austria) with a fluorescence microplate reader (GeniosPro, Tecan, Austria) at 485/535 nm (excitation/emission wavelength). TEER in Ohm/cm^2 and permeability coefficients including blank insert values were calculated as previously published (Neuhaus et al., 2008; Novakova et al., 2014). Due to comparison reasons, values of normoxic mono-cultured cerebEND controls were set to 100%.

QUANTITATIVE REVERSE-TRANSCRIPTASE POLYMERASE CHAIN REACTION (qPCR)

For qPCR experiments, cerebEND and C6 cells were seeded in gelatine (0.5%, Serva, Germany) coated 6-well plates at a density of 40,000 cells/cm^2 or 20,000 cells/cm^2, respectively. Every other day growth medium was renewed. On day 6 after seeding, cells were washed with PBS twice, and 3 mL of serum-free DMEM with or without glucose was added, and normoxia or OGD treatment was applied for 4 h as described above. Four different kinds of treatments were conducted: N = normoxia, OGD = oxygen/glucose deprivation, N-C6 = normoxia of cerebEND cells with supernatant of normoxic treated C6 cells and OGD-C6 = oxygen/glucose deprivation of cerebEND cells with supernatant of OGD-treated C6 cells. In case of N-C6 and OGD-C6 treatments, C6 cells were incubated for 4 h normoxia or OGD to obtain their supernatants which were directly applied on PBS washed cerebEND cell layers to guarentee fresh soluble factors of C6 cells for further 4 h incubation. After N, N-C6, OGD or OGD-C6 treatment of cerebEND cells experimental media were removed and lysed in RA1 buffer (supplemented with 1% β-mercaptoethanol shortly before usage) of the Nucleospin-RNAII Kit (Macherey Nagel, Düren, Germany). Total RNA was isolated using the Nucleospin-RNAII Kit according to the manufacturer's instruction as described before in Neuhaus et al. (2012b). RNA concentrations were determined by means of a Nanodrop ND 2000 spectrophotometer (FisherScientific, Schwerte, Germany) at 260/280 nm. 1 µg total RNA per sample were reversely transcribed to 20 µL cDNA by means of the high capacity cDNA-kit (with random primer and RNAse inhibitor) from Applied Biosystems (Life Technologies GmbH, Darmstadt, Germany) according to the manufacturer's instruction. qPCR analysis were performed using FAM-labeled probes for all investigated targets (Taqman®, Applied Biosystems) as recently reported (Neuhaus et al., 2012b). A detailed list with the product numbers of Taqman®-probes could be found in the Supplementary part (Table 1S). Each sample was analyzed as triplicate. Relative mRNA abundances to β-actin were calculated by the ddCt method using following formula: $2^{(\text{Ct of β-actin-Ct of gene of interest})}$, where Ct is the threshold cycle value.

WESTERN BLOTTING

Cells were cultured as described above under Section Quantitative Reverse-Transcriptase Polymerase Chain Reaction (qPCR) and scraped after the treatments in 50 µL RIPA buffer per 6-well [50 mM TRIS pH 8; 150 mM NaCl, 0.1% SDS, 0.5% sodium-deoxycholate, 1% NP40 supplemented with one complete ULTRA protease inhibitor cocktail and PhosphoSTOP minitablet per 10 mL (complete ULTRA tablets, Mini, EASYpack, REF05892970001; PhosSTOP, REF04906837001, Roche Applied Science, Mannheim, Germany)] on ice after washing with ice-cold PBS twice as described previously (Neuhaus et al., 2012b). In case of membran protein enrichment, proteins were extracted with 1% Triton-X 100 in PBS (supplemented with one complete ULTRA protease inhibitor cocktail and PhosphoSTOP minitablet per 10 mL) gentle shaking at 4°C for 30 min after washing with ice-cold PBS on ice twice. Sample's protein concentrations were determined by a detergent-compatible Pierce BCA assay (FisherScientific) utilizing a BSA standard curve (Albumin Standard, FisherScientific). Before storage at −80°C 4x Laemmli buffer (8% SDS, 40% glycerol, 0.004% bromphenolic blue, 0.25 M Tris-HCl supplemented with 6% β-mercaptoethanol shortly before usage) was added to the samples. 20 µg protein of total RIPA-cell lysates and 15 µg of Triton-X 100 fractions per lane and peqGOLD prestained protein marker V (PEQLAB) were loaded onto 7.5, 10 or 12% SDS-PAGE gels (1.5 mm thick) after ultrasound treatment and 5 min denaturation at 70°C. After gel electrophoresis at 130V, proteins were immunoblotted onto polyvinylidene difluoride membranes (162–0177, Biorad, München, Germany) by a tank blotter at 40 mA per gel at 4°C overnight. Incubations with primary and secondary antibodies were carried out as previously described (Neuhaus et al., 2012b). Used primary and secondary antibodies are listed in the Supplementary Table 2S. To visualize the bands, western blots were incubated with ECL-solutions for 3 min and were developed using a FluorChem FC2 Multiimager II (Alpha Innotech, Hessisch Oldendorf, Germany). Density values of single protein bands were calculated with the software Alpha View and were related to the corresponding β-actin bands. In some cases, antibodies onto western blots were stripped by washing with H_2O dest. for 5 min followed by a treatment with 0.2 M NaOH for 5 min and a second washing step with H_2O dest. for 5 min. After an additional washing step with PBS-T, membranes were blocked with 5% milk powder before reprobing with the next primary antibody. In case of strong antibody-protein binding, antibodies were stripped by washing 10 min with PBS-T for three times and incubated with a 100 mM ß-mercaptoethanol containing buffer (62.5 mM Tris-HCl $pH = 6.8$, 2% SDS, freshly added ß-mercaptoethanol) at 56°C and gentle shaking for 20 min followed by three washing steps with PBS-T each for 10 min. Finally, membranes were blocked with 5% milk powder as before.

IMMUNOFLUORESCENCE MICROSCOPY

Immunofluorescence microscopy was carried out as published recently (Neuhaus et al., 2008, 2012b). In brief, cerebEND cell layers (cultured as explained above on 15 mm, with ethanol disinfected, PBS washed and collagen IV coated glas slides in 12-well plates) were washed for three times with PBS and fixed and permeabilized with precooled MeOH at −20°C for 20 min. After washing with PBS twice, cells were rehydrated with PBS at RT for 15 min. In case of staining with the mouse anti-occludin antibody, cells were fixed with ice-cold ethanol incubated at −20°C for 20 min followed by −20°C cold acetone at room temperature for 3 min and drying after acetone removal at room

temperature for 10 min. Then, cell layers were incubated with primary antibody solutions (see Supplementary Table 2S) at 37°C for 1 h. After washing for three times with PBS cell layers were incubated with 1:100 1% BSA/PBS solutions with the secondary antibodies (see Supplementary Table 2S) at 37°C for 30 min. For nuclei staining an additional incubation step of 10 min at 37°C with 1:3000 of 5 mg/mL DAPI in PBS was carried out. After washing with PBS for three times, cover slips were transferred to glas slides and were embedded in Vectashield Hard-Set mounting medium (Vector Laboratories LTD., Peterborough, United Kingdom). Images were generated by using an Olympus BX51 microscopy system using UPlan FLN objectives (4x/0.13/∞/-/FN26.5; 10x/0.30//∞/-/FN26.5; 20x/0.50/∞/0.17/FN26.5; 40x/0.75/∞/0.17/FN26.5; 60x/0.90/0.11-0.23/FN26.5) equipped with U-RFL-T laser and controlled by the software cellSense Dimension.

UPTAKE ASSAYS

cerebEND cells were seeded in 200 μL growth medium at a density of 40,000 cells/cm^2 in clear, gelatine (0.5%) coated 96-well plates (GreinerBioOne, Kremsmünster, Austria). On day 6 after seeding, cells were washed with 200 μL prewarmed HBSS per 96-well twice and incubated in 100 μL appropriate medium for 4 h. For normoxia (N) serum-free DMEM containing glucose, for OGD serum-free DMEM without glucose, for N-C6 sterile filtered, serum-free DMEM with glucose preincubated on washed C6 cell layers over night and for OGD-C6 sterile filtered, serum-free DMEM without glucose preincubated on washed C6 cell layers under OGD conditions over night were used. In case of OGD and OGD-C6 with cerebEND cells, medium was changed after 4 h of incubation in the hypoxia chamber to normoxic or N-C6 medium (i.e., serum-free DMEM with glucose or sterile-filtered, serum-free DMEM with glucose preincubated with C6 cells over night) for further 20 h in the normxia chamber to simulate reoxygenation/renutrition. After 24 h of incubation in total, medium in selected wells was changed with 100 μL appropriate experimental media containing specific ABC-transporter inhibitors [Abcb1: 100 μM verapamil (100 mM stock in DMSO), Abcc4: 10 μM MK571 (10 mM stock in DMSO), 100–500 μM db-cAMP (100 mM stock in H_2O), Abcg2: 5 μM Ko143 (5 mM stock in DMSO)] for 15–30 min preincubation at 37°C. After that, uptake of specific substrates (end concentrations: 1 μM calcein-AM for Abcb1, 10 μM fluo-cAMP for Abcc4 (Reichel et al., 2010), 0.5 μM Bodipy-FL-prazosin for Abcg2) was started by addition of 100 μL of appropriate experimental media containing 2-fold substrate concentrations and in case additionally one-fold inhibitor concentration. Stock solutions of substrates (1 mM Calcein-AM in DMSO, 2 mM fluo-cAMP in H_2O, 500 μM Bodipy-FL-prazosin in DMSO) were thawed in darkness shortly before addition to ice-cold experimental media prepared in darkness to prevent degradation of light-sensitive substrates. After 45 (Abcb1), 90 (Abcc4) or 120 (Abcg2) minutes of incubation at 37°C, cells were washed on ice with 200 μL/well ice-cold HBSS for three times and lysed with 1% Triton-X 100 solution (in HBSS) for at least 1 h during gently shaking in darkness. Then, fluorescence of uptaken substrates was measured at 485/535 nm excitation/emission wavelength by means of a fluorescence microplate reader (GeniosPro, Tecan, Austria), substracted by background fluorescence of cells and related to their protein concentration determined by BCA method as described above in Section Western Blotting. Control wells after 24 h of normoxia were set to 100% and effects of added inhibitors under each condition were calculated. Changes of efflux functionality were presented as differences in inhibitor dependent uptake percentages in order to minimize the influence of other unkown, possibly regulated transporters on the total uptake of the used substrates. Prelimenary tests with used transporter inhibitors verapamil (Abcb1), MK571 (Abcc4) and Ko143 (Abcg2) confirmed their specificity for investigated transporters in cerebEND cells (see Supplementary Figure 2S).

MEASUREMENT OF ENZYME ACTIVITIES

To measure enzyme activity of matrixmetalloproteinases (MMP) or of tissue plasminogen activator (t-PA), medium supernatants of cerebENDs after N, N-C6, OGD or OGD-C6 treatments or C6 cells after N or OGD treatment were collected and stored at −80°C until measurement. 6 mL medium pooled from two six-wells were concentrated to 200 μL by centrifugation at 4000 g and 4°C for 40 min using 10 kDa Amicon ultrafiltration tubes (UFC901024, Millipore, Germany). In case of MMP activity, 80 μL of retentates were mixed with 50 μL of 1:10 diluted MMP substrate with assay buffer (520 MMP FRET substrate SB-14, 100 μM stock dissolved in 10% DMSO in assay buffer, 60581-01, Anaspec, USA) in a black 96-well microplate (GreinerBioOne, Kremsmünster, Austria). The MMP substrate is sensitive to detect activity of MMP-1, -2, -3, -7, -8, -9, -12, and -13. Increasing fluorescence was recorded over 120 min at 37°C and 485/535 nm in a microplate reader (GeniosPro, Tecan, Austria). Control blank values with pure DMEM were substracted from MMPs containing media and slopes between 10 and 60 min were calculated after linear regression analysis. Values were corrected for minor deviations of obtained retentat volumina after the centrifugation before. Slopes of media supernatants of cerebEND cells treated for 4 h of normoxia (N) were set to 100%. In case of t-PA activity, 80 μL of retentats were mixed with 50 μL of 1:100 diluted t-PA substrate with assay buffer (component A of Sensolyte AMC t-PA activity assay kit, 72160, Anaspec, USA) in a black 96-well microplate and fluorescence was recorded over 120 min at 37°C and 360/460 nm in a microplate reader. Control blank values with pure DMEM were substracted from t-PA containing media and slopes between 10 and 120 min were calculated after linear regression analysis. Values were corrected for minor deviations of obtained retentat volumina after the centrifugation before. Slopes of media supernatants of cerebEND cells treated for 4 h of normoxia (N) were set to 100%.

SURFACE NANO-TEXTURE ANALYSIS (nAnostic)

Contact mode Atomic force microscopy (AFM) on cultivated cells was performed as described before (Jungmann et al., 2008). In this study, cells were chemically stabilized by glutardialdehyde fixation (1% final concentration). Briefly, AFM measurements were carried out in PBS-buffered solution (pH 7.4) using a Multimode AFM equipped with Nanoscope III controller and software version 5.30sr3 (Digital Instruments, Santa Barbara, CA, USA). Silicon-nitride tips on V-shaped gold-coated cantilevers were

used (0.01 N/m, MLCT, VEECO, Mannheim, Germany). Imaging was performed at ambient temperature with forces less than 1 nN at 1–3 scan lines per second (1–3 Hz) with 512*512 pixels resolution. For texture analysis, subcellular scan areas of $(20\,\mu m)^2$ are recorded. Topographical data of the cell surfaces were analyzed using the nAnostic™-method applying custom-built, proprietary algorithms (Serend-ip GmbH, Münster, Germany). The method principle has been described before (Thoelking et al., 2010). Briefly, each nanostructure protruding from the mean surface level is morphometrically evaluated. Then, they are filtered by their size and shape through computer vision; here, only structures of positive local deviational volume (LDV), with LDV less than 1 cubed µm (10^3 nm height, 10^3nm*10^3nm area) were considered. The average volume of thousands of objects analyzed per sample was 0.0021 μm^3. Values are given for the count of such objects (per 20 µm image). Ten $(20\,\mu m)^2$ areas per slide were arbitrarily chosen and evaluated from two independent experiments. To comply with inbuilt biological heterogeneity the number of analyzed areas was set to $n = 20$ for statistical significance.

RESULTS

ASTROCYTES INCREASE OGD-INDUCED DAMAGE OF THE BLOOD-BRAIN BARRIER

Based on preliminary experiments and literature data of OGD-treated murine brain endothelial cells (Kleinschnitz et al., 2011), it was decided to concentrate on 4 h OGD-treatments with 1% O_2 in glucose-, and serum-free medium. Prior studies showed that cerebENDs reacted on 4 h OGD-treatment with significant upregulation of HIF1α (1.5-fold) and VEGFa (11-14-fold) (Supplementary Figure 3S, further targets angiopoietin-2, caveolin-1, Lrp-1, neuropilin-1 and Tie-2 see Supplementary Table 3S), while cell viability remained unaffected (Neuhaus et al., 2012a). Differences of electrical resistance raw data between cell and blank inserts in serum-free media before OGD-treatment were in average about 80–82 Ω for mono-cultured and 62–63 Ω for cerebENDs co-cultured with C6 cells resulting in calculated TEER values in average of 250 Ω/cm^2 or 200 Ω/cm^2, respectively. Measuring changes of TEER before and after OGD-treatment revealed no significant decrease of TEER of mono-cultured cerebEND layers [100 ± 12% normoxia ($n = 42$) vs. 98 ± 16% OGD ($n = 11$)] (**Figure 1A**). On the contrary, comparison of cerebENDs co-cultivated with C6 cells showed a significant increase in TEER to 117 ± 20% ($p < 0.05$, $n = 24$) after 4 h serum-free normoxia and a significant decrease to 61 ± 23% ($p < 0.05$, $n = 59$) after 4 h OGD treatment. Data of fluorescein permeability studies under normoxic conditions after TEER measurement—equivalent with a 1 h reoxygenation phase—confirmed the deleterious effects of C6 cells on tightness of cerebEND cell layers during OGD, but also uncovered harmful effects of OGD on mono-cultured cerebENDs (**Figure 1B**). In detail, fluorescein permeability was significantly increased by OGD to 152 ± 31% ($p < 0.05$, $n = 11$) in comparison to normoxic controls (100 ± 20%, $n = 31$). In case of C6 co-cultered cerebENDs, fluorescein permeability was 131 ± 21% ($n = 18$) under normoxic conditions in comparison to the normoxic mono-cultured cerebEND layers and was significantly elevated to 242 ± 61% by OGD treatment ($p < 0.05$, $n = 50$).

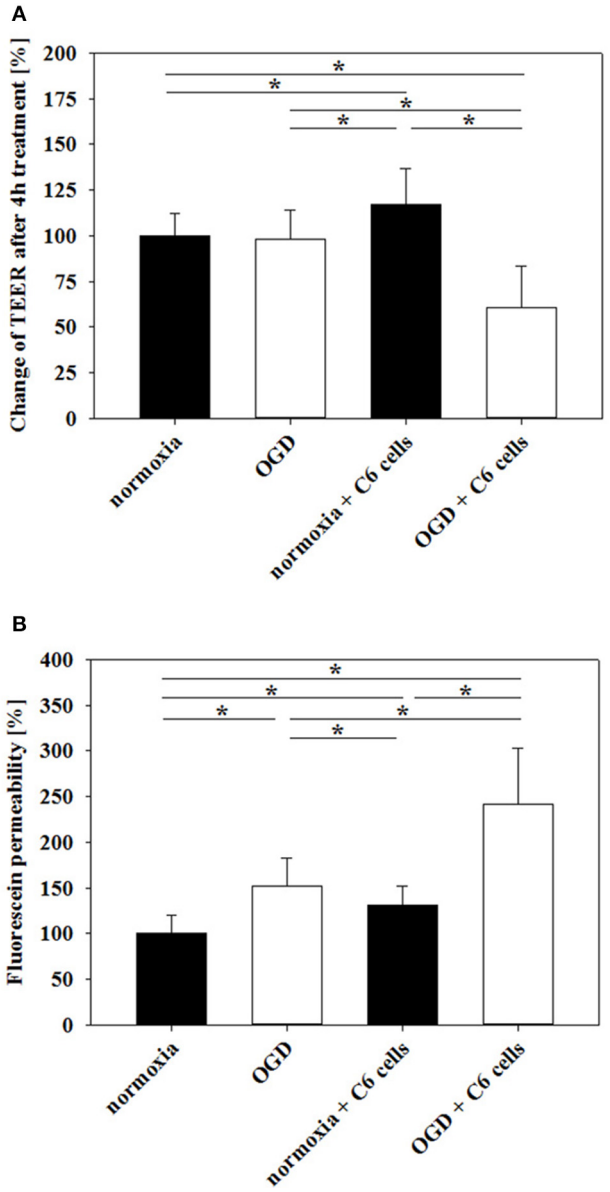

FIGURE 1 | Influence of 4 h oxygen/glucose deprivation (OGD) treatment on barrier functionality of mono-cultured cerebEND layers or co-cultured with astrocytes (C6) on Transwell inserts measured by TEER (A) or fluorescein permeability (B). Statistical significance was labeled with *($p < 0.05$, two-sided student's *t*-test with same variances). Data are presented as means ± *SD* ($n = 11$–59).

INFLUENCE OF ASTROCYTES AND OGD ON EXPRESSION OF TIGHT JUNCTION PROTEINS

First, influence of astrocytes and OGD on mRNA levels of claudin-3, claudin-5, claudin-12, occludin and ZO-1 was measured by qPCR (**Table 1**). Claudin-3 was only downregulated by addition of astrocyte factors (73%, $p < 0.05$). In case of claudin-5, it was significantly downregulated by OGD and OGD-C6 to

Table 1 | Influence of astrocytes and OGD on mRNA expression of tight junction molecules of cerebEND cells.

	Normoxia	OGD	N-C6	OGD-C6
Claudin-3	1.00 ± 0.06	1.01 ± 0.11 §	0.73 ± 0.05*#	0.82 ± 0.07
Claudin-5	1.00 ± 0.01	0.65 ± 0.08*§	1.04 ± 0.04#	0.55 ± 0.05*§
Claudin-12	1.00 ± 0.05	1.05 ± 0.05	1.02 ± 0.09	1.17 ± 0.09
Occludin	1.00 ± 0.02	0.38 ± 0.04*§	1.08 ± 0.11#	0.24 ± 0.02*#§
ZO-1	1.00 ± 0.05	0.74 ± 0.04*§	1.18 ± 0.11#	0.67 ± 0.07*§

*Normoxia, cerebEND cells 4 h normoxia; OGD, cerebEND cells 4 h OGD; N-C6, cerebEND cells 4 h normoxia with C6-medium; OGD-C6, cerebEND cells 4 h OGD with C6-OGD medium. Data are presented as means ± s.e.m. (n = 8–10). Statistical significance was labeled with *vs. normoxia, #significant vs. OGD, §significant to N-C6 (p < 0.05, two-sided student's t-test with same variances).*

65% and to 55% ($p < 0.05$), respectively, whereas mRNA expression of claudin-12 was not affected by any of the treatments. Similar to claudin-5, occludin was also significantly decreased by OGD and OGD-C6 to 38% and to 24%, respectively ($p < 0.05$). Finally, mRNA expression of ZO-1 was downregulated by OGD and OGD-C6 to 74% and to 64% ($p < 0.05$). In comparison to mRNA data western blots of total cell lysates revealed not such a significant regulation of tight junction protein expression (**Figure 2**). Claudin-3 was decreased by OGD-C6 to 81%, whereas claudin-5 protein expression was upregulated by N-C6 to 1.53-fold after 4 h treatment ($p < 0.05$). Claudin-12 was hardly detectable on western blots. Similar to mRNA regulation occludin was downregulated by OGD to 83% ($p < 0.05$), whereas ZO-1 was upregulated significantly by OGD and OGD-C6 to 1.34-fold and to 1.36-fold, respectively ($p < 0.05$). In contrast to western blots of total cell lysates, western blotting data with membrane enriched Triton-X 100 fractions were similar to mRNA expression data of claudins. In case of claudin-3, a reduction was only observed after N-C6 treatment (69%, $p < 0.05$). Claudin-5 expression was decreased after OGD, N-C6 and OGD-C6 to 70, 67, and 70%, respectively ($p < 0.05$). On the contrary, occludin expression of the 65 kDa band remained unchanged and ZO-1 also increased after OGD to 1.27-fold. Western blotting data proposed a change in localization of most tight junction proteins, which was confirmed by immunofluorescence microscopic images (**Figure 3**). Claudin-3 was significantly lost after treatment with astrocyte factors (N-C6), whereas it formed distinct continuous tight junction strands under other conditions. In case of claudin-12, no continuous tight junction strands surrounding the total cell were detected. OGD treatment seemed to cause broader tight junction strands of claudin-5, occludin and ZO-1, which could be due to a movement of tight junction proteins apart from the cell-cell contacts. Images of OGD-C6 treated cerebENDs showed a generally weaker staining density of tight junction strands in comparison to normoxia samples.

INFLUENCE OF ASTROCYTES AND OGD ON EXPRESSION AND FUNCTIONALITY OF ABC-TRANSPORTERS

mRNA expression of Abcb1a was upregulated by astrocyte factors, OGD and OGD-C6 to 1.44-fold, 5.71-fold and 5.24-fold, respectively ($p < 0.05$) (**Table 2**). On the contrary, mRNA expression of Abcc4 was only signifcantly reduced by OGD-C6 treatment to 66% ($p < 0.05$). In case of Abcg2, mRNA expression was downregulated by OGD and OGD-C6 to 48 and 54%, respectively ($p < 0.05$). Western blotting of total cell lysates for Abcb1 confirmed the trend of mRNA data (**Figure 4**). Abcb1 protein was significantly upregulated by OGD (1.55-fold, $p < 0.05$) and OGD-C6 (1.56-fold, $p < 0.05$). In contrast to mRNA expression, Abcc4 protein was also upregulated by OGD (1.39-fold, $p < 0.05$) and OGD-C6 (1.44-fold, $p < 0.05$). In case of Abcg2 no significant regulation was found on the protein level after 4 h treatment. Immunofluorescence images confirmed that Abcg2 was the strongest expressed of the investigated Abc-transporters in cerebENDs, followed by Abcc4 and Abcb1 (**Figure 4**).

To test transporter functionality, uptake assays were performed for Abcb1, Abcc4, and Abcg2 with specific substrates (Calcein-AM, Bodipy-FL-Prazosin, fluo-cAMP). No significant effects on transporter functionalities were found after treatment for 4 h. Therefore, uptake assays were carried out after 24 h normoxia or 4 h OGD followed by 20 h normoxia. Verapamil increased the uptake of Abcb1 substrate calcein-AM by 69.53 ± 4.08% ($n = 16$) after normoxic treatment for 24 h (**Figure 5**). In concordance to upregulated Abcb1 expression, Abcb1 functionality was increased after OGD, N-C6 and OGD-C6 treatment resulting in a significant higher raise by verapamil by 118.92 ± 10.43% (OGD, $n = 16$), by 125.26 ± 8.36 (N-C6, $n = 8$) and by 105.81 ± 6.79% (OGD-C6, $n = 8$), respectively ($p < 0.05$). Uptake of Abcc4 substrate fluo-cAMP was increased by MK571 by 90.91 ± 5.14 ($p < 0.05$, $n = 16$) after 24 h normoxia. OGD treatment did not change the amount of increased uptaken fluo-cAMP by MK571 (89.21 ± 4.39, $n = 16$), whereas fluo-cAMP uptake differences by MK571 were elevated by N-C6 and by OGD-C6 by 213.38 ± 8.16% and by 161.59 ± 11.65%, respectively ($p < 0.05$, $n = 8$). In case of Abcg2, OGD reduced significantly the Ko143 increased uptake under normoxic conditions from 80.94 ± 2.71% ($n = 16$) to 28.31 ± 5.56% ($p < 0.05$, $n = 16$), whereas N-C6 and OGD-C6 treatments did not lead to significant changes. According to calcein-AM uptake data, western blotting showed upregulation of Abcb1 protein after 4 h OGD and subsequent 20 h normoxia, after 24 h N-C6 and 24 h OGD-C6 to 1.64-fold, to 1.45-fold and 1.52-fold ($p < 0.05$, $n = 6$), respectively. In case of Abcc4, OGD, N-C6 and OGD-C6 increased Abcc4 protein expression after 24 h to 1.59-fold ($p < 0.05$, $n = 6$), to 1.50-fold ($p < 0.05$, $n = 6$) and to 1.86-fold ($p < 0.05$, $n = 6$). In contrast to uptake data, Abgc2 western blots of total cell lysates revealed an increase after OGD (1.32-fold) and N-C6 (1.22-fold, $p < 0.05$), but interestingly a decrease after OGD-C6 (0.88-fold, $p < 0.05$). To elucidate the reason for the mismatching of functionality and Abcg2 protein expression data, western blots after 24 h treatment were repeated with membrane protein enriched fractions extracted with Triton-X 100. Protein expression data of Triton-X 100 fractions were similar to total protein lysates for Acbb1 and Abcc4 and corresponded well to functional uptake data (Abcb1: 1.52-fold OGD-C6; Abcc4: 1.48-fold OGD, 1.90-fold OGD-C6; $p < 0.05$, $n = 6$). In case of Abcg2, protein expression was significantly decreased by OGD to 0.73 ± 0.12-fold ($p < 0.05$, $n = 4$), whereas N-C6 nor OGD-C6 showed no significant differences in comparison to the normoxic control.

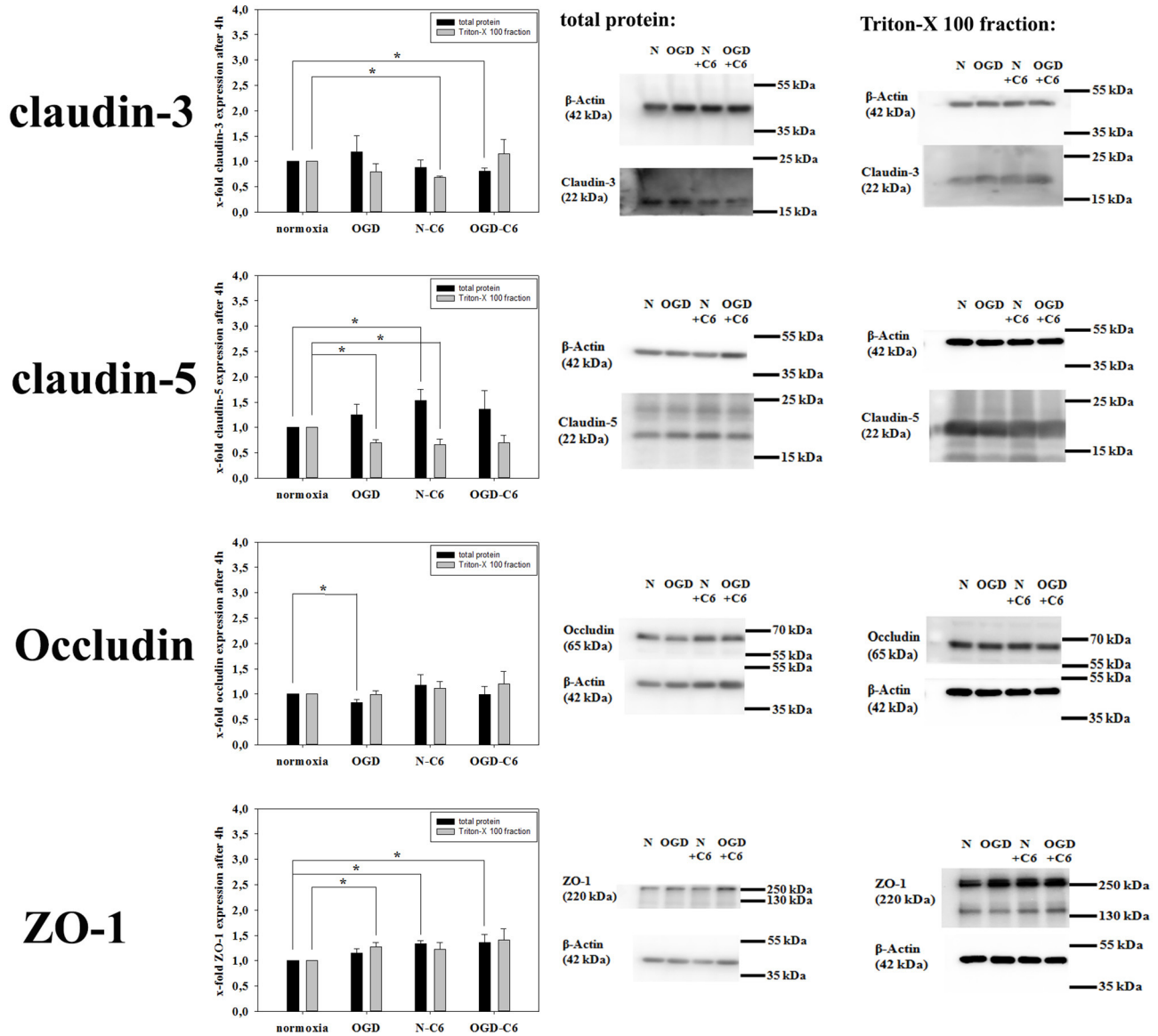

FIGURE 2 | Influence of 4 h oxygen/glucose deprivation (OGD) treatment and presence of astrocyte soluble factors on protein expression of tight junction proteins claudin-3, claudin-5, occluding, and ZO-1 of cerebEND cells. On the left side results of densitometric analysis derived from western blots of total protein cell lysates as well as of Triton-X 100 membrane enriched fractions are presented, on the right side according representative western blot images are depicted. N, cerebEND normoxic control; OGD, cerebENDs treated under OGD conditions for 4 h; N−C6 or N+C6, cerebENDs treated for 4 h with C6 conditioned medium derived from C6 cells which were treated under normoxic control conditions for 4 h; OGD−C6 or OGD+C6, cerebENDs treated for 4 h OGD with C6 conditioned medium derived from C6 cells which were treated under OGD conditions for 4 h. Statistical significance was labeled with *($p < 0.05$, two-sided student's *t*-test with same variances). Data are presented as means ± s.e.m. ($n = 4$–6).

INFLUENCE OF ASTROCYTES AND OGD ON EXPRESSION AND FUNCTIONALITY OF MMPs

Matrixmetalloproteinases (MMPs) play a pivotal role in the degradation processes of basal lamina as well as of tight junction proteins at the blood-brain barrier during ischemic insults (reviewd in Jin et al., 2010). Therefore, changes of MMP2, MMP3, and MMP9 as well as from their inhibitors TIMP1 and TIMP3 of cerebENDs cells were investigated. Results were summarized in **Table 3**. Four hours of OGD significantly decreased TIMP-1 mRNA expression 88%, whereas increased TIMP-3 mRNA 1.39-fold ($p < 0.05$, $n = 6$). Addition of astrocyte factors (N-C6) increased MMP-3 expression 3.93-fold and TIMP-1 expression

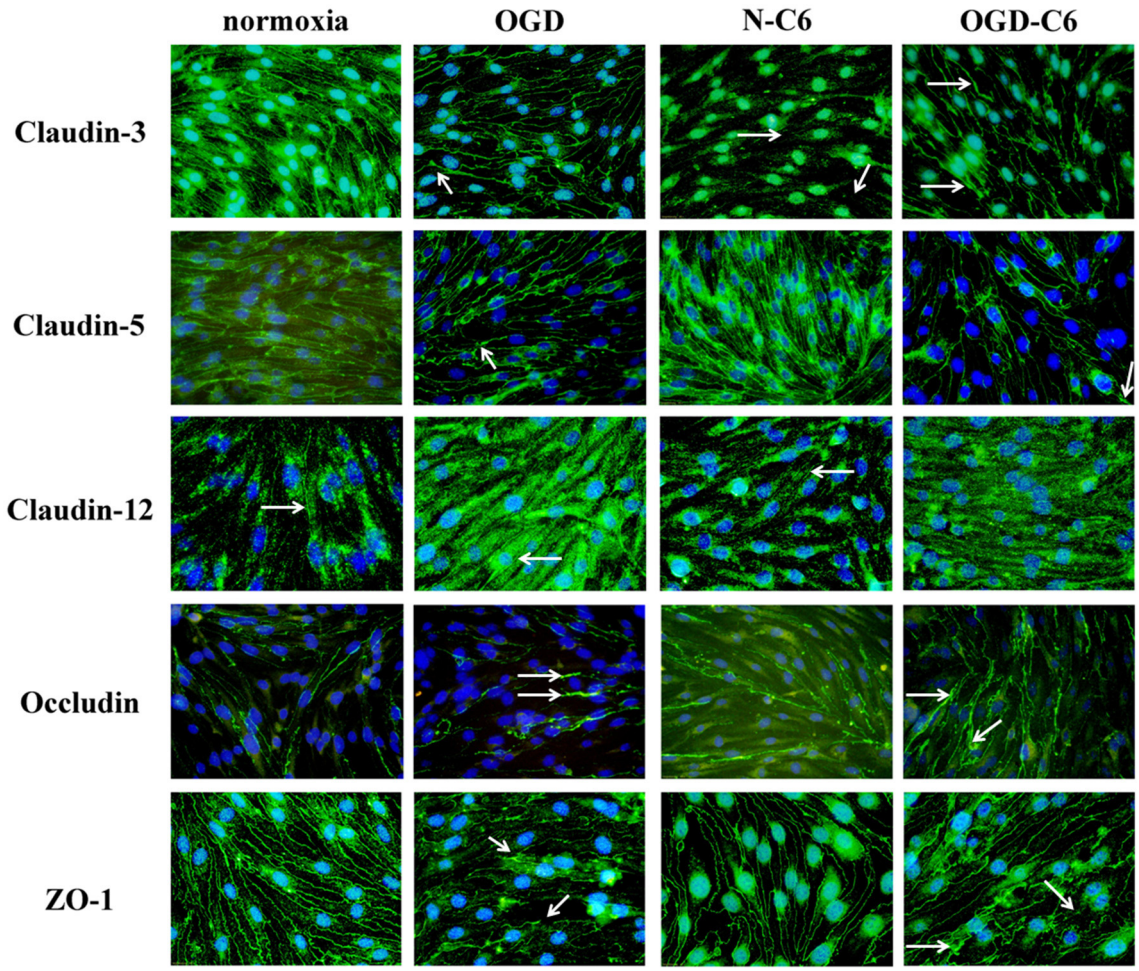

FIGURE 3 | Representative immunofluorescence images of tight junction proteins of cerebENDs treated for 4 h under normoxic or OGD conditions in the presence or absence of astrocyte soluble factors. Normoxia, cerebEND normoxic control for 4 h; OGD, cerebENDs treated under OGD conditions for 4 h; N-C6, cerebENDs treated for 4 h with C6 conditioned medium derived from C6 cells which were treated under normoxic control conditions for 4 h; OGD-C6, cerebENDs treated for 4 h OGD with C6 conditioned medium derived from C6 cells which were treated under OGD conditions for 4 h. White arrows indicate lost tight junctions strands in case of claudin-3 N-C6, broadened tight junctions strands after OGD and OGD-C6 in case of claudin-3, claudin-5, and occludin, broadening and leaks in tight junction strands of ZO-1 OGD and OGD-C6 and non-continuous tight junction strands in case of claudin-12.

1.47-fold, but decreased TIMP-3 expression to 79% ($p < 0.05$, $n = 8$). In case of OGD treatment with astrocyte factors (OGD-C6), MMP-2 expression was lowered to 92%, whereas TIMP-1 was upregulated to 1.39-fold ($p < 0.05$, $n = 8$). In order to test the functional relevance of these complex regulations, total enzyme activity of MMPs in medium supernatants was measured (**Figure 6**). Four hours OGD treatment of cerebEND cells significantly increased MMP activity to 164.31 ± 36.01% compared to normoxic control (100.00 ± 27.08%, $p < 0.05$, $n = 8$). Four hours of normoxic incubation of cerebENDs with astrocyte medium supernatants, which was incubated for 4 h under normoxic conditions before, led to an increase to 587.60 ± 105.47%, whereas addition of medium supernatants of OGD-treated astrocytes increased MMP-activity still significantly, but only to 446.07 ± 14.35% ($p < 0.05$). Analysing MMP-activity of C6 supernatants revealed a distinct higher activity for C6 supernatants in comparison to that obtained from cerebEND cells. Normoxic treated C6 cells secreted a MMP activity of 2694.62 ± 383.77% ($p < 0.05$, $n = 14$) in comparison to normoxic control of cerebENDs. OGD-treatment of C6 cells resulted in a significant decrease to 1271.67 ± 221.88% ($p < 0.05$, $n = 12$). Considering these MMP-activity start values for cerebEND incubation with C6 supernatants, a net increase of MMP-activity between N-C6 and OGD-C6 treatment of cerebEND cells of 60.86% could be calculated. In summary, OGD treatment increased MMP activity released by cerebENDs regardless of the presence of astrocytes.

INFLUENCE OF ASTROCYTES AND OGD ON EXPRESSION AND FUNCTIONALITY OF t-PA

t-PA activity plays a major role in proteases cascades responsible for plasmin and MMP activation and following degradation of

Table 2 | Influence of astrocytes and OGD on mRNA expression of Abc-transporters of cerebEND cells.

	Normoxia	OGD	N-C6	OGD-C6
Abcb1a	1.00 ± 0.07	$1.44 \pm 0.17^{*\S}$	$5.71 \pm 0.46^{*\#}$	$5.25 \pm 0.72^{*\#}$
Abcc4	1.00 ± 0.02	0.89 ± 0.15	1.07 ± 0.06	$0.66 \pm 0.02^{*\#\S}$
Abcg2	1.00 ± 0.03	$0.48 \pm 0.03^{*\S}$	$0.89 \pm 0.06^{\#}$	$0.54 \pm 0.05^{*\S}$

*Normoxia, cerebEND cells 4 h normoxia; OGD, cerebEND cells 4 h OGD; N-C6, cerebEND cells 4 h normoxia with C6-medium; OGD-C6; cerebEND cells 4 h OGD with C6-OGD medium. Data are presented as means ± s.e.m. (n = 8–10). Statistical significance was labeled with *vs. normoxia, #significant vs. OGD, §significant to N-C6 ($p < 0.05$, two-sided student's t-test with same variances).*

the basal lamina. Therefore, expression and functionality of t-PA and its inhibitor PAI-1 were assessed under normoxic and OGD conditions. **Figure 7A** shows an upregulation of t-PA mRNA expression in cerebEND cells by OGD treatment to 4.40-fold, by N-C6 to 1.36-fold and by OGD-C6 to 5.81-fold ($p < 0.05$, $n = 6$–8), whereas PAI-1 remained unregulated by any of the treatments (**Figure 7B**). To assess functional consequences of regulated t-PA, t-PA activity was measured in medium supernatants of cerebEND cells. Four hours of OGD significantly increased t-PA activity to 161.90 ± 8.17% in comparison to 100 ± 9.92% of the normoxia control ($p < 0.05$). N-C6 treatment raised t-PA activity to 169.42 ± 13.34%, which was further elevated to 226.66 ± 8.83% by OGD-C6 ($p < 0.05$). Interestingly, t-PA activity of medium supernatants derived from treated C6 cells decreased after OGD from 106.64 ± 22.61% to 47.11 ± 13.68% ($p < 0.05$, **Figure 7C**). These C6-medium supernatents were subsequently used to incubate cerebEND cells. Including the t-PA activity starting values of the C6-medium supernatants in the comparison of N-C6 vs. OGD-C6 data resulted in a real net t-PA activity increase of about 116% caused by cerebENDs.

INFLUENCE OF ASTROCYTES AND OGD ON SURFACE MORPHOLOGY OF BRAIN ENDOTHELIAL CELLS

To evaluate whether different treatments changed the surface structure of cerebEND cells, their surface was analyzed at the nanoscale using atomic force microscopy (AFM). To objectively quantify the surface texture, a novel method was applied (nAnostic), which detects structures protruding from the mean surface level via computer vision. Interestingly, OGD treatment revealed a significant reduction of protruding nano-objects on the surface from 315 ± 18 to 196 ± 23 ($p < 0.05$, $n = 20$, **Figure 8**). Addition of astrocyte factors (N-C6) also reduced the number of identified nanostructures to 255 ± 22, which was further decreased by OGD-C6 to 163 ± 18 objects ($p < 0.05$).

DISCUSSION

Ischemic insults such as stroke or traumatic brain injury induce changes of several facets of the blood-brain barrier (BBB). This comprises opening of the paracellular sealing by disorganization and disruption of tight junctions which thereafter contributes to vasogenic edema formation and worsened stroke outcome. But also Abc-transporter expression and functionality are changed leading to altered defense and protection against xenobiotics (drugs) as well as endogenous substrates (hormones). In addition to edema formation, this causes distinct disturbances in brain homeostasis and consequently damage of e.g., astrocytes or neurons via penetrated blood-born proteins (albumin) or changed ion concentration. Moreover, pharmacokinetics of drugs are influenced by dysregulated Abc-transporter activities. Therefore, it is of immense importance to unravel the complex underlying mechanisms to understand the pathology and to be able to develop novel therapeutic strategies.

In this context, it is known that astrocytes play a pivotal role during stroke pathogenesis. In the non-pathological status astrocytes are crucial for the maintenance of BBB characteristics (Janzer and Raff, 1987; Neuhaus et al., 1991; Deli and Joó, 1996; Ronaldson and Davis, 2012). They are suggested to regulate BBB permeability, water and ion exchange (Ballabh et al., 2004; Abbott et al., 2006; Mathiisen et al., 2010). In case of ischemic insults, the response of astrocytes is multifaceted. Astrocyte end feet cover over 95% of the brain capillary surface on the brain side. The injury of astrocytes results in a compromised BBB. Their local loss leads to a disassembly of the tight junction network and decreased expression of tight junction proteins such as occludin, claudin-5 or ZO-1. This correlates directly to paracellular leakiness (Willis et al., 2004, 2013; Shin et al., 2013). In addition, ischemia injured/activated astrocytes secrete chemokines (MCP-1, Rantes), pro—and anti-inflammatory cytokines (IL-1α, IL-1β, TNFα, interferon-γ) and growth factors (VEGF, bFGF, TGF-β). These molecules are known to directly activate processes at the brain endothelium to disrupt the BBB, promote angiogenesis or regulate transporters at the BBB (Lau and Yu, 2001; Lee et al., 2007; Doyle et al., 2010; Strecker et al., 2011; Vangilder et al., 2011; Ronaldson and Davis, 2012). Although these relations are known, no comprehensive *in vitro* study exist to unravel the effects of astrocytes, OGD or their combination on brain endothelial cells. Consequently, we studied the influence of astrocytes and OGD on an established blood-brain barrier (BBB) *in vitro* model based on murine cerebEND cells (Silwedel and Förster, 2006). CerebENDs represent an adequate BBB cell culture model forming significant tight monolayers with high TEER values comparable to models based on mouse primary brain endothelial cells (Silwedel and Förster, 2006; Takeshita et al., 2014). Moreover, cerebEND cells were successfully used for OGD studies and responded to TNFα-stimulus with a rapid increase of paracellular permeability (Silwedel and Förster, 2006; Neuhaus et al., 2012a). In the presented study permeability coefficients for the paracellular marker fluorescein were between 0.4–0.9*10^{-3} cm/min, which was in a similar range of other paracellular markers with a similar molecular weight (sucrose, Lucifer yellow) used for OGD *in vitro* studies with primary brain endothelial cells (Mysiorek et al., 2009). This range of permeability for such integrity markers (below 1*10^{-3} cm/min) was reported as adequate for validated BBB *in vitro* models in terms of *in vitro*/*in vivo* correlations (Lundquist et al., 2002; Culot et al., 2008; Mysiorek et al., 2009).

