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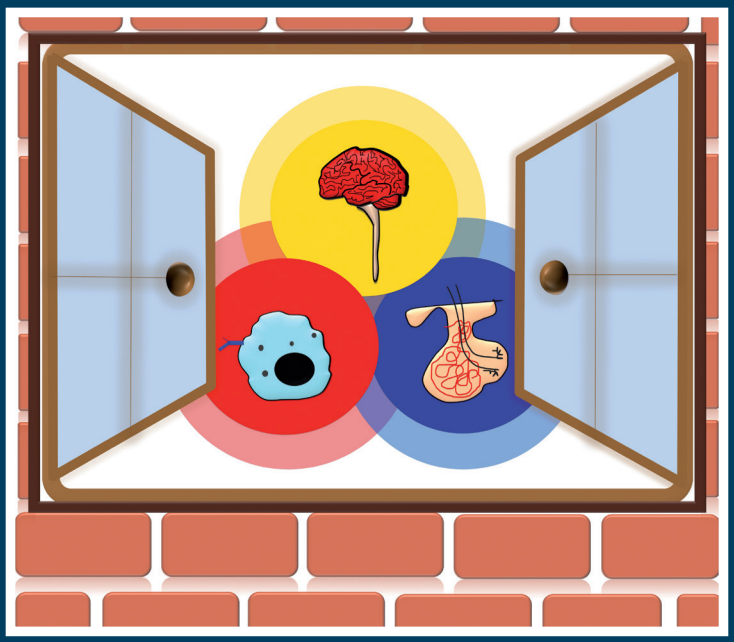

BEYOND THE BORDERS: THE GATES AND FENCES OF NEUROIMMUNE INTERACTION

Topic Editors
Javier Velázquez-Moctezuma,
Emilio Domínguez-Salazar and
Beatriz Gómez-González

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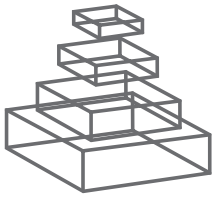

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ISSN 1664-8714
ISBN 978-2-88919-274-8
DOI 10.3389/978-2-88919-274-8

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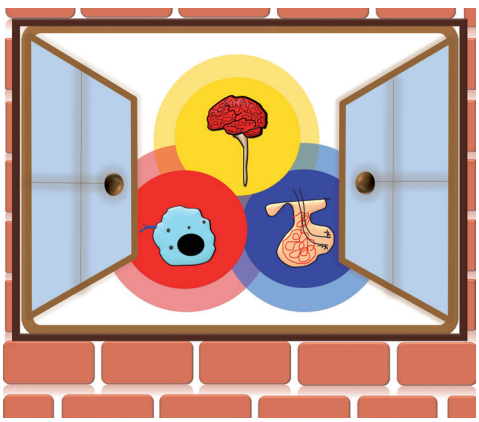

Neuro-immuno-endocrine network

Neuroimmunology is a rapidly growing emerging field at which two old sciences have converged to integrate two different types of responses into a single coherent response involving the coordinated action of both systems, neural and immune. During long time it was thought that both systems worked separately and in divergent pathways. The brain was considered an immunoprivileged site and the immune organs were deemed as independent of any neural influence and also of nervous innervation. Time has gone and has proven that the borders between both systems were merely artificial. Since the beginning of Neuroimmunology in the 1980s much work has been done to elucidate the gates and fences in neuro-immune interactions. Brain was shown to be under the continuous surveillance of the immune system, even under basal physiological conditions in the absence of any pathology. Likely, it was found a profuse nervous innervation of lymphoid organs and even of single immune cells.

Gates for direct neural immune communication were found both centrally and peripherally. Centrally, the gates, but also the fences, were situated at the brain barriers, the blood-brain barrier and the blood-cerebrospinal fluid barrier, and at the circunventricular organs. Peripherally, the fences constituted the apparent diverse nature of molecules involved in neural and immune signaling; however, time proved that both system were capable of producing the same signaling molecules and also systematically responded to the molecules released by the other system. Therefore, the gates were open for direct neural-immune communication at the peripheral level.