As first important parameter we have investigated the functionality of the physical barrier by measuring TEER and fluorescein permeability directly after OGD treatments. In experimental models of focal cerebral ischemia it was shown that sucrose, a vascular marker with low molecular weight, was able to

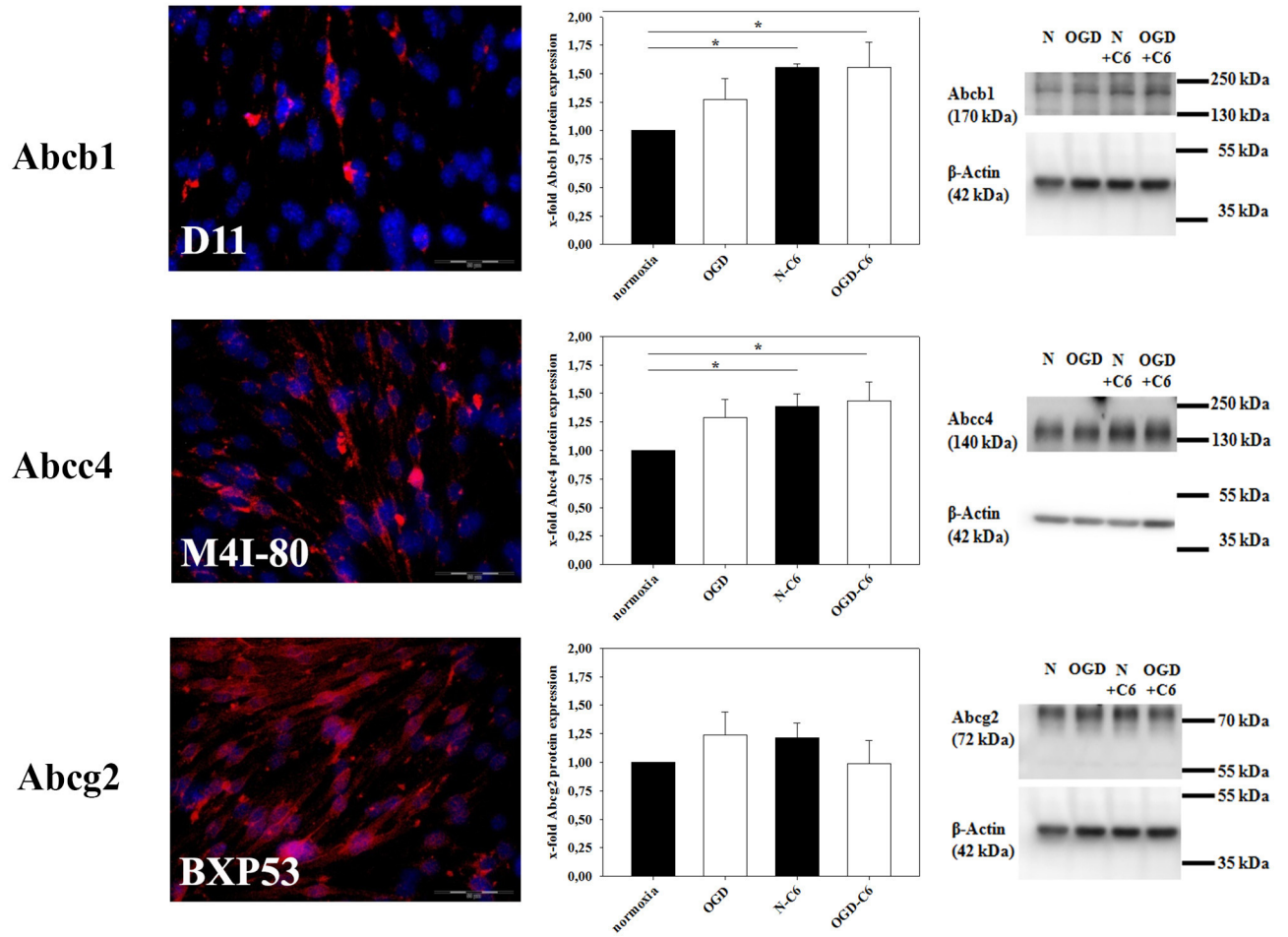

FIGURE 4 | Influence of 4 h oxygen/glucose deprivation (OGD) treatment and presence of astrocyte soluble factors on protein expression of Abc-transporters Abcb1, Abcc4, and Abcg2 of cerebEND cells. Immunofluorescence images of cerebEND cells under normoxic conditions show the distribution of Abcb1, Abcc4, and Abcg2. In the middle, results of densitometric analysis derived from western blots of total protein cell lysates are presented, on the right side according representative western blot images are depicted. N, cerebEND normoxic control; OGD, cerebENDs treated under OGD conditions for 4 h; N−C6 or N+C6, cerebENDs treated for 4 h with C6 conditioned medium derived from C6 cells which were treated under normoxic control conditions for 4 h, OGD−C6 or OGD+C6, cerebENDs treated for 4 h OGD with C6 conditioned medium derived from C6 cells which were treated under OGD conditions for 4 h. Statistical significance was labeled with *($p < 0.05$, two-sided student's *t*-test with same variances). Data are presented as means ± s.e.m. ($n = 4$-6).

permeate into the ipsilateral parenchymal hemisphere after transient middle cerebral artery occlusion (tMCAO) (Pfefferkorn and Rosenberg, 2003). Moreover, 2 h after tMCAO leaks in the BBB were found even for bigger molecules such as Evans Blue-albumin complexes (>60,000 Da) indicating a rapid opening of the BBB after stroke (Jiao et al., 2011). In concordance to this, we were able to show a significant BBB breakdown after OGD treatment in our *in vitro* model. Moreover, loss of barrier functionality was significantly increased by the presence of astrocytes during the OGD-treatment. Our data confirmed a previous OGD-study with a BBB co-culture model consisting of primary mouse endothelial and rat glial cells in which the presence of glial cells was necessary to disrupt the barrier significantly (Brillault et al., 2002; Mysiorek et al., 2009). In this context, vascular endothelial growth factor (VEGF) could be one major factor for BBB breakdown in our model. It was shown that VEGF was significantly upregulated in astrocytes after OGD or hypoxia, and it was postulated that this is responsible for BBB damage (Redzic et al., 2013). With regard to our model, C6 cells secreted significant amounts of VEGF which was further upregulated via hypoxia (Boveri et al., 2005; Yeh et al., 2008). Under our experimental conditions OGD increased mRNA levels of VEGF in C6 cells approximately two-fold (data not shown). In this context, it should be noted that cerebEND layers co-cultured with C6 cells exhibited lower TEER values in comparison to the mono-culture set-up before OGD experiments (presumably due to high basal VEGF levels and MMP activity (see **Figure 6**) in C6 medium supernatants). This fact has to be considered for data interpretation

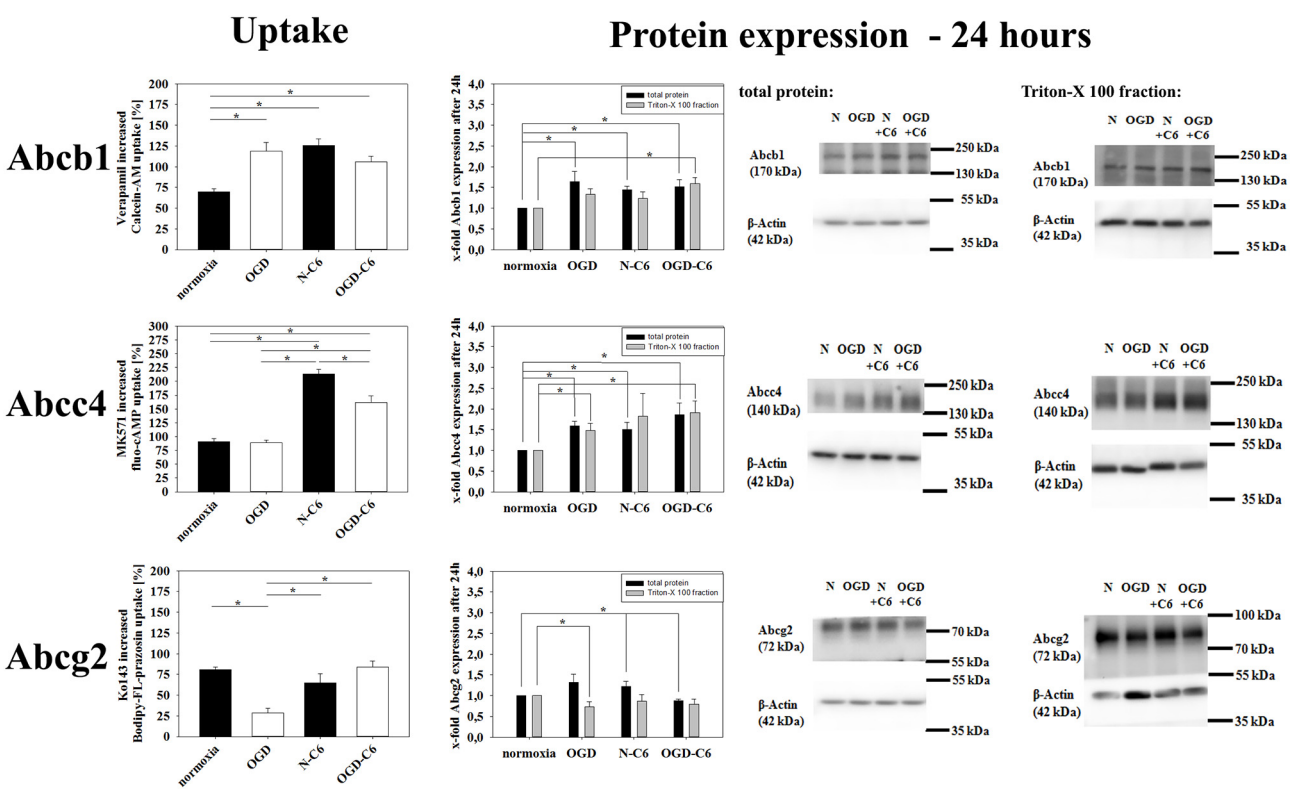

FIGURE 5 | Influence of 4 h oxygen/glucose deprivation (OGD) treatment with subsequent 20 h reoxygenation phase and presence of astrocyte soluble factors on Abc-transporter activity and protein expression. On the left side results of uptake assays with specific substrates for Abcb1 (calcein-AM), Abcc4 (fluo-cAMP), and Abcg2 (Bodipy-FL-prazosin) are presented. In the middle densitometric analysis derived from western blots of total protein cell lysates as well as Triton-X 100 membrane enriched fractions are shown, on the right side according representative western blot images are depicted. N, cerebEND normoxic control; OGD, cerebENDs treated under OGD conditions for 4 + 20 h reoxygenation/renutrition; N−C6 or N+C6, cerebENDs treated for 24 h with C6 conditioned medium derived from C6 cells which were treated under normoxic control conditions for 4 h; OGD−C6 or OGD+C6, cerebENDs treated for 4 h OGD with C6 conditioned medium derived from C6 cells which were treated under OGD conditions for 4 h with a subsequent incubation of cerebENDs for 20 h with N−C6 medium. Statistical significance was labeled with *($p < 0.05$, two-sided student's *t*-test with same variances). Data are presented as means ± s.e.m. ($n = 8$–16 for uptake assays, $n = 4$–6 for protein expression data).

Table 3 | Influence of astrocytes and OGD on mRNA expression of matrixmetalloproteinases MMP-2, -3, and -9, and TIMP-1 and TIMP-3 of cerebEND cells.

	Normoxia	OGD	N-C6	OGD-C6
MMP-2	1.00 ± 0.02	0.95 ± 0.09	1.11 ± 0.06	0.92 ± 0.03*§
MMP-3	1.00 ± 0.03	0.88 ± 0.14	3.93 ± 1.07*#	3.12 ± 1.12
MMP-9	1.00 ± 0.02	0.95 ± 0.17	0.89 ± 0.19	1.10 ± 0.22
TIMP-1	1.00 ± 0.01	0.88 ± 0.04*§	1.47 ± 0.18*#	1.39 ± 0.15*#
TIMP-3	1.00 ± 0.05	1.39 ± 0.07*§	0.79 ± 0.04*#	1.02 ± 0.09#§

*normoxia, cerebEND cells 4 h normoxia; OGD, cerebEND cells 4 h OGD; N-C6, cerebEND cells 4 h normoxia with C6-medium; OGD-C6, cerebEND cells 4 h OGD with C6-OGD medium. Data are presented as means ± s.e.m. (n = 6–8). Statistical significance was labeled with *vs. normoxia, #significant vs. OGD, §significant to N-C6 ($p < 0.05$, two-sided student's t-test with same variances).*

of fluorescein experiments (**Figure 1B**). In comparison to normoxia controls, N-C6 (normoxia+C6 cells) treatment resulted in increased TEER, but also apparently contradictory in elevated fluorescein permeability. TEER was determined before and after the experiments, whereas fluorescein was only measured at the end of the tests. Hence, fluorescein data reflected the absolute tightness status after the incubation periods, whereas TEER data presented the relative changes between before and after the treatments. Consequently, fluorescein permeability of N-C6 (normoxia+C6 cells) was higher than normoxic control (**Figure 1B**), because basal tightness of co-cultured cerebENDs was lower in the beginning of the OGD-experiment in comparison to mono-cultured cerebENDs. However, comparison of normoxic and OGD treated co-cultured cerebEND cells (normoxia+C6 cells vs. OGD+C6) revealed a very significant increase of fluorescein permeability after OGD-treatment, confirmed corresponding TEER data and thus demonstrated the additionally deleterious effects of astrocytes on BBB breakdown *in vitro*.

Increased BBB permeability for paracellular markers (sucrose, dextrans, Evans Blue-albumin) was directly correlated with changed expression or localization of tight junction proteins such as claudin-5, occludin or ZO-1 *in vivo* (Witt et al., 2003; Jiao et al., 2011). Redistribution of tight junction molecules were detected even after 2 h of reperfusion in a tMCAO rat model (Jiao et al., 2011). In concordance to this, OGD decreased mRNA expression

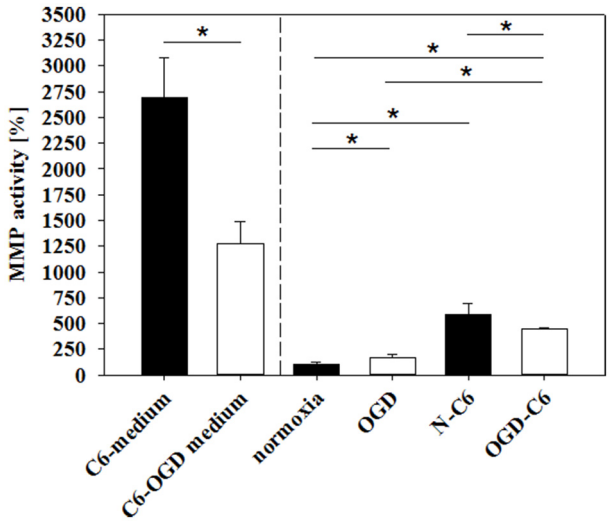

FIGURE 6 | Influence of 4 h oxygen/glucose deprivation (OGD) treatment and presence of astrocyte soluble factors on total MMP activity in medium supernatants. C6-medium, supernatants of C6 cells after 4 h normoxia; C6-OGD medium, supernatants of C6 cells after 4 h OGD; normoxia, supernatants of cerebEND cells after 4 h normoxia; OGD, supernatants of cerebEND cells after 4 h OGD; N-C6, supernatants of cerebEND cells after 4 h normoxia treated with C6-medium; OGD-C6, supernatants of cerebEND cells after 4 h OGD treated with C6-OGD medium. Statistical significance was labeled with *($p < 0.05$, two-sided student's *t*-test with same variances). Data are presented as means ± s.e.m. ($n = 8$–19).

of claudin-5, occludin and ZO-1 in our model significantly. Interestingly, presence of astrocytes did not influence mRNA levels of these tight junction proteins. Moreover, protein expression of tight junction proteins in total cell lysates was not strongly reduced. This was in concordance to results of Engelhardt et al. (2014) who showed that total protein expression of claudin-5 (up to 48 h), occludin (up to 48 h) and ZO-1 (up to 8 h) were not decreased after several hours of hypoxia (1% O_2) although TEER was reduced. Similar to our analysis, immunofluorescence images uncovered a delocalization of the investigated tight junction proteins. They and others reported that increased tyrosine-phosphorylation of tight junction molecules such as occludin or claudin-5 occurred which probably caused the disappearance of tight junction molecules from the cell boarders (András et al., 2007; Engelhardt et al., 2014). In this context, western blots of membrane enriched Triton-X 100 fractions confirmed the reduction of claudin-5 by OGD in our model and supported mRNA data as well as immunofluorescence images. Few data exist about claudin-3 and stroke. A recent study showed downregulated claudin-3 after 24 h after the surgery in a mouse model of intracerebral hemorrhage (Krafft et al., 2013). In our model expression of claudin-3 was not changed after 4 h of OGD treatment. Other claudins (claudin-1, claudin-12) are thought to be not significantly important for the barrier function in our model. Claudin-1 was hardly detectable by western blotting and was not found at cell-cell boarders on immunofluorescence images (data not shown). Claudin-12 formed non-continuous tight junction

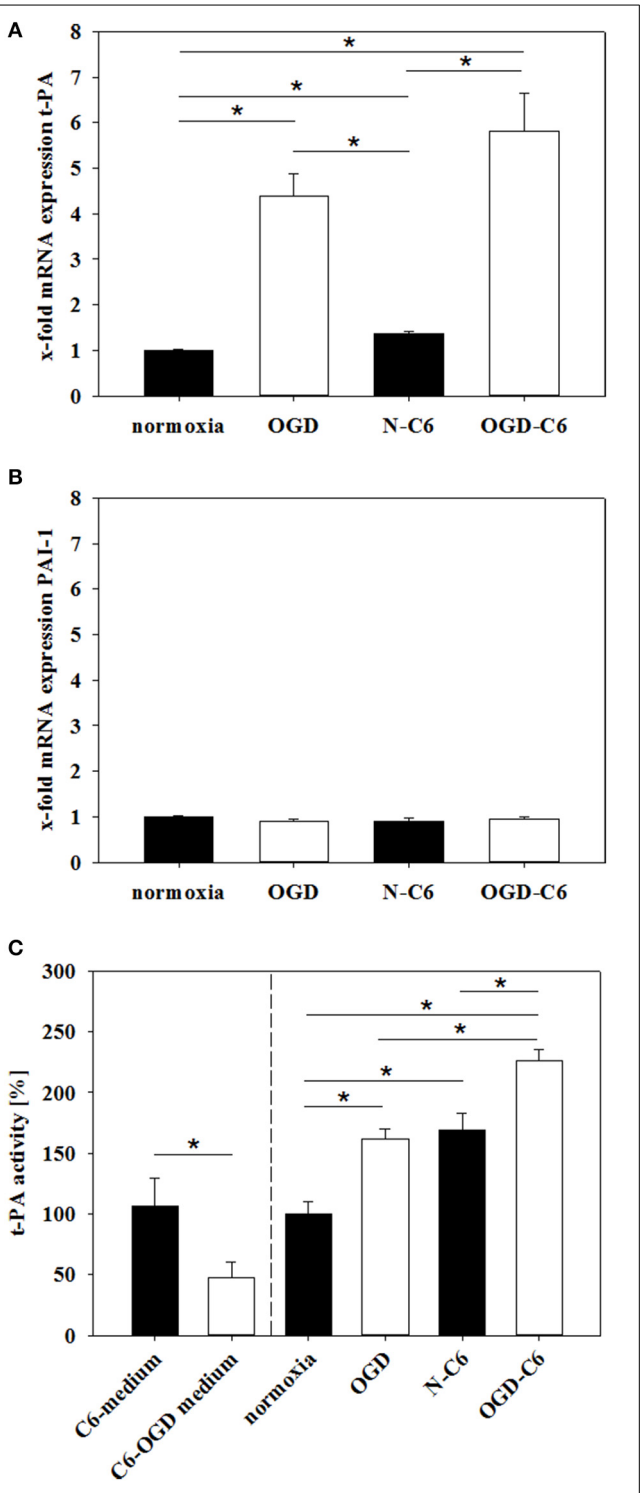

FIGURE 7 | Influence of 4 h oxygen/glucose deprivation (OGD) treatment and presence of astrocyte soluble factors on mRNA expression of t-PA (A) and PAI-1 (B) of cerebENDs and t-PA activity in medium supernatants (C). For mRNA expression data: N, cerebEND normoxic control; OGD, cerebENDs treated under OGD conditions for 4 h; N-C6, cerebENDs treated for 4 h with C6 conditioned medium derived from C6 cells which were treated under normoxic control conditions for 4 h,

(Continued)

FIGURE 7 | Continued
OGD-C6, cerebENDs treated for 4 h OGD with C6 conditioned medium derived from C6 cells which were treated under OGD conditions for 4 h; for t-PA activity data: C6-medium, supernatants of C6 cells after 4 h normoxia; C6-OGD medium, supernatants of C6 cells after 4 h OGD; normoxia, supernatants of cerebEND cells after 4 h normoxia; OGD, supernatants of cerebEND cells after 4 h OGD; N-C6, supernatants of cerebEND cells after 4 h normoxia treated with C6-medium; OGD-C6, supernatants of cerebEND cells after 4 h OGD treated with C6-OGD medium. Statistical significance was labeled with *($p < 0.05$, two-sided student's *t*-test with same variances). Data are presented as means ± s.e.m. ($n = 6$–8 for mRNA analysis, $n = 3$–13 for t-PA activity).

strands according to the postulation that claudin-12 itself can not form tight junctions (Piontek et al., 2011).

As next key property of the BBB, the expression and functionality of ABC-transporters were investigated. Few *in vivo* data are available about the regulation of ABC-transporters after stroke. Most of the reports showed an increase of expression as well as functionality of Abcb1 (Spudich et al., 2006; Lazarowski et al., 2007; Ueno et al., 2009). On the contrary, Dazert et al. (2006) found no differences in the regulation of Abcb1 on the mRNA level in his rat stroke model. In our model Abcb1 was significantly upregulated by C6 cells under normoxic conditions as well as OGD treatment. This was in concordance to other studies revealing that primary astrocytes as well as C6 cells were able to increase Abcb1 expression and functionality of brain endothelial cells (El Hafny et al., 1997; Gaillard et al., 2000; Berezowski et al., 2004). In case of Abcg2 few data exist about its regulation *in vivo* after stroke. For example, Dazert et al. (2006) published that Abcg2 was significantly upregulated at mRNA level after 14 days of reperfusion. In our model we were able to show for the first time that Abcg2 was downregulated by OGD in brain endothelial cells. In concordance to our *in vitro* data, own *in vivo* stroke studies revealed that Abcg2 protein expression of total brain samples was significantly decreased after 1 h tMCAO followed by 24 h reperfusion in male C57Bl/6 mice (unpublished data). Previous reports showed that inflammatory processes were mediated by pro-inflammatory cytokines such as TNFα within 2–6 h after the ischemic insult (Ronaldson and Davis, 2012). In this context and completely concordant to our expression as well as functionality data, it was shown that TNFα increased expression of ABCB1, but decreased expression of ABCG2 in a human BBB *in vitro* model (Poller et al., 2010). However, related to our *in vitro* data it can not be excluded that Abcg2 expression and functionality would be increased after a longer reperfusion phase after OGD as shown by Dazert et al. (2006).

Several studies reported that matrix metalloproteinases (MMPs) and tissue-type plasminogen activator (tPA) were involved in BBB disruption during stroke (Adibhatla and Hatcher, 2008; Bauer et al., 2010). As mentioned before, MMPs could degrade tight junction proteins claudin-5 and occludin as well as components of the basal lamina such as fibronectin, laminin and collagen leading to a disintegrated BBB and brain edema (Adibhatla and Hatcher, 2008; Cunningham et al., 2005; Yang and Rosenberg, 2011). Previously, MMP-2, MMP-3 and MMP-9 were shown to be the major MMPs in BBB disruption with a time-dependent activation and a very complex regulation (Jin et al., 2010). Moreover, MMP-9 levels were significantly increased after acute stroke in patients of a clinical study (Brouns et al., 2011). In our model, no significant regulation was found on the mRNA level of the investigated MMPs by OGD. However, according to the literature enzymatic activity of total MMPs of cerebEND cells was significantly upregulated by OGD in our model. This suggested that the MMP regulation took place mainly on the protein level. Moreover, recently Lenglet et al. (2014) proved the relevance of active MMP-1, MMP-10 and MMP-13 during stroke. These MMPs were not analyzed on the mRNA level in the presented study. Furthermore, protease t-PA can induce MMP-9 mediated BBB disruption and increase the MMP-1/TIMP-2, MMP-2/TIMP-2, MMP-8/TIMP-2 and MMP-9/TIMP-2 ratios in the hyperacute phase of reperfusion (Wang et al., 1998; Tsuji et al., 2005; Lenglet et al., 2014). In our model, expression as well as activity of t-PA of cerebEND cells was significantly upregulated by OGD according to the literature and can therefore also contribute to the observed barrier breakdown *in vitro*. Interestingly, in contrast to MMP activity, presence of astrocyte factors increased t-PA activity of cerebEND cells after OGD.

The novel Nanostic-AFM method enabled us to detect and quantify changes of brain endothelial cells after OGD treatments via a morphological approach even at the nanometer scale. Of note, the topographical alterations focus on local height distributions which are non-detectable for light microscopy. With the brain endothelial cells investigated here, OGD significantly reduced the number of microvilli-like objects. In epithelial cell layers the number of counted nanostructures (objects) on the cell surface was related to the cytoskeletal organization and the degree of cellular differentiation (Thoelking et al., 2010). In brain endothelial cells tight junction proteins are connected to the cytoskeleton (Abbott et al., 2006; Ronaldson and Davis, 2012). In our model, the link between the number of objects to an underlying cytoskeletal remodeling has to be shown in further studies. However, it could be hypothesized that a decreased number of nanostructures could be associated to the already shown redistribution of tight junction proteins and loss of barrier after OGD.

In summary, we were able to show that the influence of astrocytes in our BBB *in vitro* stroke model is of immense importance comprising the physical and the transport barrier as well as major BBB damaging proteases. Consequently, we would recommend to include astrocytes in BBB *in vitro* stroke models according to the concept of the neurovascular unit and its role during cerebral ischemia (Berezowski et al., 2012). However, it has to be pointed out that the brain endothelium *in vivo* is additionally regulated by blood flow and several other cell types of its microenvironment (pericytes, microglia, oligodendrocytes, blood cells). *In vivo* processes causing BBB disruption and repair during and after stroke take place in a complex regulatory network which is not possible to be simulated entirely in our model. Also, we can not exclude that results obtained from a BBB model with a 3D capillary architecture based on the same cells as used in this study would differ from the presented ones. Therefore, future studies with *in vivo* stroke models have to elucidate concordance and relevance of our *in vitro* findings.

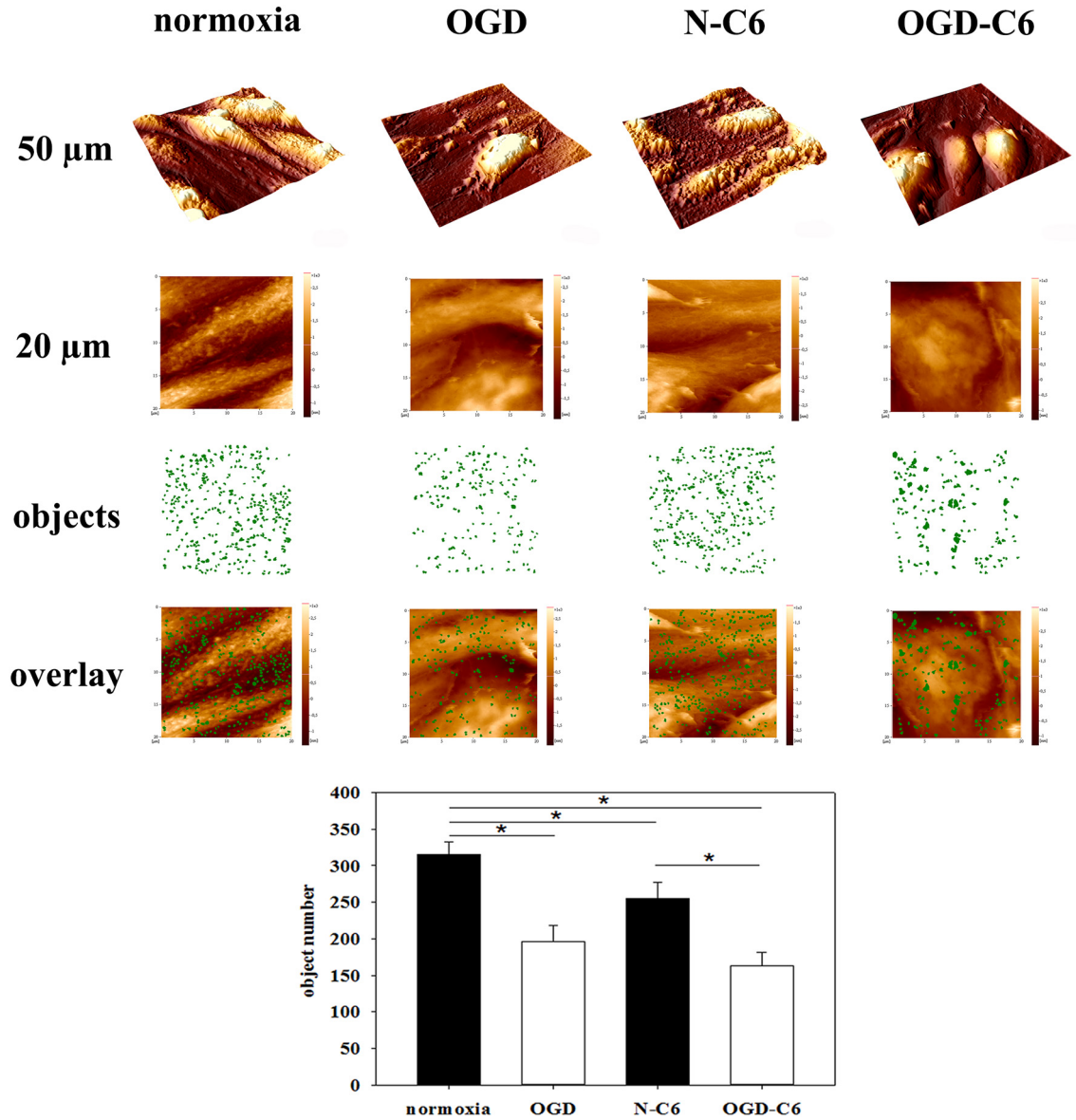

FIGURE 8 | Nanoscale surface topography analysis (nAnostic). CerebEND cells after 4 h oxygen/glucose-deprivation (OGD) and/or incubation with astrocyte conditioned medium were fixed by addition of glutardialdehyde (1% final conc.) and subjected to topography recording through atomic force microscopy (AFM) in buffer fluid—without any drying or labeling procedure. Quantitative analysis of protruding nano-objects is performed by computer vision. Shown are 3D-overviews of 50 µm (upper row), 20 µm raw data as taken for quantitative analysis (2nd row), a mask of identified nano-objects in green (3rd row) and the overlay of the two latter (lower row). Normoxia, cerebEND cells 4 h normoxia; OGD, cerebEND cells 4 h OGD; N-C6, cerebEND cells 4 h normoxia with C6-medium; OGD-C6, cerebEND cells 4 h OGD with C6-OGD medium. Statistical significance was indicated with an *($p < 0.05$ two-sided student's *t*-test with same variances). Data are presented as means ± s.e.m. of $n = 20$ images of $(20\,\mu m)^2$ area.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: http://www.frontiersin.org/journal/10.3389/fncel.2014.00352/abstract

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Blood brain barrier dysfunction and delayed neurological deficits in mild traumatic brain injury induced by blast shock waves

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Mild traumatic brain injury (mTBI) resulting from exposure to blast shock waves (BSWs) is one of the most predominant causes of illnesses among veterans who served in the recent Iraq and Afghanistan wars. Such mTBI can also happen to civilians if exposed to shock waves of bomb attacks by terrorists. While cognitive problems, memory dysfunction, depression, anxiety and diffuse white matter injury have been observed at both early and/or delayed time-points, an initial brain pathology resulting from exposure to BSWs appears to be the dysfunction or disruption of the blood-brain barrier (BBB). Studies in animal models suggest that exposure to relatively milder BSWs (123 kPa) initially induces free radical generating enzymes in and around brain capillaries, which enhances oxidative stress resulting in loss of tight junction (TJ) proteins, edema formation, and leakiness of BBB with disruption or loss of its components pericytes and astrocyte end-feet. On the other hand, exposure to more intense BSWs (145–323 kPa) causes acute disruption of the BBB with vascular lesions in the brain. Both of these scenarios lead to apoptosis of endothelial and neural cells and neuroinflammation in and around capillaries, which may progress into chronic traumatic encephalopathy (CTE) and/or a variety of neurological impairments, depending on brain regions that are afflicted with such lesions. This review discusses studies that examined alterations in the brain milieu causing dysfunction or disruption of the BBB and neuroinflammation following exposure to different intensities of BSWs. Furthermore, potential of early intervention strategies capable of easing oxidative stress, repairing the BBB or blocking inflammation for minimizing delayed neurological deficits resulting from exposure to BSWs is conferred.

Keywords: blast-related brain injury, blast shock waves, blood-brain barrier leakage, chronic traumatic encephalopathy, mild traumatic brain injury, neuroinflammation, oxidative stress, vascular lesions

INTRODUCTION

Exposure to shock waves stemming from ignition of explosive devices can produce considerable injury to both torso and brain (Rosenfeld et al., 2013; Kovacs et al., 2014). The danger for such exposures is extremely great to military personnel in contemporary warfare but they can also occur to civilians in circumstances such as bomb detonations by terrorists. The use of individual body protection systems by military personnel has diminished blast-related fatal thoracic and abdominal injuries in the recent Operation Iraqi Freedom and Operation Enduring Freedom wars in Afghanistan (Rosenfeld et al., 2013; Kovacs et al., 2014). However, a significant fraction of military personnel exposed to blast shock waves (BSWs) exhibit mild traumatic brain injury (mTBI; Ling and Ecklund, 2011). Persons exposed to BSWs display diverse neurological deficits depending upon the severity of shock waves and the region of brain affected by these shock waves. The symptoms may range from temporary mild cognitive problems to a more serious and continuing brain dysfunction characterized by significant memory and mood impairments, post-traumatic epilepsy or coma (Kovacs et al., 2014). Injuries from blast waves have been categorized as primary, secondary, tertiary and quaternary types (see Kovacs et al., 2014 for more details). Primary injury refers to brain damage happening directly from exposure to the explosive blast wave, secondary injury is brain damage owing to being hit by bomb constituents (e.g., fragments, rocks) driven by blast waves, and tertiary injury is a crash related brain damage resulting from being physically thrown into other objects or the ground. Quaternary injury refers to all other forms of injury ensuing through a blast, which include fireball related burns, exposure to toxic fumes and radiation released at blast sites (Phillips, 1986; Kovacs et al., 2014).

Multiple experimental studies have revealed that blast overpressure results from a sudden discharge of energy that produces rapid expansion of high-pressure gas into the ambient

atmosphere. When a pressure pulse of random form cruises through a medium, higher-pressure components of the pulse travel faster than lower pressure parts, which causes the wave components to add gainfully and generate a sudden increase in pressure, or a shock (Cullis, 2001; Yeoh et al., 2013). However, once rarefaction waves catch up to the shock front, the shock begins to degrade into a blast wave. The resulting pressure time wave shape makes a Friedlander curve where pressure falls swiftly after the peak and then transitorily plunges lower than the atmospheric level (Cullis, 2001; Yeoh et al., 2013). The area of the curve denoting greater pressure than atmospheric pressure is called the positive phase whereas the region indicating lower pressure than the atmospheric pressure is called the negative phase (Yeoh et al., 2013).

Several mechanisms have been proposed for the primary brain injury caused by BSWs. A widely accepted hypothesis is that, shock waves traverse the brain tissue causing its acceleration and deformation; the degree of brain damage would depend upon the shape of BSW, its peak overpressure and pulse duration and the tissues' natural resonant frequencies (Desmoulin and Dionne, 2009; Magnuson et al., 2012; Kovacs et al., 2014). Another supposition is that shock waves first impact the torso, the kinetic energy of these waves gets transferred into hydraulic energy in the cardiovascular system and causes a rapid physical displacement of blood which moves through blood vessels from the high pressure body cavity to the low-pressure cranial cavity, causing damage to tiny cerebral blood vessels and blood-brain barrier (BBB; Chen et al., 2013). Studies in animal models have suggested that both direct and indirect mechanisms (when torso is not protected with a body armor such as Kevlar) contribute to the pathophysiology of blast-related mTBI through dysfunction or disruption of the BBB (Säljö et al., 2008; Long et al., 2009; Abdul-Muneer et al., 2013; Yeoh et al., 2013). The goal of this review is to confer studies that examined changes in the brain milieu causing BBB dysfunction or disruption and neuroinflammation following exposure to different intensities of BSWs. Moreover, potential of early intervention strategies capable of easing oxidative stress, repairing the BBB or blocking inflammation for diminishing delayed neurological deficits ensuing from exposure to BSWs is discussed.

BLOOD-BRAIN BARRIER—STRUCTURE AND FUNCTION

The BBB, a multicellular vascular structure, acts as a diffusion barrier to prevent the inflow of most compounds from blood to brain and thereby maintains brain homeostasis (Ballabh et al., 2004; Obermeier et al., 2013). The endothelial cells that form the walls of the capillaries are the primary components of the BBB in the mammalian brain and spinal cord (**Figure 1A**). The combined surface area of these capillaries forms the principal interface for blood-brain exchange (Abbott et al., 2010). Anatomically, the BBB is composed of: (i) brain endothelial cells; (ii) tight junctions (TJs) between endothelial cells; (iii) capillary basement membrane (BM); (iv) pericytes; and (v) astrocyte end-feet (**Figure 1A**). Contrasting to endothelial cells in the rest of the body, endothelial cells making up the BBB do not display fenestrations and undergo low rates of transcytosis. However, they have more extensive TJs, which are the locations of fusion linking the outer leaflets of plasma membrane of adjacent endothelial cells (**Figure 2A**).

The diffusion barrier is a consequence of the selectivity of TJs to impede the passage of most blood-borne substances from inflowing into the brain (Ballabh et al., 2004; Abbott et al., 2010). The TJs are composed of transmembrane proteins such as occludin, claudins, and junctional adhesion molecule (**Figure 2A**). All of these proteins are anchored into the endothelial cells by cytoplasmic protein complex comprising zonula occludens proteins 1–3 (ZO-1-3) and cingulin (Ballabh et al., 2004; Engelhardt and Sorokin, 2009). The TJs limit the flux of hydrophilic molecules across the BBB while smaller lipophilic substances such as O_2 and CO_2 diffuse freely across plasma membranes along their concentration gradient (Grieb et al., 1985). The other functions of the BBB include maintaining ionic compositions at optimal levels for synaptic signaling function via specific ion channels and transporters, keeping the pools of central and peripheral neurotransmitters separate from each other, preventing macromolecules from entering the brain and shielding the CNS from neurotoxic substances circulating in the blood (Bernacki et al., 2008; Abbott et al., 2010). Transporters mediate the entry of nutrients such as glucose and amino acids across the BBB whereas receptor-mediated endocytosis facilitates the uptake of larger molecules such as insulin, leptin, and iron transferrin (Pardridge et al., 1985; Zhang and Pardridge, 2001; Ballabh et al., 2004). Pericytes ensheath the abluminal surfaces of cerebral vessel walls (**Figure 1A**). They are important for angiogenesis, structural integrity and differentiation of endothelial cells and formations of TJs (Allt and Lawrenson, 2001; Bandopadhyay et al., 2001; Ballabh et al., 2004), as injury to pericytes can result in microaneurysms (Lindahl et al., 1997). Pericytes are also believed to have a unique synergistic relationship with brain endothelial cells in the regulation of capillary permeability through secretion of cytokines, chemokines, nitric oxide, matrix metalloproteinases (MMPs), and by means of capillary contraction (Hurtado-Alvarado et al., 2014).

On the other hand, perivascular astrocyte end-feet (also referred to as glia limitans) encircle the abluminal side of cerebral vessels (**Figure 1A**). They are highly specialized and polarized structures having orthogonal arrays of intramembranous particles consisting of the most abundant water channel aquaporin-4 (AQP-4; Obermeier et al., 2013). Astrocyte end-feet are necessary for the induction and maintenance of the TJ barrier (Rubin et al., 1991; Ballabh et al., 2004). Taken together, every constituent cell type makes a crucial contribution to the BBB's integrity. When one member of the BBB fails, the barrier can break down and lead to dramatic consequences, and neuroinflammation and neurodegeneration can ensue.

MECHANISMS OF BBB DISRUPTION IN DISEASE CONDITIONS

The barrier function of the BBB is not always rigid, as it undergoes modulation and regulation, both in physiology and pathology (Abbott et al., 2010). The barrier dysfunction can range from mild and transient TJ opening to chronic barrier breakdown (Förster, 2008). Chronic BBB dysfunction can exacerbate the overall brain pathology and contribute to persistent neurological deficits, as it

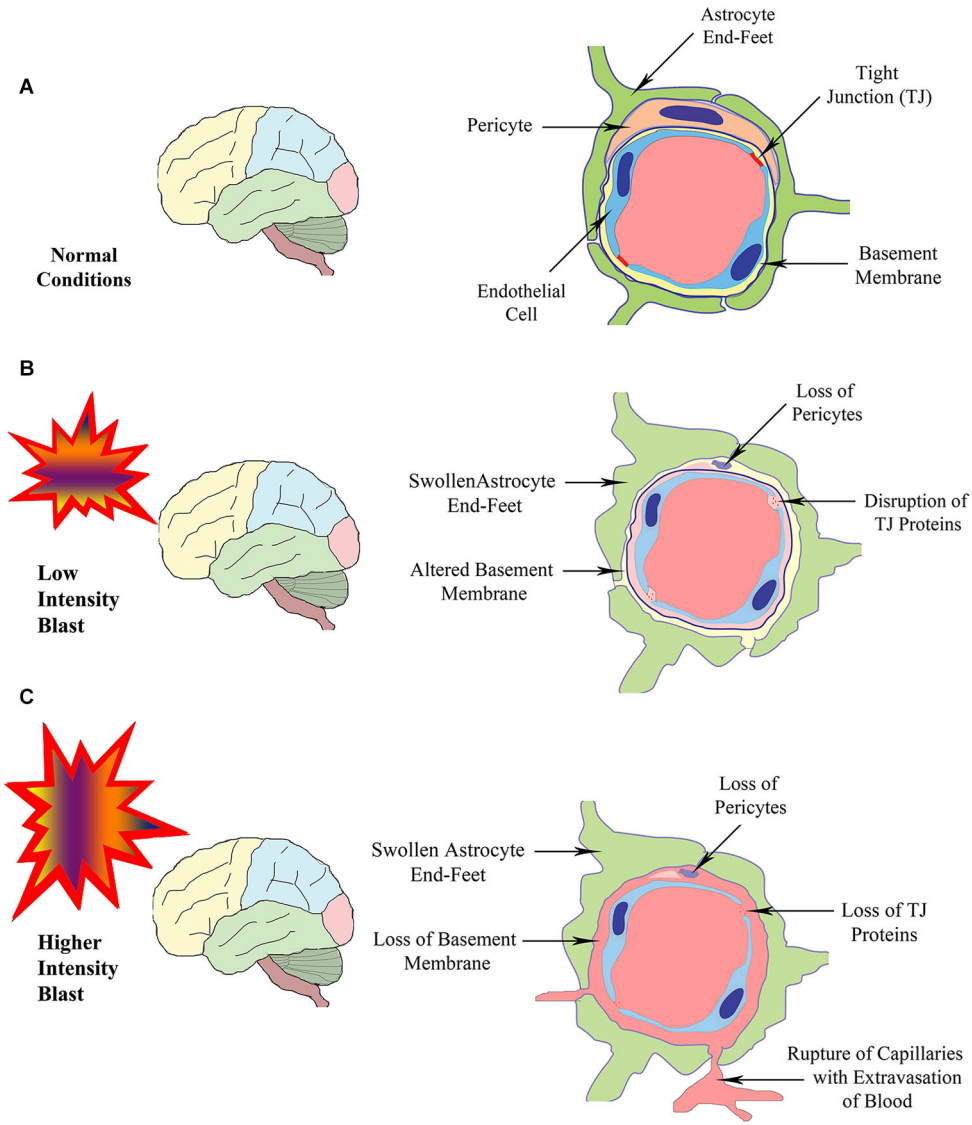

FIGURE 1 | Schematic illustrating the structure of the BBB in normal conditions (A), and following exposure to BSWs of lower intensity (B) or higher intensity (C). Exposure to lower intensity BSWs likely result in disruption of TJ proteins, damage or changes to the BM, detachment or loss of pericytes and swelling of astrocyte end-feet in brain microvessels **(B)**. In contrast, exposure to higher intensity BSWs likely cause direct rupture of brain microvessels causing the loss of TJ proteins, BM, pericytes and endothelial cells, swelling of astrocyte end-feet and extravasation of blood (hemorrhage) into perivascular areas **(C)**.

leads to increased extravasation of immune cells, poorly regulated flux of molecules and ions, impaired transport processes (Abbott et al., 2010; Obermeier et al., 2013). Under normal situations, a small number of mononuclear leukocytes, monocytes and macrophages may enter the adult CNS through the cytoplasm of endothelial cells (via a process called diapedesis) (Engelhardt and Wolburg, 2004). This low-level leukocyte trafficking across the BBB is believed to be for immune surveillance and to effect responses to brain infections (Obermeier et al., 2013). However, in pathological conditions (such as trauma, ischemia, stroke, status epilepticus, multiple sclerosis etc.), these cells infiltrate in large numbers into the CNS and perform roles similar to the resident microglia such as debris clearing (Scholz et al., 2007; Davoust et al., 2008; Abbott et al., 2010).

Classically, sites of BBB inflammation attract circulating neutrophils and mononuclear cells. It is believed that pericytes control the migration of leukocytes in response to inflammatory mediators by upregulating the expression of adhesion molecules and releasing chemoattractants (Hurtado-Alvarado et al., 2014). Neutrophils and mononuclear cells penetrate the barrier and form cuffs in the perivascular space particularly around small vessels. It is believed that the perivascular space acts as a specific niche for synchronized immune reaction (Bechmann et al., 2001; Konsman et al., 2007; Abbott et al., 2010). A multitude

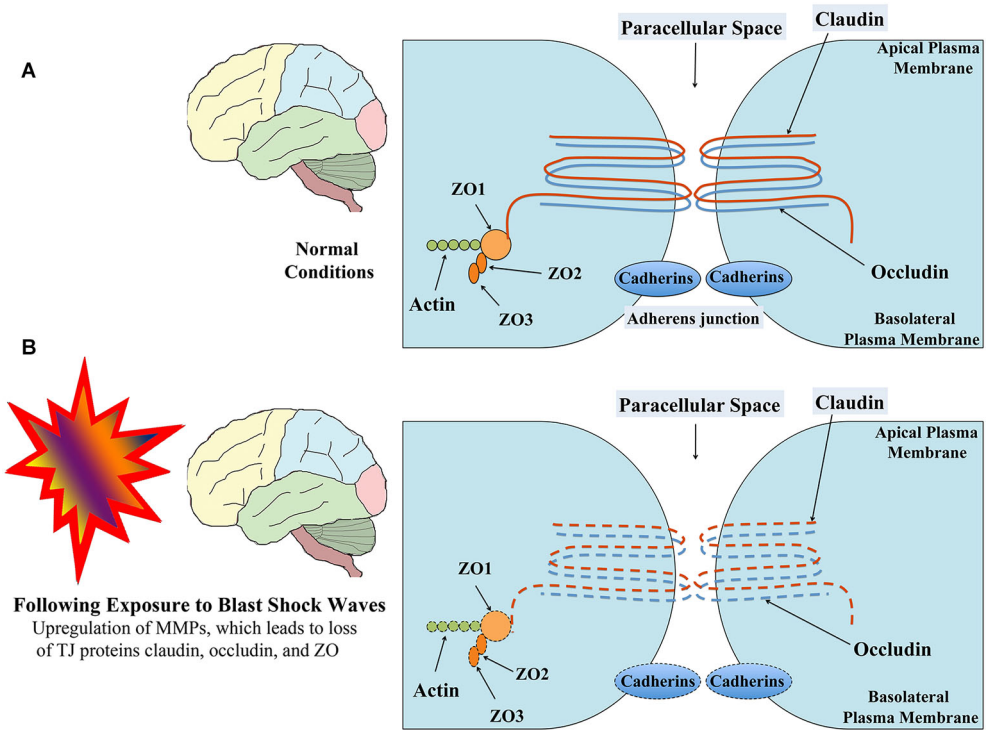

FIGURE 2 | Schematic showing the structure of TJ between brain endothelial cells in normal conditions (A) and following exposure to low-intensity BSWs (B). TJs mainly comprise trans-membrane proteins claudin and occuldin anchored to cytoplasmic proteins zona occludens-1 (ZO1), ZO2 and ZO3. Following exposure to shock waves, both trans-membrane and cytoplasmic proteins of TJ undergo modifications or loss resulting in leaky BBB. *Figure adapted from Ballabh et al. (2004) and Engelhardt and Sorokin (2009).*

of factors can disrupt the BBB, which include secreted elements to immune cells and pathogens, reactive oxygen species (ROS), activation of MMPs, and chronic up-regulation of angiogenic factors and pro-inflammatory cytokines. When BBB integrity is compromised, it may manifest initially as increased barrier permeability with reduced expression of TJ proteins. However, depending upon the severity, it may show other features such as pericyte detachment, astrocyte end-feet swelling or loss, and disrupted BM (Obermeier et al., 2013). Disruption of the BBB eventually culminates in neuronal dysfunction, neuroinflammation and neurodegeneration (Obermeier et al., 2013).

MECHANISMS OF BBB DISRUPTION FOLLOWING EXPOSURE TO BLAST INDUCED SHOCK WAVES

An *in vitro* study, utilizing a shock tube driven by compressed gas to generate operationally relevant, ideal pressure profiles consistent with improvised explosive devices, examined the effects of blast waves on the integrity of BBB function (Hue et al., 2013). Several measures demonstrated that barrier function of an in vitro BBB model gets disrupted with exposure to a range of controlled blast loading conditions. This was evidenced through: (i) an acute decrease in trans-endothelial electrical resistance (TEER) in a dose-dependent manner (which correlated with impulse rather than peak overpressure or duration); (ii) increased hydraulic conductivity and solute permeability post-injury across the barrier; and (iii) compromised ZO-1 immunoreactivity. This study provided the evidence that immediate disruption of BBB can occur with exposure to primary blast waves.

Studies using animal models have also reported the BBB dysfunction and disruption with exposure to BSWs. A recent study suggests that low intensity BSWs initially induce oxidative and nitrosative damage, which in turn causes BBB disruption and leads to cerebrovascular inflammation (**Figure 1B**; Abdul-Muneer et al., 2013). This study, using a 9-inch square cross-section shock tube and young rats, investigated the kinetic profile of one-time 123-kPa intensity blast exposure on the underlying mechanisms of cerebrovascular injury at 1, 6, 24 and 48 h post-exposure. They found the following changes in brain capillaries. First, there was considerable oxidative stress at 1–24 h after exposure to BSWs. This comprised induction of: (i) free-radical generating enzymes, NADPH oxidase 1 (NOX1) and inducible nitric oxide synthase (iNOS); (ii) oxidative/nitrosative damage markers such as 4-Hydroxynonenl (4-HNE, a major end product of lipid peroxidation) and 3-nitrotyrosine (3-NT, a product of tyrosine nitration mediated by ROS). These results imply that, single shock-wave exposure of 123-kPa intensity is sufficient to induce considerable oxidative/nitrosative stress in brain capillaries. Second, oxidative stress in capillaries progressed into BBB disruption (**Figure 1B**). This was evidenced through considerable loss of TJ proteins

occludin, claudin-5, and zonula occluden 1 (ZO-1), and reduced expression of PDGFR-beta (a marker of pericytes) (**Figure 2B**). Third, oxidative stress also activated the expression of several matrix metalloproteinases (MMP2, MMP3, MMP9) capable of digesting TJs, BM proteins and degradation of perivascular units (Abdul-Muneer et al., 2013). Fourth, the expression of AQP-4 (a water channel protein typically associated with astrocyte end-feet at the BBB) was upregulated in perivascular regions as well as the cortical tissue causing edema around cerebral vessels. Fifth, adhesion and infiltration of macrophages were increased in the microvessels and plasma samples showed higher levels of S-100 beta protein (at 6 h post-exposure) and neuron-specific enolase (appeared at 6 h and continued beyond 24 h post-exposure). Sixth, significant numbers of endothelial cells expressed caspase-3 and some endothelial cells expressed TUNEL implying that they are undergoing apoptosis. Thus, exposure to even a relatively low intensity BSWs can cause BBB disruption, inflammation and neurodegeneration.

In agreement with the above findings, another rat study using a blast overpressure of 129 kPa (Cho et al., 2013) showed increased ROS generation in the brain as early as 4 h and persistence of upregulated ROS until 2 weeks post-exposure. This study also found enhanced expression of genes encoding inflammation (interferon gamma [IFNγ] and monocyte chemoattractant protein-1 [MCP-1]) at 4 h and IFNγ and MCP-1 proteins at 24 h post-exposure. Additionally, animals displayed memory impairment in a novel object recognition test and increased density of Iba-1+ activated microglia in brain regions at 2 weeks post-exposure.

Several other studies have examined the effects of higher intensity shock waves. Yeoh et al. (2013) quantified cerebrovascular injury in rats exposed to moderate to intense BSWs (145, 232 and 323 kPa), using a rifle primer-driven shock tube. Cerebrovascular injury was quantified via measurements of the areas of extravasation of immunoglobulin G (IgG) around brain capillaries. They found small lesions (i.e., areas of IgG extravasation) scattered throughout the brain. It was also observed that both size and number of lesions increased with peak overpressure level (Yeoh et al., 2013). Red blood cells were associated with some extravasations implying some minor hemorrhage or coagulation of blood within capillaries. However, no significant difference was seen between acute and 48 h survival times, implying that all vascular lesions are acute and represent primary effects of the exposure to shock waves rather than delayed BBB opening associated with inflammation (Yeoh et al., 2013). Thus, exposure to high intensity shock waves causes acute cerebrovascular injury resulting in immediate BBB disruption, evidenced through extravasation of IgG and hemorrhage around capillaries (**Figure 1C**).

Another study by Tompkins et al. (2013) using a rat model and a blast overpressure of 80 psi (equivalent to ∼552 kPa) reports that polymorphonuclear leukocytes and lymphocytes infiltrate the brain parenchyma within an hour after exposure to BSWs. Furthermore, cells (neurons/glia) immunoreactive for cyclo-oxygenase-2 (COX-2, an inflammatory mediator involved in the cyclo-oxygenase pathway), interleukin-1 beta and tumor necrosis factor-alpha (pro-inflammatory cytokines) and 4-HNE (a marker of lipid peroxidation) could be seen as early as an hour after the exposure to BSWs. Most of these cells persisted for at least 3 weeks after the exposure. Cells immunoreactive for cleaved caspase-3 were also seen 3 weeks after the exposure. Additionally, magnetic resonance imaging showed hyper-intense regions in the somatosensory area within an hour after the exposure. The animals exposed to BSWs also exhibited hippocampus-dependent cognitive dysfunction at 5–12 days and axonal damage at 3 weeks post-exposure.

Taken together, it appears that exposure to relatively lower intensity BSWs causes oxidative stress and MMP activation in brain capillaries, which then evolves into disruption of the BBB and vascular edema formation with apoptosis of ECs and leads to inflammation with infiltration of leucocytes and macrophages. In support of these findings, an earlier study using a mouse model has shown specific neurodegeneration developing in perivascular areas following exposure to BSWs (Goldstein et al., 2012). Thus, in situations involving exposure to low-intensity shock waves, oxidative stress precedes the BBB disruption and/or inflammation (Abdul-Muneer et al., 2013; Cho et al., 2013). However, in circumstances where exposures to higher intensity shock waves occur, disruption of the BBB appears to occur swiftly through acute rupture of cerebral blood vessels, which is immediately followed by increased oxidative stress and early inflammation (Tompkins et al., 2013; Yeoh et al., 2013). Thus, BBB disruption appears to be the major primary injury that leads to neuroinflammation and neurodegeneration following exposure to milder to more intense BSWs.

LINKS BETWEEN SHOCK-WAVE INDUCED EARLY CHANGES SUCH AS BBB DISRUPTION AND DELAYED NEUROLOGICAL DISORDERS

mTBI incurred through single or repeated exposure to BSWs can lead to a variety of neurological problems at months or years after the incident. The symptoms may include headache, sensitivity to light and noise, behavioral changes, attention and memory deficits, loss of problem solving abilities, anxiety, post-traumatic stress disorder (PTSD), and post-traumatic epilepsy (Trudeau et al., 1998; Hicks et al., 2010; Rosenfeld and Ford, 2010; Bogdanova and Verfaellie, 2012; Tomkins et al., 2013). Furthermore, individuals with mTBI appear to have increased mental stress or depression and are likely to display higher tendency for alcohol misuse and/or drug abuse (Wilk et al., 2010; MacDonald et al., 2014). Based on animal model studies, early changes after exposure to BSWs mainly include increased intracranial pressure (Leonardi et al., 2011), deformation of brain areas in some cases (Bayly et al., 2006), considerable oxidative stress in brain capillaries and BBB disruption. This is typically followed by neuroinflammation typified by the appearance of activated microglia and increased concentration of pro-inflammatory cytokines and neurodegeneration in perivascular regions (Kaur et al., 1995, 1996, 1997; Abdul-Muneer et al., 2013), and atypical distribution of phosphorylated neurofilaments in neurons (Säljö et al., 2000). Furthermore, a mouse study has reported that multifocal axonal injury in white matter tracts typically occurs only when the torso of animal is not shielded during the exposure to shock waves (Koliatsos et al., 2011). However, a diffusion

tensor imaging (DTI) study in rats with chest protection noted significant interactions in axial and radial diffusivity in a number of subcortical structures at 2 h after exposure to BSWs but not at 42 days post-exposure (Kamnaksh et al., 2014). Likewise, another investigation found that some of the behavioral abnormalities (depression, anxiety) observed a day after the exposure to multiple shock waves spontaneously recovered by 16 days post-exposure although the histology showed apoptotic cells as early as 2 h after exposure in the dorsal and ventral hippocampus and persistence of apoptotic cells in the ventral hippocampus until 22 days post-exposure (Kamnaksh et al., 2012). A recent study, in addition, demonstrates that the hippocampus is vulnerable to high (165 kPa) as well as low (69–97 kPa) intensity BSWs, based on the occurrence of one or more signs of neurodegeneration such as activation of cleaved caspase-3 and loss of neurons, activation of microglia and hypertrophy of astrocytes at 7 days post-injury (Sajja et al., 2014). Interestingly, this study also revealed increased expression of genes encoding neurotrophic factors and antioxidants in the hippocampus following exposure to BSWs, implying that a healthy brain attempts to self-repair or minimize adverse alterations through activation of innate neuroprotective mechanisms.

Long-term structural changes resulting from exposure to BSWs include diffuse axonal degeneration in white matter tracts and inflammation. A study by Goldstein et al. (2012) demonstrated chronic traumatic encephalopathy (CTE) in postmortem brains of US military veterans exposed to blasts. Chronic traumatic encephalopathy, a tau protein-linked progressive neurodegenerative disease associated with memory loss, impaired judgment and depression, is typically seen in people who undergo repetitive brain concussions. However, emergence of CTE may take months, years or even decades after the last concussion (Maroon et al., 2014). Interestingly, Goldstein et al. (2012) found changes similar to CTE in a mouse model 2 weeks after exposure to BSWs, which comprised phosphorylated tauopathy, myelinated axonopathy, microvasculopathy, chronic neuroinflammation and neurodegeneration in the absence of macroscopic tissue damage or hemorrhage. Behavioral studies demonstrated hippocampus-dependent learning and memory deficits for a month after the exposure, which correlated with impairments in axonal conduction and activity-dependent long-term potentiation of synaptic transmission (Goldstein et al., 2012). Moreover, head immobilization during blast exposure prevented blast-induced learning and memory deficits, implying that head acceleration caused by BSWs plays a major role in inducing brain pathology and cognitive impairments.

Furthermore, a recent behavioral study at an extended time-point (6 months) after the exposure to BSWs using a mouse model suggests that single exposure of moderate BSWs to head is adequate for developing chronic cognitive and mood dysfunction (Mishra et al., 2014). Cognitive impairments were seen for hippocampus-dependent spatial memory retrieval function in a water maze test, perirhinal cortex-dependent object recognition function in a novel object recognition test, and dentate gyrus dependent pattern separation function (i.e., ability to discern minor changes in the environment) in a pattern separation test. Mood impairments were evidenced through novelty suppressed feeding and forced swim tests (Mishra et al., 2014). Analyses of the hippocampus at 8 months after the exposure revealed considerably decreased neurogenesis, a substrate important for maintenance of hippocampus-dependent cognitive function (particularly for pattern separation function) and mood (Shetty et al., 2014). Additionally, DTI of fixed brains *ex vivo* revealed significant white matter (corpus callosum) alterations in a subset of animals, which are typified by increased radial diffusivity (a marker of myelin degradation) and decreased relative anisotropy (implying asymmetry of water mobility) (Shetty et al., 2014). Thus, exposure to BSWs can dampen hippocampus neurogenesis and initiate progressive myelin degradation in white matter tracts on a long-term basis. These changes likely contribute to cognitive and mood dysfunction observed in people exposed to BSWs.

From the above perspectives, it appears that multiple acute changes observed in the brain after exposure to BSWs (such as oxidative stress, BBB disruption, neuroinflammation, diffuse axonal injury and sporadic neurodegeneration) initiate lasting pathological cascades that eventually evolve into persistent neurological dysfunction typified by cognitive and mood impairments (Terrio et al., 2009; Elder et al., 2010; Gavett et al., 2010; Cho et al., 2013). Nonetheless, it is currently unclear whether these early changes trigger long-standing secondary changes (such as alterations in structure and function of neurons and synapses, neuron-glia communication and neurochemistry, diminished hippocampus neurogenesis and axonal degeneration) or just persist at lower levels for prolonged periods and interfere with the normal brain function. First, it remains to be determined whether the repair of BBB occurs completely over days/weeks after exposure to BSWs or remains somewhat leaky or dysfunctional for protracted periods after exposure because of alterations in astrocyte end-feet, loss of pericytes and malfunction of TJs. Second, it is unclear whether increased oxidative stress seen in perivascular regions and in some cases within brain parenchyma persists at variable levels for prolonged periods. For example, decreased hippocampus neurogenesis observed at 8 months post-exposure (Shetty et al., 2014) could be because of oxidative stress induced loss of neural stem/progenitor cells in the early period after exposure and/or lingering oxidative stress and inflammation impairing neural stem/progenitor cell activity in the hippocampus. Proteomic analyses of plasma from blast injured animals however showed some indirect evidence regarding the persistence of oxidative stress triggered by hypoxia (based on markers 4-HNE, hypoxia-inducible factor-1α and ceruloplasmin) and vascular abnormalities such as BBB leakiness (based on the presence of von Willebrand Factor) at 42 days post-exposure (Ahmed et al., 2013). Thus, correlative histological, biochemical and behavioral studies at multiple time-points after exposure to BSWs are needed in the future. These studies may help in determining whether early pathological changes (oxidative stress, BBB disruption and neuroinflammation and axonal injury): (i) trigger secondary pathological changes in neurons and glia; (ii) impair the function of neural stem/progenitor cells to decrease net hippocampus neurogenesis; (iii) persist for prolonged periods; and (iv) progressively expand to involve more regions of the brain.

CAN MITIGATION OF SHOCK WAVE INDUCED EARLY CHANGES (BBB DISRUPTION AND NEUROINFLAMMATION) RESTRAIN DELAYED NEUROLOGICAL COMPLICATIONS?

Neuroprotective drugs capable of halting or mitigating secondary changes following blast shock wave induced oxidative stress and/or BBB disruption may considerably ease or slow down the development of subsequent neurological and neuropsychiatric impairments such as cognitive problems, memory dysfunction, non-specific mental and emotional symptoms, chronic motor deficits and PTSD (Chen et al., 2013). While the clinical management of blast related brain injury comprises treatment for reducing cerebral edema, intracranial hemorrhage and cerebral vasospasm (Chen et al., 2013), an efficient neuroprotective drug therapy capable of blocking secondary neurological complications of blast-related brain injury is yet to be identified. Hitherto, efficacy of only a few neuroprotective drugs has been assessed in animal models of blast injury. Kovesdi et al. (2012) investigated whether acute treatment with the non-steroidal anti-inflammatory drug minocycline can mitigate the neurobehavioral abnormalities resulting from exposure to BSWs. In this study, 4 h after a single exposure to mild blast overpressure, animals received minocycline (at 50 mg/Kg, once daily for 4 days). Interestingly, memory and anxiety analyses performed at 8 and 45 days post-exposure revealed that blast exposed animals receiving minocycline have similar memory and anxiety scores as control animals whereas blast exposed animals receiving vehicle displayed memory dysfunction and increased anxiety. Furthermore, minocycline treatment normalized serum and tissue levels of several selected

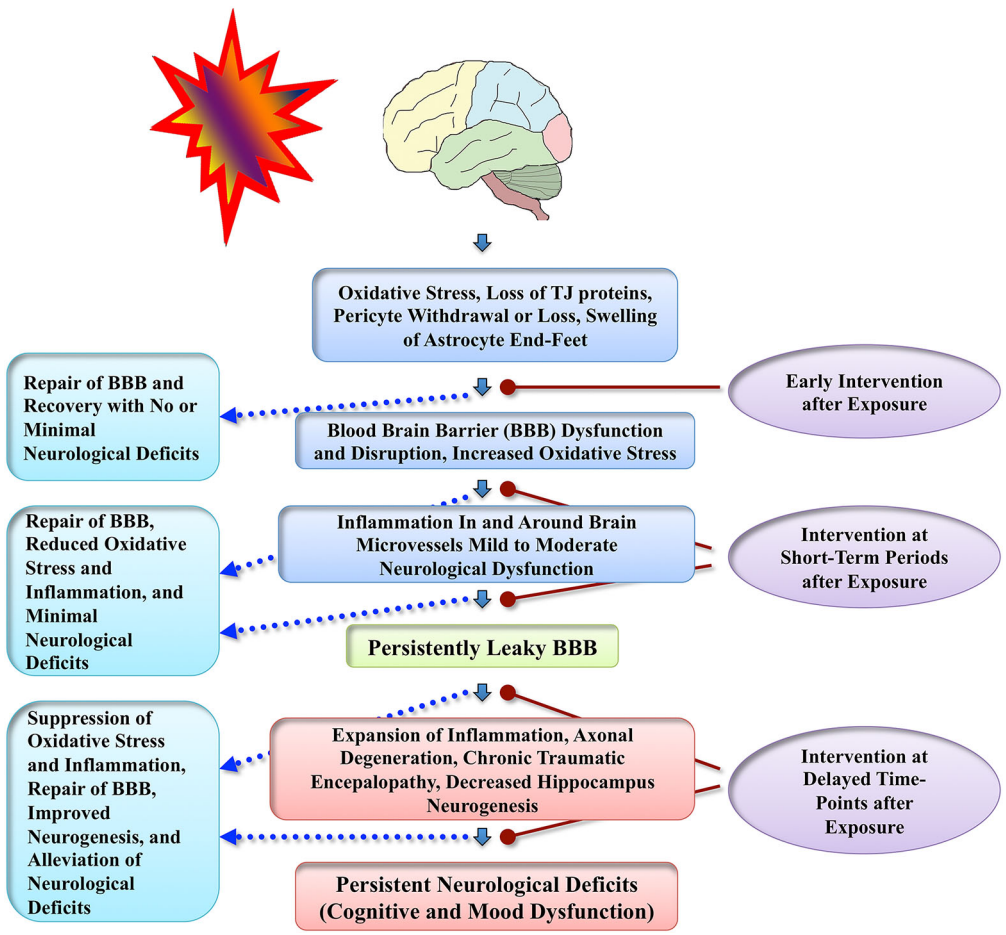

FIGURE 3 | Schematic illustrates conceived sequence of events that precede persistent neurological deficits following exposure to BSWs. Adverse changes likely commence with increased oxidative stress causing dysfunction or disruption of the BBB and inflammation around brain microvessels, which then, depending on the extent of initial injury, evolve into multiple chronic changes such as persistently leaky BBB, expansion of neuroinflammation, axonal degeneration, CTE, and decreased hippocampus neurogenesis. These chronic changes likely underlie neurological impairments seen in patients with mTBI resulting from exposure to BSWs. Figure also illustrates the possible benefits of apt neuroprotective interventions applied at different time-points after the exposure to BSWs. Efficient early neuroprotective interventions (hours after exposure) may repair the BBB quickly and facilitate both structural and functional recovery with no or minimal neurological deficits. Interim interventions (days or weeks after exposure) may be useful for suppressing oxidative stress and inflammation, which would likely repair the BBB and prevent the evolution of initial injury into long-term neurological deficits. Delayed interventions (months or years after exposure) may suppress the chronic oxidative stress, inflammation and axonal degeneration and thereby alleviate neurological deficits that are already present.

inflammatory, vascular, neuronal, and glial markers, implying that blockage of inflammation cascade occurring early after blast-induced BBB damage has promise for easing blast shock wave exposure induced cognitive and mood dysfunction (Kovesdi et al., 2012).

Du et al. (2013) examined the efficacy of antioxidant treatment for blast-related brain injury in a rat model. Rats received a combination of antioxidants (2,4-disulfonyl α-phenyl tertiary butyl nitrone and N-acetylcysteine), an hour after exposure to 14 psi blast overpressure and then twice a day for the following 2 days. Antioxidant treatment reduced 4-HNE, amyloid precursor protein (APP) and neurofilament 68 (NF-68) expression in the hippocampus, 4-HNE expression in the corpus callosum, c-fos expression in the retrosplenial cortex, APP and NF-68 expression in the auditory cortex and medial geniculate nucleus. Although the effects of antioxidants on blast shock wave induced long-term behavioral abnormalities were not examined in this study, the results suggest that antioxidant therapy has promise to ease neurological deficits associated with blast-related brain injuries. Another recent study using a rabbit model suggests that hyperbaric oxygen therapy starting 12 h after exposure to BSWs is neuroprotective, based on observations such as maintenance of the BBB integrity, and inhibition of brain edema, apoptosis and inflammation (Zhang et al., 2014). Overall, neuroprotective studies in blast-induced brain injury models are still in nascent stages partly because pathophysiological sequences of blast-related brain injury are still being worked out in animal models of distinct blast-related brain injuries. However, we will likely see multiple neuroprotective studies in blast-related brain injury models in the coming years, as the number of neuroscientists working on this field has been growing considerably over the last few years. A schematic in **Figure 3** illustrates the possible outcome of interventions applied at different time-points after exposure to BSWs.

CONCLUSIONS

Aspects such as activation of MMPs, and increased levels of ROS and pro-inflammatory cytokines in brain capillaries observed after exposure to BSWs can disrupt the BBB function. Even relatively moderate alterations in the BBB function, such as reduced expression of TJ proteins observed after exposure to milder intensity shock waves, can increase barrier permeability and cause moderate levels of inflammation (e.g., perivascular cuffing) and neuronal dysfunction. On the other hand, a major disruption of the BBB with the loss of pericytes, considerable swelling of astrocyte end-feet and disruption of the BM expected after exposure to moderate to higher intensity of BSWs can cause more robust and long-lasting neuroinflammation and lead to substantial neurodegeneration. If spontaneous repair of the BBB does not occur over days or weeks after such injuries, persistently leaky BBB can contribute to expansion of neuroinflammation, neuronal dysfunction and neurodegeneration in larger areas of the brain. Such changes may cause persistent neurological impairments such as cognitive problems, memory dysfunction, sleep disorder, depression and anxiety, depending upon the regions of brain afflicted with such changes.

However, it remains to be determined whether early changes in cerebral vessels observed after exposure to BSWs trigger long-standing secondary changes or just persist at lower levels for prolonged periods and interfere with the normal brain function. Therefore, to understand BSW induced sequential pathological changes occurring in the brain over days to months, multidisciplinary studies that not only assess structural and neurochemical changes in the brain but also the associated behavioral deficits and alterations in electrical and synaptic properties of neurons will be needed in animal models at multiple early and extended time-points after exposure to BSWs. While animal models may not exhibit all features of blast-related brain injury occurring in humans, they are nonetheless useful for unraveling sequential pathological changes that occur in the brain following an exposure to different intensities of BSWs. Therefore, rigorously examining compounds and drugs that have shown ability to repair the BBB injury, suppress neuroinflammation and provide neuroprotection in other disease models, using well established and reproducible blast-related brain injury models would be beneficial to determine whether an early intervention therapy after exposure to BSWs can block or at least restrain the development of delayed neurological deficits.

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Role of IFN-γ and LPS on neuron/glial co-cultures infected by *Neospora caninum*

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Neospora caninum causes cattle abortion and neurological symptoms in dogs. Although infection is usually asymptomatic, classical neurological symptoms of neosporosis may be associated with encephalitis. This parasite can grow in brain endothelial cells without markedly damages, but it can modulate the cellular environment to promote its survival in the brain. In previous studies, we described that IFN-γ decreased the parasite proliferation and down regulated nitric oxide (NO) production in astrocyte/microglia cultures. However, it remains unclear how glial cells respond to *N. caninum* in the presence of neurons. Therefore, we evaluated the effect of 300 IU/mL IFN-γ or 1.0 mg/mL of LPS on infected rat neuron/glial co-cultures. After 72 h of infection, LPS did not affect the mitochondrial dehydrogenase activity. However, IFN-γ decreased this parameter by 15.5 and 12.0% in uninfected and infected cells, respectively. The number of tachyzoites decreased 54.1 and 44.3% in cells stimulated with IFN-γ and LPS, respectively. Infection or LPS treatment did not change NO production. On the other hand, IFN-γ induced increased nitrite release in 55.7%, but the infection reverted this induction. IL-10 levels increased only in infected cultures (treated or not), meanwhile PGE_2 release was improved in IFN-γ/infected or LPS/infected cells. Although IFN-γ significantly reduced the neurite length in uninfected cultures (42.64%; $p < 0.001$), this inflammatory cytokine reverted the impairment of neurite outgrowth induced by the infection (81.39%). The results suggest a neuroprotective potential response of glia to *N. caninum* infection under IFN-γ stimulus. This observation contributes to understand the immune mediated mechanisms of neosporosis in central nervous system (CNS).

Keywords: *Neospora caninum*, neuron/glial co-culture, immune response, parasite NO downmodulation, neurite impairment

INTRODUCTION

Neospora caninum is a protozoan that causes cattle abortion and neurological symptoms in dogs (Wouda et al., 1998; Dubey, 1999; Jolley et al., 1999). Classical neurological symptoms are related with severe multifocal necrotizing encephalitis associated with mononuclear cell infiltration (Malaguti et al., 2012). However *N. caninum* infection is generally latent and asymptomatic, due to a formation of cysts with bradyzoites (chronic and latent parasite stage) that can be found in any organ, but is more frequently found in animal brains (Kobayashi et al., 2001; Dubey et al., 2004, 2007).

The blood-brain barrier (BBB) protects the brain against exogenous agents and is constituted by physical, metabolic, and active mechanisms (El-Bachá and Minn, 1999). However, this parasite overcomes these mechanisms and is able to infect glial cells inducing an immune regulation during protozoan infection in central nervous system (CNS) tissues (Yamane et al., 2000; Pinheiro et al., 2006a,b). Recently, Elsheikha et al. (2013) showed *that N. caninum* is able to grow in brain microvascular endothelial cells (fundamental component of the BBB) without markedly disrupting their normal proliferation or mitochondrial integrity and it was associated with an increase in infected cell respiration.

The immunopathogenesis of neosporosis is complex and only partially understood. Considering any intracellular microbial agent, cellular stress increases as the infection progresses, and host cells normally develop strategies to compensate it by metabolic shifts as an attempt to maintain energy homeostasis and cell viability, avoiding tissue damages (Elsheikha et al., 2013). Inflammatory mediators can modulate the physiology of the BBB during parasite infection, which could play important roles in CNS inflammation (Abbott, 2005). Buxton et al. (2002) and Hemphill et al. (2006) discussed that the progression of *N. caninum* infection was directed related with a balance between the tachyzoite's ability to penetrate and multiply into host cells and

the host's ability to inhibit parasite multiplication. To clarify the pathogenesis and immune response to this parasite, some authors have studied these mechanisms in cells of the CNS. Yamane et al. (2000) observed a reduction in tachyzoite numbers in bovine cerebellar cells previously infected and stimulated with IFN-γ and TNF-α. However, the mechanism of proliferation inhibition remained unclear, except by determining that it should be independent of nitric oxide (NO) release. Despite this, the production of NO by peripheral immune response may favor the parasite penetration in the brain, since free radicals induce endothelial permeability changes (Lagrange et al., 1999).

Following these findings, Pinheiro et al. (2006a,b) proposed a rat astrocyte primary culture as a new model to study *N. caninum* infection *in vitro*. These authors observed that parasite stimulated astrogliosis and production of IL-10, TNF-α and NO. Thereafter, these authors found similar results with mixed cultures of astrocytes and microglia, observing the production of high levels of IL-6 and no detection of IFN-γ (Pinheiro et al., 2010).

In vitro experiments have revealed that astrocytes are necessary to establish the expression of several proteins and enzymes by brain endothelial cells (Bart et al., 2000). Therefore, infected astrocytes could interfere in the BBB function. Accurate knowledge about interactions between neuron and glial cells and *N. caninum* is required to learn how the infection can disturb the brain homeostasis. Recent studies of our research group proved that the stimulation of glial cells (astrocyte and microglia) with IFN-γ and TNF-α controlled the parasite proliferation independent of NO production, since it was synergically inhibited by IFN-γ and tachyzoites. Additionally, an increase in PGE_2 release was observed in infected cultures, while IL-10 and TGF-β depletion seems to play a possible role on parasite persistence in infected cells. Moreover, while both regulatory cytokines did not interfere in the modulation of NO synthesis, IL-10 could stimulate the release of PGE_2 (Jesus et al., 2013).

To continue these studies, it is necessary to understand how glial cells respond to *N. caninum* infection when neurons are present. Some studies have showed that glia can affect neurons by releasing neurotransmitters and other extracellular signaling molecules. Indeed, it is known that the interplay between resident cells of the CNS and peripheral immune response is complex and it can lead to neurotoxic or neuroprotecting effects (reviewed by Kerschensteiner et al., 2009). On the other hand, the parasite could also act in the modulation of this response. As suggested by Elsheikha et al. (2013), it is possible that the parasite could modify the cellular environment to promote its own intracellular survival.

This is necessary to understand how the interaction between *N. caninum*, neurons and glial cells affects immunopathogenic mechanisms and the response to infection. Therefore, the aim of the present study was to evaluate the effect of inflammatory stimulus (IFN-γ and LPS) on neuron/glial co-cultures infected with *N. caninum* in order to understand aspects of mediators release and their influence on neurotoxic/neuroprotective effects induced by the parasite infection.

MATERIAL AND METHODS

NEURON/GLIAL CO-CULTURES

Mixed glial cells (astrocytes and microglia) were first obtained from brain cortices of newborn rats (<48 h of age) by mechanical dissociation of the tissue. The cultures were maintained in Dulbecco's modified Eagle's medium-F12 (DMEM-F12) supplemented with 10% (v/v) fetal bovine serum, 100 IU/mL penicillin G, 100 g/mL streptomycin, 2 mM L-glutamine, 0.011 g/L pyruvate, 3.6 g/L Hepes and 12 mM glucose, incubated at 37°C in a humid atmosphere with 5% CO_2. All of these reagents were purchased from Gibco/Invitrogen.

These cultures were initially seeded onto culture dishes with the diameter of 100 mm (TPP, Switzerland) and after 14 days, they were re-seeded (5×10^4) in tissue culture plates with 24-wells for assays. This culture was previously characterized as containing about 86% of astrocytes and 12% of microglia (Pinheiro et al., 2010).

At this time, embryos were removed from pregnant rats on the 17th or 18th gestational day by cesarian section. Cells were dissociated from embryo brain cortices in DMEM/F-12 as described above. Neurons (2.5×10^4/well) were plated on astrocyte/microglia monolayer and the cultures were maintained with regular DMEM/F-12 changed every 48 h for 7 days, when the experiments were performed. All animal procedures were performed in accordance with the local Ethical Committee for Animal Experimentation.

CULTURE OF *NEOSPORA CANINUM*

N. caninum tachyzoites of the NC-1 strain were maintained in Vero cells in RPMI 1640 medium (Gibco BRL, USA) supplemented with 10% (v/v) fetal bovine serum (Gibco BRL, USA), 100 IU/mL penicillin G and 100 g/mL streptomycin (CULTILAB, Brazil). To obtain the parasites, the Vero cells were first washed with phosphate buffered saline (PBS) and then mechanically disrupted. Thereafter, tachyzoites were purified using a 5.0 μm filter (Millipore, Carrigtwohill, Ireland) as described by Pinheiro et al. (2010).

NEURON/GLIAL CO-CULTURE INFECTION AND TREATMENT

Neuron/Glial co-cultures were treated with 300 IU/mL of recombinant rat IFN-γ (R&D Systems, USA) or 1.0 mg/mL of LPS from *Escherichia coli* 0111:B4 (Sigma-Aldrich, USA) diluted in culture medium. Cells were treated only with fresh medium in control conditions. Twenty-four hours after treatment, neuron/glial co-cultures were infected with tachyzoites of *N. caninum* (host:parasite ratio of 1:1). Analyses were performed 72 h post-infection as determined in previous studies (Jesus et al., 2013).

MTT ASSAY

The MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide] assay was performed to evaluate the energetic metabolic activity of cells. The assay is based in the ability of alive cells to convert yellow MTT in purple formazan crystals by mitochondrial dehydrogenases. The experiment was performed in 96-well plates (TPP, Switzerland). Briefly, cells (1×10^4 cells/well)

under different culture conditions were incubated with MTT at a final concentration of 1.0 mg/mL for 2 h. Thereafter, cells were lyzed with 20% (w/v) sodium dodecyl sulfate (SDS), 50% (v/v) dimethyl formamide (DMF) (pH 4.7), and plates were kept overnight at 37°C in order to dissolve formazan crystals. The optical density was quantified at 580 nm (Hansen et al., 1989). Three independent experiments were carried out with eight replicate wells for each analysis. Results are shown as mitochondrial activity percentage compared to untreated/uninfected control cultures, considered as 100%.

DETERMINATION OF PARASITE NUMBER

To quantify the parasite in cultures, the number of tachyzoites was counted in each culture 72 h after infection. To ensure that all parasites (intra- and extracellular) were counted, culture monolayers were scraped with their culture media, the cells were ruptured by three passages through a 22-gauge needle and tachyzoites were counted using a hemocytometer, as described by Yamane et al. (2000). Three independent experiments were performed in triplicate by two independent investigators in a blind assay. The results are expressed as the mean of tachyzoite percentages compared with the untreated control cultures (considered as 100%).

MEASUREMENT OF NITRITE LEVELS

Supernatants from neuron-glial co-cultures were assayed in triplicate for nitrite content, which reflects NO production, using the Griess reagent (1% sulfanilamide and 0.1% naphthyl-ethylenediamine dihydrochloride in 2.5% phosphoric acid in equal volumes). After 15 min of incubation at room temperature, the absorbance was measured at 560 nm using a microtiter plate reader (Biotek instruments, Inc., USA). Nitrite concentrations were calculated by comparison with a standard calibration curve of sodium nitrite ($NaNO_2$:1.26–100 mM/L) with DMEM-F12 as the baseline control.

PGE_2 LEVELS DETERMINATION

Culture supernatants from the different treatments were assayed for PGE_2 levels using an enzyme immunoassay kit (Cayman Chemical Co., USA), according to manufacturer instructions. This assay has a detection limit of 15 pg/mL.

CYTOKINES DETERMINATION

TNF-α and IL-10 were measured in the culture supernatants by using a commercial kit (Sandwich ELISA, R&D, USA), according to manufacturer instructions. Cell culture medium (three samples of three independent experiments) was collected 72 h after infection, centrifuged at 3500 g during 5 min and stored at −70°C until the time of assay. Results are expressed as percentage of concentration means compared to untreated control cultures, considered as 100%.

MORPHOMETRY ANALYSIS

β-III tubulin immunocytochemistry was performed to detect neurites. Cells were incubated with mouse monoclonal anti-β-III tubulin (Santa Cruz Biotechnology Inc., USA) diluted 1:400 in TBS (tris buffer solution), overnight at 4°C in a humid chamber. Then, these cells were incubated with goat anti-mouse IgG peroxidase (1/400 in TBS, Bio-Rad, Hercules, CA) and the immunoreactivity was visualized using a peroxidase-conjugated substrate kit according to manufacturer's instructions (Bio-Rad, Hercules, CA). Co-staining (a blue panchromic differential staining to nuclei and other cytoplasmic components) was performed by using the protocol established by Rosenfeld (1947). The Rosenfeld's reagent (1 mL) was added and incubated for 20 min at room temperature. Thereafter, the plates were rinsed with water, air dried, analyzed and photographed in an optical phase microscope (Nikon TS-100) using a digital camera (Nikon E-4300).

Neurite lengths were determined using NIH software Image J, with Neuron J plug-in (Copyright from Erik Meijering). Three independent experiments were performed and neurites of each neuron were measured in five randomly chosen fields per sample. Results are shown as percentage of mean total neurite length compared to untreated/infected control neurons, considered as 100%.

Statistical analysis

The results are expressed as the mean ± the standard deviation (SD). The comparisons between the experimental groups and the corresponding controls were performed with GraphPad Prism 6 for Mac OS X (GraphPad Software, Inc.) using a two-way ANOVA, except to parasite number evaluation that one-way ANOVA followed by a Tukey post-test was performed. Probability values (p) of 0.05 or less were considered significant.

RESULTS

IFN-γ DECREASED THE MITOCHONDRIAL ACTIVITY

MTT assay was performed to evaluate whether the experimental treatment with cytokines induced a metabolic challenge to cells. Under experimental conditions, untreated and LPS-treated cultures (infected or not) did not show reduction in mitochondrial dehydrogenase activities. However, IFN-γ decreased mitochondrial dehydrogenase activities by 15.5 in uninfected cells (**Figure 1**).

IFN-γ AND LPS DECREASED THE PARASITE NUMBER

The inflammatory microenvironment in neuron/glial co-cultures induced by the experimental treatment reduced the parasite number. After 72 h of infection, the number of *N. caninum* tachyzoites decreased 54.1 and 44.3% in cells stimulated with IFN-γ and LPS, respectively (**Figure 2**).

IFN-γ INDUCED NO PRODUCTION, BUT THE PARASITE ABOLISHED THIS EFFECT

Nitrite levels were measured in culture media under inflammatory stimulus as a parameter to evaluate NO production. In untreated/infected cultures, the nitrite concentration in supernatant did not change. As expected, IFN-γ induced NO production (increased 55.7%) in uninfected cultures, but the infection decreased this induction, significantly. Meanwhile, LPS stimulus did not change nitrite levels compared with control conditions (**Figure 3A**).

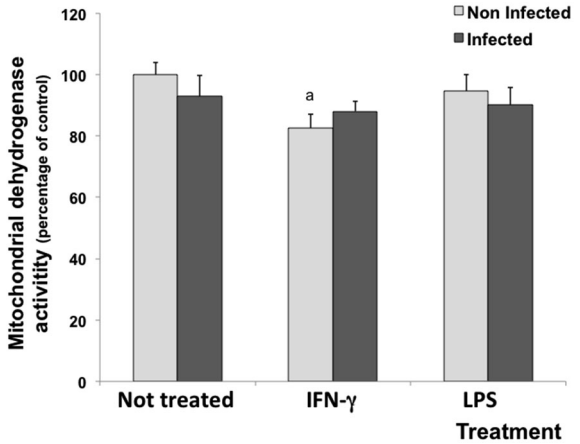

FIGURE 1 | Percentage of cell viability measured by the MTT assay in rat neuron/glial cell co-cultures treated with 300 IU/mL of IFN-γ and 1.0 mg/mL of LPS and infected with *Neospora caninum* tachyzoites (ratio cell:parasite 1:1). The results are expressed as the percentage of cell viability observed in different treatment conditions and the respective standard deviation compared with untreated/uninfected control cultures (considered as 100%) 72 h post infection. The results are expressed as the mean of the percentage and the respective standard of eight samples, in three independent experiments. "a" represents a significant statistical difference when compared to untreated/uninfected control cultures; (Two-way ANOVA/Tukey's Multiple Comparison Test—$p < 0.05$).

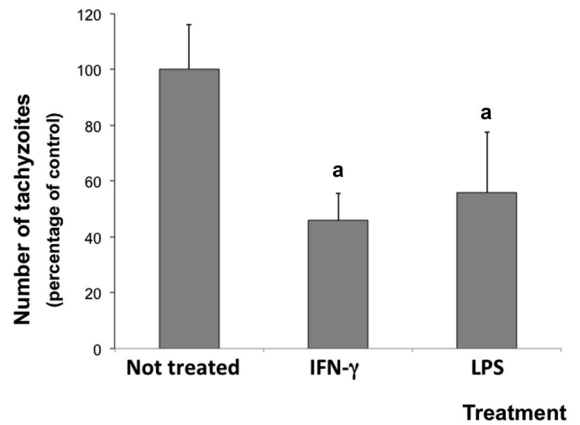

FIGURE 2 | Number of tachyzoites in rat neuron/glial cell co-cultures treated with 300 IU/mL IFN-γ or 1.0 mg/mL LPS and infected with *Neospora caninum* tachyzoites (ratio cell:parasite 1:1), 72 h post infection. Results are expressed as means (± standard deviations) of tachyzoite percentages compared to non-treated/infected control cultures (considered as 100%) in three independent experiments carried out in triplicate. "a" represents a significant statistical difference when compared to not treated/infected cultures (One Way ANOVA/Tukey's Multiple Comparison Test—$p < 0.05$).

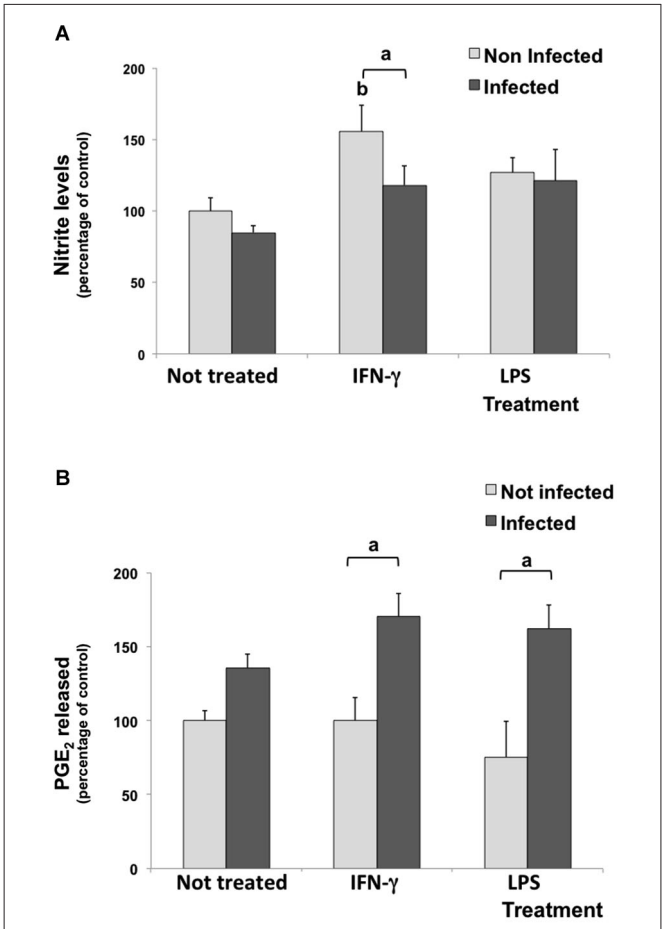

FIGURE 3 | Nitrite (A) and Prostaglandin E2 (PGE_2) (B) levels in culture media of rat neuron/glial cell co-cultures treated with 300 IU/mL of IFN-γ and 1 mg/mL of LPS and infected with *Neospora caninum* tachyzoites (ratio cell:parasite 1:1), 72 h post infection. Results are expressed as the means (±SD) of the percentage of nitrite **(A)** or PGE_2 **(B)** compared to control conditions (considered as 100%) in three independent experiments carried out in triplicate. "a" represents a significant statistical difference between the same treatment group; "b" represents a significant statistical difference when compared to untreated/uninfected control cultures (Two-way ANOVA/Tukey's Multiple Comparison Test—$p < 0.05$).