This Research Topic aimed to include original reports, reviews and technical reports regarding the description of the gates and fences in neural immune interactions. We intended to provide an extensive view of the mechanisms governing central and peripheral neural-immune interactions, and the role of the borders, the blood-neural barriers, in the regulation of the neural-immune communication.

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frontiers in
INTEGRATIVE NEUROSCIENCE

EDITORIAL
published: 13 March 2014
doi: 10.3389/fnint.2014.00026

Beyond the borders: the gates and fences of neuroimmune interaction

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Edited by:
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Keywords: neuroimmunomodulation, neuroimmunology, neuroendocrinology, brain barriers, neuroimmunoendocrinology

Historically, in most organisms the nervous, immune, and the endocrine systems have been studied as independent components. However, during the last decades, growing evidence supports the notion that these are three parts of a unique system, the neuro-immune-endocrine system (Besedosky and Rey, 2007). Both clinical observations and experimental data obtained from animals reveal a close relationship among the three components of the system. This is the theme of this Research Topic.

The literature contains a large number of reports concerning the relationship between two of the three components: neuro-immune, neuro-endocrine, and immune-endocrine. More recently, the third component of the triad has been added to the study of stress (Baumann and Turpin, 2010) and depression (Hernández et al., 2013). A similar situation has been reported for neuro-immune mechanisms in which an endocrine component is now disclosed, e.g., irritable bowel syndrome (Stasi et al., 2012). Thus, if we consider that the neuro-immune-endocrine system is just one complex regulatory system, then the understanding of the interactions among the three systems can lead us to analyze many pathological states, which have usually been studied as a single disequilibrium of one of these three components, such as rheumathoid arthritis (del Rey et al., 2010), and depression (Hernández et al., 2013).

As part of their independent study, the three systems were characterized as having highly specialized signaling molecules that constituted a fence against mutual interaction; neurotransmitters were described for neural communication, hormones for endocrine communication and cytokines and chemokines for immune signaling. However, as the characterization of both the signaling molecules and the receptor systems progressed, the fences transformed into gates for direct neuro-immune-endocrine communication; receptors for neural derived signals were found in both the endocrine and immune systems. Cytokine production was described inside the central nervous system and the hormones were shown to signal both the neural and immune cells. The only fences left were the barriers precluding direct contact between the cellular and molecular components of the three systems, particularly the brain barriers. Those barriers were shown to have localized fences that allowed selective interaction among the cellular and molecular components of the three systems in a highly regulated manner.

This Research Topic includes original reports, reviews and minireviews regarding the description of the gates and fences in neuro-immune-endocrine interactions. It contains four sections; in the first section three papers describe the gates and fences for neuroimmune interactions directly at the central nervous system. Stolp et al. (2013) describe the changes in neuro-immune interactions through the brain barriers during early development and ageing. Hurtado-Alvarado et al. (2013) present evidence on the role of pericytes, a blood-brain barrier cellular component, in the regulation of the immune response in the brain under both physiological and pathological conditions. Chavarría and Cárdenas (2013) review the influence of neurons and glial cells on the immune response once immune cells have trespassed the brain barriers, molecules promoting an immuno-modulatory environment in the brain are described.

The second section includes two reviews emphasizing the role of hormones on neuro-immune interactions. Quintanar and Guzmán-Soto (2013) describe the role of hypothalamic neurohormones in peripheral immune responses, including the clinical relevance of those hormones. Monasterio et al. (2013) discuss the participation of prolactin and progesterone in the regulation of immune responses in the central nervous system of pregnant and lactating females.