IFN-γ AND LPS INDUCED PGE_2 RELEASE IN INFECTED CELLS

Culture media were also assayed for PGE_2 levels. Although the inflammatory treatment with IFN-γ or LPS did not change the PGE_2 release in uninfected cells, levels increased by 70.4% and 86.5% in IFN-γ-treated/infected and in LPS-treated/infected cultures, when compared to IFN-γ and LPS treated cultures, respectively (**Figure 3B**).

LPS INCREASED THE RELEASE OF TNF-α AND LPS INCREASED THE RELEASE OF IL-10

To investigate glial immune response in this culture infection model, the release of TNF-α and IL-10 cytokines was measured in cell culture supernatants (**Figure 4**). The infection or the IFN-γ treatment did not change the basal level of TNF-α measured in untreated/uninfected control cultures. However, the LPS treatment significantly increased TNF-α release both in uninfected and infected cultures (**Figure 4A**).

In infected cultures, the IL-10 amount in supernatant increased about 45% when compared with untreated/uninfected cultures (**Figure 4B**). However, IFN-γ and LPS did not change it.

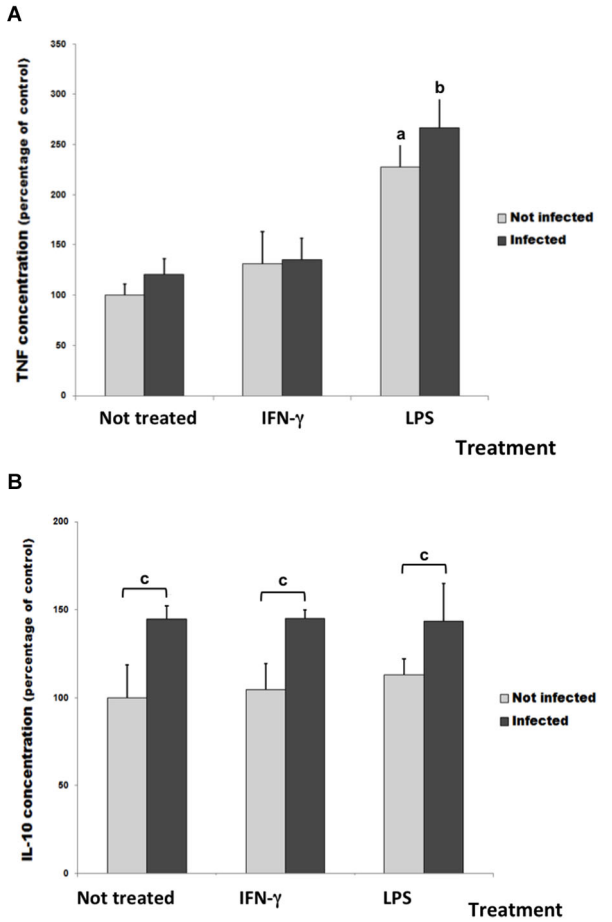

FIGURE 4 | TNF-α (A) and IL-10 levels (B) in culture medium of rat neuron/glial cell co-cultures treated with 300 IU/mL of IFN-γ or 1 mg/mL of LPS and infected with *Neospora caninum* tachyzoites (ratio cell:parasite 1:1), 72 h post infection. Data represent the percentage of concentration means and its respective standard deviation compared to untreated control cultures (considered as 100%) three independent experiments carried out in triplicates. "a" represents a significant statistical difference when compared to untreated/uninfected control cultures; "b" represents a significant statistical difference when compared to untreated/infected cultures; "c" represents a significant statistical difference between the same treatment group; (Two-way ANOVA/Tukey's Multiple Comparison Test—$p < 0.05$).

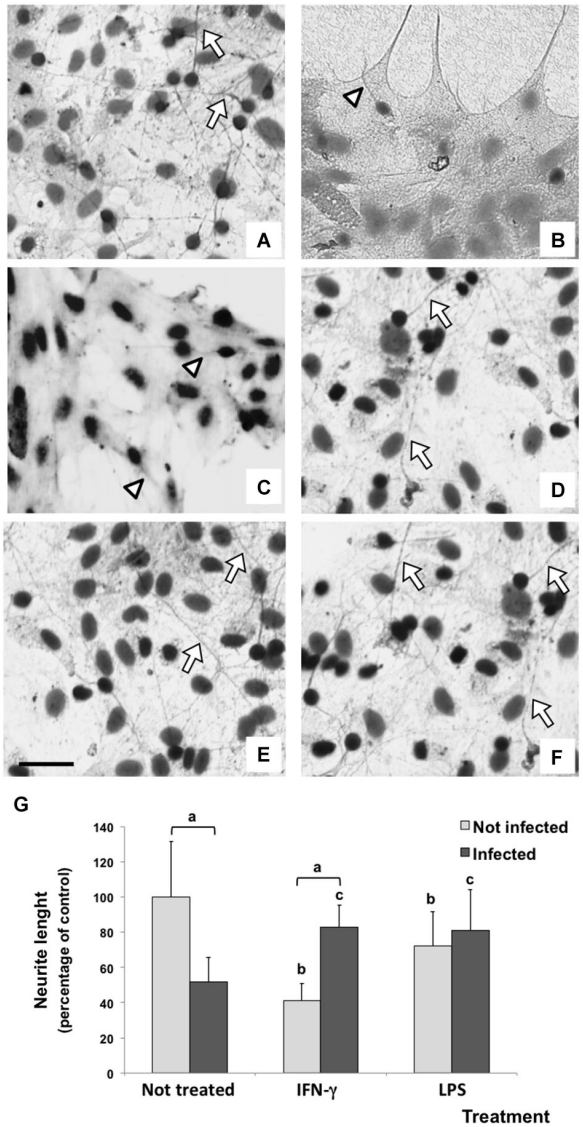

FIGURE 5 | Immunodetection (immunoperoxidase) of tubulin βIII of rat neuron/glial cells co-culture treated with 300 IU/mL of IFN-γ and 1 mg/mL of LPS and infected with *Neospora caninum* tachyzoites (ratio cell:parasite 1:1), 72 h post infection. Control cells maintained in fresh medium exhibit a basal neurite outgrowth (arrow) **(A)**; a drastic impairment of neurite (arrowhead) was observed in *N. caninum* infected culture **(B)** and IFN-γ treated cells **(C)**; neurite length (arrow) in IFN-γ/infected cultures **(D)**; LPS/uninfected and LPS/infected cells did not exhibit changes in neurite lenght **(E,F)**. Scale bar = 50 μm. **(G)** Measurement and statistical analysis of neurite length (percentage of control) and its respective standard deviation compared to untreated/uninfected cultures (considered as 100%) in three samples in three independent experiments. "a" represents a significant statistical difference between the same treatment group; "b" represents a significant statistical difference when compared to untreated/uninfected control cultures; "c" represents a significant statistical difference when compared to untreated/infected cultures (Two-way ANOVA/Tukey's Multiple Comparison Test—$p < 0.05$).

IFN-γ RESTORED NEURITE OUTGROWTH IN INFECTED CELLS

In this study, neurite outgrowth length was used as a parameter to evaluate the neuronal ability to maintain the dynamics of the tubulin and actin cytoskeletons (**Figure 5**), which is essential for the establishment of synapses. The basal neurite length under control conditions was considered as 100%. Untreated/infected neuron/glial co-cultures showed a drastic impairment of neurite outgrowth (reduction of 51.47%; $p < 0.001$), which can represent a possible deleterious effect of parasite infection. Although IFN-γ significantly reduced the neurite length in uninfected cultures (42.64%; $p < 0.001$), this inflammatory cytokine reverted the impairment of neurite outgrowth induced by the infection (81.39%).

A similar result was observed in LPS treated cultures. While neurite length reduced in LPS treated/uninfected cultures (72.3%;

$p < 0.001$), LPS also inhibited a further impairment of neurite outgrowth induced by the infection (increase of 29.4% when compared with untreated/infected cultures).

DISCUSSION

Previous studies of our group described that astrocyte/microglia cultures infected with *N. caninum* presented a decreased parasite proliferation, release of PGE_2 and down modulation of NO after IFN-γ stimulation (Jesus et al., 2013). These events were associated with a parasite escape mechanism and an anti-inflammatory pattern of response by infected glial cells, suggesting a possible glial protective role on nervous tissue during this parasite infection. However, the knowledge of these response effects in the presence of neurons remains unclear. In this study, we have studied the glial response to *N. caninum* infection in a neuron/glial co-culture model and their consequences.

Different from previously observed in astrocyte/microglia mixed cultures, neuron/glial co-cultures showed reduced mitochondrial dehydrogenase activities when stimulated with 300 IU/mL IFN-γ, independent of infection. Therefore, this suggests a toxic effect of this cytokine on neuron/glial co-cultures, affecting the mitochondrial function.

The BBB is more than a physical barrier constituted by tight junctions of brain endothelial cells. Enzymes involved in the detoxification of the metabolism of numerous endogenous and exogenous compounds, such as UDP-glucuronyltransferases (EC 2.4.1.17), are expressed in microsomes of the brain (El-Bachá et al., 2000), constituting a metabolic barrier. Furthermore, some studies have shown that reactive astrocytes release a wide array of mediators, including pro- and anti-inflammatory cytokines, neurotrophic or neurotoxic factors, chemokines, complement factors and reactive oxygen species (ROS; Liberto et al., 2004; Farina et al., 2007; Sofroniew and Vinters, 2010; Allaman et al., 2011). This means that there is also an immunological barrier protecting neurons against biological agents. We previously showed that *N. caninum* induced the expression of IL-10 in rat astrocyte primary cultures, but these cells did not release IFN-γ (Pinheiro et al., 2006b). However, IFN-γ is one of the most important cytokines involved in the control of *N. caninum* growth, because IFN-γ-deficient mice succumb to acute infection with tachyzoites (Nishikawa et al., 2001). Moreover, IFN-γ inhibited *N. caninum* growth in human astrocytoma cells (Spekker et al., 2009). In the present work, IFN-γ decreased the activity of mitochondrial dehydrogenases even in uninfected cells. Therefore, this may challenge neurons.

On the other hand, the inflammatory stimulus was able to reduce the parasite number in infected neuron/glial co-culture. Some previous studies have shown reduction in tachyzoite number mediated by inflammatory stimulus both *in vitro* and *in vivo* (Innes et al., 1995, 2000; Khan et al., 1997; Tanaka et al., 2000). In brain cells, Yamane et al. (2000) showed that IFN-γ and TNF-α inhibited the parasite growth in bovine cerebellar cells *in vitro*. Similarly, previous findings of our research group (Jesus et al., 2013) had already obtained a similar result in primary cultures of glial cells (astrocytes and microglia).

A large number of studies have shown the role of IFN-γ in controlling *T. gondii* proliferation, but the exact mechanism that promotes this anti-parasitic effect remains uncertain (Jun et al., 1993; Halonen et al., 1998, 2001; Halonen and Weiss, 2000; Freund et al., 2001; Scheidegger et al., 2005; Delair et al., 2009). Some evidences indicate that cytokines, mainly IFN-γ, can also activate astrocytes to inhibit the growth of *T. gondii*, but the mechanism of inhibition remains to be elucidated (Halonen et al., 1998). These authors showed that this event is not due to NO production and that the addition of tryptophan had no effect on inhibition, indicating that the mechanism was not mediated via indoleamine 2,3-dioxygenase (IDO) induction.

In the same way, studies about the role of IFN-γ in controlling *N. caninum* proliferation are controversial. Tanaka et al. (2000) indicated a NO dependent mechanism as responsible to kill tachyzoites inside macrophages. Using *in vivo* models, Baszler et al. (1999) showed the role of IFN-γ in controlling acute neosporosis in mice. However, the development of encephalitis and parasite proliferation were more related to the absence of IL-4-mediated response than a strong IFN-γ response.

The mechanisms involved on the parasite destruction induced by IFN-γ cytokine or other inflammatory stimuli still need to be clarified. Vonlaufen et al. (2002) reported that after 5 days of infection with *N. caninum* tachyzoites, IFN-γ treated slices of CNS organotypic cultures showed only small necrotic pseudocysts and many parasites supposedly dead after cell invasion. However, it is not yet possible to say whether the reduction in the number of tachyzoites reported in this study was due to a reduction in the invasiveness of the parasite and/or an increased ability to destroy the infected cells in their cytoplasm.

This study did not observe a reduction on nitrite levels in the supernatant of infected co-cultures. This disagrees with our previous findings, in which the infection reduced NO release in astrocyte/microglia mixed cultures (Jesus et al., 2013). However, in IFN-γ treated co-cultures, the infection reduced NO production. Rozenfeld et al. (2005) provided evidence that the NO production of IFN-γ-activated microglia is inhibited by *T. gondii* infection, which appears to favor neuron viability. Previously, Yamane et al. (2000) showed that NMMA (an iNOS selective inhibitor) did not reverse the inhibition of parasite growth by IFN-γ in cerebellar bovine cells. Therefore, we can suppose that the parasite reduction in IFN-γ-stimulated cultures might be induced through other mechanisms than NO. Nevertheless, further studies should be performed to elucidate the NO down-modulation mechanism by the parasite infection on glial cells.

Another possibility to be considered involves the consumption of NO to produce peroxynitrite anion—a potent oxidant and toxic agent—by NADPH oxidase stimulation in glial cells (Minghetti and Levi, 1998; Bal-Price et al., 2002; Brown and Bal-Price, 2003). However, peroxynitrite determination was not tested in this study.

These data suggests two interpretations: (1) the reduction in the tachyzoite number promoted by inflammatory stimuli should not be mediated by NO; (2) *N. caninum* infection in glial cells can induce parasite escape mechanisms, which could, among other things, decrease NO production. A possible direct action

of the parasite is reinforced by Rozenfeld et al. (2005). These authors observed that in microglia cultures treated with IFN-γ the inhibition of iNOS expression was restricted to *T. gondii* infected cells.

Despite its classic performance as a pro-inflammatory molecule, PGE_2 also plays a role in neuronal injury protection by decreasing NO production in activated microglia and modulating proinflammatory events (Aloisi et al., 1999; Zhang and Rivest, 2001). In *T. gondii* infection it is believed that PGE_2 may be especially favorable to nervous tissue, modulating the immune response and contributing to maintain the integrity of brain cells (Rozenfeld et al., 2003).

In this study, we observed an increase in PGE_2 released by IFN-γ-treated/infected and LPS-treated/infected cultures. This fact could be indirectly related with regulatory mechanisms, triggered by inflammatory stimulus, that contribute to NO down modulation in the presence of the parasite. However, further experiments should be conducted to confirm this hypothesis. PGE_2 has been also associated with an enhancement of regulatory cytokine secretion, as IL-10. Some studies showed exogenous PGE_2 inducing cAMP up-regulation, which leads to TNF-α and IL-12 inhibition and over expression of IL-10 (Aloisi et al., 1997, 1999; Levi et al., 1998; Rozenfeld et al., 2003).

The role of regulatory cytokine IL-10 in inflammatory and infectious diseases has been largely studied, as reviewed by Ouyang et al. (2011). This cytokine can also facilitate the tissue-healing process in injuries caused by infection or inflammation, repressing pro-inflammatory responses and limiting unnecessary tissue disruptions caused by inflammation. In this study, IL-10 was produced only in response to infection. This agrees with previous findings of (Pinheiro et al. (2006b, 2010)) who showed IL-10 overexpression by *N. caninum* infected astrocyte and in microglia/astrocyte cultures.

Due to these facts, a limited involvement of PGE_2 and IL-10 on glial responses to infection can be supposed. However, these mediators are only two in a large number of molecules (not yet identified) that act in the complex cellular/molecular interaction during neuroglial response to *N. caninum* infection. Kerschensteiner et al. (2009) propose that the neuro-immune crosstalk in CNS is not determined by single molecules or even classes of individual molecules, but by the integration of multiple signals that individually may favor the destruction or tissue repair.

In neuron/glial co-culture, the parasite infection inhibited drastically neurite outgrowth, showing a *N. caninum*-mediated neurotoxic effect. This fact could be associated with neurological symptoms and pathological findings in infected animal (Dubey et al., 1998; Poli et al., 1998; Reichel et al., 2007; Dubey and Schares, 2011). In the same way, IFN-γ stimulus induced a severe neuronal damage. Fortunately, this cytokine was not detected in infected astrocytes and microglia cultures (Pinheiro et al., 2006b, 2010). Lipopolysaccharide induced the same inflammatory effect in neurite outgrowth. Kitayama et al. (2011) showed neurite outgrowth inhibition by LPS stimulated microglia. Indeed, a combined effect between tachyzoite and these inflammatory stimuli can be supposed, since the neurite length was preserved in IFN-γ- or LPS treated/infected cultures.

The preservation of neurite outgrowth in IFN-γ-treated/infected co-cultures can be not associated to an indirect effect of PGE_2 and IL-10 secreted by IFN-γ-treated/infected cells as supposed by Rozenfeld et al. (2003). In this study, PGE_2 and IL-10 were also released by untreated/not infected cultures, in which neurite impairment was more evident. A direct effect of neuron parasite infection has to be more studied to clarify how the neurotoxic effect of *N. caninum* infection occurs.

The mechanism by which IFN-γ-stimulated glia can protect neurons in *N. caninum* infected cultures are still under investigation in our research group. The IFN-γ pretreatment could induce astrocyte stored glycogen, which sustains their own energy requirements and enables them to support neighboring neurons through the export of glucose or lactate (Liberto et al., 2004). Other hypothesis is the up regulation of neurotrophic factors by glia-derived cytokines. Nerve growth factor (NGF), brain-derived neurotrophic factor (BDNF), neurotrophin-3 (NT-3) and glial cell-derived neurotrophic factor (GDNF) are able to promote neuronal survival via tyrosine kinase receptors or inhibition of NO synthetase expression (Barbacid, 1995; Wang et al., 1997).

Much more knowledge are needed to elucidate the immunoregulatory glial role during *N. caninum* infection in the CNS. The results presented here should contribute with this understanding. Under IFN-γ stimulus, the parasite reduction number associated with inhibition of NO production, release of PGE_2 and IL-10, and neurite length preservation suggest a neuroprotective response. However, the mechanism triggered by IFN-γ stimulus and the parasite infection that leads to neuroprotection needs to be clarified. This observation can contribute to understand immune-mediated mechanisms of neosporosis in the CNS and contribute to further *in vivo* experiments. Furthermore, it contributes to understand how immunological mechanisms help to protect neurons, especially when the physical, metabolic and other mechanisms of the BBB fail to protect the brain against biological challenges.

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Astrocytic modulation of blood brain barrier: perspectives on Parkinson's disease

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The blood–brain barrier (BBB) is a tightly regulated interface in the Central Nervous System (CNS) that regulates the exchange of molecules in and out from the brain thus maintaining the CNS homeostasis. It is mainly composed of endothelial cells (ECs), pericytes and astrocytes that create a neurovascular unit (NVU) with the adjacent neurons. Astrocytes are essential for the formation and maintenance of the BBB by providing secreted factors that lead to the adequate association between the cells of the BBB and the formation of strong tight junctions. Under neurological disorders, such as chronic cerebral ischemia, brain trauma, Epilepsy, Alzheimer and Parkinson's Diseases, a disruption of the BBB takes place, involving a lost in the permeability of the barrier and phenotypical changes in both the ECs and astrocytes. In this aspect, it has been established that the process of reactive gliosis is a common feature of astrocytes during BBB disruption, which has a detrimental effect on the barrier function and a subsequent damage in neuronal survival. In this review we discuss the implications of astrocyte functions in the protection of the BBB, and in the development of Parkinson's disease (PD) and related disorders. Additionally, we highlight the current and future strategies in astrocyte protection aimed at the development of restorative therapies for the BBB in pathological conditions.

Keywords: BBB, astrocytes, reactive astrogliosis, endothelial cells, Parkinson disease

INTRODUCTION

The Blood Brain Barrier (BBB) is an essential regulatory component of the neural interface with the brain vasculature. It exerts a tightly regulation in the movement of ions, molecules and cells between the neural cells and the blood (Daneman, 2012; Wong et al., 2013), thus maintaining the ionic homeostasis, hormonal and transmitter levels and transport of nutrients in the brain (Luissint et al., 2012). In this aspect, BBB is important for the separation of neurotransmitters pools and neuroactive agents that regulate brain microenvironment (Abbott et al., 2006). Furthermore, the BBB supplies the brain with different nutrients, exerts a restriction of ionic substances between the blood and the brain through specific ion transporters, regulates the ISF (interstitial fluid), prevents the formation of additional injuries during diseases and cerebrovascular accidents and is an important barrier for the brain transport and metabolization of drugs (Abbott et al., 2006; Daneman, 2012; Wong et al., 2013).

The BBB is composed by brain capillary endothelial cells (ECs), with a specific phenotype located in a strong association with astrocytic endfeet processes and mesenchymal-like cells pericytes. Importantly, the BBB is characterized by the presence of tight junctions between ECs, and the expression of specific polarized transport systems (Luissint et al., 2012). On the other hand, astrocytes through their endfeet establish the link between the endothelial blood flux and neurons, and are important regulators in the formation and maintenance of the BBB (Alvarez et al., 2013). BBB dysfunction has been associated with pathological conditions and diseases including cerebral ischemia, brain trauma, glioblastoma, stroke, multiple sclerosis, epilepsy, Alzheimer and Parkinson's Disease (PD; Haseloff et al., 2005; Daneman, 2012; Alvarez et al., 2013).

PD is a progressive neurodegenerative disorder caused by neuronal death in substantia nigra (SN), degeneration of dopaminergic neurotransmission, and the presence of α-synuclein and protein inclusions in neuronal cells, also known as Lewy bodies (Nutt and Wooten, 2005; Halliday and Stevens, 2011). Main symptoms of Parkinson include asymmetrical bradikinesia, rigidity, resting tremor and postural instability (Fernandez, 2012; Singer, 2012). Initiation and progression of PD is dependent upon cellular events, such as failures in the

protein degradation machinery, oxidative stress, mitochondrial dysfunction, defects in mitochondrial autophagy (mitophagy) and the continuous accumulation of α-synuclein, driven through cell to cell interactions between glial cells and neurons that ultimately lead to apoptosis (Jenner, 2003; Halliday and Stevens, 2011; Vives-Bauza and Przedborski, 2011). Although there is not a cure for the disease, the most used and cheaper treatment for PD continues to be Levodopa, frequently accompanied by carbidopa or benserazide (Singer, 2012; Ossig and Reichmann, 2013). However, about 40% of patients developed motor fluctuations and dyskinesias after 4 to 6 years of treatment (Ogawa et al., 2005; Fernandez, 2012), demonstrating that further pharmacological research is needed in order to counterbalance these side effects.

Current research suggests that the exact cause of PD remains unknown (Hirsch et al., 2003; Fernandez, 2012; Schwartz and Sabetay, 2012). Mutations in various proteins such as LRRK2, PARK2), phosphatase and tensin homolog (PTEN)-induced putative kinase 1 (PINK1), and (DJ-1) have been observed in familiar cases of Parkinson, which only account for 10–15% of diagnosed cases (Hirsch et al., 2003; Rappold and Tieu, 2010; Pan-Montojo et al., 2010; Wang et al., 2011). Similarly, various environmental factors have been found to induce PD-like symptoms, including vascular insults to the brain, oxidative stress, neuroleptic drugs, heavy metals exposure and the exposure to pesticides like rotenone or paraquat (Betarbet et al., 2000; Brown et al., 2006; Rappold and Tieu, 2010; Tanner et al., 2011). Similarly, there is clinical and *in vitro* evidence of BBB disruption during PD development (Kortekaas et al., 2005; Hirano et al., 2008; Ohlin et al., 2011; Lee and Pienaar, 2014). In this aspect, previous studies have suggested that α-synuclein deposition has an increase in BBB permeability (Jangula and Murphy, 2013), suggesting the importance of α-synuclein in BBB disruption and PD development. (Braak et al., 2006; Halliday and Stevens, 2011).

A great body of research has shown the importance of astrocytes in the maintenance of BBB properties both during normal and pathological conditions (Ramaswamy and Kordower, 2009; Yasuda and Mochizuki, 2010; Alvarez et al., 2013). Astrocytic secreted molecules are important for the regulation of interactions between BBB components such as ECs and pericytes (Alvarez et al., 2013; Lee and Pienaar, 2014). Furthermore, astrocytes produce antioxidative molecules like GSH, ascorbate and SOD (superoxide dismutase) and a great number of growth factors and neurotrophins, important for brain cell survival during neurodegenerative processes (Dringen, 2000; Ramaswamy and Kordower, 2009; Yasuda and Mochizuki, 2010; Zheng et al., 2010; Barreto et al., 2011).

In the present review we provide a throughout overview of the astrocytic functions in the BBB and its importance during pathophysiological events elicited in PD. Additionally, we highlight the current and future strategies in astrocyte protection aimed at the development of restorative therapies for the BBB in pathological conditions.

COMPONENTS OF THE BBB

ENDOTHELIAL CELLS

ECs within the brain have a characteristic phenotype that makes them different from EC located elsewhere (Dejana, 2004; Stamatovic et al., 2008; Nag, 2011; Daneman, 2012). For example, brain ECs have similarities with epithelial cells, as they are polarized cells that express some specific transporters and in that they are connected by circumferential tight junctions that interfere with the paracellular transport of molecules and ions between cells (Nag, 2011; Daneman, 2012). As well, brain EC have an increased density of mitochondria when compared with the peripheral vasculature, suggesting a higher risk of reactive oxygen species (ROS) formation (Nag, 2011; Lee and Pienaar, 2014). Structurally, EC are in contact with astrocytic endfeet and pericyte through the basal lamina, thus forming the neurovascular unit (NVU), with neurons (Hawkins and Davis, 2005; Stanimirovic and Friedman, 2012; Najjar et al., 2013).

Among its functions in BBB maintenance, EC are important in the bidirectional transport across the brain through ion transporters, protein and peptide carriers and active efflux transport (Nag, 2011). Furthermore, EC have highly organized tight and adherent junctions which restrict the passage of polar substances including hexose sugars, amino acids, nucleosides monocarboxylic acids, and vitamins (Grammas et al., 2011; Mokgokong et al., 2014). Importantly, the integrity of tight junctions is essential to prevent the paracellular transport of many molecules and ions, and its disruption is associated with pathological events in the brain such as microbial infection, cancer, inflammatory responses, stroke, Alzheimer disease and PD (Stamatovic et al., 2008; Luissint et al., 2012). Moreover, some studies have shown alterations in endothelial tight junctions during PD development (Kim et al., 2003; Chen et al., 2008; Lee and Pienaar, 2014). For example, Chen et al. (2008) found a decrease in the tight junction proteins occludin and ZO-1 in a MPTP murine model of PD. Similarly, the exposure of murine EC to ROS increased the activity of metalloproteinase-9 (MMP-9), which caused degradation of the basal lamina and BBB disruption. This oxidative damage was reduced by the overexpression of SOD1 and catalase, suggesting the importance of oxidative stress in BBB disruption (Kim et al., 2003). Additionally, there is *in vitro* and clinical evidence of angiogenic activity in PD development caused by an upregulation in the expression of vascular endothelial growth factor (VEGF; Wada et al., 2006; Lee and Pienaar, 2014). In summary, the cellular and molecular properties of brain ECs are essential for maintaining BBB permeability through an adequate ionic balance, conservation of the junctional structure and an adequate interaction with cells of the NVU.

PERICYTES

Pericytes are enwrapping cells of blood microvessels, and are located between the EC and astrocytic endfeet and neurons (Wong et al., 2013). They are important regulatory cells for the maintenance of both homeostasis and hemostasis in the BBB (Dore-Duffy and Cleary, 2011). Additionally, pericytes are relevant in functions such as stromal regeneration, angiogenesis and neovascularization, antigen presenting cells under brain pathologies, control of EC proliferation, and promotion of neural stem cell properties (Lange et al., 2013; Elali et al., 2014; Hurtado-Alvarado et al., 2014). In this regard, pericytes have shown to differentiate *in vitro* into chondrocytes, vascular smooth muscles cells (VsMCS), osteoblasts and skeletal muscle, suggesting a

promising clinical use for pericytes in Central Nervous System (CNS) injuries and other pathologies (Armulik et al., 2010; Lange et al., 2013). Both pericytes and EC are enveloped by a basal membrane that is continuous between the two cell types, which separates pericytes from astrocyte endfeet (Sá-Pereira et al., 2012). This association is achieved through the endothelial secretion of PDGF-B and other angiogenic factors such as VEGF, TGF-β and angiopoietins (Angs), through the interaction of multiple signaling pathways (Dore-Duffy, 2008; Armulik et al., 2010; Ribatti et al., 2011).

Morphologically, pericytes exhibit an oval cell body with a great number of projections that enwrap ECs in different patterns, along the abluminal surface (Armulik et al., 2010). The two main types of pericytes, granular (95% of total pericytes) and agranular have been described in the brain according to the presence or absence of lysosome granules in the cytoplasm. Interestingly, alterations in granular pericytes have been associated with amyloid deposition, and lipid accumulation in human brain cultures, suggesting the importance of pericyte alterations in Alzheimer disease and other pathologies (Castejón, 2011).

Of greater importance are the interactions between astrocytes and pericytes. In this aspect, it has been shown that both pericytes and astrocytes are essential for brain vasculogenesis and BBB maintenance possibly through the activation of PDGFRB signaling (Dejana, 2004; Bonkowski et al., 2011). Moreover, both pericytes and astrocytes are important in the preservation of EC tight junctions through the regulation of proteins like occludin, claudin and ZO-1 (zona occludens-1, Haseloff et al., 2005; Wolburg et al., 2009; Bonkowski et al., 2011). This result suggests the importance of astrocyte-pericyte communication in brain physiology. However, further research is needed in order to understand the implications of the mentioned interactions during neurodegenerative disorders.

ASTROCYTES

Astrocytes are the most common cell type in the mammalian brain, conforming the glia with oligodendrocytes and microglia (Chen and Swanson, 2003). Among its many functions, astrocytes are essential for many metabolic processes in the brain such as the promotion of neurovascular coupling, the attraction of cells through the release of chemokines, K^+ buffering, release of gliotransmitters, release of glutamate by calcium signaling, control of brain pH, metabolization of dopamine and other substrates by monoamine oxidases, uptake of glutamate and γ-aminobutyricacid (GABA) by specific transporters and production of antioxidant compounds like glutathione (GSH) and enzymes such as superoxide dismutases (SODs; Volterra and Meldolesi, 2005; Chinta and Andersen, 2008; Hamby and Sofroniew, 2010; Kimelberg and Nedergaard, 2010; Parpura et al., 2011).

Globally, astrocytes are characterized by the expression of the intermediate filaments vimentin (Vim) and glial fibrillary acidic protein (GFAP), which are upregulated under CNS insults, in a process known as astrogliosis (Volterra and Meldolesi, 2005; Hamby and Sofroniew, 2010; Céspedes et al., 2013). Morphologically, astrocytes are characterized by a stellate shape with multiple processes and ramifications (Chen and Swanson, 2003; Volterra and Meldolesi, 2005), and become activated following brain injuries and degenerative diseases (Barreto et al., 2007, 2009, 2011, 2012; Adelson et al., 2012).

Although a great heterogeneity exists among astrocytes, two main types have been described in the CNS: protoplasmic astrocytes of the grey matter which envelope neuronal bodies and synapses, and fibrous astrocytes from the white matter that interact with the nodes of Ranvier and oligodendroglia (Halliday and Stevens, 2011; Oberheim et al., 2012). Current research has suggested that only protoplasmic astrocytes have an increase in the accumulation of α-synuclein, and these are of importance for PD development (Braak et al., 2006; Halliday and Stevens, 2011). Interestingly, protoplasmic astrocytes are arranged in non-overlapping domains forming a syncytial network that may contact approximately 160.000 synapses, thus integrating neural activity with the vascular network (Bushong et al., 2002; Barreto et al., 2011). This architecture is altered under pathological events such as Alzheimer and Epilepsia and has been associated with reactive astrogliosis (Oberheim et al., 2012), suggesting the importance of structural alterations during damaging processes.

Astrocytic terminal processes, known as endfeet, contact the brain vasculature surface facing ECs and pericytes and enwrap the neuronal synapses, enabling the modulation of both neuronal activity and cerebral blood flow, following an elevation in intracellular Ca^{2+} levels in the endfeet (Zonta et al., 2003; Maragakis and Rothstein, 2006). Importantly, astrocytic endfeet express specialized molecules such as Kir4.1 K^+ channels and aquaporin 4 that regulate BBB ionic concentrations, and protein transporters such as glucose transporter-1 and P-glycoprotein, suggesting the importance of the endfeet in astrocyte polarization (Abbott et al., 2006; Nag, 2011). Additionally, astrocytes communicate between each other through gap junctions forming a functional syncitium with well-coordinated responses (Theis et al., 2005; Alvarez et al., 2013). In this aspect, it has been suggested that the astrocytic mechanisms that regulate vasodilation and vasoconstriction are transmitted through this inter-astroglial gap junctions (Alvarez et al., 2013). Furthermore, astrocytes are important in the development and maintenance of BBB characteristics in ECs through the release of growth factors like VEGF, glial cell line-derived neurotrophic factor (GDNF), basic fibroblast growth factor (bFGF), and ANG-1 (Alvarez et al., 2013; Wong et al., 2013). These growth factors are important in the formation of tight junctions, the promotion of enzymatic systems and the polarization of transporters (Wong et al., 2013). Astrocyte-secreted growth factors are also important for neuronal growth and maintenance, and have survival properties during brain damaging processes like PD (Hamby and Sofroniew, 2010).

EXTRACELLULAR MATRIX (ECM)

In addition to the different cell types which constitute the BBB, the extracellular Matrix (ECM) is an important structural element of the BBB that serves as an anchor for the endothelium through the interaction of endothelial integrin receptors and matrix proteins such as laminin (Hawkins and Davis, 2005). In the brain, the ECM is composed of hyaluronan, hyaluronic acid, lecticans, proteoglicans and tenascins, which are important

for the maintenance of the paracellular diffusion in the BBB (Hawkins and Davis, 2005; Wong et al., 2013). Previous studies have suggested that the disruption of the ECM is strongly associated with an increase in BBB permeability during pathogenic states such as glioblastoma multiforme, ischemia and hemorrhagic necrosis of the brain. For example, during ischemia, the basement membrane suffers a breakdown caused by the increased expression of the matrix metalloproteinases (MMPs) MMP9 and MMP2 which in addition may cause microglial activation (del Zoppo and Milner, 2006; Lau et al., 2013). Furthermore, increased expression of MMP9 and GFAP in astrocytes was observed in a parkinsonian mouse model with MPTP (1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine; Annese et al., 2014). These results suggest the importance of ECM breakdown in glial activation during neurodegeneration and PD.

PARKINSON DISEASE AND BBB

CAUSES OF DISRUPTION OF THE BBB

Several processes may affect the integrity of the BBB, including an increase in ROS production, elevated levels of proinflammatory cytokines, inappropriate clearance of Ab peptide and other toxic substances (Minagar and Alexander, 2003; Popescu et al., 2009; van Sorge and Doran, 2012). Previous studies have shown an increase in BBB permeability associated with age that is in part responsible for pathological alterations such as white matter lesions (Simpson et al., 2007; Popescu et al., 2009). In this aspect, it has been reported that elder individuals and senescence mouse models have a higher albumin and IgG concentration than younger individuals caused by a leakage through the BBB (Popescu et al., 2009). Moreover, ageing is also associated with an increased production of ROS and proinflammatory cytokines in vascular ECs, which have been linked with memory and learning impairment in mouse models (Fukui et al., 2001; Popescu et al., 2009; Enciu and Popescu, 2013). Aged people have shown a diminished activity of the P-glycopotein efflux transporter that is associated with a limited removal of toxic substances from the brain (Popescu et al., 2009), demonstrating an important correlation between ageing processes (such as the increased expression of ROS) and BBB dysfunction. Taking into account that PD is associated with both age and ROS production, it is important to explore the cellular and molecular mechanisms that are activated during BBB disruption in this pathology and its protective mechanisms.

DISRUPTION OF BBB IN PARKINSON DISEASE

Disruption of BBB in PD has been quite controversial. It was initially assumed that BBB remained unaltered during the development of the pathology, as observed in animal models and permeability studies of PD drugs such as levodopa and benserazide (Kurkowska-Jastrzebska et al., 1999; Haussermann et al., 2001). More recently, clinical studies have presented evidence of BBB disruption in PD patients (Kortekaas et al., 2005; Hirano et al., 2008; Ohlin et al., 2011; Lee and Pienaar, 2014). For example, an early study (Kortekaas et al., 2005) pointed out an increase in the brain uptake of drugs that usually do not cross the BBB including benzerazide and [^{11}C] verapamil in PD patients and rat models, suggesting a possible BBB breakdown. Additionally, a PET study (positron emission tomography) found deficiencies in cerebral blow flow in PD patients that were highly associated with dyskinesias and levodopa treatment (Hirano et al., 2008). These changes in cerebral blood flow have been associated with an increased BBB permeability and angiogenesis that are mediated by VEGF (Kortekaas et al., 2005; Ohlin et al., 2011). Similarly, various toxin-induced PD models have shown BBB disruption, including 6-OHDA treated rats and MPTP-treated mice (Carvey et al., 2005; Chen et al., 2008). On the other hand, a growing body of evidence has shown the importance of ABC multidrug transporters such as P-gp in BBB disruption (Kortekaas et al., 2005; Bartels et al., 2008; Bartels, 2011). In this aspect, KO mice for P-glycoprotein have shown an increased accumulation of neurotoxin ivermectin and the carcinostatic drug vinblanstine in the brain, suggesting the importance of P-glycoprotein in the clearance of toxic substances and a possible BBB disruption in PD (Schinkel et al., 1994). Additionally, Kortekaas et al. (2005) has suggested that Parkinson patients have a reduced P-gp (glycoprotein) function in the midbrain, which is associated with a BBB disruption. Interestingly, some PET studies reported a decrease in BBB P-gp function in several brain regions during aging, demonstrating that elder people are more susceptible to the accumulation of toxin compounds in the brain. Taking into account that α-synuclein accumulation is associated with PD pathogenesis, it is possible that a reduction of P-gp could be related with an accumulation of α-synuclein in the brain (Bartels, 2011). However, further research is needed to assess the importance of P-gp in this process. Finally, the release of proinflammatory cytokines by microglia and astrocytes during PD is associated with both an increased neuronal death and protein rearrangements in tight junctions on EC surface (**Figure 1;** Desai Bradaric et al., 2012). For example, increased levels of the cytokines IL-6, IL-1B and TNF-A and a decrease in proteins ZO-1 and occludin in tight junctions have been associated with a reduction in the trans-endothelial electrical resistance, suggesting an alteration in BBB permeability (Wong et al., 2004). Importantly, the loss of signaling interactions between astrocytes and CNS vasculature through changes in protein expression in astrocytic endfeet is associated with morphological changes including hypertrophy, upregulation of GFAP and vimentin and therefore triggering the induction of astrocytes to a more reactive state (Robel et al., 2009; Alvarez et al., 2013). These results highlight the importance of astrocytes in the modulation of BBB properties and the involvement of the reactive astrogliosis during BBB disruption (**Figure 1**).

REACTIVE ASTROGLIOSIS IN PD

Reactive astrogliosis is the main reaction of astrocytes following brain insults such as infection, inflammatory processes, trauma, α-synuclein accumulation, ischemia and neurodegenerative diseases (Barreto et al., 2007, 2009; Gu et al., 2010; Hamby and Sofroniew, 2010; Xiong et al., 2011; Adelson et al., 2012). This process involves both molecular and morphological changes in astrocytes, which include the hypertrophy of cell bodies and glial processes, increased expression of proteins like GFAP, vimentin, nestin, tenascin-C and chondroitin sulfate proteoglycans (CSPGs; Alvarez et al., 2013). Other characteristics of the process are the increased uptake of glutamate caused by an alteration of vesicular transporters of GABA (vGAT) and glutamate (vGLUT),

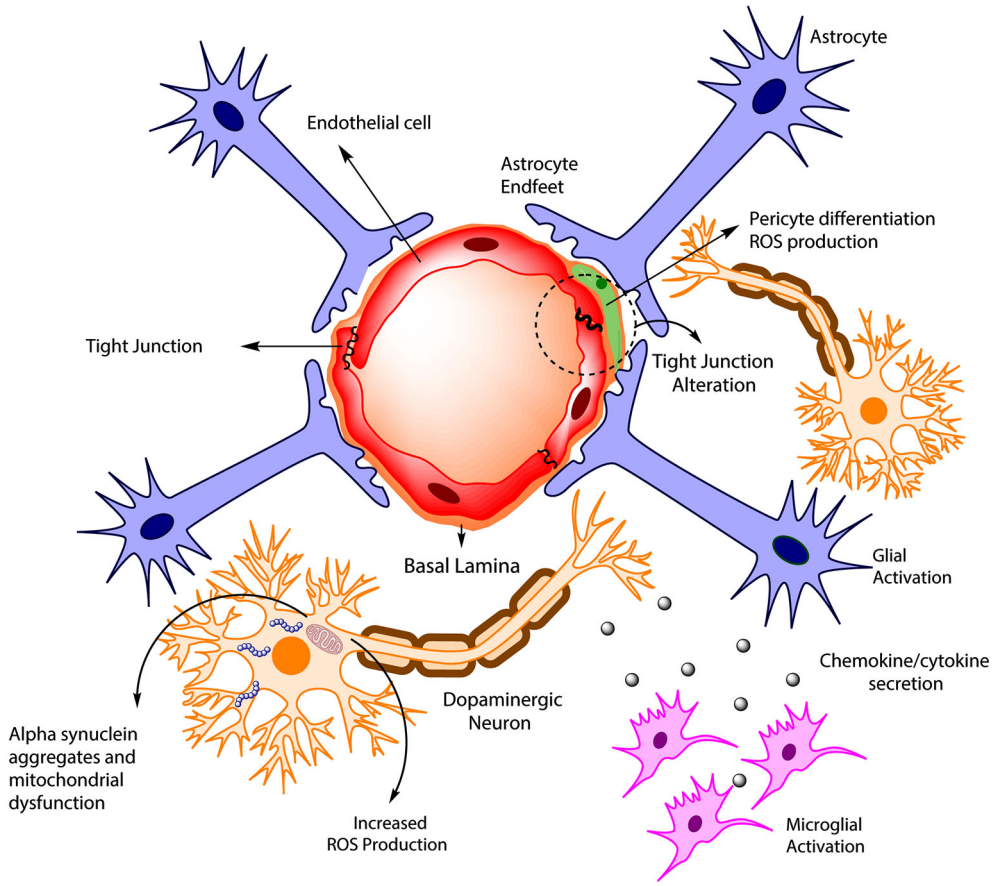

FIGURE 1 | BBB disruption in PD. During PD development, increased ROS production leads to the accumulation of α-synuclein in DAneurons, and this is accompanied by mitochondrial dysfunction and increased neuronal death. Concurrently, astrocyte and microglia became activated, promoting cytokine release, which in turn affects endothelial tight junctions, pericyte phenotype and BBB permeability.

production of cytokines and chemokines that have a modulatory effect on microglia (Croisier and Graeber, 2006), and in some cases the formation of glial scar (Hirsch et al., 2003; Hamby and Sofroniew, 2010; Kang and Hebert, 2011; Colangelo et al., 2014).

Importantly, reactive astrogliosis is a mechanism highly dependent on the cellular and molecular context of the events triggering it, therefore it may have both beneficial and detrimental effects on surrounding neural and non-neural cells (Hamby and Sofroniew, 2010). For example, the glial scar produced after severe astrogliosis may separate necrotic tissue from healthy one, but also has the detrimental effect of impairing axonal regeneration through the expression of molecules like CSPGs, semaphorins and ephrin (Fitch and Silver, 2008; Duffy et al., 2009).