The third section contains three papers that describe interactions between the brain and gut. Montiel-Castro et al. (2013) describe the role of the immune system in the cross-talk between the gut microbiota and the brain, focusing in the description of the regulatory effects of microbiota on brain physiology and behavior. Campos-Rodríguez et al. (2013) present evidence regarding the role of stress hormones on the intestinal immune response, both at the cellular and molecular levels. Garzoni et al. (2013) review the neuro-immune mechanisms mediating the development of antenatal intestinal inflammatory response, with special emphasis on the role of the cholinergic anti-inflammatory pathway in the generation of necrotizing enterocolitis.

Finally, the fourth section includes two papers discussing the alteration in neuro-immuno-endocrine interactions in disease. The paper by Meraz-Ríos et al. (2013) discusses the role of inflammatory signals in the exacerbation of the hallmark pathophysiological changes in Alzheimer's disease. In addition, they also discuss the outcomes of the use of anti-inflammatory drugs and immunotherapy to prevent and/or reduce neuroinflammation in patients suffering Alzheimer's disease. Finally, León-Cabrera et al. (2013) describe the relationship among leptin levels, inflammatory mediators and metabolic changes in the Mexican population, aiming to establish a profile of neuro-immune-endocrine factors ensuing the generation of metabolic syndrome.

All the papers included in this volume are just a sample of the large amount of research that should be done in the forward years

to understand the mechanisms underlying the gates and fences of neuro-immuno-endocrine interactions. As editors, we would like to express our gratitude to all the scientists that collaborated to finally get this volume, both authors and reviewers; the effort and careful work of all of them undoubtedly led to the high academic value of this volume.

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Received: 02 February 2014; accepted: 25 February 2014; published online: 13 March 2014.

Citation: Velázquez-Moctezuma J, Domínguez-Salazar E and Gómez-González B (2014) Beyond the borders: the gates and fences of neuroimmune interaction. Front. Integr. Neurosci. ***8****:26. doi: 10.3389/fnint.2014.00026*

This article was submitted to the journal Frontiers in Integrative Neuroscience.

frontiers in
INTEGRATIVE NEUROSCIENCE

REVIEW ARTICLE
published: 23 August 2013
doi: 10.3389/fnint.2013.00061

Immune responses at brain barriers and implications for brain development and neurological function in later life

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For a long time the brain has been considered an immune-privileged site due to a muted inflammatory response and the presence of protective brain barriers. It is now recognized that neuroinflammation may play an important role in almost all neurological disorders and that the brain barriers may be contributing through either normal immune signaling or disruption of their basic physiological mechanisms. The distinction between normal function and dysfunction at the barriers is difficult to dissect, partly due to a lack of understanding of normal barrier function and partly because of physiological changes that occur as part of normal development and ageing. Brain barriers consist of a number of interacting structural and physiological elements including tight junctions between adjacent barrier cells and an array of influx and efflux transporters. Despite these protective mechanisms, the capacity for immune-surveillance of the brain is maintained, and there is evidence of inflammatory signaling at the brain barriers that may be an important part of the body's response to damage or infection. This signaling system appears to change both with normal ageing, and during disease. Changes may affect diapedesis of immune cells and active molecular transfer, or cause rearrangement of the tight junctions and an increase in passive permeability across barrier interfaces. Here we review the many elements that contribute to brain barrier functions and how they respond to inflammation, particularly during development and aging. The implications of inflammation–induced barrier dysfunction for brain development and subsequent neurological function are also discussed.

Keywords: blood-brain barrier, choroid plexus, cerebrospinal fluid, inflammation, development

INTRODUCTION

It is a long-held belief that the central nervous system (CNS) is an immune-privileged site, due in part to the muted inflammatory response and presence of several protective brain barriers at the CNS-peripheral interface. In contrast to this earlier dogma, it is now evident that the CNS does contain immune capabilities, and that neuroinflammation is likely to play an important role in most, if not all, neurological disorders. In addition, the protective barriers of the brain contribute to these altered functions through either normal immune signaling, or disruption of the basic physiological barrier mechanisms. Recent work has shown that the peripheral immune response contributes to neuroinflammatory conditions (Anthony et al., 2011). This has been particularly well-established in conditions such as multiple sclerosis (and the corresponding animal model—experimental autoimmune encephalomyelitis, EAE) and similar findings have been reported in models of amyotrophic lateral sclerosis, stroke, and epilepsy among others (Campbell et al., 2007b, 2010; Serres et al., 2009; Auvin et al., 2010).

In all these conditions, changes in blood-brain barrier structure and function have been reported. The brain barriers play an important role in maintaining the homeostatic environment of the CNS, and damage to the various structural and functional components of the barrier systems may contribute significantly to disease etiology or progression. What is currently unclear is how (a) the brain barriers themselves contribute to inflammatory signaling in neurological disease? and (b) which specific barrier mechanisms are altered in response to inflammation? Of particular interest to us is the importance of these pathological mechanisms in the developing brain.

In a typical adult inflammatory state, cells mediating the inflammatory response arrive at the site of inflammation or infection and release a large number of mediators that act to control the accumulation and activation of other cell types (both locally and migrating). The key features of CNS inflammation include a range of responses: glial activation, edema, major histocompatibility complex expression, systemic acute phase response (general

Abbreviations: CSF, cerebrospinal fluid; CNS, central nervous system; EAE, experimental autoimmune encephalitis; E, embryonic; Itga6, integrin a6; JAM, junctional adhesion molecule; IL, interleukin; PGP, P-glycoprotein; PKC, protein kinase C; SVZ, subventricular zone; TNF, tumor necrosis factor; VCAM, vascular cell adhesion molecule; VZ, ventricular zone, ZO, zonular occludin.

inflammation and acute phase protein synthesis), complement activation, synthesis of inflammatory mediators (e.g., cytokines, free radicals, prostaglandins) expression of adhesion molecules and the invasion of circulating immune cells (Perry et al., 1995). Due to the protective nature of both the blood-brain and blood-CSF barriers, there are two questions for consideration. Firstly, are mediators of the inflammatory response captured within the CNS space unable to be removed rapidly by the bloodstream? And secondly, does the functional tightness of the barrier impede the entry of immune cells, thereby slowing the immune response? Though the brain can mount its own defense by the activation of resident cells such as astrocytes and microglia (both cell types that are able to produce and secrete and number of cytokines), there is still a major reliance on peripheral immune cells. There is a continued argument about the balance between protection and damage in the CNS that results from a neuroinflammatory response, given its limited regenerative capacity (Aguzzi et al., 2013).

Differentiating the role of inflammatory mediators in pathogenesis is particularly difficult early in development, as a number of immune mediators play an important role in normal brain development. Neuropoietic cytokines contribute to proliferation of neural precursors, fate determination and differentiation, migration of neurons and glia, as well as cell survival and activity-dependent alteration of synaptic function (Stolp, 2013). Inflammation during development therefore, may cause widespread injury to the brain—not only due to the damaging effects of the inflammatory response itself, but also by interfering with the normal balance of cytokine signaling and therefore CNS development.

It is now well-documented that the barrier systems in the brain are well-established during early development and are essential for the normal functioning of the brain (see Saunders et al., 2012 for review). However, other research suggests that the brain barriers may be more susceptible to inflammation-induced changes in the developing brain (Anthony et al., 1997; Stolp et al., 2005a) in turn contributing to the pathology of serious neurodevelopmental disorders such as autism, cerebral palsy and epilepsy (Stolp and Dziegielewska, 2009). Alterations in signaling through barrier systems following inflammatory injury may lead to changes in many elements of brain development—contributing to these serious developmental disorders [reviewed by Stolp (2013)] or they may change the susceptibility of the brain to later onset conditions such as schizophrenia or neurodegenerative disease [reviewed by Stolp and Dziegielewska (2009); Bilbo and Schwarz (2012)].