Experimental evidence using cellular and animal models have shown that environmental and biological toxins, like α-synuclein, LPS (lipopolysaccharides), herbicides and pesticides like rotenone or MPTP (1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine), can induce both astrogliosis and microgliosis, which is accompanied by altered striatal neuronal morphology, neuronal death, mitochondrial dysfunction and nuclear fragmentation (Langston et al., 1999; Samantaray et al., 2007; Niranjan et al., 2010). Additionally, injection of LPS in rat brains was followed by an increase in the inducible nitric oxide synthase (iNOS), suggesting that chronic glial activation can cause oxidative stress in the brain, similarly to that in neurodegenerative processes like AD and Parkinson (Sugaya et al., 1998; Hirsch et al., 2003; de Oliveira et al., 2011). Similarly, there is clinical evidence showing that astrogliosis is present in different areas of the brain in PD patients, including the SN, the putamen and the hippocampus (Baxendale et al., 1998; Dickson et al., 2002; Dickson, 2012). Finally, some studies have shown that activated glial cells can participate in the death of dopaminergic neurons, probably via activation of apoptosis by cytokines like TNF-α, IL-1B, IL-6 (**Figure 2**) and interferon-γ and the subsequent production of nitric oxide by the iNOS that may diffuse toward the neurons and induce lipid peroxidation, DNA strands breaks and inhibition of mitochondrial metabolism (Hirsch et al., 2003; Rappold and Tieu, 2010). Released cytokines may bind to TNFR1 and 2, specific receptors in dopaminergic neurons, and activate proapoptotic mechanisms through the activation of caspase 3, caspase 8, and cytochrome

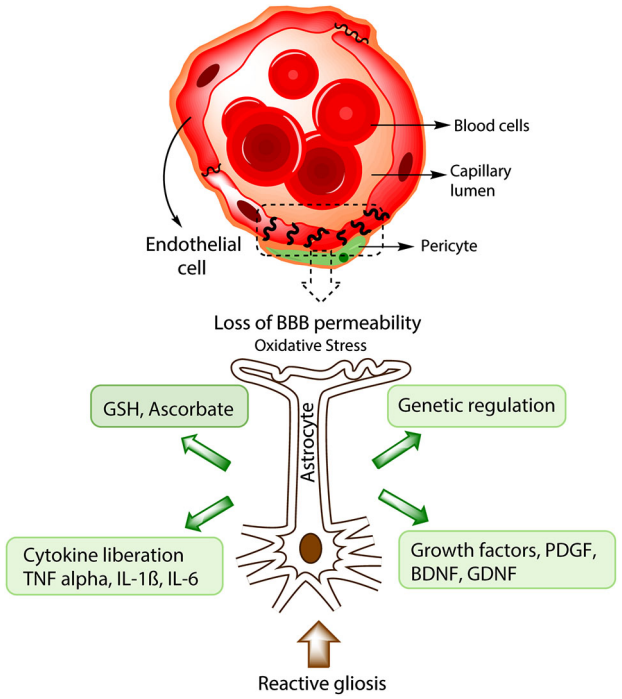

FIGURE 2 | Protective strategies of astrocytes during BBB disruption. In advanced stages of PD, BBB disruption takes place and causes a lost in barrier permeability, entrance of toxic substances and in some instances immune cell infiltration. Processes such as increased ROS production, reactive gliosis and cellular death will inevitably occur. Astrocytic response to BBB disruption includes the production of antioxidative molecules like GSH and ascorbate, generation of growth factors like Brain derived neurotrophic factor (BDNF) and GDNF that could alleviate the cellular death and promote angiogenesis. Furthermore, astrocytes are important in the genetic regulation of endothelial proteins from the tight junction like Occludin and ZO-1. During chronic brain damage, astrocytes also induce the liberation of cytokines like TNF-α, IL-1B, IL-6, important in microglial activation and neuronal death.

C (Hirsch et al., 2003). These results suggest that both the glial reaction and the consequent inflammatory processes could be considered as a promising therapy to reduce neuronal damage during PD (Hirsch et al., 2003).

PROTECTION STRATEGIES OF ASTROCYTES IN BBB DISRUPTION

Over the last years, much research has focused on specific molecules produced by astrocytes as promising neuroprotective strategies in neuropathologies. These molecules include antioxidant enzymes such as SODs, growth factors, peptide hormones and heat shock proteins (Dringen, 2000; Zheng et al., 2010; Barreto et al., 2011). Many of them have shown protective effects both in dopaminergic neurons and glial cells, and have been used in animal models and clinical trials with remarkable results (Ramaswamy and Kordower, 2009; Yasuda and Mochizuki, 2010). In the last section of our review we discuss the current methods used in neuroprotection based on astrocyte molecules. Additionally, we highlight the future strategies in astrocyte protection aimed at the development of restorative therapies for the BBB in pathological conditions.

ASTROCYTIC ANTIOXIDANTS AND PD

Astrocytes secrete beneficial antioxidant molecules, including GSH, (SODs 1, 2 and 3), and ascorbate, which are important for cell survival during neurodegenerative processes (see **Figure 2,** Anderson and Swanson, 2000; Dringen, 2000; Lindenau et al., 2000; Sims et al., 2004; Mythri et al., 2011). The tripeptide GSH is the main antioxidant in the brain, which is needed for the conversion of methylglyoxal into d-lactate by glyoxalase 1 (Dringen, 2000; Bambrick et al., 2004). Furthermore, GSH is also important in limiting and repairing the deleterious actions of NO and other ROS in the brain such as nitrations and fibril formations of α-synuclein (Chinta and Andersen, 2008). Interestingly, astrocytes possess a greater concentration of GSH (3.8 mmol/L) than neurons (2.5 mmol) probably due to their higher content of γ-glutamylcysteine-synthetase (Rappold and Tieu, 2010). In this aspect, some studies demonstrated that neurons co-cultured with astrocytes exhibit higher levels of GSH compared to neurons cultured alone, suggesting that astrocytes may provide further antioxidant defenses to neurons and BBB cells (Maier and Chan, 2002; Slemmer et al., 2008; Giordano et al., 2009). Additionally, an increase in GSH peroxidase-containing cells showed to be inversely correlated with the severity of dopaminergic cell loss in cell populations from patients with PD, demonstrating that the quantity of GSH peroxidase in cells might be critical for a protective effect against oxidative stress during PD (Damier et al., 1993). Different murine models have shown the importance of GSH in BBB protection, including maintenance of BBB permeability and oxidative protection of mouse pericytes (Shukla et al., 1993; Agarwal and Shukla, 1999; Price et al., 2012). Similarly, ascorbate was shown to protect BBB integrity in a rat ischemic model by preventing changes in BBB permeability and increased ROS production (Lin et al., 2010). One important problem with the use of GSH as a possible therapeutic agent is that its precursor, N-acetycysteine (NAC), does not cross the BBB in significant amounts, therefore various strategies have been used to improve the GSH transport into the brain, such as the use of liposomes, nanoparticles and L-dopa conjugates (Smeyne and Smeyne, 2013). However, further research is needed to address the use of GSH in BBB disruption.

Previous studies showed that SODs exert neuroprotection in PD and other oxidative-related events (Chen and Swanson, 2003). For example, the overexpression of Cu/Zn SOD (SOD1) was able to rescue dopaminergic neurons and diminishes locomotor disabilities in a *Drosophila* mutant model for α-synuclein overexpression (Botella et al., 2008). Interestingly, a specific increase in SOD levels in the SN, with no changes in activities of GSH peroxidase, catalase and GSH reductase, is observed in PD patients (Chinta and Andersen, 2008). A similar increase was noted in the mitochondrial isoform of SOD (SOD2) in motor cortex from PD patients (Radunovic et al., 1997), suggesting that SODs have a greater importance than other antioxidant enzymes during PD development. Furthermore, the reduction or induced mutation of SOD1 in astrocytes has been shown to induce neuronal degeneration and injury in ischemic and amyotrophic lateral sclerosis

(ALS) murine models (Kondo et al., 1997; Kim et al., 2001; Blackburn et al., 2009; Papadeas et al., 2011). Finally, it was also reported that the overexpression of SOD1 in a transgenic mouse model attenuated BBB disruption by superoxide anion during ischemia (Kim et al., 2001). Altogether, these results emphasize the importance of antioxidant enzymes for the treatment of PD and BBB disruption.

GROWTH FACTORS AND BBB PROTECTION IN PD

Several neurotrophic and growth factors secreted by astrocytes have been extensively used in animal models of neurodegenerative disorders for exerting protection of dopaminergic neurons and glial cells against toxins and ROS during injury through the activation of specific signaling pathways that are responsible for cell survival, induction of antioxidant enzymes, and axonal sprouting (See **Figure 2,** Ramaswamy and Kordower, 2009; Yasuda and Mochizuki, 2010; Proschel et al., 2014). Some of them like GDNF and neurturin (NRTN) have been tested in clinical trials for PD and other neurodegenerative diseases (Peterson and Nutt, 2008; Ramaswamy and Kordower, 2009).

BDNF, from the neurotrophin family, has been shown to be critical in the survival of cortical, hippocampal and serotonergic neurons. Reduction in BDNF levels is associated with many pathological conditions such as PD, AD, Huntington Disease, ALS, depression and schizophrenia (Allen et al., 2013). Furthermore, BDNF protects neurons against excitotoxicity through activation of the transcription factor NF-kB, which induces expression of antioxidant enzymes such as Mn-SOD and the anti-apoptotic proteins, Bcl-2 and inhibitor of apoptosis proteins IAPs (Mattson, 2008; Lee et al., 2009). Endogenous administration of BDNF was demonstrated to protect neurons in SN following 6-OHDA and MPTP toxicity in rat and primate PD models (Ramaswamy and Kordower, 2009).

The family of GDNF comprises ligands, such as GDNF, NRTN, artemin (ARTN) and persephin. GDNF, secreted by astrocytes and pericytes, is essential for the survival of dopaminergic neurons, peripheral motor neurons and neurons from the locus coeruleus (Yasuda and Mochizuki, 2010; Allen et al., 2013). In this aspect, GDNF administration by catheter increases dopaminergic neuronal resistance against 6-OHDA toxicity, with preservation of motor functions in rat and rhesus monkey models (Safi et al., 2012). More recently, GDNF was shown to increase the expression of claudin-5 and the transendothelial electrical resistances of brain microvascular ECs, suggesting that it may improve the barrier function of the BBB (Sano et al., 2007). However, clinical trials in patients that were administered GDNF in different regions of the brain have shown mixed results, in part due to the mechanism of administration, and the growth factor inability to cross the BBB, therefore further research is needed in order to surpass this obstacle (Gill et al., 2003; Ramaswamy and Kordower, 2009; Allen et al., 2013).

The family of the fibroblast growth factors (FGF) includes 22 structurally related signaling molecules in humans, such as acid FGF, and bFGF, which are important in processes like angiogenesis, wound healing and embryonic development (Itoh and Ornitz, 2011; Huang et al., 2012). Different studies have shown that bFGF protects hippocampal and cortical neurons against glutamate toxicity by changing the expression of *N*-methyl-D-aspartic acid (NMDA) receptors and antioxidant enzymes like SODs and GSH reductase (Timmer et al., 2004; Mattson, 2008). Furthermore, a co-culture of transgenic Schwann cells overexpressing FGF-2 with dopaminergic neurons improved neuronal survival and the behavioral outcome in a parkinsonian rat model lesioned with 6-OHDA (Timmer et al., 2004). Additionally, bFGF preserves BBB endothelial adherens junctions in a mouse model of intracerebral hemorrhage through the inhibition of RhoA protein, suggesting that bFGF maintains BBB integrity (Huang et al., 2012). Finally, there are other neurotrophic factors with potential effects on BBB protection including insulin-like growth factors (IGFs), vascular endothelial growth factor (VEGF-B), hepatocyte growth factor (HGF), mesencephalic astrocyte-derived neurotrophic factor and platelet derived growth factor (PDGF; Aberg et al., 2006; Ramaswamy and Kordower, 2009; Pang et al., 2010; Yasuda and Mochizuki, 2010; Sullivan and Toulouse, 2011). For instance, VEGF has shown to improve cerebral blood flow and the pericyte coverage of brain ECs in a murine ischemic model (Zechariah et al., 2013). Also, PDGF-BB impairment in mice has been associated with a reduced number of pericytes, edema formation and murine embryonic lethality, suggesting its importance in BBB development and maintenance (Bergers and Song, 2005; Bonkowski et al., 2011).

The main obstacle with the use of growth factors as therapeutic agents in neurodegenerative diseases seems to be their inability to cross the BBB thoroughly (Peterson and Nutt, 2008). In this regard, different strategies have been used including injections into the lumbar or ventricular CSF, viral vectors with growth factor genes, the temporal disruption of the BBB with hyperosmotic agent like mannitol, the use of linked peptides or peptidomimetic monoclonal antibodies or nanoparticles (Allen et al., 2013). For example, a recent methodology using magnetic nanocarriers for the transport of BDNF was able to cross the BBB without affecting cell viability seems promising (Pilakka-Kanthikeel et al., 2013). A different approach seems to be the transplantation of dopaminergic neurons or glial precursor cells into the injured regions of the brain, which increases the expression of growth factors like BDNF, GDNF, and IGF (Hauser, 2011; Jankovic and Poewe, 2012; Proschel et al., 2014). In this aspect, a recent study by Proschel et al. (2014) has demonstrated that the transplantation of glial precursor cells in 6-OHDA injured rats causes the recovery of DA neurons of the striatum by an increase in the levels of GSH, GDNF, and BDNF. These results suggest that growth factors are essential in the recovery of BBB injuries and related pathologies.

CONCLUSIONS AND FUTURE PERSPECTIVES

Based on the past studies, it seems to be of greater importance to understand the role of BBB in neurodegenerative diseases. It is likely that the maintenance of the BBB and the NVU will decrease the accumulation of Lewy bodies, α-synuclein fibrils and ROS that worsen the effects of PD. It is important to determine the extent of BBB disruption in PD, and how this disruption may allow the transport of growth factors and antioxidant molecules to the site of injury. The combination of novel drug therapies, such as the use of growth factors, antioxidant molecules or nanoparticles combined with a better understanding

of the astrocytic functions in the BBB, and the use of other therapies that increase astrocyte survival and its antioxidant function may shed light on a prospective cure of PD in the near future.

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Cerebrospinal fluid biochemical studies in patients with Parkinson's disease: toward a potential search for biomarkers for this disease

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The blood-brain barrier supplies brain tissues with nutrients and filters certain compounds from the brain back to the bloodstream. In several neurodegenerative diseases, including Parkinson's disease (PD), there are disruptions of the blood-brain barrier. Cerebrospinal fluid (CSF) has been widely investigated in PD and in other parkinsonian syndromes with the aim of establishing useful biomarkers for an accurate differential diagnosis among these syndromes. This review article summarizes the studies reported on CSF levels of many potential biomarkers of PD. The most consistent findings are: (a) the possible role of CSF urate on the progression of the disease; (b) the possible relations of CSF total *tau* and phospho*tau* protein with the progression of PD and with the preservation of cognitive function in PD patients; (c) the possible value of CSF beta-amyloid 1-42 as a useful marker of further cognitive decline in PD patients, and (d) the potential usefulness of CSF neurofilament (NFL) protein levels in the differential diagnosis between PD and other parkinsonian syndromes. Future multicentric, longitudinal, prospective studies with long-term follow-up and neuropathological confirmation would be useful in establishing appropriate biomarkers for PD.

Keywords: Parkinson's disease, cerebrospinal fluid, biological markers, neurotransmitters, oxidative stress, tau protein, alpha-synuclein, beta-amyloid

INTRODUCTION

The diagnosis of Parkinson's disease (PD) in live patients is fundamentally clinical, and is based on the presence of its cardinal signs (rest tremor, rigidity, bradykinesia, and postural instability), and the absence of atypical data for idiopathic PD. The final confirmation of the diagnosis is made by post-mortem neuropathological analysis. To date, there are no definitive biomarkers to make an accurate differential diagnosis with other parkinsonian syndromes.

Because the cerebrospinal fluid (CSF) is in close contact with the extracellular space of the brain, it is believed that many of the biochemical modifications in the brain should be reflected in the CSF. Therefore, CSF has been widely investigated in PD and in other parkinsonian syndromes with the aim of acquiring knowledge on the pathogenesis of this disease. This article summarizes the data on analyses performed in the CSF of patients diagnosed with PD compared with controls, with regard to: (1) concentrations of neurotransmitters (mainly monoamines and their metabolites), neuromodulators, and related substances as possible biological markers of the disease itself or its complications; (2) concentrations of endogenous neurotoxins; (3) status of oxidative stress markers or substances which could be related with the induction of oxidative stress or with "neuroprotection" against it; (4) status of inflammation and immunological markers, neurotrophic and growth factors, and (5) concentrations of proteins related with the pathogenesis of PD or other compounds.

The aim of this review is to provide an extensive descriptive overview of studies published on this issue (including references to many reports in the last six decades which have historical interest).

SEARCH STRATEGY

References for this review were identified by searching in PubMed from 1966 until June 20, 2014. The term "*Parkinson's disease*" was crossed with "*cerebrospinal fluid*" and "*blood brain barrier*," and the related references were selected. **Table 1** summarizes a classification of the diverse types of compounds which have been analyzed in the CSF of PD patients in accordance with the search.

NEUROTRANSMITTERS, NEUROMODULATORS, AND RELATED SUBSTANCES

DOPAMINE METABOLITES

Because the main neurochemical finding in PD is the depletion of dopamine (DA) in the nigroestriatal system (Benito-León et al., 2008), it is to be expected that the CSF concentrations of the main metabolites of DA, dihydroxyphenyl-acetyc acid (DOPAC)

Table 1 | Relation and classification of compounds measured in CSF of PD.

(A) Neurotransmitters, neuromodulators, and related substances
(1) Dopamine (DA) metabolites: dihydroxyphenylacetic acid (DOPAC) and homovanillic acid (HVA), 3-orthomethylDOPA (3-OMD)
(2) Serotonin (5-hydroxytryptamine or 5-HT) metabolites or precursors: 5-hydroxytryptophan (5-HTP), 5-hydroxyindoleacetic acid (5-HIAA), kynurenine, 3-hydroxykynurenine
(3) Noradrenalin (norepinephrine or NE) metabolites or precursors: 3-methoxy-4-hydroxy-phenylethylenglycol (MHPG), dopamine-beta-hydroxylase (DBH)
(4) Acetylcholine (Ach) and related substances: choline, acetylcholine-esterase (AchE), butiryl-cholin-esterase (BchE)
(5) Neurotransmitter amino acids: gamma-amino butyric acid (GABA), glutamate, aspartate, glycine
(6) Neuropeptides: substantia P (SP), cholecystokinin-8 (CCK-8), met-enkephalin (MET-ENK), leu-enkephalin (LEU-ENK), dynorphin A(1-8), somatostatin, neuropeptide Y (NPY), beta-endorphin, arginine-vasopressine (AVP), vasoactive intestinal peptide (VIP), delta sleep-inducing peptide (DSIP), alpha-melanocyte-stimulating hormone-like, diazepam-binding inhibitor, neurokinin A, corticotropin-releasing hormone (CRH), adrenocorticotropin hormone (ACTH), beta-lipotropine, angiotensin, chromogranins A and B, secretogranin II, orexin-A/hypocretin-1
(7) Other neurotransmitters: endogenous cannabinoids, β-phenylethylamine
(8) Cyclic nucleotides: cyclic adenosine 3′5′ monophosphate (cAMP), cyclic guanosine 3′5′ monophosphate (cGMP)
(9) Biopterin derivatives and other cofactors
(B) Endogenous neurotoxins
(1) Tetrahydroisoquinolin (TIQ) derivatives: 2-methyl-6,7-dihydroxy1,2,3,4-TIQ (2-MDTIQ), 1-MDTIQ (salsolinol). 1-benzyl-1,2,3,4-TIQ
(2) β-carbolinium cations (BC+s)
(C) Oxidative stress markers
(1) Lipid peroxidation markers: Malonyl-dialdehyde (MDA) (E)-4-hydroxynonenal (HNE) Low density lipoprotein (LDL) oxidation products Schiff bases, conjugated dienes, oxidized proteins, and aldehyde polymers
(2) DNA oxidation markers: 8′-hydroxy-2′deoxyguanine (8-OHdG) 8-hydrosyguanosine (8-OHG) 8-OHdG/8-OHG ratio
(3) Transition metals and related proteins: iron, ferritin, transferring, copper, cerulopasmin, ferroxidase, manganese, zinc
(4) Other metals: selenium, chromium, magnesium, calcium, aluminum, silicon, cobalt, tin, lead, barium, bismuth, cadmium, mercury, molibdenum, nichel, antimony, strontium, thallium, vanadium, wolfram, and zirconium
(D) Inflamatory and immunological markers
(1) Inteleukins (IL)
(2) Tumor necrosis alpha (TNF-α)
(3) Other: leukotrienes. α-1-antichymotrypsin
(E) Growth and neurotrophic factors
(1) Brain-derived neurotrophic factor (BDNF)
(2) Transforming Growth Factors: TGF-α, TGF-β1, TGF-β2
(3) Insulin-like growth factor-1 (IGF-1) and IGF-binding proteins (IGFBPs)
(4) Neuroregulins (Epidermal Growth Factor or EGF family)
(F) Proteins involved in the pathogenesis of PD
(1) Microtubular-Associated Protein *Tau* (MAPT)
(2) Alpha-synuclein
(3) Amiloyd beta
(4) Neurofilament proteins
(5) Other proteins: DJ-1, UCH-L1
(G) Other compounds

and homovanillic acid (HVA), should be decreased. Indeed, many classical studies have shown variable degrees of decrease in the CSF HVA levels of PD patients compared with controls (Bernheimer et al., 1966; Guldberg et al., 1967; Johansson and Roos, 1967; Olsson and Roos, 1968; Gottfries et al., 1969; Curzon et al., 1970; van Woert and Bowers, 1970; Godwin-Austen et al., 1971; Mones et al., 1972; Papeschi et al., 1972; Pullar et al., 1972; Cox et al., 1973; Voto Bernales et al., 1973; Weiner and Klawans, 1973; Granerus et al., 1974; Davidson et al., 1977; Tabaddor et al., 1978; Lovenberg et al., 1979; Cunha et al., 1983; Mann et al., 1983; Cramer et al., 1984; Mena et al., 1984; Pezzoli et al., 1984; Burns et al., 1985; Gibson et al., 1985; Jolkkonen et al., 1986; Liu, 1989; Hartikainen et al., 1992; Strittmatter and Cramer, 1992; Chia et al., 1993; Mashige et al., 1994; Eldrup et al., 1995; Cheng et al., 1996; Strittmatter et al., 1996; Kanemaru et al., 1998; Goldstein et al., 2008). Engelborghs et al. (2003) reported normal CSF DA and HVA, and decreased DOPAC levels. González-Quevedo et al. (1993) described normal CSF HVA levels, Espino et al. (1994)

found decreased HVA only in advanced but not in early PD, Parkinson Study Group DATATOP Investigators found normal levels in early PD (LeWitt et al., 2011). Zubenko et al. (1986) described a non-significant trend toward decreased CSF HVA levels in demented PD patients compared with controls. Tohgi et al. (1993a) found correlation of CSF DA and HVA levels with akinesia and freezing of gait.

Although levodopa treatment usually increases CSF HVA levels according to the majority of studies, this is not related with clinical improvement, with some exceptions (Durso et al., 1989), and pre-treatment CSF HVA levels does not predict levodopa response (Weiner et al., 1969; Chase, 1970; Curzon et al., 1970; Bertler et al., 1971; Casati et al., 1973; Cox et al., 1973; Mones, 1973; Weiner and Klawans, 1973; Granerus et al., 1974; Davidson et al., 1977; Liu, 1989; Nishi et al., 1989; Strittmatter et al., 1996; Antkiewicz-Michaluk et al., 1997; Durso et al., 1997; Krygowska-Wajs et al., 1997), except in one study which described an association between relatively high pre-treatment CSF HVA levels and a better response to levodopa (Gumpert et al., 1973). One study failed to show changes in ventricular CSF HVA levels after a single acute administration of levodopa (Moussa et al., 1992). On the other hand, dopamine agonists such as piribedil and bromocriptine decreased significantly both the basal level (McLellan et al., 1975; Rinne et al., 1977) and probenecid-induced accumulations of HVA in CSF (Rinne et al., 1975, 1977), indicating that the drugs reduced the turnover of endogenous dopamine. Amantadine did not change HVA levels (Cox et al., 1973). Tetrahydrobiopterin (Dissing et al., 1989) and L-threo-3,4-dihydroxyphenylserine (precursor or noraderenalin or norepinephrine –NE) (Maruyama et al., 1994) increased CSF HVA levels in PD patients, but to a lesser extent than levodopa.

Friedman et al. (Friedman, 1985) reported an HVA/5-HIAA ratio in PD patients who developed levodopa-induced dyskinesias (LID) which was significantly higher than in PD patients under levodopa therapy and in controls, but Lunardi et al. (2009) found similar HVA/DA ratios in patients with and without LID. CSF DA, levodopa, and HVA levels were similar in PD patients treated with levodopa with wearing-off motor fluctuations to those without this complication of levodopa therapy, while CSF 3-ortho-methyldopa (3-OMD) levels were higher in the fluctuating patients (Tohgi et al., 1991a). CSF DOPAC and HVA were similar in PD patients with and without depression (Kuhn et al., 1996a), and in patients with major depression with PD than in those without PD (Pålhagen et al., 2010). CSF HVA levels were correlated with striatal uptake in PD patients measured with PET imaging with carbon-11-labeled 2β-carbomethoxy-3β-(4-fluorophenyl)-tropane (^{11}C-FT) (Ishibashi et al., 2010).

Tohgi et al. (1991b, 1997) found a significant increase in tyrosine, and a significant decrease in CSF levodopa, DA, and 3-OMD in PD patients, which was related with levodopa dosage, and described an additional decrease in 3-OMD in subjects treated with tolcapone (Tohgi et al., 1995a). Other authors reported increased CSF 3-OMD related with levodopa therapy (Antkiewicz-Michaluk et al., 1997; Krygowska-Wajs et al., 1997). On the other hand, Chia et al. (1993) found normal CSF 3-OMD concentrations. Moser et al. (1996) described increased CSF levodopa/3-OMD ratio in PD patients with hallucinations. Iacono et al. (1997) found similar HVA levels in PD patients with postural instability and gait disorders to PD patients without these symptoms.

Although many of the studies of DA metabolites were performed on patients with different types of parkinsonism, with different degrees of severity, and the fact that many of these studies were made using small sample sizes, there is a general consensus that CSF HVA levels are decreased in untreated PD patients and rise after levodopa therapy starts (decreased HVA may not be present in early stages of PD). It is to be expected that low CSF HVA levels should be a reflection of DA depletion in the nigroestriatal system. However, CSF DA metabolite levels are not useful to distinguish between different parkinsonian syndromes and could be normal in early stages of the disease. To our knowledge, no studies have been published regarding the correlation of CSF DA metabolite levels and brain DA levels, although the observation of a correlation between CSF HVA levels and striatal uptake of DA markers in PET imaging (Ishibashi et al., 2010), suggests this correlation.

SEROTONIN (5-HYDROXYTRYPTAMINE OR 5-HT) METABOLITES

Several studies have described neuronal loss, and presence of Lewy body in serotonergic raphe nuclei in PD patients (Benito-León et al., 2008). Tohgi et al. (1993b,c, 1997) reported a 15–20% reduction of CSF 5-HT, tryptophan (precursor of 5-HT), kynurenine and 3-hydroxykynurenine (metabolites of tryptophan) levels in PD patients. CSF 5-HT levels showed a negative correlation with the severity of bradykinesia, rigidity and freezing of the gait, and decreased after levodopa therapy. This group also found a correlation between CSF 5-HIAA levels and akinesia and freezing of gait (Tohgi et al., 1993a). In contrast, Engelborghs et al. (2003) described increased 5-HT levels. LeWitt et al. (2013) described increased CSF 3-hydroxykynurenine levels, and Widner et al. (2002) described an increased CSF kynurenine/tryptophan ratio in PD patients.

Several studies have shown reduced CSF levels of 5-hydroxyindoleacetic acid (5-HIAA), the main metabolite of 5-HT, in PD patients (Guldberg et al., 1967; Johansson and Roos, 1967, 1971; Olsson and Roos, 1968; Gottfries et al., 1969; Chase, 1970; Rinne and Sonninen, 1972; Rinne et al., 1973; Davidson et al., 1977; Mayeux et al., 1984, 1986, 1988; Kostić et al., 1987; Tohgi et al., 1993c, 1997; Mashige et al., 1994; Strittmatter et al., 1996; Engelborghs et al., 2003). Other authors report normal CSF 5-HIAA levels (Papeschi et al., 1970, 1972; Godwin-Austen et al., 1971; Granerus et al., 1974; Davidson et al., 1977; Tabaddor et al., 1978; Cramer et al., 1984; Burns et al., 1985; Chia et al., 1993; González-Quevedo et al., 1993; Volicer et al., 1985; Fukuda et al., 1989). Liu et al. (1999) described lower ventricular CSF 5-HIAA levels in patients with rigid-akinetic PD than in patients with tremoric PD, and a negative correlation between CSF 5-HIAA levels and PD severity.

CSF 5-HIAA levels seem to be unchanged by therapy with levodopa (Godwin-Austen et al., 1971; Davidson et al., 1977), bromocriptine (Gumpert et al., 1973), or piribedil (Gumpert et al., 1973), or were found decreased by levodopa therapy (Casati et al., 1973). Gumpert et al. (1973) described an

association between relatively low pre-treatment CSF 5-HIAA levels with a good response to levodopa, while Davidson et al. (1977) reported this association with higher CSF 5-HIAA levels, and others found no such relation (Granerus et al., 1974). Tetrahydrobiopterin increased (Dissing et al., 1989), and L-threo-3,4-dihydroxyphenylserine decreased (Maruyama et al., 1994) CSF 5-HIAA levels.

Some authors have described decreased CSF 5-HIAA (Mayeux et al., 1984, 1986, 1988; Mena et al., 1984; Kostić et al., 1987) and 5-HT levels (Mena et al., 1984) in PD patients with depression, while others have described normal CSF 5-HIAA in depressed PD patients (Granerus et al., 1974; Kuhn et al., 1996a), and others still have reported similar CSF 5-HIAA levels in patients with major depression with PD tothose without PD (Pålhagen et al., 2010). Moser et al. (1996) described increased CSF 5-HIAA in PD patients with hallucinations. Iacono et al. (1997) found higher CSF 5-HT and 5-HIAA and lower 5-HTP levels in PD patients with postural instability and gait disorders than in PD patients without these symptoms.

Studies on the correlation of CSF 5-HT metabolite levels and brain 5-HT levels are lacking. The majority of studies report results on CSF 5-HIAA levels, with the controversial results based on short series of cohorts of patients with PD or other parkinsonian syndromes. Current data do not lend support to the role of CSF 5-HIAA as an unequivocal marker of depression linked to PD.

NORADRENALIN (NOREPINEPHRINE OR NE) METABOLITES

Neurons containing NE in the brain, mainly in the dorsal nuclei of vagus nerve, are involved in the degenerative process of PD (Benito-León et al., 2008). CSF NE levels have been found normal (Turkka et al., 1987; Chia et al., 1993; Kuhn et al., 1996a; Engelborghs et al., 2003) or decreased (Martignoni et al., 1992; Eldrup et al., 1995) in PD patients. CSF levels of 3-methoxy-4-hydroxy-phenylethyleneglycol (MHPG), the main metabolite of NE, have been reported to be normal (Wilk and Mones, 1971; Davidson et al., 1977; Mann et al., 1983; Mena et al., 1984; Hartikainen et al., 1992; Martignoni et al., 1992; Chia et al., 1993; González-Quevedo et al., 1993; Mashige et al., 1994; Kuhn et al., 1996a; Engelborghs et al., 2003) or decreased (Granerus et al., 1974) in PD patients. CSF MHPG levels do not increase either after treatment with levodopa (Wilk and Mones, 1971; Davidson et al., 1977) or with the NE precursor L-Threo-3,4-dihydroxyphenylserine (L-threo-DOPS) (Yamamoto et al., 1986; Teelken et al., 1989), while L-threo-DOPS increases CSF NE levels (Tohgi et al., 1990, 1993d).

Several authors have described a negative correlation between CSF MHPG levels and cognitive functioning (Mann et al., 1983) and bradyphrenia (Mayeux et al., 1987) in PD patients, and others have described a relationship between CSF NE levels with severity of PD assessed by Hoehn & Yahr staging, akinesia scores, and freezing of the gait (Tohgi et al., 1993a). Pålhagen et al. reported decreased CSF MHPG levels in patients with major depression with PD compared to those without PD (Pålhagen et al., 2010).

CSF activity of dopamine-β-hydroxylase (DBH), an enzyme involved in NE synthesis, has been found decreased in PD patients when compared with controls (Matsui et al., 1981; Hurst et al., 1985).

The normality of CSF MHPG levels found in nearly all studies with PD or other parkinsonian syndromes indicates that this is not a useful marker of PD. The correlation between CSF MHPG and brain NE is unknown.

ACETYLCHOLINE (Ach) AND RELATED SUBSTANCES

CSF levels of Ach (Duvoisin and Dettbarn, 1967; Welch et al., 1976; Yamada et al., 1996) and its precursor choline (Aquilonius et al., 1972; Welch et al., 1976; Nasr et al., 1993) have been reported to be similar in PD patients to controls with the exception of one study in which lower CSF choline levels were described in PD patients (Manyam et al., 1990).

CSF activity of acetylcholine-esterase (AchE), the main enzyme involved in Ach degradation, has been reported to be similar in PD patients and controls (Jolkkonen et al., 1986; Ruberg et al., 1986; Zubenko et al., 1986; Sirviö et al., 1987; Yoshinaga et al., 1989; Manyam et al., 1990; Hartikainen et al., 1992), although there are studies which have described increased (Ruberg et al., 1986), decreased (Konings et al., 1995), or normal activity (Zubenko et al., 1986; Sirviö et al., 1987) in demented patients, and decreased activity only in those patients with the most severe disease (Hartikainen et al., 1992).

CSF activity of butirylcholine-esterase (BchE) have been found to be similar in PD patients and controls (Ruberg et al., 1986; Sirviö et al., 1987), but increased in demented PD patients in a single study (Ruberg et al., 1986). Data on CSF Ach and related substances are scarce and based on short series of patients, and do not permit valid conclusions.

GAMMA-AMINO BUTYRIC ACID (GABA) AND OTHER NEUROTRANSMITTER AMINO ACIDS

CSF GABA levels in PD patients have been found to be decreased, when compared with controls, by many authors (Lakke and Teelken, 1976; Manyam et al., 1980, 1988; Kuroda et al., 1982; Manyam, 1982; Teychenné et al., 1982; Kuroda, 1983; de Jong et al., 1984; Araki et al., 1986; Tohgi et al., 1991c), while others have found this value to be normal (Enna et al., 1977; Abbott et al., 1982; Bonnet et al., 1987; Perschak et al., 1987; Mally et al., 1997; Engelborghs et al., 2003) or even increased (Jiménez-Jiménez et al., 1996). Manyam and Tremblay (1984) found reduced CSF free GABA levels and normality of conjugated levels. Abbot et al. (Perschak et al., 1987) found decreased CSF GABA levels in PD patients treated with levodopa, but not in "*de novo*" PD patients, while other authors found decreased CSF GABA in untreated PD patients (Manyam, 1982; de Jong et al., 1984), with CSF GABA normal (de Jong et al., 1984; Tohgi et al., 1991c) or slightly decreased (Manyam, 1982) in PD patients under levodopa therapy, suggesting that levodopa increases CSF levels. Teychenné et al. (1982) described low CSF GABA especially in PD patients with poor response to therapy or suffering from "on-off" motor fluctuations.

Normality of CSF glutamate levels has been reported by most investigators (Van Sande et al., 1971; Gjessing et al., 1974; Lakke and Teelken, 1976; Lakke et al., 1987; Perschak et al., 1987; Espino et al., 1994; Jiménez-Jiménez et al., 1996; Kuiper et al.,

2000), although 3 groups described decreased CSF glutamate levels (Gründig and Gerstenbrand, 1980; Tohgi et al., 1991c; Mally et al., 1997), while CSF glutamine (the main precursor of glutamate) has been found to be normal (Gjessing et al., 1974; Lakke and Teelken, 1976; Manyam et al., 1988; Jiménez-Jiménez et al., 1996) or increased (Mally et al., 1997).

CSF aspartate levels have been reported as normal (Lakke and Teelken, 1976; Manyam, 1982; Araki et al., 1986; Perschak et al., 1987; Mally et al., 1997; Jiménez-Jiménez et al., 1996; Engelborghs et al., 2003), except in the study by Tohgi et al. (1991c) who reported decreased CSF aspartate; CSF asparagine (the main metabolite of aspartate) has been found normal (Lakke and Teelken, 1976; Manyam, 1982; Araki et al., 1986; Perschak et al., 1987; Jiménez-Jiménez et al., 1996; Mally et al., 1997; Engelborghs et al., 2003).

The results on CSF glycine levels have been reported as normal by most investigators (Gjessing et al., 1974; Perschak et al., 1987; Manyam et al., 1988; Jiménez-Jiménez et al., 1996; Mally et al., 1997; Engelborghs et al., 2003), although two groups found them increased (Lakke and Teelken, 1976; Araki et al., 1986; Lakke et al., 1987), and another decreased (Tohgi et al., 1991c). In agreement with Tohgi et al. (1991c), our group reported lower glycine levels in untreated PD patients when compared with PD patients under levodopa therapy or with controls (Jiménez-Jiménez et al., 1996).

Data regarding other (non-neurotransmitter) amino acids are even more controversial. CSF levels of neutral and basic amino acids have been reported to be both increased (Van Sande et al., 1971; Lakke and Teelken, 1976; Lakke et al., 1987), and decreased (Molina et al., 1997a). Two groups reported decreased (Molina et al., 1997a; Engelborghs et al., 2003) and another increased CSF levels of taurine (Lakke and Teelken, 1976; Araki et al., 1986; Lakke et al., 1987). Ornithine, citruline, and arginine (implicated in the urea cycle, and the two latter in the synthesis of nitric oxide) have been found to be increased (Van Sande et al., 1971; Lakke and Teelken, 1976; Lakke et al., 1987), normal (Kuiper et al., 2000), or decreased (Molina et al., 1997a). Another group described increased CSF levels of total homocysteine but normal ones of free homocysteine in PD patients (Isobe et al., 2005), with an additional increase after treatment with levodopa, while total methionine levels decreased after this therapy (Isobe et al., 2010a).

In general, the results on CSF amino acid levels in PD patients are inconclusive, because they might be influenced by selection of study subjects, sample size, lack of adequate matching between cases and controls in many studies, differences in antiparkinsonian therapy, and differences in study techniques, storage and handling of the samples (Jiménez-Jiménez et al., 1996; Molina et al., 1997a).

NEUROPEPTIDES

Neuropeptides modulate neuronal communication by acting on cell surface receptors. Many of them are co-released with classical neurotransmitters. There have been reports of a number of changes in the concentrations of several neuropeptides in PD brain, which are mainly significant decreases in (Jiménez-Jiménez, 1994): (a) met-enkephalin (MET-ENK), substantia P (SP), and cholecystokinine 8 (CCK-8) in the substantia nigra; (b) MET-ENK and leu-enkephalin (LEU-ENK) in the putamen and globus pallidus; (c) MET-ENK in the ventral tegmental area; (d) SP, somatostatin and neurotensin in the neocortex, and (e) somatostatin and neurotensin in the hippocampus. It is likely that many of these changes are related with dopaminergic deficit, and the only clear relationship between a neuropeptide and a clinical feature of PD is that of somatostatin with the presence of cognitive impairment (Jiménez-Jiménez, 1994). **Table 2** summarizes the findings of classical studies on CSF neuropeptide levels in PD patients. Most of these studies enrolled limited series of patients.

In recent years, there has been increased interest in the possible role of orexin-A/hypocretin-1, a neuropeptide hormone implicated in the pathogenesis of narcolepsia, on the development of excessive daytime sleepiness in PD patients. Since the first report by Drouot et al. (2003), who described decreased ventricular CSF orexin levels in PD patients, which were related with the severity of the disease, other authors have confirmed decreased CSF orexin in PD (Fronczek et al., 2007; Asai et al., 2009) and in other neurodegenerative parkinsonisms (Yasui et al., 2006), and the relation of CSF orexin with severity of PD (Asai et al., 2009), and with the presence of sleep attacks (Asai et al., 2009). In contrast, Compta et al. (2009a) found no significant differences in CSF orexin levels between demented PD patients, non-demented PD patients, and healthy controls, and found no relation between CSF orexine and Epworth sleepiness scale or Mini-Mental State Examination. Drouot et al. (2011) found a lack of association between low ventricular CSF orexin and sleepiness in PD, and a relation between high levels of orexin-A in PD associated with loss of REM muscle atonia (Bridoux et al., 2013), while Wienecke et al. (2012) reported association between low CSF orexin levels and sleepiness in PD. Finally, Pålhagen et al. (2010) described similar CSF orexin levels in patients with major depression with or without concomitant PD. The results regarding orexin A are controversial, and await confirmation.

OTHER NEUROTRANSMITTERS

Pisani et al. (2005, 2010) found increased CSF levels of the endogenous cannabinoid anandamide in untreated PD patients, which were unrelated to the severity of the disease (Pisani et al., 2005) and reversed by chronic dopaminergic replacement (Pisani et al., 2010). Zhou et al. (1997) found decreased CSF β-phenylethylamine (PEA) levels in PD patients which were correlated negatively with Hoehn & Yahr stage.

CYCLIC NUCLEOTIDES

These compounds act as intracellular second messengers of neurotransmitters or other compounds such as nitric oxide (NO). The most important are cyclic adenosine 3′5′ monophosphate (cAMP) and cyclic guanosine 3′5′ monophosphate (cGMP). Belmaker et al. (1978) reported a 40–50% decrease of CSF cAMP and an 80–90% decrease of CSF cGMP levels in PD patients who were not related with levodopa therapy. Decreased CSF cAMP levels in PD have also been reported in another study (Volicer et al., 1986), while others found this value to be normal (Cramer et al., 1973, 1984; Covicković-Sternić et al., 1987; Oeckl et al., 2012), both in PD patients with and without dementia (Oeckl et al., 2012). Four further studies described normal CSF cGMP levels (Volicer et al., 1986; Covicković-Sternić et al., 1987; Ikeda et al.,

Table 2 | Alterations in CSF neuropeptide levels in PD patients compared with controls.

Neuropeptide	References	PD patients/ Controls	Cerebrospinal fluid levels
Substantia P (SP)	Pezzoli et al., 1984	12/10	Increased 5-fold
	Cramer et al., 1989	15/9	Normal
	Cramer et al., 1991	23/9	Decreased by 30% (controls were essential tremor patients)
Cholecystokinin-8 (CCK-8)	Lotstra et al., 1985	20/68	Decreased by 50%
Met-enkephalin (MET-ENK)	Pezzoli et al., 1984	12/10	Increased 3-fold in PD patients with slight or moderate disability ($n = 6$)
	Yaksh et al., 1990	8/9	Decreased by 37%
	Baronti et al., 1991	16/19	Decreased by 31.7%
Leu-enkephalin (LEU-ENK)	Liu, 1989	22/19	Increased by 122% in untreated PD patients without further modification by levodopa therapy
Dynorphin A(1-8)	Baronti et al., 1991	16/19	Normal
Somatostatin	Jolkkonen et al., 1986	35/19	Decreased by 22% ($p < 0.01$), especially in demented patients
	Strittmatter and Cramer, 1992	38/12	Decreased by 27.5% ($p < 0.01$)
	Strittmatter et al., 1996	35/11	Decreased $p < 0.05$, similar in untreated vs. treatment with levodopa
	Cramer et al., 1989	15/9	Decreased by 39%
	Dupont et al., 1982	39/29	Decreased by 40%
	Christensen et al., 1984	48/32	Decreased by 40%
	Cramer et al., 1985	50/6	Decreased by 34%(controls were patients with essential tremor)
	Masson et al., 1990	35/11	Decreased ($p < 0.02$), especially in untreated patients and in those with more severe disease
	Jost et al., 1990	68/6	Decreased by 28%
	Hartikainen et al., 1992	35/34	Normal
	Volicer et al., 1986	10/9	Normal
	Beal et al., 1986	6/84	Normal
	Poewe et al., 1990	22/11	Normal in PD patients with dementia ($n = 11$) and without dementia ($n = 11$)
	Espino et al., 1995	23/26	Increased by 47%, especially in demented patients
Neuropeptide Y (NPY)	Martignoni et al., 1992	10/20	Decreased by 31%
	Yaksh et al., 1990	8/9	Normal
Beta-endorphin	Nappi et al., 1985	24/15	Decreased ($p < 0.005$) both in 14 untreated and 10 treated PD patients
	Jolkkonen et al., 1987	36/35	Normal
Arginine-vasopressine (AVP)	Sundquist et al., 1983	11/21	Decreased by 68%
	Olsson et al., 1987	12/32 OND	Decreased by 71%
Vasoactive intestinal peptide (VIP)	Sharpless et al., 1984	19/12	Normal
Delta sleep-inducing peptide (DSIP)	Ernst et al., 1987	9/20	Decreased by 28.7% (Ferrero et al., 1988)
Alpha-melanocyte-stimulating hormone-like	Rainero et al., 1988	9/12	Increased by 2-fold

(Continued)

Table 2 | Continued

Neuropeptide	References	PD patients/ Controls	Cerebrospinal fluid levels
Diazepam-binding inhibitor	Ferrero et al., 1988	25/82	Increased by 42.5% (80% in depressed PD patients and normal in non-depressed PD patients
	Ferrarese et al., 1990	28/10	Decreased by 50% in PDD ($n = 14$), normal in PDND ($n = 14$)
Neurokinin A	Galard et al., 1992	12/11	Decreased by 24%
Corticotropin-releasing hormone (CRH)	Suemaru et al., 1995	10/5	Normal
ACTH	Nappi et al., 1985	24/15	Normal
Beta-lipotropine	Nappi et al., 1985	24/15	Normal
Angiotensin converting enzyme (ECA)	Konings et al., 1994	88 PDND/18 PDD/20	Increased in PDND patients under levodopa therapy ($p < 0.05$). Normal in untreated PDND and in PDD
	Zubenko et al., 1985	10 PDD/30	Decreased by 27% in demented PD patients
	Zubenko et al., 1986	15/10	Decreased by 24%
Chromogranin A and B and secretogranin II	Eder et al., 1998	8/29	Normal

OND, other neurological diseases; PDD, Parkinson's disease demented; PDND, Parkinson's disease non-demented.

1995; Oeckl et al., 2012), while another found a non-significant trend toward higher CSF cGMP levels in PD patients when compared with controls and higher levels in levodopa-treated PD patients compared with those without levodopa treatment (Navarro et al., 1998).

BIOPTERIN DERIVATIVES AND OTHER COFACTORS

Biopterins act as cofactors for aromatic amino acid hydroxylases, which produce a number of neurotransmitters including DA, NE, epinepherine, and 5-HT and are also required for the production of NO. CSF levels of neopterin and biopterin have been found decreased in PD patients by several groups, especially in those with early-onset PD (Fujishiro et al., 1990; Furukawa et al., 1992), and in carriers of the *PARK8* mutation (Koshiba et al., 2011), which was negatively correlated with duration of illness in those patients with akinetic-rigid PD (Furukawa et al., 1991). In contrast, another group found increased CSF neopterin in PD (Widner et al., 2002).