The aim of this review is to introduce the brain barrier mechanisms and the response and contribution of these barriers to inflammation in the CNS. We shall initially discuss these issues in the context of adult disease, before exploring the developmental barrier systems and their contribution to neurodevelopmental disorders.

BARRIER MECHANISMS IN THE ADULT AND DEVELOPING BRAIN

The brain develops and functions within a well-defined internal environment, which is determined by regulation of interchange between the main compartments of the CNS, brain, cerebrospinal fluid (CSF) and the blood, by a combination of physical and functional mechanisms. These mechanisms, often referred to by the generic term of "blood-brain barrier," are present at three main interfaces in the brain, both in the adult and in the embryo, although there are some important age-related differences between them. These interfaces, illustrated in **Figure 1**, are: (i) the blood-brain barrier proper at the level of the cerebral endothelial cells, (ii) the blood-CSF barrier at the epithelial cells of the choroid plexuses within the four cerebral ventricles and (iii) the pia arachnoid. There is also an additional barrier interface (iv), present only in the early brain development, between the CSF and the brain interstitial fluid. In both the adult and developing brain the essential morphological feature of the blood-brain barrier proper (i) lies in the presence of tight junctions between the cerebral endothelial cells of the vasculature of the brain both within the parenchyma and over the surface in the pia-arachnoid. Compared with other blood vessels there is also a lack of pinocytotic vesicles in the cerebral endothelial cells, although there is some evidence that they may be more frequent in endothelial cells in the developing brain (Dziegielewska et al., 1979). In the choroid plexuses (ii), tight junctions are found between intimately apposed epithelial cells. The tight junctions prevent the intercellular (paracellular) passage of small molecules even in the very early stages of the developing brain (Ek et al., 2003, 2006). An important functional consequence of this is that the presence of tight intercellular junctions enables the cerebral endothelial cells and choroid plexus epithelial cells to have effective one-way transport mechanisms (Liddelow et al., 2009; Ek et al., 2010), which are essential for establishing and maintaining the internal environment of the brain separate from that of the rest of the organism. The morphology of the barrier interface over the surface of the brain (iii) is more complex during early development. Thus, in addition to the adult barrier of tight junctions linking the endothelial cells of blood vessels in the pia arachnoid, there is a wide array of specialized intercellular junctions over the pial surface of the brain, which has been described in the rat embryo (Balslev et al., 1997). From embryonic day 14 (E14), the progressive appearance of distinct junctional structures between the glial end feet was observed. Analysis of albumin distribution at the electron microscopic level suggested that these junctions may contribute to restriction of diffusion between the subarachnoid space and the brain interstitial space. However, at E12 and E14, the intercellular basis for this barrier appeared incomplete, so it was suggested that basement membrane may be an important component of this functionally effective barrier interface (Balslev et al., 1997). At the CSF-brain interface (iv) lining the cerebral ventricles, early in embryonic development, the cells of the neuroependyma (neuroepithelium) are linked by strap junctions (Møllgård et al., 1987), which are an effective limitation to intercellular diffusion at least for large molecules (Fossan et al., 1985). During brain development these strap junctions disappear, and in the adult the cells lining the ventricles (ependymal cells) are linked by gap junctions (Møllgård et al., 1987) that do not provide a significant restraint to diffusion of even large molecules from CSF to brain interstitial fluid (Fossan et al., 1985). Consequently, in the embryonic brain only, there appear to be barrier mechanisms that restrict entry of proteins from CSF into

the brain interstitial fluid. These proteins may be contributing to some aspects of early brain development such as neurogenesis and cellular differentiation in the ventricular zone (VZ), either by uptake of individual proteins or ligands bound to them (see below).