CSF concentration of hydroxylase cofactor, predominantly composed of tetrahydrobiopterin (BH_4), has also been found decreased (Williams et al., 1980a,b).

Thiamine is an essential cofactor for several important enzymes involved in brain oxidative metabolism. Our group found normal CSF levels of thiamine-diphosphate, thiamine-monophosphate, free thiamine, and total thiamine in PD patients (Jiménez-Jiménez et al., 1999).

ENDOGENOUS NEUROTOXINS

One of the classical etiological hypotheses of PD is related with the presence of endogenous substances which share structural similarities with 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP), a neurotoxin that induces a parkinsonism resembling PD.

Moser et al. (Moser and Kömpf, 1992; Moser et al., 1995) identified two tetrahydroisoquinolin (TIQ) derivatives in the CSF of PD patients, but not in healthy controls, 2-methyl and 1-methyl-6,7-dihydroxy1,2,3,4-TIQ (2-MDTIQ and 1-MDTIQ or salsolinol). This group described a relation between high salsolinol levels and the presence of visual hallucinations (Moser et al., 1996), and reported an increased HVA/3OMD ratio in PD patients in which 2-MDTIQ was detected when compared with those PD in which it was not detectable.

CSF salsolinol levels have been reported to be increased in PD patients compared with controls by other groups (Maruyama et al., 1996; Antkiewicz-Michaluk et al., 1997; Krygowska-Wajs et al., 1997; Naoi and Maruyama, 1999), especially in demented PD patients (Antkiewicz-Michaluk et al., 1997), and in those patients with more severe parkinsonism (Krygowska-Wajs et al., 1997), although other authors have described a trend toward decrease in CSF salsolinol levels with the progression of the disease (Maruyama et al., 1999). In contrast, another group reported similar CSF salsolinol (Müller et al., 1999a,b), but higher levels of harman and norharman β-carbolines (structural analogs of MPTP as well) in PD patients than in controls (Kuhn et al., 1996b). CSF levels of 1-benzyl-1,2,3,4-TIQ have also been found by another group to be increased (Kotake et al., 1995).

Matsubara et al. (1995) measured β-carbolinium cations (BC+s) in the lumbar CSF of 22 PD patients and 11 age-matched controls, and found the 2,9-dimethylnorharmanium cation (2,9-Me2NH+) in 12 PD patients but not in controls. This group described decreased activity of nicotinamide N-methyltranserase (NNMT), an enzyme that catalyzes the N-methylation of nicotinamide and other pyridines in the CSF of younger PD patients compared with younger controls, and a trend toward decrease with aging in PD patients (Aoyama et al., 2001).

The results of studies on neurotoxins related with the risk for PD are based on small series and are not conclusive.

OXIDATIVE STRESS MARKERS

Because there is much evidence on the contribution of oxidative stress in the pathogenesis of PD (**Figure 1**) (Alonso-Navarro et al., 2008), the measurement of oxidative stress markers and substances related with oxidative and defense against oxidative phenomena in the CSF of PD patients is useful. Data regarding lipid peroxidation markers are controversial, while DNA oxidation markers have been found to be increased (**Table 3**).

Transition metals such as iron, copper, and manganese, act as prooxidant agents, although copper is also essential for the

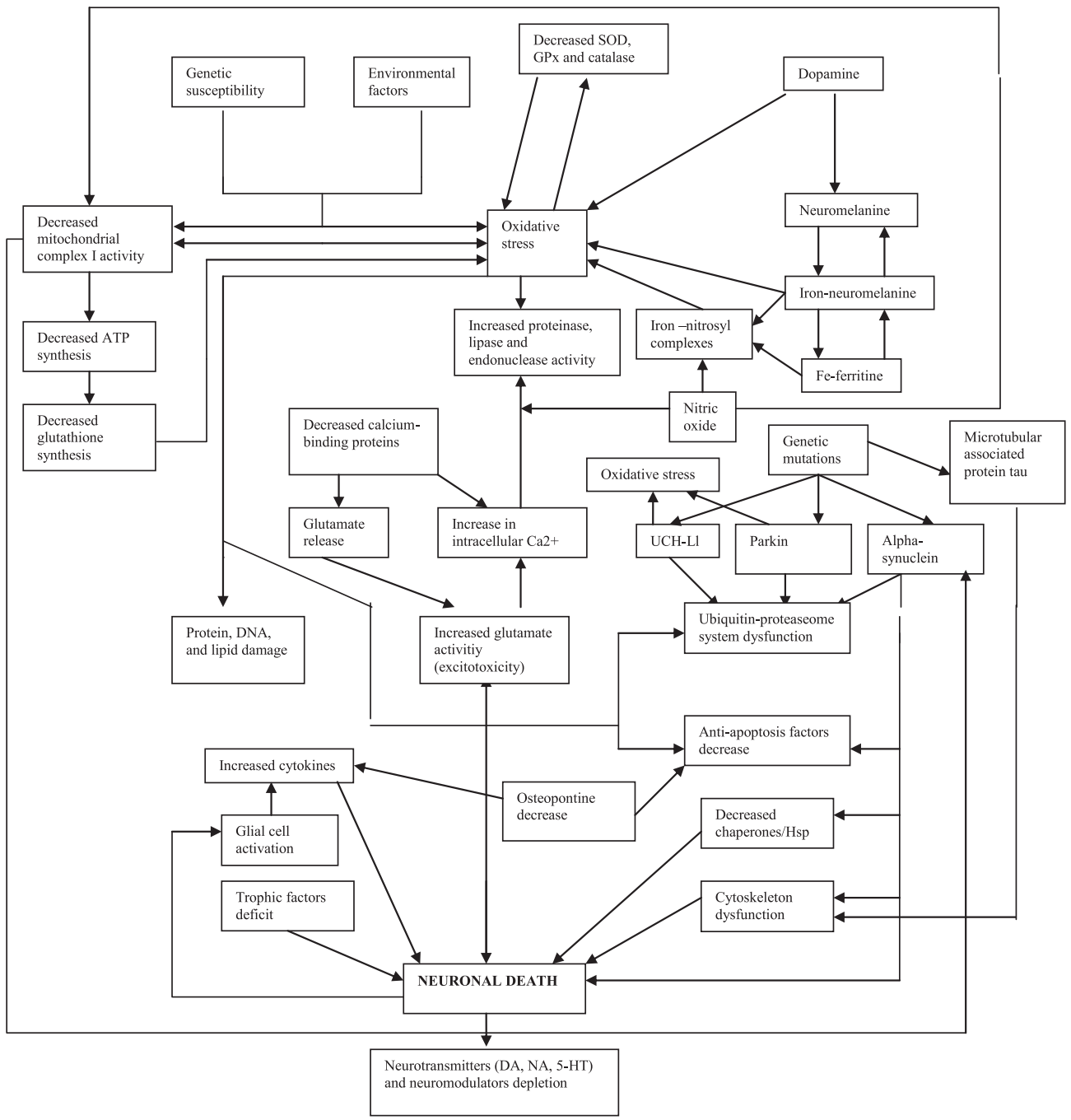

FIGURE 1 | Pathogenical mechanisms proposed for Parkinson's disease (modified from Alonso-Navarro et al., 2008).

antioxidant function of the protein ceruloplasmin, and copper and manganese are constituents of the cytosolic Cu^{+2}/Zn^{+2} and the mitochondrial Mn^{+2}-superoxide-dismutases (SOD, protective against oxidative processes). Zinc has antioxidant activity and is a constituent of Cu^{+2}/Zn^{+2}-SOD (Jiménez-Jiménez et al., 1998). The results of studies with CSF levels of iron and copper are controversial (**Table 3**), but a recent meta-analysis showed similar values in PD patients to controls (Mariani et al., 2013), thus suggesting that these metals are not useful as markers of PD.

Together with its role in glutamate excitotoxity, NO could contribute to oxidative stress mechanisms in the pathogenesis of PD by interacting with ferritin to release iron, inducing mitochondrial complex I damage (Molina et al., 1998), and by inducing nitrosylation of proteins (Fernández et al., 2013). However, studies on CSF levels of nitrates and nitrites have given controversial results (**Table 3**).

Among other antioxidant enzymes and substances (**Table 3**), one study involving an important number of early PD patients showed the relationship between the presence of relatively higher levels of urate and the slower rates of clinical decline (Ascherio et al., 2009), despite the fact that CSF urate levels were found to be similar in PD patients and controls in the same study.

INFLAMMATORY AND IMMUNOLOGICAL MARKERS

CSF interleukin (IL) 1-β levels were found to be normal in one study (Pirttila et al., 1994) and increased in three (Blum-Degen et al., 1995; Mogi et al., 1996a; Mogi and Nagatsu, 1999), CSF IL-2 normal (Blum-Degen et al., 1995) or increased (Mogi et al., 1996a; Mogi and Nagatsu, 1999), IL-4 increased (Mogi and Nagatsu, 1999), and CSF IL-10, IL-12, and interferon-gamma levels have been reported to be similar in PD patients and controls (Rota et al., 2006). CSF IL-6 levels have been found to be decreased in PD patients with major depression in comparison with patients with major depression without PD in one study (Pålhagen et al., 2010), while another 4 found higher CSF IL-6 in PD patients than in healthy controls (Blum-Degen et al., 1995; Mogi et al., 1996a; Müller et al., 1998; Mogi and Nagatsu, 1999), and in one of them CSF IL-6 was correlated with PD severity (Müller et al., 1998). CSF tumor necrosis α (TNF-α) levels have been found to be increased (Mogi et al., 1994; Mogi and Nagatsu, 1999), leukotriene 4 (Irkeç et al., 1989), and α-1-antichymotrypsin normal (Pirttila et al., 1994), and β-2-microglobuline decreased in PD (Mogi et al., 1989; Mogi and Nagatsu, 1999). The CSF levels of the cytokine fractalkine have been found to be normal in PD patients and increased in multiple system atrophy (MSA), and Flt3 ligand normal in these two diseases (Shi et al., 2011). The presence of certain syalilated isoforms of Serpin A1 in the CSF has been related with the development of dementia in PD patients (Jesse et al., 2012).

CSF levels of pros-methylimidazol acetic acid, an isomer of the histamine metabolite tele-methylimidazol acetic acid, have been found to be decreased in PD (Prell et al., 1991), and were highly positively correlated with the severity of the disease (Prell and Green, 1991).

CSF complement 3 (C_3) and factor H (FH) levels were reported to be normal in one study (Wang et al., 2011), while another described a decrease in several isoforms of $C_{3b,}C_{4b}$, FH, and factor B (Finehout et al., 2005), and another normal C_{4d} (Yamada et al., 1994). CSF levels of heat shock proteins Hsp65 and Hsp70 have been found to be increased (Fiszer et al., 1996), and PD patients have shown higher HLA-DR expression in CSF monocytes in comparison with controls (Fiszer et al., 1994a).

Oligoclonal IgG bands have not been detected in the CSF of PD patients (Chu et al., 1983), but antibodies against DA neurons have been detected in 78% of PD patients and in only 3% of controls (Carvey et al., 1991), and the CSF of PD patients has shown a higher proportion of gamma-delta-T+ cells than in controls (Fiszer et al., 1994b).

The results of studies on inflammatory and immunological markers in PD have a low number of patients and controls enrolled, and are inconclusive.

GROWTH AND NEUROTROPHIC FACTORS

CSF Brain Derived Neurotrophic Factor (BDNF) levels have been found to be similar in PD patients with major depression to those in patients with major depression without PD in one study (Pålhagen et al., 2010), while another described this value as increased in PD patients compared with controls (Salehi and Mashayekhi, 2009). CSF Transforming Growth Factor α (TGF-α) has been found to be increased in juvenile parkinsonism (Mogi and Nagatsu, 1999). TGF-β1 has been found to be increased (Mogi et al., 1995, 1996a; Vawter et al., 1996; Mogi and Nagatsu, 1999) or normal (Rota et al., 2006), and TGF-β2 increased (Vawter et al., 1996). CSF insulin-like growth factor-1 (IGF-1) and IGF binding proteins (IGFBPs) expression is increased in PD patients (Mashayekhi et al., 2010). Finally, a single study found a non-significant trend toward increased CSF levels of neuroregulins (which belong to the Epidermal Growth Factor or EGF family) in PD patients (Pankonin et al., 2009). The results of studies on growth and neurotrophic factors in PD, involving a low number of patients and controls, do not permit definitive conclusions.

PROTEINS INVOLVED IN THE PATHOGENESIS OF PARKINSON'S DISEASE

MICROTUBULAR ASSOCIATED PROTEIN *Tau* (MAPT)

Because *MAPT* gene is one of the main genes involved in the risk for PD (Alonso-Navarro et al., 2014), the measurement of CSF protein *tau* levels are hypothetically useful as a marker of this disease. *Tau* protein is important for maintaining the stability of axonal microtubules involved in the mediation of fast axonal transport of synaptic constituents. Hyperphosphorylation of *tau* causes reduces binding affinity for microtubules, leading to their malfunction. Following neuronal damage, tau is released into extracellular space and may be increased in the CSF. *Tau* is an important component of the neurofibrillary tangles (pairwise, helical protein filaments which are found in the cytoskeleton or neuronal cells in Alzheimer's disease (AD) brains. CSF *tau* protein levels are increased in AD patients, and so are a useful marker for this disease. The high risk of PD patients of developing cognitive impairment or dementia patients makes measurement of CSF *tau* reasonable as a possible marker of this disease.

Many studies have shown similar CSF total *tau* and phosphorylated *tau* (phospho*tau*) in PD patients to controls (Blennow et al., 1995; Molina et al., 1997c; Jansen Steur et al., 1998; Sjögren et al., 2002; Mollenhauer et al., 2006; Parnetti et al., 2008, 2011,

Table 3 | Alterations in the CSF levels of oxidative stress markers and substances related with oxidative stress in PD patients compared with controls.

		References	PD/Controls	Cerebrospinal fluid levels
Lipid peroxidation markers	Malonyl-dialdehyde (MDA)	Ilić et al., 1998	31/16	Increased ($p < 0.001$)
		Ilic et al., 1999	33/16	Increased ($p < 0.001$)
		Shukla et al., 2006	21/20	Normal
	(E)-4-hydroxynonenal (HNE)	Selley, 1998	10/10	Increased 4-fold
	Low density lipoprotein (LDL) oxidation products	Buhmann et al., 2004	70/60 OND/31 HC	Increased 3-fold with –SH decreased 1.5-fold
	Schiff bases, conjugated dienes, oxidized proteins, and aldehyde polymers	Boll et al., 2008	22/41	Increased 1,5 fold (Isobe et al., 2010b)
DNA oxidation markers	8′-hydroxy-2′deoxyguanine (8-OHdG)	Kikuchi et al., 2002	48/22	Increased ($p < 0.0001$)
		Isobe et al., 2010b	20/20	Increased ($p < 0.0001$)
	8-hydrosyguanosine (8-OHG)	Kikuchi et al., 2002	48/22	Increased
		Abe et al., 2003	24/15	Increased 3-fold ($p < 0.001$)
	8-OHdG/8-OHG ratio	Kikuchi et al., 2002	48/22	Increased 2-fold ($p < 0.0005$)
Transition metals and related proteins	Iron	Campanella et al., 1973	13/5	Normal
		Pall et al., 1987	24/34	Normal
		Gazzaniga et al., 1992	11/22	Normal
		Takahashi et al., 1994	20/25	Normal
		Pan et al., 1997	NS/NS	Normal
		Jiménez-Jiménez et al., 1998	37/37	Normal
		Hozumi et al., 2011	20/15	Normal
		Forte et al., 2004	26/13	Decreased ($p < 0.05$)
		Alimonti et al., 2007	42/20	Decreased ($p < 0.05$)
		Qureshi et al., 2006	36/21	Increased
	Ferritin	Campanella et al., 1973	13/5	Normal
		Dexter et al., 1990	26/11	Normal
		Pall et al., 1990	24/21	Normal
		Kuiper et al., 1994a	72 PDND/15 PDD/20 HC	Normal
	Transferrin	Loeffler et al., 1994	12/11	Normal
	Copper	Campanella et al., 1973	13/5	Normal
		Gazzaniga et al., 1992	11/22	Normal
		Takahashi et al., 1994	20/25	Normal
		Pan et al., 1997	NS/NS	Increased ($p < 0.05$)
		Jiménez-Jiménez et al., 1998	37/37	Normal
		Forte et al., 2004	26/13	Normal
		Alimonti et al., 2007	42/20	Normal
		Qureshi et al., 2006	36/21	Normal
		Boll et al., 2008	22/41	Increased 2-fold
		Pall et al., 1987	24/34	Increased ($p < 0.001$)
		Hozumi et al., 2011	20/15	Increased 2-fold ($p < 0.01$)
		Boll et al., 1999	49/26 (35 PD untreated)	Increased 1,5 fold

(Continued)

Table 3 | Continued

		References	PD/Controls	Cerebrospinal fluid levels
	Ceruloplasmin	Campanella et al., 1973	13/5	Normal
		Loeffler et al., 1994	12/11	Normal
	Ferroxidase	Boll et al., 2008	22/41	Decreased activity by 20%
		Boll et al., 1999	49/26 (35 PD untreated)	Decreased activity by 1.5-fold
	Manganese	Gazzaniga et al., 1992	11/22	Normal
		Pan et al., 1997	NS/NS	Normal
		Jiménez-Jiménez et al., 1998	37/37	Normal
		Forte et al., 2004	26/13	Normal
		Alimonti et al., 2007	42/20	Normal
		Hozumi et al., 2011	20/15	Increased 1.5-fold ($p < 0.05$)
	Zinc	Takahashi et al., 1994	20/25	Normal
		Pan et al., 1997	NS/NS	Normal
		Forte et al., 2004	26/13	Normal
		Jiménez-Jiménez et al., 1998	37/37	Decreased ($p < 0.05$)
		Qureshi et al., 2006	36/21	Decreased
		Hozumi et al., 2011	20/15	Increased 3-fold ($p < 0.01$)
Other metals	Selenium	Takahashi et al., 1994	20/25	Normal
		Qureshi et al., 2006	36/21	Increased
		Aguilar et al., 1998	28/43	Increased only in untreated PD patients ($p < 0.01$)
	Chromium	Aguilar et al., 1998	28/43	Normal
		Alimonti et al., 2007	42/20	Decreased by 50%
	Magnesium	Hozumi et al., 2011	20/15	Normal
		Forte et al., 2004	26/13	Normal
		Alimonti et al., 2007	42/20	Normal
	Calcium	Pan et al., 1997	NS/NS	Normal
		Forte et al., 2004	26/13	Normal
		Alimonti et al., 2007	42/20	Normal
	Aluminum	Forte et al., 2004	26/13	Decreased ($p < 0.05$)
		Alimonti et al., 2007	42/20	Normal
	Silicon	Forte et al., 2004	26/13	Normal
		Alimonti et al., 2007	42/20	Decreased ($p < 0.05$)
	Cobalt	Alimonti et al., 2007	42/20	Decreased ($p < 0.05$)
	Tin	Alimonti et al., 2007	42/20	Decreased ($p < 0.05$)
	Lead	Alimonti et al., 2007	42/20	Decreased by 50%
	Various	Alimonti et al., 2007	42/20	Normal levels of barium, bismuth, cadmium, mercury, molibdenum, nickel, antimony, strontium, thallium, vanadium, wolfram, and zirconium

(Continued)

Table 3 | Continued

		References	PD/Controls	Cerebrospinal fluid levels
Nitric oxide metabolites/nitroxidative stress	Nitrates	Ikeda et al., 1995	11/17	Normal
		Molina et al., 1996	31/38	Normal
		Kuiper et al., 1994b	103/20	Decreased
		Boll et al., 2008	22/41	Increased 2-fold
	Nitrites	Ikeda et al., 1995	11/17	Normal
		Ilic et al., 1999	33/?	Normal
		Kuiper et al., 1994b	103/20	Normal
		Boll et al., 2008	22/41	Increased 2-fold
		Qureshi et al., 1995	16/14	Increased 2-fold both in untreated ($n = 6$) and in levodopa-treated ($n = 10$) PD patients. Controls were young
	Nitrotyrosine-containing proteins	Fernández et al., 2013	54/40	Increased ($p < 0.01$)
		Aoyama et al., 2000	10/6	Increased 1.8-fold
Antioxidant enzymes or substances	Total superoxide-dismutase (SOD)	Marttila et al., 1988	26/26 OND	Normal
		De Deyn et al., 1998	12/58	Normal
	Cu/Zn-SOD (SOD-1)	Ilić et al., 1998	31/16	Increased ($p < 0.05$)
		Ilic et al., 1999	33/16	Increased ($p < 0.05$)
		Boll et al., 2008	22/41	Decreased ($p = 0.021$)
	Mn-SOD (SOD-2)	Aoyama et al., 2000	10/6	Normal
	Catalase	Marttila et al., 1988	26/26 OND	Normal
	Glutathione peroxidase (GPx)	Marttila et al., 1988	26/26 OND	Normal
	Glutathione reductase (GR)	Ilić et al., 1998	31/?	Increased
		Ilic et al., 1999	33/?	Increased
	Reduced glutathione (GSH)	Marttila et al., 1988	26/26 OND	Normal
		Tohgi et al., 1995b	22/15	Increased ($p < 0.02$) in L-dopa treated patients ($n = 8$)
		Konings et al., 1999	71 PD/13 PDND/21 HC	Normal
	Oxidized glutathione (GSSG)	LeWitt et al., 2013	48/57	Decreased ($p < 0.01$)
		Tohgi et al., 1995b	22/15	Decreased ($p < 0.001$) in untreated patients ($n = 14$)
	Alpha-tocopherol (vitamin E)	Buhmann et al., 2004	70/60 OND/31 HC	Decreased by 44–48%
		Tohgi et al., 1995b	22/15	Normal
		Molina et al., 1997b	34/47	Normal
	Alpha-tocopherol-quinone	Tohgi et al., 1995b	22/15	Decreased ($p < 0.001$) in untreated patients ($n = 15$)
	Urate	Tohgi et al., 1993e	11/14	Normal
		Constantinescu et al., 2013	6/18	Normal
		Ascherio et al., 2009	713/0	Relation of higher CSF levels of urate with slower rates of clinical decline

(Continued)

Table 3 | Continued

	References	PD/Controls	Cerebrospinal fluid levels
Xantine (uric acid precursor)	LeWitt et al., 2011	217/26	Normal
Ascorbate	Buhmann et al., 2004	70/60 OND/31 HC	Normal
Carnitine	Jiménez-Jiménez et al., 1997	29/29	Normal
Oxidized coenzyme Q10/total Q10 ratio	Isobe et al., 2010b	20/20	Increased 18% ($p < 0.05$)
	Isobe et al., 2007	20/20	Increased 18% ($p < 0.05$)
Osteopontine	Maetzler et al., 2007	30/30	Increased 2-fold ($p < 0.002$)

OND, other neurological controls; HC, healthy controls; PDND, Parkinson's disease non-demented.

2014a,b; Ohrfelt et al., 2009; Compta et al., 2009b; Alves et al., 2010; Montine et al., 2010; Aerts et al., 2011; van Dijk et al., 2013a; Herbert et al., 2014). Several of these studies have shown increased CSF *tau* in demented PD patients (Mollenhauer et al., 2006; Compta et al., 2009b). The 33 KDa/55 KDa *tau* isoforms ratio have also been found to be normal in PD (Borroni et al., 2008, 2009), but decreased in progressive supranuclear palsy (PSP), and normal in patients with diffuse Lewy body disease (DLBD), demented PD patients (PDD), AD, and frontotemporal dementia (FTD) (Borroni et al., 2008, 2009).

Some authors have found decreased CSF total *tau* and phospho*tau* levels when compared with controls (Mollenhauer et al., 2011; Shi et al., 2011; Kang et al., 2013) and similar levels in PD to PSP, DLBD, and MSA (Mollenhauer et al., 2011), while others found higher CSF *tau* in DLBD compared with PDD patients (Andersson et al., 2011), and still others higher CSF total *tau* in MSA than in PD patients (Herbert et al., 2014). Hall et al. (2012) reported decreased CSF total *tau* and normal phospho*tau* both in PD and PDD, while total *tau* was increased in CBD and normal in PSP, DLBD, and MSA, and phospho*tau* was decreased in PSP and MSA in comparison with controls.

Přikrylová Vranová et al. (2010) found increased CSF *tau* levels in PD patients with less than 2 years of evolution, and increased CSF *tau* levels which were higher in patients with PDD than in PD, and in PD than in controls, and similar CSF *tau* in DLDB than in controls (Vranová et al., 2014). This group and others found increased CSF total *tau* levels in patients with non-tremor variants of PD as compared to tremor-dominant PD and controls (Jellinger, 2012; Přikrylová Vranová et al., 2012). Compta et al. (2011) described increased CSF *tau* levels in PD patients carrying the allele rs242557A. Siderowf et al. (2010) showed a lack of association between baseline CSF *tau* levels and cognitive decline in PD patients. Patients with corticobasal degeneration (CBD) and PSP have shown higher CSF total and phospo*tau* levels (Aerts et al., 2011), and patients with DLBD showed similar CSF *tau* levels to PD patients in one study (Ohrfelt et al., 2009), while other authors found higher CSF *tau* levels in AD than in DLBD, in DLDB higher than in PDD, and in PDD higher than in PD (Parnetti et al., 2008).

Baseline CSF levels of total and phospho*tau* in the DATATOP study, involving 403 early PD patients, were negatively correlated with disease progression assessed with the Unified PD Rating Scale (UPDRS) (Zhang et al., 2013).

Beyer et al. (2013) reported a lack of correlation between CSF levels of total and phospho*tau*, and ventricular size in 73 non-demented PD patients and 18 PD patients with mild cognitive impairment.

The results of the studies reported on CSF *tau* levels in PD are summarized in **Table 4**. Although these results are not conclusive, CSF *tau* levels could be related to the progression of the disease (Zhang et al., 2013), and to the preservation of cognitive function in PD patients (Stewart et al., 2014).

ALPHA-SYNUCLEIN

Alpha-synuclein (α-synuclein) is a 140 amino acid-long presynaptic protein, which is the major component of the Lewy bodies (the neuropatologic hallmark of PD), and has been implicated in the pathogenesis of PD and in synucleinopathies such as MSA and DLBD. Mutations of the α-*synuclein* (*SNCA*) gene are related with early-onset monogenic familial PD and are associated with increased risk for sporadic PD (Alonso-Navarro et al., 2014). Although early studies failed to detect the native form of α-synuclein in the CSF of PD and control patients (Jakowec et al., 1998), later studies have detected monomeric SNC in the CSF, with similar levels in PD patients and controls (Borghi et al., 2000). Several studies have found similar CSF total α-synuclein levels in PD patients and in controls (Woulfe et al., 2002; Ohrfelt et al., 2009; Park et al., 2011; Parnetti et al., 2011; Tateno et al., 2012) and others decreased CSF α-synuclein in PD (Tokuda et al., 2006; Hong et al., 2010; Mollenhauer et al., 2011, 2013; Hall et al., 2012; Wang et al., 2012; Kang et al., 2013; Wennström et al., 2013; Parnetti et al., 2014a,b; Mondello et al., 2014; van Dijk et al., 2014), DLBD (Parnetti et al., 2011; Wennström et al., 2013), MSA (Wang et al., 2012; Mondello et al., 2014), and PSP (Wang et al., 2012). Four studies have reported increased CSF oligomeric α-synuclein levels in PD compared with controls (Tokuda et al., 2010; Park et al., 2011; Parnetti

Table 4 | Results of studies on CSF tau and phosphotau levels in PD, other parkinsonian syndromes and controls.

References	Cases/Controls	Main findings
Blennow et al., 1995	44 AD, 31 controls, 17 VAD, 11 FTD, 15 PDND, major depression	CSF total tau and phosphorylated tau (phosphotau) higher in AD than in controls, VAD, FTD, PDND, and major depression (PDND similar than controls)
Molina et al., 1997c	26 PDND, 25 controls	CSF total tau similar in PD and controls
Jansen Steur et al., 1998	115 PD (48 with MMSE lower than 25) 15 controls	CSF total and phosphotau similar in PD (not related with MMSE scores) and controls
Sjögren et al., 2002	19 AD, 14 FTD, 11 ALS, 15 PD, 17 controls	CSF total tau and phosphotau increased in AD compared with FTD ($p < 0.001$), ALS ($p < 0.001$), PD ($p < 0.001$), and controls ($p < 0.001$)
Mollenhauer et al., 2006	73 PDD, 23 PDND, 41 controls (non-demented neurological patients)	CSF total tau significantly higher in PDD than in PDND and controls. This observation was most marked ($p < 0.05$) in a subgroup of patients with PDD carrying the apolipoprotein genotype epsilon3/epsilon3
Parnetti et al., 2008	19 DLBD, 18 PDD, 23 AD, 20 PDND, 20 controls	CSF total tau of DLBD patients significantly lower than in AD patients, but twofold to threefold higher than in PDD, PDND, or control subjects CSF total tau levels similar in PDD and PDND Phosphotau increased in the AD group only
Borroni et al., 2008	21 PSP, 20 CBD, 44 FTD, 29 AD, 10 PDND, 15 DLBD, 27 controls	CSF tau 33/55 kDa ratio significantly reduced in PSP when compared to controls and to patients with other neurodegenerative conditions CSF tau 33/55 kDa ratio decrease correlated significantly with brainstem atrophy
Borroni et al., 2009	78 patients with neurodegenerative disorders and 26 controls	CSF tau 33/55 kDa ratio significantly decreased in patients with PSP (0.46 ± 0.16) when compared to healthy controls ($p = 0.002$), AD ($P < 0.001$), FTD, CBD, PD, and DLBD (values in PD similar to those of controls)
Ohrfelt et al., 2009	66 AD, 15 PD, 15 DLBD, 55 controls	CSF total tau and phosphotau increased significantly in AD, similar levels in PD, DLBD, and controls
Compta et al., 2009b	20 PDND, 20 PDD, 30 controls patients	CSF total tau and phosphotau higher in PDD than in PDND and controls ($P < 0.05$). High CSF total tau and phospho-tau were associated with impaired memory and naming
Alves et al., 2010	109 PDND, 36 controls, 20 mild AD	CSF total tau and phosphotau similar in PD and controls CSF tau did not correlate with cognitive measures
Montine et al., 2010	150 controls (115 >50 years; 24 amnestic Mild Cognitive Impairment (aMCI), 49 AD, 49 PD, 11 PDD 62 PD-CIND (cognitive imparment non-demented)	CSF total tau and phospho181-tau significantly increased in AD and aMCI in comparison with the other groups Total tau similar in PDD, PDD and PD-CIND and controls Phospho181-tau slightly decreased when compared with controls >50 years

(Continued)

Table 4 | Continued

References	Cases/Controls	Main findings
Přikrylová Vranová et al., 2010	32 PD, 30 controls	CSF total tau and total tau/beta-amyloid (1-42) ratio higher in PD than in controls ($p = 0.045$ and 0.033, respectively)
Siderowf et al., 2010	45 PD, longitudinal follow-up at least 1 year	No association between CSF total tau and phospo181-tau and cognitive decline
Aerts et al., 2011	21 PSP, 12 CBD, 28 PD, 49 controls	CSF total tau CBD > PSP > PD = controls CSF phospotau CBD > PSP = PD = controls
Parnetti et al., 2011	38 PD, 32 DLBD, 48 AD, 31 FTD, 32 controls with other neurological diseases ($n = 32$)	CSF total tau and phosphotau AD > FTD > DLBD = PD = controls
Shi et al., 2011	137 controls, 126 PD, 50 AD and 32 MSA	CSF total tau and phosphotau AD > controls > PD = MSA
Mollenhauer et al., 2011	Cross-sectional cohort: 51 PD, 29 MSA, 55 DLBD, 62 AD, and 72 neurological controls	CSF total tau AD > DLBD > PD = controls = MSA
Mollenhauer et al., 2011	Validation cohort: 275 PD, 15 MSA, 55 66 DLBD, 8 PSP,22 normal pressure hydrocephalus (NPH) and 23 neurological controls	CSF total tau MSA < DLBD = PD < DLBD < controls
Andersson et al., 2011	47 DLBD, 17 PDD ($n = 17$)	CSF total-tau higher in DLBD than in PDD CSF phosphotau similar in DLBD and PDD
Compta et al., 2011	38 PD patients (19 PDD, 19 PDND). All cases were genotyped for a series of tau gene polymorphisms rs1880753, rs1880756, rs1800547, rs1467967, rs242557, rs2471738, and rs7521	The A-allele rs242557 polymorphism was the only tau gene variant significantly associated with higher CSF tau and phospho-tau levels, under both dominant and dose-response model. This association depended on the presence of dementia, and was only observed in individuals with low (<500 pg/mL) CSF Aβ levels
Hall et al., 2012	90 PDND, 33 PDD, 70 DLBD, 48 AD, 45 PSP, 48 MSA, 12 CBD, 107 controls	CSF total tau AD > MSA = CBD > PSP = Controls = DLBD > PDND = PDD CSF phosphotau increased in AD, AD > PDD = DLBD = controls = CBD > PDND > PSP = MSA
Přikrylová Vranová et al., 2012	48 PD (17 early-onset PD, 15 tremor dominant, 16 non-tremor-dominant), 19 neurological controls, 18 AD	CSF tau and index tau/amiloid beta42 increased in non-tremor-dominant PD compared with controls, and other PD groups, and siminar to those of AD
Jellinger, 2012	12 PD (6 tremor-dominant PD and 6 non-tremor-dominant PD), 27 AD, 17 controls	CSF total tau higher in AD compared with the other groups, and higher in tremor-dominant PD compared with non-tremor dominant PD and controls
van Dijk et al., 2013a	52 PD, 50 controls	CSF total tau and phosphotau similar in PD and controls
Kang et al., 2013	63 PD, 39 controls	CSF total tau and phosphotau181 significantly lower in PD than in controls
Zhang et al., 2013	403 early stage PD patients enrolled in the DATATOP study	Baseline CSF phosphotau/total tau and phosphotau/amyloid beta significantly and negatively correlated with the rates of the Unified Parkinson Disease Rating Scale change
Beyer et al., 2013	73 PDND, 18 PD with mild cognitive impairment	No associations between CSF total tau and phosphotau and hippocampal atrophy

(Continued)

Table 4 | Continued

References	Cases/Controls	Main findings
Herbert et al., 2014	43 PD, 23 MSA, 30 controls	CSF total tau significantly lower in PD than in MSA, but similar to those of controls CSF phosphotau similar in PD, MSA and controls
Parnetti et al., 2014a	71 PD (8 of 44 carriers of a mutation in the beta-glucocerebrosidase gene (*GBA1*) 45 controls with other neurological disases	CSF total tau and phosphotau similar in PD and controls
Parnetti et al., 2014b	44 PD and 25 controls with other neurological diseases	CSF total tau and phosphotau similar in PD and controls, and unrelated with prognosis and cognitive impairment
Vranová et al., 2014	27 PDND, 14 PDD, 14 DLBD, 17 AD 24 controls	CSF total tau AD > PDD > PDND > DLBD = controls

AD, Alzheimer's disease; PD, Parkinson's disease; VAD, vascular dementia; FTD, frontotemporal dementia; PDND, PD non-demented; PD, PD demented; MMSE, MiniMental State Examination; DLBD, diffuse Lewy body disease; PSP, progressive supranuclear palsy; CBD, corticobasal degeneration; MSA, multiple system atrophy; aMCI, Amnestic Mild Cognitive Impairment; PD-CIND, PD with cognitive imparment non-demented; NPH, normal pressure hydrocephalus.

et al., 2014a,b), and one of them showed increased CSF α-Syn in PD patients compared with patients with PSP and AD (Tokuda et al., 2010). Wang et al. (2012) found increased CSF levels of the phosphorylated α-synuclein phospho-Ser129 (PS-129) in PD patients when compared with controls, but lower levels in MSA and PSP of this protein than in PD patients and controls.

Aerts et al. (2012) found similar CSF α-synuclein levels in PD patients to DLBD, PSP, and MSA. Hall et al. (2012) found higher CSF α-synuclein in PSP than in PD, PDD, DLBD, and MSA. Tateno et al. (2012) reported similar CSF α-synuclein levels in PD, MSA, DLBD, and controls but higher CSF α-synuclein levels in AD patients, while Ohrfelt et al. (2009) found higher CSF α-Syn levels in AD than in DLDB and PD, and in DLBD than in PD patients. Foulds et al. (2012) found similar post-mortem CSF total α-synuclein levels in PD, MSA, DLBD, and PSP, but increased CSF levels of phosforylated oligomers in MSA.

van Dijk et al. (2014) reported a lack of relation between CSF α-synuclein levels and striatal dopaminergic deficit measured by dopamine transporter binding and single photon emission computed tomography. In addition, a recent study by Shi et al. (2012) described a lack of relation between the loss of striatal dopaminergic function, assessed by positron emission tomography (PET), and CSF α-synuclein levels, in asymptomatic carriers of mutations in the *LRRK2* gene. CSF neurosin (a protease that degrades α-synuclein) levels have been found to be decreased (Wennström et al., 2013).

Lower baseline CSF α-synuclein levels in the DATATOP study predicted a better preservation of cognitive function in early PD patients with up to 8 years of follow-up (Stewart et al., 2014).

The results of the studies reported on CSF α-synuclein levels in PD are summarized in **Table 5**. The majority of recent studies have shown decreased CSF α-synuclein levels both in PD and in other synucleopathies. Therefore, this should be a useful marker to distinguish this disease from controls, but not to distinguish among synucleopathies.

AMYLOID-BETA

Amyloid beta (Aβ) are a group of different lengths peptides resulting from the enzymatic cleavage of the amyloid precursor protein (APP). The most common is the 42 amino-acid long Aβ42. These peptides have a differential trend toward aggregation (specially Aβ1-42) to form amyloid plaques, one of the pathological hallmarks of AD and DLBD. The increased risk for developing cognitive impairment and dementia of PD patients in comparison with the general population makes it reasonable to link AD markers such as Aβ42 to PDD. Several studies have shown similar (Holmberg et al., 2003; Mollenhauer et al., 2006; Ohrfelt et al., 2009; Přikrylová Vranová et al., 2010; Aerts et al., 2011; Parnetti et al., 2011; van Dijk et al., 2013a) or decreased (Sjögren et al., 2002; Compta et al., 2009b; Mollenhauer et al., 2011; Shi et al., 2011; Kang et al., 2013; Nutu et al., 2013a; Vranová et al., 2014) CSF Aβ1-42 (Aβ1-42) in PD patients, with the exception of one study which reports increased levels (Parnetti et al., 2014b). Other found decreased CSF Aβ-1-42 (Mollenhauer et al., 2006; Compta et al., 2009b; Alves et al., 2010; Montine et al., 2010; Siderowf et al., 2010) and Aβ1-40 (Alves et al., 2010) and Aβ1-38 (Alves et al., 2010) only in PDD patients or in PD patients with memory impairment.

Baseline CSF Aβ levels in the DATATOP study, were negatively correlated with disease progression assessed with UPDRS (Zhang et al., 2013). Baseline CSF levels of Aβ1-42 in two studies (Siderowf et al., 2010; Parnetti et al., 2014b); and the combination of lower baseline CSF Aβ, worse verbal learning, semantic fluency and visuoperceptual scores, and thinner superior-frontal/anterior cingulated in precentral regions by 3T-brain-Magnetic Resonance Imaging in another (Compta et al., 2013) have been associated with further cognitive decline in PD patients.

CSF Aβ1-42 levels have been reported as decreased (Parnetti et al., 2008; Andersson et al., 2011; Parnetti et al., 2011) or similar (Ohrfelt et al., 2009; Nutu et al., 2013a) in DLBD than in PDD and PD patients, decreased in MSA (Holmberg et al., 2003; Shi et al., 2011), and decreased in DLBD in comparison with PD,

Table 5 | Results of studies on CSF alpha-synuclein and phosphotau levels in PD, other parkinsonian syndromes and controls.

References	Cases/Controls	Main findings
Borghi et al., 2000	12 PD, 10 controls	Identification of a 19 kDa band that corresponds to monomeric α-synuclein (similar levels in PD and controls)
Woulfe et al., 2002	5 PD, 4 controls	Similar anti-α-synuclein antibodies in PD and controls
Tokuda et al., 2006	33 PD, 38 controls (9 healthy and 29 with OND)	CSF α-synuclein levels significantly lower in PD than in controls ($p < 0.0001$)
Ohrfelt et al., 2009	66 AD, 15 PD, 15 DLBD, 55 controls	CSF α-synuclein AD > Controls = DLBD = PD
Hong et al., 2010	117 PD, 132 controls, 50 AD	CSF α-synuclein PD < Controls = AD (after correcting for hemoglobin levels)
Tokuda et al., 2010	32 PD, 28 controls (12 healthy and 16 with OND)	CSF α-synuclein oligomers and oligomers/total-α-synuclein ratio in CSF higher in PD group ($p < 0.0001$)
Tokuda et al., 2010	25 PD, 18 PSP, 35 AD, 43 controls	CSF α-synuclein PD > PSP = Controls > AD
Parnetti et al., 2011	38 PD, 32 DLBD, 48 AD, 31 FTD, 32 controls with other neurological diseases ($n = 32$)	CSF α-synuclein Controls > PD > DLBD = AD = FTD
Mollenhauer et al., 2011	Cross-sectional cohort: 51 PD, 29 MSA, 55 DLBD, 62 AD, and 72 neurological controls	CSF α-synuclein PD < DLBD < MSA < controls < AD
Kang et al., 2013	Validation cohort: 275 PD, 15 MSA, 55 66 DLBD, 8 PSP, 22 NPH, and 23 neurological controls	CSF α-synuclein MSA < DLBD = PD < NPH = PSP < controls
Park et al., 2011	23 PD, 29 neurological controls	CSF α-synuclein oligomer significantly higher in PD than in neurological controls
Kang et al., 2013	63 PD, 39 controls	Slightly, but significantly, lower CSF levels of α-synuclein in PD compared with healthy controls Lower levels of CSF α-synuclein associated with increased motor severity
Hall et al., 2012	90 PDND, 33 PDD, 70 DLBD, 48 AD, 45 PSP, 48 MSA, 12 CBD, 107 controls	CSFα-synuclein AD > PSP = Controls > PDD = DLBD = MSA = CBD = PDND
Tateno et al., 2012	9 AD, 6 DLBD, 11 PD, 11 MSA, 11 neurological controls	CSFα-synuclein levels in AD higher than in controls ($P < 0.05$), and significantly lower in PD ($P < 0.001$), DLBD ($P < 0.01$), and MSA ($P < 0.05$) when compared with AD
Wang et al., 2012	Discovery series: 93 PD, 26 AD, 78 controls, 33 PSP, 16 MSA Replication series: 116 PD, 50 AD, 126 controls, 27 PSP, 25 MSA	CSF Phosphorylated α-synuclein (PS-129) PD > Controls > AD > MSA = PSP CSFα-synuclein MSA < PD < PSP > AD = Controls CSF PS-199/α-synuclein ratio MSA > PK > AD > PSP = Controls
Aerts et al., 2012	58 PD, 47 MSA, 3 DLBD, 22 Vascular Parkinsonsim, 10 PSP, 2 CBD, 57 controls	CSFα-synuclein did not differ significantly among the study groups
Foulds et al., 2012	13 PDND, 10 PD with cognitive impairment, 16 PDD, 17 DLBD, 12 PSP, 8 MSA, 20 controls (ventricular CSF obtained post-mortem)	CSF total α-synuclein, oligomeric α-synuclein and phosphorylated α-synuclein similar in PDND, PDCI, PDD, DLBD, PSP, MSA, and control groups CSF oligomeric phosphorylated α-synuclein significantly higher in MSA ($p < 0.001$) when compared with the other study groups
Shi et al., 2012	8 symptomatic and 18 asymptomatic carriers of the G2019 mutation in the *LRRK2* gene	Lack of correlation between PET scan evidence of loss of striatal dopaminergic and CSF α-synuclein levels

(Continued)

Table 5 | Continued

References	Cases/Controls	Main findings
Mollenhauer et al., 2013	78 PD (drug naive), 48 controls	CSF α-synuclein lower in PD than in controls
Wennström et al., 2013	52 controls, 46 AD,38 PDND, 22 PDD, 33 DLBD	AD > controls > DLBD > PD > PDD
Parnetti et al., 2014a	71 PD (8 of 44 carriers of a mutation in the beta-glucocerebrosidase gene (*GBA1*) 45 controls with other neurological diseases	CSF α-synuclein lower and oligomeric/total α-synuclein ratio higher in PD than in controls
Parnetti et al., 2014b	44 PD and 25 controls with other neurological diseases	CSF total α-synuclein lower and oligomeric α-synuclein higher in PD than in controls. No relation with prognosis and cognitive impairment
van Dijk et al., 2014	53 PD, 50 controls	CSF α-synuclein levels reduced in patients with PD, but not correlated with measures of disease severity, and striatal dopaminergic deficit assessed with neuroimaging
Mondello et al., 2014	22 controls, 52 PD, 34 MSA, 32 PSP, 12 CBD	CSF α-synuclein MSA < PD < PSP < CBD < Controls
Stewart et al., 2014	304 early PD patients enrolled in the DATATOP study. Longitudinal follow-up	CSF α-synuclein showed a longitudinal decrease over follow-up period CSF α-synuclein was not correlated with the rate of clinical progression of the motor symptoms Lower basal levels of CSF α-synuclein were associated with better preservation of cognitive function

AD, Alzheimer's disease; PD, Parkinson's disease; FTD, frontotemporal dementia; PDND, PD non-demented; PD, PD demented; OND, Other neurological diseases; DLBD, diffuse Lewy body disease; PSP, progressive supranuclear palsy; CBD, corticobasal degeneration; MSA, multiple system atrophy; NPH, normal pressure hydrocephalus.