In terms of functional exchanges at the brain barrier interfaces, these have only been studied in any detail at the blood-brain and blood-CSF barriers, although much less so during early development than in the adult brain. Some recent studies have used molecular screening techniques to elucidate the range of genes coding for various proteins involved in transport mechanisms that are expressed at these interfaces during fetal or neonatal stages of brain development. Daneman et al. (2010) used Affymetrix genechip arrays to compare expression patterns in neonatal and adult mouse cerebral endothelial cells, while Liddelow et al. (2012, 2013) used both Affymetrix arrays and high-throughput RNA sequencing to compare gene expression in mice and rats from E15 embryos and adult choroid plexuses. These studies are complimented by those of Kratzer et al. (2012) who used Affymetrix arrays to study gene expression in rat choroid plexus at several ages between E18 and adult, and by Marques et al. (2009) of adult mouse choroid plexus using Illumina whole genome beadchips. Collectively, these studies have revealed expression of an astonishing array of transcripts for proteins known to be associated with tight junctions, transporters (both influx and efflux) and ion channels, as well as numerous enzymes in various metabolic and signal transduction pathways. Many of these genes are expressed at a higher level (in some cases two orders of magnitude higher) in the developing cerebral endothelial and choroid plexus epithelial cells than in the adult. A major problem, however, is determining whether these high levels of expression also reflect a higher level of transport. By comparing published data from *in vivo* transport from blood to brain or CSF, for example for glucose and amino acids in neonatal animals (such experiments in fetuses have so far proved to be a technically intractable problem), it is clear that these high levels of expression are likely to reflect higher levels of transport in the developing brain (Saunders et al., 2013). However, because of the overlap in substrates for different transporters it is not yet possible to be sure that higher expression always equates to greater transport. Some examples comparing expression of individual transporter genes with data on *in vivo* transport of various amino acids are shown in **Table 1**.

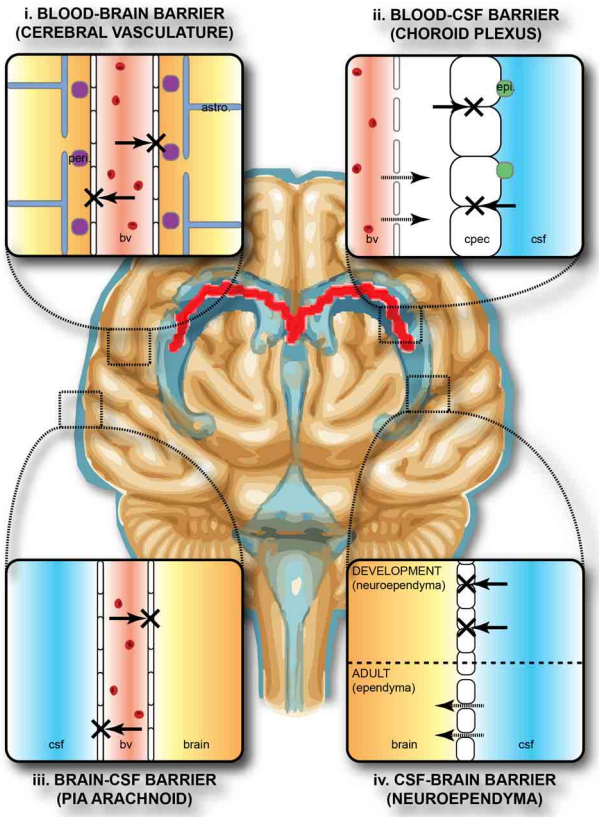

FIGURE 1 | Protective barriers of the brain. The collective term "blood-brain barrier" is used to describe four main interfaces between the central nervous system and the periphery. (i) The blood-brain barrier proper formed by tight junctions between the endothelial cells of the cerebral vasculature. It is thought that pericytes (peri.) are sufficient to induce some barrier characteristics in endothelial cells, while astrocytes (astro.) are able to maintain the integrity of the blood-brain barrier postnatally. (ii) The blood-CSF barrier formed by tight junctions between epithelial cells of the choroid plexus epithelial cells (note the plexus vasculature is fenestrated). Resident epiplexus (epi.) immune cells are present on the CSF-surface of the plexus epithelium. (iii) The outer CSF-brain barrier and the level of the pia arachnoid, formed by tight junctions between endothelial cells of the arachnoid vessels. (iv) The inner CSF-brain barrier, present only in early development, formed by strap junctions between the neuroependymal cells lining the ventricular surfaces. In the adult this barrier is no longer present. Both the blood-brain and CSF-brain barriers extend down the spinal cord. The CSF-filled ventricular system is depicted in blue, while CNS brain tissue is in brown. The lateral ventricular choroid plexuses are shown in red. Abbreviations: astro, astrocyte; bv, blood vessel; cpec, choroid plexus epithelial cell; csf, cerebrospinal fluid; peri, pericytes.

Early in brain development, the choroid plexuses are much more substantial structures compared to the limited level of vascularization of the brain (Saunders et al., 2013). It seems reasonable to propose that the plexuses may be more important than the sparse blood vessels for the supply of nutrients and other essential molecules to the early developing brain [as originally proposed by Klosovskii and Zhukova (1963)]. If this is the case, it is not clear whether the access for these materials or immune cells to the CNS is via diffusion across the CSF-brain interfaces (internal and external) or if there are also transport mechanisms in the cells of these interfaces. Such a mechanism for plasma proteins has recently been proposed for choroid plexus epithelial cells (Liddelow et al., 2012).

So very little is known about the cellular and molecular properties of the CSF-brain interface in the developing brain that is not even clear if the transport mechanisms at this interface function similarly to other barriers. It is known that at some early stages of brain development, at a time when strap junctions are present at the CSF-brain interface (see above), plasma-derived proteins found in the CSF (which are present in a much higher concentration than in adult CSF, Dziegielewska et al., 1988) are taken up by some of the neuroependymal cells lining the ventricular system (**Figure 2**). This phenomenon has

been described for both endogenous proteins (e.g., alpha 2-HS glycoprotein in human fetuses—Møllgård et al., 1988) and exogenously administered non-native proteins (e.g., human albumin injected into the wallaby—Dziegielewska et al., 1988; or rat—Balslev et al., 1997). Examples of plasma protein staining in different animal species are shown in **Figure 2**. It is not known, however, if this is selective with respect to individual proteins (as is seen in the choroid plexus, Liddelow et al., 2009), regions of the ventricular system or stage of brain development. It is also unclear whether the functional significance of this protein uptake lies in the ligands known to be bound to many of these proteins (e.g., growth factors, vitamins) or in some specific properties of the individual proteins themselves. It is also unknown if this internal barrier is able to impede any CNS immune response.

As a result of the complex array of barrier mechanisms (both physical and biochemical) that surround the brain both in the adult and during development, there is great control of both the passive barrier to diffusion, and of the dynamic transport system controlling the internal environment of the CNS. As more evidence is provided for the movement of ions, plasma proteins, drugs and other molecules both into and out of the CNS, there is increasing support for the notion that an interaction with the immune system is additionally one of the many functions of these barrier systems.

Table 1 | Comparison of expression of influx transporters and published reports on transport function in the developing brain.

Transporter	Fold change	Transport function
Slc16a10	66.8	Iodothyronines T3, T4[a]
Slc6a15	11.4	Neutral amino acids[b]
**Slc40a1*	9.6	Iron[c]
Slc7a11	7.1	Cysteine, glutamate[b]
Slc4a1	5.5	Anion transporter[d], (Cl^--HCO_3 exchange)[e]
Slc6a13	4.6	GABA transporter[f]
Slc1a4	4.4	Glutamate, neutral amino acids[g]
Slc38a4	4.2	Acidic and neutral amino acids[b,g]
Slc6a6	4.1	Taurine[b]
Slc4a4	4.1	Na^+-HCO_3^- cotransporter[d]
Slc7a1	4.1	Acidic amino acids[b]
Slc39a8	3.3	Zinc transporter[h]