PDD (Hall et al., 2012; Vranová et al., 2014), PSP, MSA, and CBD (Hall et al., 2012).

Alves et al. (2013) reported that patients with PD with the postural instability-gait disorders (PIGD) phenotype had significantly reduced CSF Aβ42, Aβ38, Aβ42/40, and Aβ38/40 levels compared with patients with the tremor-dominant phenotype and controls.

Nutu et al. (2013b) described lower CSF levels of Aβ1-15/16 in PD, PDD, PSP, and MSA compared to CBD, AD, and controls.

Beyer et al. (2013) reported a correlation between CSF levels of Aβ38, Aβ40, and Aβ42, and ventricular size in 73 non-demented PD patients and 18 PD patients with mild cognitive impairment.

The results of the studies reported on CSF Aβ levels in PD are summarized in **Table 6**. Many of these studies suggest the potential usefulness of CSF Aβ1-42 levels to predict cognitive impairment in PD patients.

NEUROFILAMENT PROTEINS

Abnormal accumulation in the cytoplasm of neurofilaments (NF), members of the cytoskeleton proteins expressed by neurons, have been detected in neurodegenerative diseases including AD, MSA, DLBD, and PD. CSF levels of neurofilament light (NFL) proteins have been found normal in PD patients (Constantinescu et al., 2010; Hall et al., 2012), and increased in patients with PSP (Holmberg et al., 1998; Constantinescu et al., 2010; Hall et al., 2012), MSA (Holmberg et al., 1998; Constantinescu et al., 2010; Hall et al., 2012), CBD (Constantinescu et al., 2010; Hall et al., 2012), and PDD (Hall et al., 2012).

CSF neuronal thread protein (NTP) levels have been found increased when compared with controls and decreased when compared with AD patients in one study (de la Monte et al., 1992), and similar to those of controls in another (Yamada et al., 1993). CSF annexine V has been found to be decreased in PD (Vermes et al., 1999). Glial fibrilar acidic protein (GFAP) has been found to be normal in the CSF of PD, MSA, PSP, and CBD patients (Constantinescu et al., 2010). CSF levels of the glial activation marker YKL-40 have been found to be decreased in PD, MS, PSP, and CBD (Olsson et al., 2013).

OTHER PROTEINS

Defects in the gene encoding DJ-1 protein cause an autosomal recessive early-onset PD, PARK7 (Alonso-Navarro et al., 2014). This protein is also a marker of oxidative stress. CSF levels of DJ-1 protein have been found to be increased in PD in 2 studies (Waragai et al., 2006; Herbert et al., 2014) and decreased in another 2 (Shi et al., 2011; Hong et al., 2010). One of these studies described decreased CSF DJ-1 in MSA as well (Shi et al., 2011), and other increased DJ-1 in MSA compared with PD and with controls (Herbert et al., 2014). Shi et al. (2012) described a lack of relation between the loss of striatal dopaminergic function and CSF DJ-1 levels in asymptomatic carriers of mutations in the *LRRK2* gene (PARK8). The results on DJ-1 are, therefore, inconsistent and should not be considered as a marker of PD.

Table 6 | Results of studies on CSF amiloyd beta (Aβ) levels in PD, other parkinsonian syndromes and controls.

References	Cases/Controls	Main findings
Sjögren et al., 2002	19 AD, 14 FTD, 11 ALS, 15 PD, 17 controls	CSF Aβ42 markedly decreased in AD = ALS < FTD < PD < controls
Holmberg et al., 2003	36 MSA, 48 PD, 15 PSP, 32 controls	CSF Aβ42 MSA < PSP = controls = PD
Mollenhauer et al., 2006	73 PDD, 23 PDND, 41 controls (non-demented neurological patients)	CSF Aβ42 lower in the PDD patients compared to PDND patients and controls. This observation was most marked ($p < 0.05$) in a subgroup of patients with PDD carrying the apolipoprotein genotype epsilon3/epsilon3
Parnetti et al., 2008	19 DLBD, 18 PDD, 23 AD, 20 PDND, 20 controls	DLBD showed the lowest mean CSF Aβ42 levels, with a negative association to dementia duration. PDD patients had mean CSF Aβ42 similar to those seen in PD patients
Ohrfelt et al., 2009	66 AD patients, 15 PD patients, 15 patients with dementia with Lewy bodies (DLBD) and 55 cognitively normal controls	CSF Aβ42 AD < DLBD < PD = Controls
Compta et al., 2009b	20 PDND, 20 PDD, 30 controls patients	CSF Aβ42 ranged from high (controls) to intermediate (PDND) and low (PDD) levels ($P < 0.001$). In all PD and PDD patients, in PDND, CSF Aβ42 was related with phonetic fluency
Alves et al., 2010	109 PDND, 36 controls, 20 mild AD	CSF Aβ42 (19%; $p = 0.009$), Aβ40 (15.5%; $p = 0.008$), and Aβ38 (23%; $p = 0.004$) significantly decreased in PD compared with controls CSF Aβ42 reductions in PD less marked than in AD (53%; $p = 0.002$) Associations between CSF levels of Aβ42 (β = 0.205; $p = 0.019$), Aβ40 (β = 0.378; $p < 0.001$), and Aβ38 (β = 0.288; $p = 0.001$) and memory impairment, but not executive-attentional or visuospatial dysfunction
Montine et al., 2010	150 controls (115 >50 years; 24 amnestic Mild Cognitive Impairment (aMCI), 49 AD, 49 PD, 11 PDD 62 PD-CIND (cognitive imparment non-demented)	CSF Aβ42 levels reduced in AD ($p < 0.001$), PD-CIND ($P < 0.05$), and PDD ($P < 0.01$), and similar to those of controls in PD
Přikrylová Vranová et al., 2010	32 PD, 30 controls	CSF Aβ1-42 similar in PD and controls
Siderowf et al., 2010	45 PD, longitudinal follow-up at least 1 year	Lower baseline CSF Aβ1-42 associated with more rapid cognitive decline Subjects with CSF Aβ1-42 levels =192 pg/mL declined an average of 5.85 (95% confidence interval 2.11–9.58, $p = 0.002$) points per year more rapidly on the DRS-2 than subjects above that cutoff, after adjustment for age, disease duration, and baseline cognitive status
Aerts et al., 2011	21 PSP, 12 CBD, 28 PD, 49 controls	CSF Aβ1-42 similar in CBD, PSP, PD, and controls
Parnetti et al., 2011	38 PD, 32 DLBD, 48 AD, 31 FTD, 32 controls with other neurological diseases	CSF Aβ1-42 controls = PD > DLBD = AD = FTD
Shi et al., 2011	137 controls, 126 PD, 50 AD and 32 MSA	CSF Aβ1-42 controls = PD = _MSA > AD
Mollenhauer et al., 2011	Validation cohort: 275 PD, 15 MSA, 55 66 DLBD, 8 PSP, 22 NPH, and 23 neurological controls	CSF Aβ1-42 DLBD < MSA = NPH = PD < controls < PSP
Andersson et al., 2011	47 DLBD, 17 PDD	Aβ42 lower in DLBD than in PDD
Kang et al., 2013	63 PD, 39 controls	Slightly, but significantly, lower levels of Aβ1-42 in PD compared with controls

(Continued)

Table 6 | Continued

References	Cases/Controls	Main findings
Hall et al., 2012	90 PDND, 33 PDD, 70 DLBD, 48 AD, 45 PSP, 48 MSA, 12 CBD, 107 controls	CSF Aβ1-42 AD < DLBD = PDD = PSP = MSA = CBD = PDND = Controls
Přikrylová Vranová et al., 2012	48 PD (17 early-onset PD, 15 tremor-dominant, 16 non-tremor-dominant), 19 neurological controls, 18 AD	CSF Aβ42 lower in AD than in the other groups, and lower in non-tremor-dominant PD compared with controls
Jellinger, 2012	12 PD (6 tremor-dominant PD and 6 non-tremor-dominant PD), 27 AD, 17 controls	CSF Aβ42 lower in tremor-dominant PD than in non-tremor-dominant PD and AD, and lower in these three groups than in controls
van Dijk et al., 2013a	52 PD, 50 controls	CSF Aβ42 similar in PD and controls
Zhang et al., 2013	403 early stage PD patients enrolled in the DATATOP study	CSF baseline levels of Aβ42 weakly but negatively correlated with baseline Unified Parkinson Disease Rating Scale total scores
Beyer et al., 2013	73 PDND, 18 PD with mild cognitive impairment	Association between CSF Aβ38, Aβ40, and Aβ42 with the radial distance of the occipital and frontal horns of the lateral ventricles in PDND. Negative association between CSF Aβ38 and Aβ42 with enlargement in occipital and frontal horns of the lateral ventricles in the pooled sample, and with enlargemente of the occipital horns in PD with mild cognitive impairment
Nutu et al., 2013a	43 PDND, 33 PDD, 51 DLBD, 48 AD, 107 controls	CSF Aβ1-40 AD < DLDB < PDD < PDND = controls CSF Aβ1-42 PDD = DLBD = PDND < controls = AD CSF Aβ1-40/Aβ1-42 ratio AD < DLDB < PDD = controls = PD
Compta et al., 2013	27 PDND, longitudinal following (11 developed dementia)	Lower CSF amyloid-β predicted development of dementia together with worse verbal learning, semantic fluency and visuoperceptual scores, and thinner superior-frontal/anterior cingulate and precentral regions
Alves et al., 2013	99 PD *de novo* (39 with postural instability/gait disorders –PIGD—and 60 tremor-dominant—TD), 46 controls	CSF Aβ42, Aβ38, Aβ42/40, and Aβ38/40 levels significantly reduced in PIGD phenotype compared with TD phenotype and with controls (TD similar to controls)
Nutu et al., 2013b	90 PDND, 32 PDD, 68 DLBD, 48 AD, 45 PSP, 46 MSA, 12 CBD, 107 controls	Significantly lower levels of Aβ1-15/16 were detected in PD, PDD, PSP, and MSA compared to other neurodegenerative diseases and controls
Parnetti et al., 2014b	44 PD and 25 controls with other neurological diseases	CSF Aβ42 lower in PD than in controls. This value was related with cognitive impairment
Vranová et al., 2014	27 PDND, 14 PDD, 14 DLBD, 17 AD 24 controls	CSF Aβ42 PDND > PDD > DLBD >AD > controls

AD, Alzheimer's disease; PD, Parkinson's disease; ALS, amyotrophic lateral sclerosis; FTD, frontotemporal dementia; PDND, PD non-demented; PD, PD demented; DLBD, diffuse Lewy body disease; PSP, progressive supranuclear palsy; CBD, corticobasal degeneration; MSA, multiple system atrophy; aMCI, Amnestic Mild Cognitive Impairment; PD-CIND, PD with cognitive imparment non-demented; NPH, normal pressure hydrocephalus; PIGD, Postural instability and gait disorder; TD, tremor-dominant.

Defects in the gene encoding ubiquitin carboxy-terminal hydrolase 1 (UCH-L1) cause familial PD, PARK5. A recent study found decreased CSF UCH-L1 levels in PD, MSA, and PSP compared with controls (Mondello et al., 2014).

Among proteins related with apoptosis, Bcl-2 protein has not been detected in the CSF of PD patients (Mogi et al., 1996b; Mogi and Nagatsu, 1999). CSF levels of clusterin have been reported to be increased (Přikrylová Vranová et al., 2010; Vranová et al., 2014) or normal (van Dijk et al., 2013a), tissue transglutaminase (Vermes et al., 2004) increased in PD, and cystatin C normal in PD (Přikrylová Vranová et al., 2010; Yamamoto-Watanabe et al., 2010) and decreased in MSA (Yamamoto-Watanabe et al., 2010).

Studies measuring CSF levels of lysosomal hydrolases (involved in the α-Syn degradation) found decreased (Balducci

et al., 2007), normal (van Dijk et al., 2013b), or increased (Parnetti et al., 2014a) β-hexosaminidase, increased cathepsin E (van Dijk et al., 2013b), decreased α-mannosidase (Balducci et al., 2007), decreased (Balducci et al., 2007) or normal β-mannosidase (Mollenhauer et al., 2011; van Dijk et al., 2013b), decreased α-fucosidase (van Dijk et al., 2013b), β-glucocerebrosidase decreased (Balducci et al., 2007; Parnetti et al., 2014a) or normal (van Dijk et al., 2013b), β-galactosidase increased (van Dijk et al., 2013b) or normal (Balducci et al., 2007; Parnetti et al., 2014a), and cathepsin D normal (van Dijk et al., 2013b) in PD patients compared with controls.

CSF Prion protein (PrP) (Meyne et al., 2009) and tetranectin (involved in tissue remodeling) (Hong et al., 2010) levels have been found to be decreased, and apolipoprotein A-1 normal (Wang et al., 2010) in PD patients. CSF levels of transthyretin (TTR, a clearance protein produced in the choroid plexus) have been found to be increased in Lewy body diseases, including PD, PDD, and DLBD in relation with controls (Maetzler et al., 2012). CSF levels of the soluble proteoglycan NG2 (sNG2), involved in proliferation, migration, and differentiation of perycites and NG2 cells in the brain, have been found to be similar in PD patients and controls, and decreased in DLBD (Nielsen et al., 2014).

In PD patients there are reports of decreased CSF post-proline cleaving enzyme (Hagihara and Nagatsu, 1987), increased dipeptidyl-aminopeptidase II (Hagihara et al., 1987), normal dipeptidyl-aminopeptidase IV (Hagihara et al., 1987), and normal glutamic oxaloacetic transaminase (GOT) (Steen and Thomas, 1962; Weiss et al., 1975; Qureshi et al., 1995) and glutamic pyruvic transaminase (GPT) (Weiss et al., 1975) levels.

OTHER COMPOUNDS

In patients with PD there have been reports of normal CSF levels of the proteoglycan N-acetyl neuraminic acid (Lipman and Papadopoulos, 1973), and CSF insulin levels (Jiménez-Jiménez et al., 2000) have been found normal in PD patients.

The CSF levels of corticosterone (Pålhagen et al., 2010) and neuroactive steroids such as allopregnanolone (THP) and 5 α-dihydroprogesterone (DHP) (di Michele et al., 2003) have been found to be decreased in PD. Björkhem et al. (2013) reported that 10% of the PD patients were found to have increased CSF levels of 24S-hydroxycholesterol, and that there was a significant correlation between this value and duration of the disease. Lee et al. (2008) described a significant increase in the CSF levels of the polyunsaturated fatty acid eicosapentanoic acid (EPA) in patients with PD and MSA.

Paik et al. (2010) measured several polyamines in the CSF of patients with PD, MSA and controls. These substances are important for cell growth, and act as important modulators of a variety of ion channels, including glutamate NMDA and AMPA receptors. CSF total polyamine, N^1acetyl-cadaverine, and cadaverine levels were increased both in PD and MSA, but PD patients showed higher CSF putrescine and lower CSF spermidine levels than MSA and controls, and MSA patients showed lower CSF N^1acetylputrescine than PD and controls. CSF N^8-acetylspermidine levels were higher in PD patients than in controls, and in MSA than in PD patients and controls.

CONCLUSIONS

(A) The majority of classical biochemical studies on neurotransmitter and related substances have described decreased CSF HVA, and normal NE, MHPG, ACh, AChE, glutamate, aspartate, and glycine levels in patients with PD. Results on CSF GABA and 5-HIAA levels are controversial. Many of these classical studies included patients with different types of Parkinsonism and had a limited number of patients and controls.

(B) Studies on the possible value of endogenous neurotoxins, oxidative stress markers, inflammatory and immunological markers, and growth and neurotrophic factors as biological markers of PD should be considered as inconclusive. The most consistent finding related with these issues is the possible role of CSF urate on the progression of the disease (Ascherio et al., 2009).

(C) Data regarding the role of CSF total *tau* and phospho*tau* as biological markers for PD are inconsistent. The most interesting findings are the possible relations of these markers with the progression of the disease (Zhang et al., 2013), and with the preservation of cognitive function in PD patients (Stewart et al., 2014).

(D) CSF α-synuclein levels have been found to be decreased in most, but not all, studies in PD patients compared with controls. This marker should be useful for the differential diagnosis between synucleopathies and other parkinsonian syndromes, but its usefulness to differentiate among synucleopathies (PD, PDD, DLBD, and MSA), remains to be elucidated.

(E) CSF Aβ1-42 levels could be considered as a useful marker of the presence of further cognitive decline in PD patients.

(F) CSF NFL protein levels should be useful for the differential diagnosis of PSP, MSA, CBD, and PDD from PD, but not to discriminate between PD and healthy controls.

FUTURE APPROACHES

While possible biomarkers for PD in classical studies have been hypothesis-driven, attempts to develop effective procedures for the differential diagnosis of PD in its early stages have led to the performance of CSF multianalyte methods including systematic measurements of patterns of variation in proteins (proteomics) or small molecules (metabolomics). These methods have led to the identification of possible unexpected biomarkers of diseases involved in neurodegenerative processes. However, the results of these types of studies, which are briefly described below, are not clearly established and await replication.

Guo et al. (2009), in a proteomic analysis of the CSF of PD patients and controls, found significantly higher CSF levels of apolipoprotein E, autotoxin, and some SOD1 isoforms, and lower levels of complement C_4 when compared with controls, while Pigment epithelium-derived factor (PEDF or serpin F1) was not significantly increased, and complement C_3 and haptoglobin were similar in PD patients and controls.

Zhang et al. (2008) performed a proteomics-discovered multianalyte profile (MAP) in CSF on 95 control subjects, 48 patients with probable AD, and 40 patients with probable PD, and concluded that the optimal MAP leading to the correct diagnosis was

composed of the following proteins in order of contribution: tau, BDNF, IL-8, Aβ42, β2-microglobulin, vitamin D binding protein, apoA2, and apoE.

Maarouf et al. (2012) analyzed ventricular CSF from PD and controls obtained in the immediate post-mortem period using a two-dimensional difference gel electrophoresis (2D-DIGE) coupled with mass spectrophotometry protein identification, and found differences between the 2 groups in 6 molecules: fibrinogen, transthyretin, apoE, clusterin, apoA1, and glutathione-S-transferase-Pi (GSTP).

Trupp et al. (2014) reported a generally lower level of metabolites in PD as compared to controls, with a specific decrease in 3-hydroxyisovaleric acid, tryptophan, and creatinine, a significant decrease in the levels of Aβ-38 and Aβ-42, and an increase in soluble amyloid peptide precursor α (APPα) in CSF of patients.

Ideally, future studies should fulfill the following conditions: (a) a multicenter and prospective design; (b) inclusion of patients diagnosed with PD and other types of parkinsonism according to standardized criteria; (c) measurement of multiple potential biological markers in the CSF; (d) a very long-term follow-up period (till death as end-point), with assessment of both clinical features and serial determinations of the biological markers; and (e) final neuropathological confirmation by examination of the brains of patients at death (this is lacking in most of the studies published).

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An association study between *Heme oxygenase-1* genetic variants and Parkinson's disease

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The blood-brain barrier (BBB) supplies brain tissues with nutrients, filters harmful compounds from the brain back to the bloodstream, and plays a key role in iron homeostasis in the human brain. Disruptions of the BBB are associated with several neurodegenerative conditions including Parkinson's disease (PD). Oxidative stress, iron deposition and mitochondrial impaired function are considered as risk factors for degeneration of the central nervous system. Heme oxygenase (HMOX) degrades heme ring to biliverdin, free ferrous iron and carbon monoxide being the rate-limiting activity in heme catabolism. The isoform HMOX1 is highly inducible in response to reactive oxygen species, which induce an increase in BBB permeability and impair its pathophysiology. Consequently, an over- expression of this enzyme may contribute to the marked iron deposition found in PD. We analyzed the *HMOX1* SNPs rs2071746, rs2071747, and rs9282702, a microsatellite $(GT)_n$ polymorphism and copy number variations in 691 patients suffering from PD and 766 healthy control individuals. Copy number variations in the *HMOX1* gene exist, but these do not seem to be associated with PD risk. In contrast two polymorphisms that modify the transcriptional activity of the gene, namely a VNTR $(GT)_n$ and the SNP rs2071746, are strongly associated with PD risk, particularly with the classic PD phenotype and with early onset of the disease. This study indicates that *HMOX1* gene variants are associated to the risk of developing some forms of PD, thus adding new information that supports association of *HMOX* gene variations with PD risk.

Keywords: Parkinson's disease, Heme oxygenase, polymorphisms, copy number variations, biomarkers, blood-brain barrier, iron homeostasis

INTRODUCTION

The blood-brain barrier (BBB) supplies brain tissues with nutrients, filters harmful compounds from the brain back to the bloodstream, and plays a key role in iron homeostasis in human brain. Disruptions of the BBB are associated with several neurodegenerative diseases including Parkinson's disease (PD). Oxidative stress, iron deposition and mitochondrial impaired function are considered as risk factors for degeneration of the central nervous system (Schipper, 2004; Alonso-Navarro et al., 2008). In addition, it has been postulated that BBB inflammation may be related to PD risk by causing over-activation of microglia, increased production of cytokines and release of reactive oxygen species (Whitton, 2007), and the possibility that PD may be related to blood borne toxins could be the cause of these neurodegenerative disorders if the integrity of BBB is compromised (for a recent review, see Kim et al., 2012).

PD is one of the most common neurodegenerative movement disorders; current demographic trends indicate a lifetime risk approaching 4% (Schapira, 2013). The mean age of onset is 70 years, although 4% of patients develop early-onset, before the age of 50 (Schrag and Schott, 2006). This disease is characterized by rest tremors, rigidity, slowness of movement and postural imbalance. The primary pathologic abnormalities are the loss of the pigmented cells of the substancia nigra pars compacta, and the dopaminergic neurons of the striatum, which decreases in dopamine levels (Lu'o'ng and Nguyen, 2012). Other central and peripheral dopaminergic and non-dopaminergic systems are also involved. Regarding the pathogenesis of neurodegeneration in PD, patients show increased levels of oxidized lipids and a decrease in levels of glutathione (Zeevalk et al., 2008; Obeso et al., 2010; Schapira, 2012). In these patients, concentration of phospholipids and polyunsaturated free fatty acids, which are highly susceptible to oxidants, is decreased, whereas malondialdehyde, a

marker of lipid oxidation, is increased (Dexter et al., 1989; Zhou et al., 2008). Several studies have suggested the occurrence of genetic factors related to PD risk, but so far no major genetic risk factors have been identified, and it is widely accepted that PD is likely to be related to genetic plus environmental interaction (Schrag and Schott, 2006; Obeso et al., 2010; Trinh and Farrer, 2013; Alonso-Navarro et al., 2014) and that further genetic association studies based on solid functional mechanisms related to the pathogenesis of the disease are required.

A relevant feature of the development of PD is abnormal iron deposition in the substantia nigra of PD patients, as has been demonstrated both in post-mortem studies (Sofic et al., 1988) and in *in vivo* studies (Hochstrasser et al., 2004; Berg and Hochstrasser, 2006). Some consequences of elevated nigral iron levels are well known. The intranigral administration of iron produces selective dopaminergic neuronal loss and elicits parkinsonian symptoms (Youdim, 2003). Alteration of iron homeostasis, dysfunction in molecules involved in sequestering excess iron, as well as ageing, are considered as possible causes of elevated iron deposition. BBB protects the brain against circulating iron, however the specific mechanisms involved in the regulation of iron release and transport that lead to iron accumulation in aging and neurological disorders such as PD, are not completely understood. The involvement of dysregulation of brain iron homeostasis in PD has been recently revised (Weinreb et al., 2013). It was suggested that alterations in brain vascularization in certain areas may cause high levels of iron (Faucheux et al., 1999).

Heme oxygenase (HMOX) enzyme activity degrades heme ring to biliverdin, free ferrous iron and carbon monoxide being the rate-limiting activity in heme catabolism (Ryter and Tyrrell, 2000). Two HMOX isoforms have been described in the mammalian central nervous system: HMOX1 is present in neurons and neuroglia (Benjamini and Hochberg, 1995), and it is inducible or repressible by pro-oxidant and other microenvironment stimuli (Kinobe et al., 2006; Loboda et al., 2008). HMOX2 is ubiquitously and constitutively expressed throughout the mammalian neuraxis, predominantly in substantia nigra, septum and hippocampus (Verma et al., 1993; Maines, 1997). A third isoform unique to rats, designated as HMOX3, exists (Scapagnini et al., 2002). The role of single nucleotide polymorphisms (SNPs) in the *HMOX2* gene in patients with PD has been reported, the *HMOX2* SNP rs2270363 being associated with PD risk (Ayuso et al., 2011). In contrast, little is known about the role of genetic variations in the *HMOX1* gene in PD, and controversial findings justify the analysis of this gene in a large sample population (Funke et al., 2009; Infante et al., 2010). *HMOX1* is a plausible candidate gene for PD risk because immunoreactive HMOX1 has been identified in dopaminergic neurons of PD patients (Castellani et al., 1996; Schipper, 1998). In addition, neurotoxins, cytokines and nitric oxide are considered as inducers of astroglial *HMOX1* expression (Rieder et al., 2004; Schipper, 2004). Moreover, under stress conditions in astroglia, HMOX1 promotes mitochondrial sequestration of non-transferrin iron and may contribute to the pathological iron deposition described in PD (Schipper et al., 2009). In this study we analyze common variations and copy number variations in the *HMOX1* gene, selected according to their allelic frequencies and putative functional effect on enzyme activity, in a large sample of patients with PD and healthy controls.

METHODS

Subjects were recruited from several centers in Spain. The study group was composed of 691 PD patients and 766 healthy controls, which were included in the study after obtaining informed consent. From these, 460 patients with PD and 459 control individuals were recruited from the Clínica Universidad de Navarra (Pamplona, Spain). In addition, 231 patients with PD were recruited from the Hospital de La Princesa (Madrid, Spain) and 307 controls were included from the latter hospital and from the Infanta Cristina University Hospital (Badajoz, Spain). Most participants had been included in previous studies by our group (Agundez et al., 2008a; Garcia-Martin et al., 2008; Ayuso et al., 2011; Lorenzo-Betancor et al., 2011; Jimenez-Jimenez et al., 2014). All the participants were Caucasian Spanish individuals as stated in the self-report. All consecutive patients diagnosed by consultant neurologists according to the criteria recommended by Hughes et al. (1992) were requested to participate and all of them agreed to do so. Selection criteria for patients with PD included bradykinesia and at least one of the following symptoms: rigidity, resting tremor, postural instability, a positive response to dopaminergic therapy, and the absence of atypical features or other causes of parkinsonism (Hughes et al., 1992). From the total of patients, only 36 of the 691 patients with PD had a positive family history of the disease and monogenic forms of PD were excluded. Patients were divided in three groups according to different phenotypes: tremor-dominant patients with PD (TD-PD), classical phenotype patients with PD (C-PD), or akinetic-rigid patients with PD (AR-PD) when such information was available (Lorenzo-Betancor et al., 2011). As the classification of motor PD phenotype subgroups can be arbitrary, we considered those individuals with rest tremor as dominant and an initial feature of their disease to have a TD-PD. AR-PD phenotype was considered for those individuals with predominant bradykinesia or signs of rigidity, but mild or no tremor at rest. The patients with comparable severity of tremor, rigidity, and bradykinesia were considered patients with C-PD. A medical examination was performed to identify individuals in good health. The study was approved by the ethics committees of the institutions involved in the study. For all participants, genomic DNA was obtained from peripheral leukocytes and purified according to standard procedures. **Table 1** summarizes the demographic data of the subgroups analyzed in the study.

Four polymorphisms in the *HMOX1* gene, located in chromosome 22q12, were selected for genotyping on the basis of putative functional effects as well as expected allele frequency in Caucasian individuals as reported in public databases (http://www.ncbi.nlm.nih.gov/projects/SNP/snp_ref.cgi?showRare=on&chooseRs=all&go=Go&locusId=3162). These polymorphisms included a variable number tandem repeat (VNTR), consisting of alternating the purine-pyrimidine sequence $(GT)_n$ which has the potential to acquire the Z-DNA conformation, a structure which is thermodynamically unfavorable compared with the B-DNA structure. This formation has been described as a factor that negatively affects transcriptional

Table 1 | Demographic data of the sample analyzed in this study.

Group	Controls (n = 766)	PD				
		Entire PD (n = 691)	TD-PD (n = 30)	C-PD (n = 328)	AR-PD (n = 94)	Unclassifiable PD[a] (n = 239)
Age, y, mean (*SD*)	64.67 (14.28)	67.09 (10.62)	69,51 (8.09)	67.06 (10.68)	66.00 (10.79)	67.32 (11.76)
Age range, y	17–102	22–95	49–87	22–95	39–87	41–88
AAO, y, mean (*SD*)	NA	59.57 (12.61)	62.82 (8.13)	57.55 (11.58)	56.81 (11.59)	63.51 (13.65)
AAO range, y	NA	17–90	46–76	17–84	34–84	18–90
Female %	45.7%	42.6%	43.3%	36.8%	42.5%	50.2%

PD, Parkinson's disease; y, years; AAO, age at onset; SD, standard deviation; NA, not available; TD-PD, tremor-dominant Parkinson's disease; C-PD, classical PD phenotype; AR-PD, Akinetic-rigid PD.

[a] *PD subjects with no information on motor features available.*

activity (Naylor and Clark, 1990; Delic et al., 1991) and that causes differences in transcriptional activity of the promoter of *HMOX1* with different numbers of repeats. For the association study of the *HMOX1* $GT_{(n)}$, the alleles were divided into two classes: class S including short repeat sequences ($n < 25$) and class L including long repeat sequences ($n \geq 25$) (Yamada et al., 2000; Tiroch et al., 2007). The presence of this VNTR polymorphism was analyzed after PCR amplification (forward primer 5′-AGAGCCTGCAGCTTCTCAGA-3′ and reverse primer 5′-CTCTGGCTTCCTAGCAGGG-3′) and quantification of number of repetitions by using GeneGel HyRes Denaturing gels, and DNA staining kit Silver staining kit (GE Healthcare, Madrid, Spain).

Three single nucleotide polymorphisms were studied by means of TaqMan probes (Applied Biosciences Hispania, Alcobendas, Madrid, Spain): The SNP rs2071746 (A/T) is an upstream variant located at 22:35380679, rs2071747 is a missense mutation G/C (Asp/His) located at 22: 35381192, which corresponds to exon 1 of the *HMOX1* gene, and rs9282702 consists of a missense mutation C/T (Pro/Leu) located at 22:35386857, which corresponds to exon 3 of the *HMOX1* gene. Besides these SNPs, no other non-synonymous *HMOX1* SNPs have been reported to occur in Caucasian individuals at frequencies over 1%. Custom primers were designed to analyze the SNPs rs9282702 and rs2071747, whereas commercial primers were used for the detection of the SNPs rs2071746 (C__15869717_10, Applied Biosciences Hispania, Alcobendas, Madrid, Spain). The detection was carried out by qPCR in an Eppendorf Realplex thermocycler by using fluorescent probes. The amplification conditions were as follows: After a denaturation time of 10 min at 96°C, 45 cycles of 92°C 15 s 60°C 90 s were carried out and fluorescence was measured at the end of each cycle and at endpoint. All samples were determined in triplicate and genotypes were assigned both by gene identification software (RealPlex 2.0, Eppendorf) and by analysis of the reference cycle number for each fluorescence curve, calculated by the CalQPlex algorithm (Eppendorf). For technical validation purposes, the amplified fragments for 20 individuals carrying every genotype (AA, AT and TT for rs2071746 and GG, GC and all carriers of the CC genotype for rs2071747 were sequenced, and in all cases the genotypes fully corresponded with those detected with fluorescent probes.

Copy number variations (CNVs) of the *HMOX1* gene were analyzed using the TaqMan copy number assay Hs00774483_cn (Applied Biosciences Hispania), which was designed to hybridize within the open reading frame in *HMOX1* gene exon 3 (chromosome location 22:35782935). Amplification was carried out in an Applied Biosystems 7500 real-time thermocycler as described by the manufacturer, using as a copy number reference assay RNAse P. All reactions were carried out in quadruplicate. Results were analyzed by means of the CopyCaller Software (Applied Biosciences Hispania). According to standard procedures in CNV analyses, we designed as heterozygous (null/present) those samples with a single copy of the *HMOX1* gene.

STATISTICAL ANALYSIS

The Hardy–Weinberg equilibrium was analyzed with the DeFinetti program (http://ihg.gsf.de/cgi-bin/hw/hwa1.pl). Allelic and genotype analyses were performed with the PLINK software (Purcell et al., 2007). Haplotype reconstruction was performed using the program PHASE v2.1.1 (Stephens et al., 2001). This reconstruction was carried out to detect putative association of at risk haplotypes. We used the default model for recombination rate variation with 1000 iterations, 500 burn-in iterations, and a thinning interval of 1. Diplotypes were obtained from the combination of haplotypes in the best run (the one that showed the maximum consistency of results across all runs); further details are provided elsewhere (Agundez et al., 2008b). Statistical analyses were performed using the SPSS 15.0 for Windows (SPSS Inc., Chicago, Illinois, USA). Intergroup comparison values were calculated by using the χ^2 or Fisher tests when appropriate. The 95% confidence intervals were also calculated. The threshold for statistical significance was $p < 0.05$. Correction for multiple testing was done according the false discovery rate (FDR) procedure as described elsewhere (Benjamini and Hochberg, 1995). The statistical power was determined from variant allele frequencies observed in control individuals with a genetic model analyzing the frequency for carriers of the disease gene with a relative risk (RR) value = 1.5 ($p = 0.05$). The statistical power for two-tailed associations for the presence of any of the two SNPs identified in this study was 99.9% for overall patients with PD, 98.3% for patients with C-PD and 95.4% for patients with

an unknown PD phenotype. Testing for heterogeneous association (homogeneity test) was analyzed by using the Breslow–Day test.

RESULTS

At the first stage, because of DNA shortage, we analyzed the occurrence of the four *HMOX1* gene polymorphisms, including the VNTR and the three SNPs described under methods, in 100 randomly selected samples from PD patients and 150 samples from healthy individuals. The SNP rs9282702 was monomorphic. The rest of gene variations analyzed were found to be polymorphic in the population study, and therefore we extended the analyses to all patients and control individuals participating in the study. CNV analyses in the whole study group revealed the occurrence of three patients with PD and six control individuals with a single copy of the *HMOX1* gene. All these individuals were carriers of one null allele in heterozygosity. Individuals with zero or more than two gene copies were not identified in the study group. To our knowledge this is the first description of the occurrence of CNVs in the *HMOX1* gene, but the occurrence of CNVs does not seem to have a major association with PD risk.

The combined genotypes obtained after SNP and CNV analyses are summarized in **Table 2**. No deviation from Hardy–Weinberg equilibrium was found for the SNPs or CNVs analyzed either in the subgroups of patients suffering from PD or in the control group. The risk of PD associated with the SNPs and CNVs was estimated considering the major allele as the risk allele, by comparison of allelic, heterozygous, and homozygous dominant and recessive models. The major alleles for the SNPs rs2071746 and rs2071747 were A and G, respectively, in agreement with the allele frequencies shown in the 1000 genomes catalog for European individuals (http://browser.1000genomes.org/Homo_sapiens/Info/Index). The best fit was observed with the dominant and the allelic models. We identified a statistically significant difference of the rs2071746 genotypes between patients with C-PD and healthy individuals. This difference was observed both in the dominant model and in the allelic model. After correction for the three PD presentations (TD-PD, C-PD, and AR-PD), these associations remained statistically significant in the C-PD group. In addition, a weak statistically significant difference in the frequency of *HMOX1* rs2071746 genotypes between patients with TD-PD and healthy individuals was observed. This difference followed a dominant model, but the difference was not statistically significant in the allelic model (**Table 2**). When the 20 patients with C-PD and positive family history of PD were excluded, the statistical significance of the OR values were similar (not shown). No association with the SNP rs2071747 was identified in any of the PD phenotypes. The SNPs, rs2071746, and rs2071747 did not show linkage disequilibrium ($D' = 0.652$, $r = 0.147$), and no particular risk haplotypes were identified in any of the study groups.

As has been described previously, patients with the phenotypes TP-PD and C-PD may have an earlier age at the onset than the AR-PD group (Rajput et al., 2009), so we analyzed whether *HMOX1* rs2071746 genotype frequencies were related to age of

Table 2 | *HMOX1* genotype and allele frequencies in patients with Parkinson's disease according to phenotypes.

SNP	Patients	Patients subgroups	No.	Genotype frequencies (%)					P (dominant model)	OR (95% CI)	Allele frequencies (%)		P (allelic model)	OR (95%CI)
rs2071746				**A/A**	**A/T**	**T/T**	**Null/A**	**Null/T**			**A**	**T**		
	PD		691	31.2	47.6	20.6	0.4	0	0.019	0.76 (0.61–0.96)	55.1	44.9	0.054	0.87 (0.75–1.00)
		TD-PD	30	44.8	27.6	27.6	0	0	0.010; $Pc = 0.040$	0.39 (0.18–0.81)	60.0	40.0	0.193; $Pc = 0.308$	0.71 (0.42–1.20)
		C-PD	328	35.3	47.9	16.5	0.3	0	0.0009; $Pc = 0.007$	0.62 (0.47–0.83)	59.3	40.7	0.0006; $Pc = 0.005$	0.72 (0.60–0.87)
		AR-PD	94	35.6	49.4	13.6	1.4	0	0.045; $Pc = 0.090$	0.63 (0.40–0.99)	59.6	40.4	0.017; $Pc = 0.045$	0.69 (0.51–0.94)
		Unknown phenotype	239	21.3	49.2	29.5	0	0	0.110; $Pc = 0.176$	1.26 (0.89–1.79)	46.0	54.0	0.039; $Pc = 0.078$	1.24 (1.01–1.53)
	Controls		766	25.4	51.2	22.6	0.3	0.5	Ref	Ref	51.0	49.0	Ref	Ref
rs2071747				**G/G**	**G/C**	**C/C**	**Null/G**	**Null/C**			**G**	**C**		
	PD		691	92.5	6.9	0.2	0.4	0	0.172	0.77 (0.53–1.12)	96.0	4.0	0.163	0.78 (0.54–1.11)
		TD-PD	30	90.0	10.0	0.0	0	0	0.931; $Pc = 0.931$	1.06 (0.31–3.56)	95.0	5.0	1.000; $Pc = 1.000$	1.02 (0.31–3.34)
		C-PD	328	91.5	8.2	0.0	0.3	0	0.603; $Pc = 0.804$	0.89 (0.56–1.40)	95.6	4.4	0.525; $Pc = 0.653$	0.87 (0.56–1.35)
		AR-PD	94	89.3	7.9	1.3	1.5	0	0.731; $Pc = 0.835$	1.13 (0.56–2.27)	93.1	6.9	0.571; $Pc = 0.653$	1.21 0.63–2.32)
		Unknown phenotype	239	95.5	4.5	0.0	0	0	0.016; $Pc = 0.043$	0.46 (0.24–0.88)	97.7	2.3	0.014; $Pc = 0.045$	0.46 (0.24–0.87)
	Controls		766	90.4	8.5	0.3	0.7	0.0	Ref	Ref	94.7	5.3	Ref	Ref

TD-PD, tremor-dominant Parkinson's disease; C-PD, classical PD phenotype; AR-PD, Akinetic-rigid PD; Null, Null allele according to CNV analysis; Ref, reference group; Pc, P-value after correction for multiple comparisons (FDR, eight comparisons; two SNPs and four clinical groups).

onset in the C-PD subgroup. Patients with C-PD phenotype were subdivided according to the median age at onset in this subgroup (62 years). **Table 3** summarizes these results. We observed statistically significant differences among patients with early onset of C-PD both, in the dominant model and in the allelic model, whereas such significant differences were not observed among patients with late-onset C-PD.

Regarding the HMOX-1 $GT_{(n)}$ polymorphism, we studied a subset of the total of patients and healthy subjects included in this study because of DNA shortage. Thus, 365 PD patients and 371 healthy subjects were genotyped. The number of repeats ranged from 11 to 33, with the highest frequency for $GT_{(20)}$ repeats in healthy subjects and $GT_{(22)}$ repeats in the case of PD patients. Mann–Whitney comparison for means in each group revealed statistically significant differences ($p < 0.001$). The association study of this polymorphism with PD patients is shown **Table 4**.

DISCUSSION

BBB permeability is increased in the presence of reactive oxygen species, and it is widely accepted that the *HMOX1* mRNA upregulation in CNS is a marker of oxidative stress. The enhanced expression of *HMOX1* may contribute to the pathological deposition of iron which has been reported in normal aging CNS, and to a much greater extent in neurodegenerative disorders such as PD (Droge, 2002). Impaired HMOX1 activity caused by genetic variants could modify the intraneuronal production of biliverdin/bilirubin metabolites which exert an antioxidant role (Dore et al., 1999) contributing to the etiology of PD and other central nervous system disorders. Our group reported that functional variations in *HMOX2* gene are associated with PD risk (Ayuso et al., 2011). In this context and bearing in mind that HMOX1 has been postulated as a contributor to the pathological iron deposition described in PD (Schipper et al., 2009), we aimed to analyzed the possible role of *HMOX1* genetic variants in a large series of PD patients.

We identified a clinical association of the *HMOX1* rs2071746 polymorphism (A/T) with C-PD. The rs2071746 A allele was more common among PD patients with C-PD phenotype than in control individuals. Also, we observed that this association was related with an early onset of the disease. In addition, the analysis of the length of the highly polymorphic $(GT)_n$ dinucleotide repeats indicates an association of longer $(GT)_n$ repeats with susceptibility to PD. Both gene variations, rs2071746 and $(GT)_n$ dinucleotide repeats sequence, have been related to variations in the transcriptional activity of the *HMOX1* gene (Yamada et al., 2000; Tanaka et al., 2011; Kramer et al., 2013). According published evidence, the number of $(GT)_n$ repeats inversely correlates with *HMOX1* promoter activity and inducibility of the gene (Yamada et al., 2000), although controversy exist (Tanaka et al., 2011). It has been shown that the rs2071746 A allele is associated with high promoter activity (Ono et al., 2004). Our findings support an increased frequency of carriers of long ($\geq$25 $(GT)_n$) among PD patients and an increased frequency of carriers of the rs2071746 A allele among PD patients. Although these findings apparently point to opposite directions in terms of gene activity, the functional effect of these polymorphisms is not fully

Table 3 | *HMOX1* rs2071746 genotypes in C-PD subjects stratified according to age of onset.

SNP	Patients	Patients subgroups	No.	Genotype frequencies (%)					P (dominant model)	OR (95% CI)	Allele frequencies (%)		P (allelic model)	OR (95%CI)
rs2071746				A/A	A/T	T/T	Null/A	Null/T			A	T		
	C-PD	Onset < 62 years	187	37.4	46.5	15.5	0.5	0.0	0.0011; *Pc* = 0.0022	0.57 (0.41–0.80)	60.9	39.0	0.0009; *Pc* = 0.0018	0.68 (0.54–0.85)
	C-PD	Onset ≥ 62 years	138	33.3	47.8	18.8	0.0	0.0	0.056; *Pc* =0.056	0.68 (0.46–1.01)	57.2	42.8	0.075; *Pc* = 0.075	0.79 (0.61–1.03)
	Controls		766	25.4	51.2	22.6	0.3	0.5	Ref	Ref	51.0	49.0	Ref	Ref

Null, Null allele according to CNV analysis; Ref, reference group; Pc, P-value after correction for multiple comparisons (FDR, two comparisons; one SNP and two age groups).

Table 4 | Genotype and allele frequencies for $(GT)_n$ repeat polymorphisms in PD patients and healthy subjects.

Patients	Genotype frequencies (%)			P (dominant model)	OR (95% CI)	Allele frequencies (%)		P (allelic model)	OR (95%CI)
	S/S	S/L	L/L			S	L		
PD ($N = 365$)	86.3	12.3	1.4	0.0001	0.34 (0.20–0.59)	0.92	0.08	0.0001	0.37 (0.23–0.62)
Controls ($N = 371$)	94.9	4.3	0.8			0.97	0.03	Ref	

S, short repeat sequences ($n < 25$ (GT)n); L long repeats sequences ($n \geq 25$ (GT)n).

understood. Moreover, an extended model of the *HMOX1* gene along with the importance of alternative splicing in the area where these two polymorphisms are located, and its association with translational regulation has been disclosed recently (Kramer et al., 2013).

Our results agree with those by Kimpara et al. (1997) although other studies that failed to confirm these findings have been published (Funke et al., 2009). Putative discrepancy in the genetic associations may be explained because of the different ethnic origin of the participants included in each of these studies. Infante et al. (2010) reported association of the rs2071746 T allele in homozygosity as a risk factor for PD, but only when combined with another SNPs at the GSK3beta gene, and in a relatively small study group. In the study by Infante et al. however the rs2071746 T allele frequency is almost identical in patients and controls (39 and 37%). In fact, when the comparison of the rs2071746 genotypes or allele frequencies between patients and controls is made, no statistically significant differences are observed (Infante et al., 2010). In our study the association is statistically significant when analyzing genotypes or allele frequencies. Moreover, the large sample size analyzed in the present study and the fact that positive associations remained statistically significant after correction for multiple testing support the observed genetic associations. The effect of the rs2071746 SNP on different PD subgroups seems presenting consistent patterns, though some did not pass significance threshold. In fact, taking into consideration the dominant model (**Table 2**) the findings obtained in TD-PD and C-PD patients show different directions. Nevertheless it should be considered that the subgroup of TD-PD is composed of 30 patients only. These apparent discrepancies, and the lack of statistical significance in some subgroups, may be related to the small sample size of some of these subgroups. In addition, we analyzed for the first time the occurrence of CNVs in *HMOX1*, both in PD patients and in healthy individuals. We found CNVs more frequently in AR-PD patients. Although this study does not support a major role of *HMOX1* CNVs in PD risk, the demonstration of the occurrence of CNVs in the *HMOX1* gene supports further research in the functional role and clinical associations of *HMOX1* CNVs. It is of note, however, that the association cannot be observed in the subgroup of PD patients with unknown phenotype. Our findings suggest that the association of *HMOX1* genotypes and PD risk is specific of the C-PD phenotype. This reinforces the need of detailed phenotyping and categorization of patients in genetic association studies, particularly when assessing low-penetrance factors. In summary, in this study we identified two functional *HMOX1* gene variations as potential genetic biomarkers of PD. It is conceivable that these genetic biomarkers would be associated to other disorders related to oxidative stress in the CNS and in the BBB. This study adds to current knowledge of genetic biomarkers in PD (Alonso-Navarro et al., 2014) and other CNS disorders (Agundez et al., 2013) and, once these biomarkers are validated, these could be used to identify at risk individuals and to provide proof of concept regarding the mechanisms involved in the development of these diseases. Further research would be needed to analyze *HMOX1* genetic variations in an independent population to verify these associations and to analyze putative interethnic variability in the observed genetic association.

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Gliomas and the vascular fragility of the blood brain barrier

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Astrocytes, members of the glial family, interact through the exchange of soluble factors or by directly contacting neurons and other brain cells, such as microglia and endothelial cells. Astrocytic projections interact with vessels and act as additional elements of the Blood Brain Barrier (BBB). By mechanisms not fully understood, astrocytes can undergo oncogenic transformation and give rise to gliomas. The tumors take advantage of the BBB to ensure survival and continuous growth. A glioma can develop into a very aggressive tumor, the glioblastoma (GBM), characterized by a highly heterogeneous cell population (including tumor stem cells), extensive proliferation and migration. Nevertheless, gliomas can also give rise to slow growing tumors and in both cases, the afflux of blood, via BBB is crucial. Glioma cells migrate to different regions of the brain guided by the extension of blood vessels, colonizing the healthy adjacent tissue. In the clinical context, GBM can lead to tumor-derived seizures, which represent a challenge to patients and clinicians, since drugs used for its treatment must be able to cross the BBB. Uncontrolled and fast growth also leads to the disruption of the chimeric and fragile vessels in the tumor mass resulting in peritumoral edema. Although hormonal therapy is currently used to control the edema, it is not always efficient. In this review we comment the points cited above, considering the importance of the BBB and the concerns that arise when this barrier is affected.

Keywords: glioblastoma, blood-brain barrier, miRNA, exosomes, neural stem cells, tumor-related epileptic seizures, brain tumor related edema

THE BLOOD-BRAIN BARRIER

It has been one hundred years since Edwin E. Goldmann discovered the blood-brain barrier (BBB). Using trypan-blue dye intravenous injections in several animals, he observed that it spread throughout the body, except for the brain and spinal cord, which remained unstained. When trypan blue was injected into the subarachnoid space at the lumbar level, or in the cisterna magna in young rabbits, the Central Nervous System (CNS), including the choroid plexus (CP), were stained (Goldmann, 1909; Bentivoglio and Kristensson, 2014), confirming the presence of a previously unknown barrier between the blood and CNS structures.

CHOROID PLEXUS

The CP is a structure present in the CNS that also functions as a tissue barrier. It is responsible for the synthesis of the cerebrospinal fluid (CSF) and its major proteins, metabolites and a diversity of other molecules. Anatomically, the CP is a group of thin membranes located inside the lateral, third and fourth ventricles. It is basically composed of a monolayer of specialized epithelial cells derived from the ependyma that cover the ventricles walls. This monolayer coats a highly perfused stroma containing permeable fenestrated blood vessels, fibroblasts, dendritic cells and macrophages (Redzic et al., 2005). Its degree of vascularization exceeds in 10 times those of the brain parenchyma (Keep and Jones, 1990).

One of the best-described CP-secreted molecules is insulin-growth factor 2 (IGF2), which promotes neuronal proliferation and survival in the developing mouse cortex (Lehtinen et al., 2011). The presence of tight junctions along the basolateral membrane limits the exchange between the CSF and the CP connective tissue, preventing the passage of blood-derived cells and proteins (Vorbrodt and Dobrogowska, 2003). Tight junctions, combined with the expression of several transmembrane transporters, make CP cells the effectors of the blood-CP-CSF barrier (Spector, 2010). The CP is also responsible for the synthesis and generation of nearly two-thirds of the CSF total volume via the secretion of water, ions and macromolecules

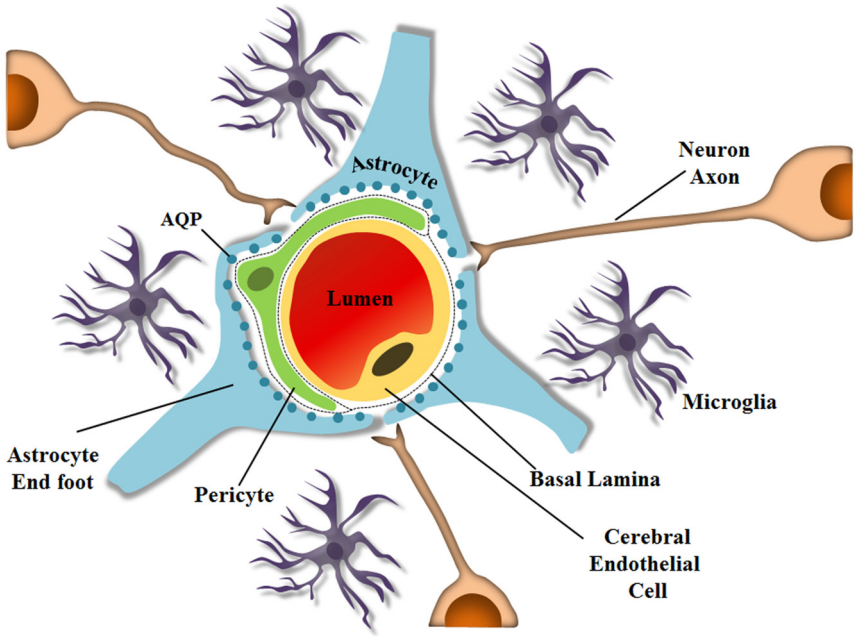

FIGURE 1 | Schematic representation of the neurovascular unit at the capillary level. The BBB is composed of several cell types and ECM molecules in close association. Highly specialized and polarized endothelial cells, basal lamina, pericytes, and astrocyte endfeet, which by wrapping the microvessel walls, establish communication with neurons in the neurovascular unit. The neurovascular unit is important to maintain optimal brain function. Pericytes and astrocytes are important in barrier induction and maintenance. Microglia are CNS-resident immune cells. (Diagram by de Spohr, TCLS). Adapted from Abbott (2013).

(Johanson et al., 2008). Aquaporins (AQPs) play a pivotal role in controlling water circulation, particularly AQP-1, which is present in the membrane of the CP cells (Benga et al., 1986).

ASTROCYTES

Astrocytes also express AQPs, especially on their endfeet, which are closely associated with endothelial cells that are also part of the BBB. Astrocytes contribute to the selection and exchange of molecules through the barrier, and although a hundred years have passed since the BBB was discovered, the mechanisms by which some drugs or parasites enter the brain still remain unsolved. The BBB is a specialized non-permeable barrier in cerebral microvessels. It consists of endothelial cells united by tight junctions, astrocytic endfeet surrounding blood vessels, pericytes embedded in the vessel basement membranes (BMs), microglia and neurons, all playing essential roles in CNS homeostasis (Abbott et al., 2010; Aryal et al., 2014), as shown schematically in **Figure 1**. Together, the components important for BBB integrity and maintenance, including the extracellular matrix, are known as the neurovascular unit, which forms a highly coordinated system that dynamically regulates the cerebral microvascular permeability (Bicker et al., 2014).

Astrocytes are involved in several other processes important for normal brain development, such as neurogenesis, myelination, synapse formation, neuronal migration, proliferation, differentiation, neuronal signaling, production of neurotrophic factors, formation of scar tissue after neuronal loss, immune activation, inflammation, and BBB integrity (Anton et al., 1997; Pfrieger and Barres, 1997; Gomes et al., 1999; Lim and Alvarez-Buylla, 1999; de Sampaio e Spohr et al., 2002; Rouach et al., 2002; Kielian, 2008; Diniz et al., 2012; Herculano-Houzel, 2014). Astrocytic endfeet protrusions interact with microvascular endothelial cells by covering the capillaries forming the *glia limitans*. A single astrocyte may contact multiple capillaries, and this interaction is required for endothelial cells to acquire properties of the BBB (Hayashi et al., 1997; Abbott, 2002; Abbott et al., 2006; Obermeier et al., 2013). Interestingly, astrocytic coverage of vessel endothelial cells and pericytes is discontinuous at only a few sites, and in these rare situations microglial processes can be observed touching the basal lamina (Lassmann et al., 1991; Mathiisen et al., 2010). Gaps in the endfeet coverage of pericytes occur more frequently, allowing neuropil elements to contact the basal lamina (Mathiisen et al., 2010). These astrocytes also interpose in the contacts between microvessels and neurons, important for coordinating oxygen and glucose transport for neural activity through regulation of local blood flow (Zonta et al., 2003; Iadecola, 2004; Takano et al., 2006; Iadecola and Nedergaard, 2007).

Astrocytes control BBB tightness by secreting soluble factors that influence endothelial cells. Additionally, the presence of numerous astrocyte endfeet close to the BBB facilitates the rapid regulation of BBB permeability, which is important for the innate immune system in the brain (Prat et al., 2001; Gimsa et al., 2013). Astrocytes participate in the formation of the BBB by inducing tight junction formation in endothelial cells, by modulating the expression and polarization of transporters, and by promoting

specialized enzyme systems (DeBault and Cancilla, 1980; Janzer and Raff, 1987; Abbott, 2002; Lee et al., 2003; Haseloff et al., 2005; Abbott et al., 2006). Astrocytes also up regulate the expression of ZO-1 and occludin and modulate the redistribution of CD31, claudin-5 and ZO-1 from a diffuse pattern to the cell borders in endothelial cells (Siddharthan et al., 2007; Colgan et al., 2008; Al Ahmad et al., 2011). The interaction between astrocytes and endothelial cells is a "two-way street", since endothelial cells also stimulate astrocyte growth and differentiation (Mi et al., 2001; Abbott et al., 2006).

Astrocytes induce and maintain barrier properties in endothelial cells postnatally, while during embryonic development these properties are first regulated by interactions with neural progenitors, and then by pericytes, since in many species, astrocyte differentiation occurs close to birth (Daneman et al., 2010). It is possible that astrocyte precursors may determine the fate of cerebral vascular endothelial cells in the immature neural environment by releasing soluble factors, as proposed based on *in vitro* co-culture experiments by Hayashi et al. (1997). Clearly, astrocytes have many features that are important to BBB physiology. The situation in the brain becomes complex when these astrocytes become malignant, as in astrocytoma. Intense neovascularization occurs in the brain around the tumor region, to contribute to tumor growth. This vascularization originates in endothelial cells, but the vessels are also constituted by the tumor cells adapted to this new function.

BBB AND GLIOMA

Gliomas comprise a group of tumors that originate in the brain. They form a special group of neoplasias for which no cure is currently available, and only modest progress has been made in understanding their biology. Their specific denomination derives from the normal stromal cells of the brain—the macroglial cells—with which they share morphological traits and molecular markers, i.e., astrocytes (astrocytomas), oligodendrocytes (oligodendrogliomas) and ependymal cells (ependymomas) (Louis et al., 2007). In adults, gliomas account for 29% of all brain tumors; 80% of malignant primary brain tumors occurring in patients 65–84 years of age (see **Figure 3**; Dolecek et al., 2012). Gliomas also affect children and the most common pediatric histological types are astrocytomas (52%), primitive neuroectodermal tumors (PNETs), medulloblastomas (21%) and high-grade gliomas (19%) (Hemmati et al., 2003). Gliomas are highly heterogeneous, infiltrative and diffuse, with different degrees of invasiveness (Alves et al., 2011b). They can penetrate through the brain, colonizing the entire organ, sending their invasive cells far beyond the principal tumor mass. Despite this considerable invasive ability, gliomas rarely leave the nervous tissue to colonize other organs, remaining confined in the skull, with only little evidence of systemic spread (Louis et al., 2007).

A universally accepted system for the classification of brain tumors was developed by the World Health Organization (WHO). This classification is intended to improve the accuracy of prognosis, and thus the effectiveness of therapeutic management of patients. Gliomas classification has long been based solely on the histopathological features of the tumor tissues, obtained through neurosurgical resections, which are whenever possible, the first line of therapeutic intervention. The diagnostic criteria include the morphology of the tumor cells, tissue architecture, and the immunohistological marker profiles (Louis et al., 2007).

The classification also includes a grading system that distinguishes four different grades of tumors: grades I, II, III and IV for astrocytomas and II or III for oligodendrogliomas and oligoastrocytomas. Lower-grade tumors (grades I and II) tend to be well differentiated and have few cellular anomalies or atypias, but in general, they closely resemble their non-neoplastic cellular counterparts. These tumors contain specific genetic alterations (Riemenschneider and Reifenberger, 2009) and evidence has been provided for the existence of progressive accumulation of additional genetic alterations. This is followed by the evolution of the tumor to higher grades, i.e., higher malignancy and progression rates (Louis, 2006).

Higher-grade tumors (grades III and IV) are anaplastic, showing signs of increased vessel density, cellular atypias, high mitotic activity and elevated cell density. Grade IV astrocytoma, better known under the name of glioblastoma (GBM) multiformis, or simply GBM, is the most common and aggressive form of glioma. Glioblastoma samples are characterized by a very high cell density, numerous atypias, areas of necrosis, and robust neoangiogenesis. Two forms of GBM have been identified: *de novo* GBM, the most frequent, and secondary GBM, which results from the evolution of a preceding low-grade astrocytoma (Louis et al., 2007). Glioblastoma accounts for 54% of all glioma subtypes (Dolecek et al., 2012). They show a highly aggressive course, poor treatment response, are incurable and patients have short-term survival expectancies (Miller and Perry, 2007).

Glioblastoma growth is closely associated with the formation of new vessels and one of the most serious clinical complication is the development of vasogenic brain edema, which dramatically increases the intracranial pressure (ICP) by BBB leakage (Noell et al., 2012). Glioblastomas are the most vascularized tumors in humans (Takano et al., 2010) and malignancy grade is directly related to endothelial proliferation (Daumas-Duport et al., 1988). Microvessel density is a standard indicator for the prognosis of patients with GBM. However, vessel formation is highly defective, resulting in vessels with abnormal morphology and function. Histologycal samples show vessels with variable diameters and permeability, heterogeneous distribution and irregular basal lamina (Dvorak, 2003).

One of the most widely accepted arguments to explain inefficient vessel formation in GBM is the relatively high amount of vascular endothelial growth factor (VEGF) present in the tumor mass (Bergers and Benjamin, 2003). Vascular endothelial growth factor is one of the most studied molecules in angiogenesis and acts mainly as a hypoxia-inducible factor; the areas expressing highest levels of VEGF are the regions of the necrotic core (Shweiki et al., 1992). Generally, VEGF is overexpressed in GBM and is responsible for the crosstalk between the tumor and the endothelial cells in order to promote angiogenesis.

Besides VEGF over expression, altered AQP expression in components of the BBB (astrocytic endfeet) has been correlated

to glioma progression. Aquaporins are a family of integral membrane transport proteins that facilitate water efflux across cell membranes in response to osmotic gradients. So far, 14 AQPs have been characterized in humans and rodents (Papadopoulos and Verkman, 2013). In gliomas, AQP1 is upregulated *in vitro* in response to increased glucose consumption and glycolysis (Hayashi et al., 2007). The expression of AQP8 in human astrocytomas is associated with the pathological grade, being directly related to the aggressiveness of the tumor (Zhu et al., 2013). AQP9, on the other hand, is highly expressed in tumor stem cells, which are found in the tumor mass and are resistant to most available treatments (McCoy and Sontheimer, 2007).

Aquaporins have been described as key regulators of the BBB integrity. The AQP4 arrangement through the membrane contributes to edema resolution and it is believed that the upregulation of AQP4 observed in gliomas *in vivo* is a mechanism of compensation for the loss of the endfeet and the increase in the perivascular space (Noell et al., 2012) observed in gliomas.

In the early stages of glioma development there is no apparent disruption of the BBB; tumor own vasculature has not yet been formed and the tumor mass is sustained by normal brain vessels. As glioma progresses and aggravates, endothelial cells derived from normal vessels are roughly separated from the vessel main structure and form new angiogenic spots associated with the tumor site. As these cells must migrate when forming new vessels, they disrupt normal vessel structure to arrive at the tumor site. Because tumors secrete many different molecules that alter the normal microenvironment, endothelial cell migration is impaired, and this impairment is reflected in the vascular architecture. In GBM, the morphological alterations of blood vessels involve the formation of fenestrations and tight junctions disruption. Besides, the thickness of the basal lamina is altered and perivascular space is increased as well as the number of pericytes associated to the vessels (Hirano and Matsui, 1975; Bertossi et al., 1997).

The disruption of the BBB can be detected through magnetic resonance imaging (MRI), using a contrast medium (CM; Sage and Wilson, 1994). The standard CM used for GBM diagnosis in MRI is gadolinium, which under normal conditions is not able to cross the intact BBB. In the case of GBM, since there is a disruption of the blood brain barrier, gadolinium can diffuse into the tissue and characteristic ring enhancing lesions are often seen.

NEURAL PROGENITOR CELLS CROSS THE BBB AND DECREASE TUMOR SIZE

It is currently accepted that GBMs can also be derived from tumor stem cells. As said before, GBMs are heterogeneous, due to the presence of non-tumor cells such as astrocytes, microglial cells and endothelial cells (Fonseca et al., 2012; Kahn et al., 2012; Lima et al., 2012). Endogenous, non-tumoral, neural stem cells are able to migrate from the subventricular zone (SVZ) toward glial brain tumors, damaged or regenerating tissue, and inflammation sites (Aboody et al., 2000; Tang et al., 2003; Synowitz et al., 2006; Walzlein et al., 2008; Díaz-Coránguez et al., 2013). Through xenotransplantation of GBM cells and experimentally induced tumors, it was shown that the number of neuroprogenitor cells (NPCs) attracted to the tumor bed decreases with age, and that this correlates with rapid tumor growth (Maslov et al., 2004; Synowitz et al., 2006; Walzlein et al., 2008). Neuroprogenitor cells of young and old mice have been shown to exhibit antitumorigenic effects by inducing GBM cell death (Glass et al., 2005; Walzlein et al., 2008). However, fewer NPCs are observed migrating toward tumors of older mice (Walzlein et al., 2008), which are larger than tumors of young mice. Chirasani et al. (2010) showed *in vitro*, that NPC-derived bone morphogenetic protein 7 (Bmp 7) acts as a tumor suppressor, decreasing the proliferation, self-renewal and tumor-initiation ability of GBM stem cells (Chirasani et al., 2010).

Surprisingly, it has also been shown that neural progenitor cells are able to reach the tumor mass when injected systemically. Neural stem cells were shown to transmigrate across the BBB and reach intracranial gliomas (Díaz-Coránguez et al., 2013). Apparently, trans-endothelial migration of NPCs is mediated by CD44 (the same molecule used by activated leucocytes during diapedesis) and can thus be blocked by soluble hyaluronic acid (its ligand) or anti-CD44 blocking antibodies (Rampon et al., 2008). Hepatocyte growth factor (HGF), VEGF, PGE_2 among other factors present in the conditioned medium of C6 glioma cells, were also found to induce NPC transmigration (Díaz-Coránguez et al., 2013). This ability to cross the barrier created by endothelial cells and astrocytes and to reach the brain parenchyma is being exploited as a possible therapeutic approach for many CNS diseases, including GBM.

EXOSOMES, EXTRACELLULAR miRNAs AND THE BLOOD-BRAIN BARRIER

Naturally existing extracellular vesicles have been exploited as carriers for therapeutics to the CNS. Exosomes represent a class of these vesicles, and emerging evidence describes them as vehicles that have the ability to cross the BBB. Exosomes are small cup-shaped extracellular vesicles with diameters ranging from 30 to 100 nm. Exosomes are originated from the endocytic pathway, generated upon fusion of multivesicular endosomes (MVBs) to the plasma membrane and the consequent release of intraluminal vesicles (ILVs) of MVBs into the extracellular environment. At this point, ILVs are referred to as exosomes (Simons and Raposo, 2009; Raposo and Stoorvogel, 2013). The potential of exosomes to cross the BBB and dispatch molecules to the brain was first demonstrated by Alvarez-Erviti et al. (2011), who designed therapeutic exosomes and delivered them into the mouse brain (Alvarez-Erviti et al., 2011). Cells from bone marrow of C57BL/6 mice were harvested and stimulated with interleukins to produce immature dendritic cells, which were engineered to express Lamp2b, an exosomal membrane resident protein, fused with the rabies virus glycoprotein (RVG), which is internalized by brain cells upon specific interaction with the acetylcholine receptor. Purified exosomes released by dendritic cells were loaded with exogenous GAPDH siRNA or BACE 1 siRNA by electroporation, and systemically injected into mice in an allogeneic way. Specific gene knockdown was observed in neurons, microglia and oligodendrocytes, suggesting efficient exosome uptake by these cells. In another report, exosomes were used to entrap anti-inflammatory

drugs, which were then delivered to the mouse brain by intranasal administration (Zhuang et al., 2011). The anti-inflammatory agents used were curcumin, a polyphenol compound that exhibits anti-inflammatory, antineoplastic and antioxidant activity; and JSI-124, a Stat3—Signal transducer and activator for transcripton 3—inhibitor. The drugs were loaded into exosomes and used in three different approaches: an LPS-induced brain inflammation model, experimental autoimmune encephalomyelitis (EAE) disease and mice bearing intracerebral GL26 tumor cells. The loaded exosomes rapidly reached the brain and were taken up by microglial cells. Curcumin exosomes led to induction of apoptosis of inflammatory microglia cells in the LPS-induced brain inflammation model. Exosomal curcumin treatment also delayed and attenuated EAE disease. JSI-124-containing exosomes significantly reduced brain tumor growth in the GL26 tumor model. Taken together, these findings suggest the possibility of using exosomes as drug carriers across biological barriers and as a promising non-immunogenic means to deliver therapeutics to the brain.

As cited above, exosomes could be used as vehicle to selectively deliver therapeutic nucleic acid based or conventional drugs, especially in those tumors that are difficult to access with chemotherapeutics due to the BBB (Ohno et al., 2013; Sun and Liu, 2014). For example, there is a number of *in vitro* and *in vivo* microRNAs (miRNAs) that had been mentioned in the context of altered signaling pathways, whose expression could represent potential therapeutic targets in GBM (Sana et al., 2011). The ability of individual miRNAs to target multiple genes and pathways could be a major advantage in GBM treatment (Purow, 2011). One of the most critical issues though, is how to deliver the agent (a miRNA mimic or inhibitor) to regions protected by the BBB (van Rooij et al., 2008; Gomes-da-Silva et al., 2013; Mizoguchi et al., 2013).

Recently, remarkable attention has been paid to the potential of circulating miRNAs profiling for cancer diagnosis and prognosis (Kosaka et al., 2010; Fujita et al., 2014). A pilot study demonstrated that miR-15b and miR-21 assessed in CSF were good markers for gliomas (Baraniskin et al., 2012). Glioblastoma tumor cells release exosomes containing miRNAs, messenger RNAs and angiogenic proteins (Skog et al., 2008; van der Vos et al., 2011; Yang et al., 2013; **Figure 2A**). microRNAs are molecules involved in gene regulation through a mechanism of inhibition or degradation of mRNAs of several known targets (Zhang et al., 2012). They can be detected in CSF, serum and plasma (Alexandrov et al., 2012; Teplyuk et al., 2012; Ilhan-Mutlu et al., 2013; Jin et al., 2013; **Figure 2**). Extracellular miRNAs are released from cells enclosed within exosomes, microvesicles, apoptotic bodies or in protein-miRNA complexes, such as high-density lipoprotein (HDL) and AGO2, the key effector protein of miRNA-mediated silencing (Valadi et al., 2007; Zernecke et al., 2009; Arroyo et al., 2011; Kosaka and Ochiya, 2011; Vickers et al., 2011). microRNAs detected in CSF of brain-cancer patients may be derived from brain-cancer cells; from surrounding brain tissues; or from extracranial tissue, as a consequence of BBB disruption and cancer treatments (Teplyuk et al., 2012). Glioblastoma derived microvesicles are likely to represent one of the mechanisms by which tumor cells change the brain microenvironment and make it more permissive for growth and invasion. As such one cannot disregard them as a powerful, non-invasive approach for diagnosing tumor progression in cancer patients (Skog et al., 2008).

Despite the large amount of studies mentioning the importance of circulating miRNAs in the diagnosis and prognosis of diseases, the exact mechanism that determines or identifies the circulating miRNAs, and their biological function remains uncertain. Two theories regarding the export and the function of circulating miRNA have been suggested. Some researches defend that circulating miRNA can be key mediators of various cell-cell communication processes, while others support the idea that all types of circulating miRNA in the biological fluids can be merely by products of cellular activity and cell death (Turchinovich et al., 2012; Turchinovich and Cho, 2014). Kirschner et al. (2013) highlighted their concern about the increasing number of reports revealing the clinical potential of using miRNA levels in body fluids as potential biomarkers. According to these authors, the field of cell-free miRNA research is still in its infancy, and it has failed to adopt a set of standardized criteria for reporting the methodology used in the quantification of miRNAs. The delivery of miRNA and the ability to target specific tissues or cells while avoiding nonspecific delivery remains challenging. For this reason, RNA carriers, such as liposomes or exosomes, are under investigation as a possible source of effective delivery strategies approaches (Santos et al., 2010; Ohno et al., 2013; **Figure 2B**). Either way, future research is needed to improve drug delivery to brain tumors (Ningaraj et al., 2007).

CLINICAL PROBLEMS RELATED TO GLIOMA TUMORS

CORTICOIDS AND MORBI-MORTALITY IN GLIOMA PATIENTS

One of the main causes of morbidity and mortality in glioma patients is the induction of severe cerebral edema, which leads to brain herniation in up to 60% of patients with GBM (Silbergeld et al., 1991). Cerebral edema, the abnormal accumulation of water inside the brain parenchyma, is commonly seen in GBM patients (**Figure 3**). Peri-tumoral edematous fluid can accumulate in amounts up to 90 ml per day in severe cases (Ito et al., 1990). Within the rigid skull, rapid augmentation of brain volume leads to a sharp increase in ICP, which can result in decreased cerebral blood flow, ischemia, brain herniation and death (Papadopoulos et al., 2004).

Brain tumor-related edema (BTRE) is caused by two main mechanisms, vasogenic and cytotoxic. While BTRE is classically associated with vasogenic edema, cytotoxic edema is being increasingly implicated in the pathophysiology of peri-tumoral swelling (Ito et al., 1990). Vasogenic edema is caused by the disruption of the BBB, which allows leakage of fluids from the blood into the brain parenchyma (Ryan et al., 2012). It is hypothesized that the disturbance of the BBB is due to two main mechanisms: (1) decreased expression of functioning tight-junctions and disruption of normally expressed tight-junctions; and (2) increased endothelial pinocytosis and endothelial fenestrations (Kröll et al., 2009). Cytotoxic edema, on the other hand, is associated with glioma-induced neuronal cell death and neurodegeneration, leading to further brain swelling and neurological deficits (Savaskan et al., 2008).

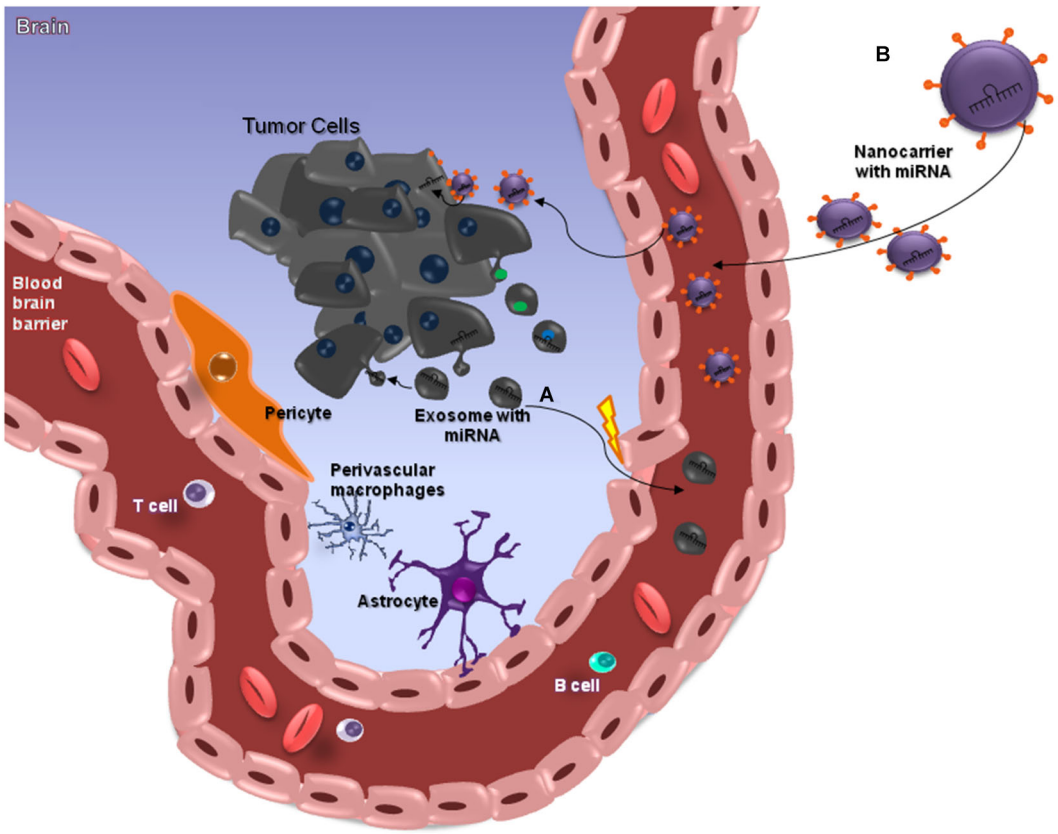

FIGURE 2 | miRNAs and the Blood-Brain Barrier. MicroRNAs (miRNAs) are small (≈22 nt) regulatory and non-codified RNAs that are frequently deregulated in cancer and have shown promise as tissue-based markers for cancer classification and prognosis. They have emerged as key regulators of several pathogenic responses, although their role is largely unknown. The clinical use of siRNA-based therapies is highly promising, although dependent on the development of nanocarriers with appropriate features for systemic administration, since these small RNAs have a unstable structure. Nanocarriers protect the RNA from nuclease degradation and promote effective regulation of target genes, especially in brain tumors such as glioblastomas (GBMs) that are somehow protected from chemotherapeutic drugs by the blood–brain barrier (BBB), which is meant to protect the brain from noxious agents. Several strategies have been employed to deliver drugs across this barrier, and some of these can cause structural damage to the BBB. The ideal method for transporting drugs across the BBB would be controllable and not damage the barrier. Among the various presently available approaches, nanobiotechnology-based delivery methods are the most promising **(A)**. This image attempts to clarify the action of miRNAs in brain-cancer cells, through nanocarriers capable of crossing through the BBB. Exosomes are a class of secreted nanoparticles that have the ability to carry RNA and proteins, and therefore to be very important mediators of intercellular communication. The altered characteristics of exosomes in many diseases, such as cancer, suggests that they could be important for both diagnostic and therapeutic purposes, for instance as drug-delivery vehicles, especially for gene therapy as emphasized in this review **(B)**.

Corticosteroids have been used for more than 50 years in the treatment of BTRE, and have revolutionized the care of brain-tumor patients (Galicich et al., 1961). Approximately 70% of glioma patients receive dexamethasone as an adjuvant treatment, with a significant decrease in deaths (Hempen et al., 2002). In humans, brain imaging studies with MRI and PET have shown decreased peritumor water content and reduced tumor capillary permeability after dexamethasone administration (Jarden et al., 1989; Sinha et al., 2004). However, although corticosteroids have been used for a long time, the mechanisms through which they reduce brain edema are still poorly understood. It has been suggested that corticosteroids reduce BTRE by decreasing the permeability of the tumor capillaries (Hedley-Whyte and Hsu, 1986; Heiss et al., 1996). The capillary permeability-reducing effect is partly explained by upregulation of the tight-junction protein occluding (Gu et al., 2009). Dexamethasone also modulates the expression of VEGF in brain-tumor cells as well as in BBB cells (Kim et al., 2008). *In vitro* experiments have shown reduced levels of VEGF and increased concentration of claudin in response to corticosteroids (Kröll et al., 2009). The corticosteroid-sparing effects of VEGF-inhibitors such as bevacizumab in GBM patients also provide indirect evidence of a VEGF-modulation mechanism of dexamethasone (Kreisl et al., 2009).

Other mechanisms implicated in steroid-reducing BTRE are reduced angiogenesis and diminished cytotoxicity, and the anti-inflammatory effect on reducing cytokine-induced BBB breakdown. Recent evidence has shown that administration of dexamethasone in a glioma-cell culture and an animal model reduced glioma-induced neuronal

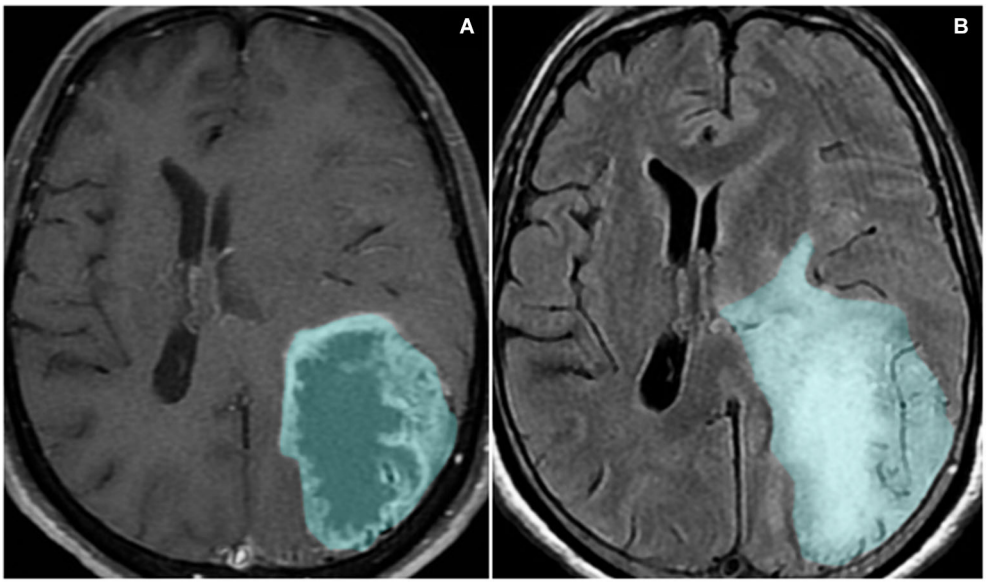

FIGURE 3 | Magnetic resonance imaging sequences T1-weighted with contrast (A) and FLAIR (B) show a left parieto-occipital mass with high signal on FLAIR and a surrounding area of vasogenic edema/tumor infiltration. The lesion presents peripheral heterogeneous contrast enhancement, representing the disruption of the blood-brain barrier.

damage and normalized vessel morphology and vessel density in the peritumoral brain area (Fan et al., 2014). Other studies have shown a 50% reduction in lymphocyte and microglia peritumoral infiltration when animals are treated with corticosteroids (Badie et al., 2000). It is hypothesized that corticosteroid inhibition of NF-κβ causes reduction of edema via inhibition of cytokine-induced barrier breakdown and decreasing the expression of cell adhesion molecules, which mediate T-cell–BBB interactions and excessive leukocyte recruitment across the BBB (Pitzalis et al., 2002).

Given the adverse effects of corticosteroids, such as osteoporosis, myopathy and immunosuppression, corticosteroid-sparing agents are being developed. Corticorelin acetate, a corticotropin-releasing factor (CRF) analog, was studied in a phase I/II trial and seems to be well tolerated. Randomized phase III trials are now envisioned (Villalona-Calero et al., 1998). Not surprisingly, given the prominent role of VEGF in the pathophysiology of BTRE, VEGF antibodies (e.g., bevazicumab) and VEGF receptor inhibitors (e.g., cediranib, sorafenib, and sunitinib) are being studied (Gerstner et al., 2009). Bevazicumab, the best-studied agent so far, has already proven to have anti-edema properties and to have the potential to be a steroid-sparing agent (Vredenburgh et al., 2010). However, because of the increased risk of intra-cerebral hemorrhage and pulmonary thromboembolism, the use of bevazicumab is not yet recommended solely for the purpose of corticosteroid-sparing (Cohen et al., 2009).

Brain tumor-related edema is a life-threatening complication of gliomas, and so far, their treatment has relied on the use of corticosteroids. Steroids have pleiotropic effects on BTRE; however, due to their numerous side effects, corticosteroid-sparing agents are being developed as alternatives for BTRE treatment.

TUMOR-ASSOCIATED EPILEPTIC SEIZURES

Epileptic seizures often occur in brain-tumor patients, with reports of seizure risk of 60–100% among low-grade tumors and 40–60% in GBM (Vecht et al., 2014). It is the presenting symptom in 30–50% of the patients, and 10–30% will continue to have recurrent seizures, which contribute to the morbidity of the disease. Clinically, tumor-related seizures manifest as simple or complex partial seizures, with or without secondary generalization. A recent study evaluating the seizure semiology and short-term developments of primary brain tumors, found that the initial seizures were tonic-clonic in 48% of the patients, focal motor in 26%, complex partial in 10% and somatosensitive in 8%. The majority of cases (60%) had isolated seizures or a low seizure frequency at the onset of the disease, whereas a high seizure frequency or status epilepticus was observed in 18% and 12% of the cases, respectively (Michelucci et al., 2013).

The incidence of seizures differs widely between low- and high-grade tumors, probably with different pathophysiological mechanisms. There is an inverse relationship between the rate of tumor growth and the risk of seizures, which are more prevalent in tumors with a slower growth rate (Schaller and Rüegg, 2003; Chang et al., 2008). Besides the growth rate, the tumor location is another important element for the development of seizures. In general, patients with seizures at the onset of disease have a more-cortical location and are among the patients with a better prognosis.

Studies suggest that specific symptoms of the disease reflect not only the location of the tumor but also its biological behavior,

because patients with low-grade glioma with early seizures in the beginning of the disease and concomitant early control have a higher survival rate than those who develop recurrent seizures (Danfors et al., 2009).

The pathogenesis of tumor-related seizures is complex, multifactorial and still not fully understood. The mechanisms of epileptogenesis differ among tumor types. The intrinsic epileptogenicity of glioneuronal tumors is supported by electrocorticography and surgical and immunocytochemical studies, suggesting the presence of a hyperexcitable neuronal component (Ferrier et al., 2006). The role of specific neuronal populations in epileptic foci was studied by comparing epileptic and nonepileptic cortex removed from patients with low-grade gliomas. Epileptic and nearby (within 1 to 2 cm) nonepileptic temporal lobe neocortex was identified using electrocorticography. Cortical specimens taken from four patients identified as epileptic and nonepileptic were all void of tumor infiltration. Somatostatin- and γ-aminobutyric acid (GABAergic)-immunoreactive neurons were identified and counted. Although there was no significant difference in the overall cell count, the authors found a significant decrease in both somatostatin- and GABAergic-immunoreactive neurons (74% and 51%, respectively) in the epileptic cortex compared to that in the nonepileptic cortex from the same patient. These findings suggest that changes in neuronal subpopulations may have a role in the onset and propagation of epileptiform activity in patients with low-grade gliomas. Gliosis and chronic inflammatory changes in the perilesional regions of these tumors seem also to participate in the generation of seizures. Some other possible mechanisms of epileptogenesis in high-grade gliomas are the perilesional focal ischemia, deafferentation of cortical areas by mass effect, and also increased iron in minor bleeding (Beaumont and Whittle, 2000).

The seizures disrupt the BBB, enhance inflammatory responses, increase glutamate excitotoxicity and cell apoptosis, and substantially increase cognitive and psychological morbidity. Several studies have described variations in neurovascular integrity and disturbances of the BBB with neuronal hypersynchronization and epileptiform activity (Liebner et al., 2000). Molecular changes in brain tumors can affect the BBB structure and functions, including decreased expression of transmembrane junctional proteins. Disruption of the BBB may also lead to abnormal extravasation of plasma protein and other substances, including glutamate, contributing to hyperexcitability and development of seizure focus (Ivens et al., 2007).

The BBB has an important role in the immune response, with elevated production of inflammatory cytokines (Vezzani and Granata, 2005). The supraregulation of adhesion molecules and metalloproteinases contributes to the changes in the BBB permeability.

Because seizures substantially increase morbidity and some patients evolve with medically refractory epilepsy, it is important to understand the mechanism of action and the effect of the drug of choice for treating these patients. The recurrence or worsening of seizures is often associated with tumor recidivism in GBM, and they require additional pharmacological treatment, which can be hard to manage because of the many drug interactions and cumulative side effects.

The current consensus is that all patients with brain tumors presenting with or having seizures should be treated with antiepileptic drugs (AEDs), because of the high risk of recurrence of seizures. The first-line AEDs, including valproate (VPA), phenytoin and carbamazepine, have demonstrated many major side effects and dangerous interactions with chemotherapeutic and antitumor drugs. The most commonly seen side effects with these drugs are cognitive impairment, liver dysfunction, dermatological reactions and bone-marrow suppression. Several studies have found that side effects are more common in patients with brain tumors than in the general epileptic population.

Drug interactions between AEDs and commonly used tumor therapies can lead to inadequate control of the seizures or subtherapeutic treatment of the tumor. Toxic effects have also been noted. Many of the common AEDs, especially carbamazepine, phenytoin and phenobarbital, induce cytochrome P450 enzymes, which accelerate the metabolism, reducing the half-life of corticosteroids and many other chemotherapy drugs that in this context exhibit decreased therapeutic effectiveness. Some chemotherapeutic agents also induce the P450 system, lowering concentrations of the AED.

Among the older AEDs, valproate, in doses of 1–3 g per day, has been claimed to be the drug of choice because of its anticonvulsive efficacy. It has an intravenous formulation, which is very useful in perioperative management and also in cases of status epilepticus, which is seen in up to 26% of these patients, with the overall mortality rate reaching 30–40%. Also, its action as a histone deacetylase inhibitor may reduce proliferation rates and promote differentiation of cancer stem cells, the main mechanism responsible for tumor recidivism in the case of GBM (Alvarez et al., 2014). Valproate may increase the hematological toxicity of the chemotherapy and may independently impair hemostasis, which is of some concern for these patients, who will often require surgical procedures.

Within the past 10 years, several new AEDs including lamotrigine, oxcarbazepine, topiramate, levetiracetam (LEV), lacosamide and zonizamide have emerged that are without clinically important drug interactions. Most of them have shown similarly good efficacy, with better tolerability for oxcarbazepine, lacosamide and lamotrigine. Most data were based on uncontrolled retrospective studies. Of this group, only levetiracetam and lacosamide have intravenous formulations.

Many studies have now proposed levetiracetam as the drug of choice in the treatment and prevention of peri- and postoperative seizures, in daily doses of 1.5–3 or 4 g. Levetiracetam has the advantages of excellent bioavailability (100%, equivalent to the venous formulation), no protein binding, no liver-metabolism or enzyme-inducer effect, and a very good tolerability profile. It has a completely different mechanism of action, binding to the Synaptic Vesicle Protein 2 A, thus inhibiting neurotransmitter release. It also has shown histone deacetylase inhibitor, anti-inflammatory and antioxidant effects. Many recent trials have demonstrated its anticonvulsive efficacy and safety in the control of perioperative (88 to 100% seizure control) and long-term epileptic seizures (91% seizure freedom), as well as in the treatment of status

epilepticus when combined with phenytoin (Claassen et al., 2003) or with phenytoin and pregabalin (trifecta trial with 70% success in halting the status epilepticus; Swisher et al., 2012).

In a retrospective comparative study between valproate and levetiracetam with 282 patients with supratentorial brain tumors (Lee et al., 2013), the two drugs showed comparable efficacy (*ca.* 7% of patients experienced postoperative seizures) and much better tolerability for levetiracetam (long-term complication rates of 26.8% for VPA and 9.8% for LEV). Moreover, 38.5% in the VPA group changed to or added another AED, whereas only 17.6% in the LEV group did so. Another retrospective comparative study (Kerkhof et al., 2013) with 291 GBM patients treated with VPA alone, with LEV alone or with a combination of the two as a second option in the refractory group, also showed a similar efficacy in seizure control (seizure freedom of 78% for VPA and 70% for LEV), although VPA was slightly superior with respect to the overall long-term survival rate, with a 2-month-longer survival. The polytherapy with VPA and LEV combined added 60% seizure freedom in both refractory monotherapy groups.

The clinical management of seizures in this context is complex, requiring experts in this field, measurement of serum levels of the antiepileptic and oncologic drugs, serial blood analysis, monitoring of clinical side effects, and keeping in mind that the recurrence of seizures may represent a tumor recurrence.

CONCLUSION AND PERSPECTIVES

In this review, we have pointed out and discussed the implications of the interactions between normal glia or glioma cells and the BBB. Since its first discovery by Edwin E. Goldman, considerable progress has been made in the understanding of BBB. Although 100 years have passed since the BBB discovery, the mechanisms by which some drugs or parasites enter the brain are still unsolved. Furthermore, the real interactions of the endothelial cells with the extra-endothelial components of the brain microenvironment remain unclear. It is not completely understood how astrocytes endfeet interact with BBB and the astrocytoma derived signals that lead to an intense vasculogenesis into the tumor. Interestingly, GBM and BBB interactions may occur via extracellular proteins. For instance, the imbalance of tenascin and fibronectin in the tumor contributes to vessel formation as we have previously demonstrated (Alves et al., 2011a). Glioblastoma, a dramatic and fatal tumor that grows into the brain, can create a scenario of seizures very difficult to control. The growth of this tumor can also promote disruption of vessels, generating edema, which complicates the health status of the patient. It is clear that novel methods to control GBM growth and BBB disruption are necessary to solve these severe complications.

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