*Expression levels for the E15 and adult mouse choroid plexus. Fold change in expression compares E15 with adult choroid plexus (positive values are enriched in the embryo). Superscript numbers indicate published studies showing transport into developing brain or CSF. *Gene product ferroportin-1 identified in choroid plexus. References: [a](Porterfield and Hendrich, 1992); [b](Lefauconnier and Trouve, 1983); [c](Morgan and Moos, 2002); [d](Damkier et al., 2010); [e](Amtorp and Sorensen, 1974); [f](Al-Sarraf et al., 1997); [g](Al-Sarraf, 2002); [h](Chowanadisai et al., 2005). Data from Liddelow et al. (2012), adapted from Saunders et al. (2013).*

ADULT RESPONSE TO INFLAMMATION

CELLULAR INFILTRATION INTO THE BRAIN

The CNS is continuously monitored by resident microglia and blood-borne immune cells such as macrophages, dendritic cells and T cells that are able to detect damaging agents that would disrupt homeostasis and optimal functioning of neurons and glia. Normal immune mechanisms in the CNS are often thought of in a manner different from that seen in the periphery—for instance the immune response in the brain can be substantial (e.g., in response to meningitis) but by contrast, a loss of immunity is also reported (e.g., cerebral infections) (for review see Ousman and Kubes, 2012) and the muted inflammatory response in the brain following injury (Andersson et al., 1992) was the original rational behind the concept of the CNS being an immune-privileged site. The developing, and ageing, brain appears to have an exacerbated immune response compared to that normally seen in the adult (Perry et al., 1995; Campbell et al., 2007a). An important example of the interaction of peripheral immune cells with the CNS is the myeloid origin of the innate CNS immune cells—microglia (Aguzzi et al., 2013). Additionally, resident bone marrow-derived perivascular cells inhabit the perivascular space, which directly communicates with the CSF-filled subarachnoid space. These cells are able to respond rapidly to inflammatory injury and

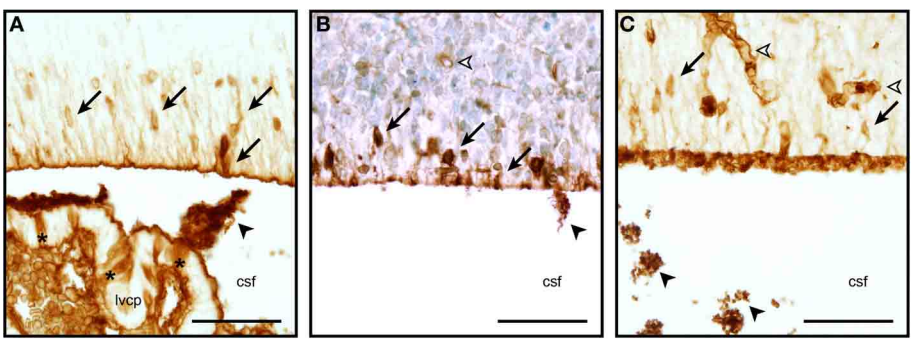

FIGURE 2 | Neuroependymal cell uptake of plasma proteins from the CSF. Coronal, paraffin-embedded section of lateral ventricular wall from sheep fetuses at embryonic day 40 (E40, **A**), E60 **(B)**, and E15 mouse **(C)** stained to detect endogenous plasma protein. The migrating neurons in the ventricular zone are strongly stained (arrows), blood vessels also show a positive staining reaction (unfilled arrowhead). Protein is also seen precipitated in the CSF (filled arrowheads). Extensive precipitation of CSF plasma protein can be seen in the embryonic mouse example displayed in panel **(C)** (arrowheads, also in **A**,**B)**. Choroid plexus epithelial cells individually positive for protein can also be seen in panel **(A)** (asterisks). Abbreviations: csf, cerebrospinal fluid; lvcp, lateral ventricular choroid plexus. Scale bars 50 μm in each.

are continuously replaced by peripheral monocytes (Hickey and Kimura, 1988). It is therefore essential that the brain-barriers are able to facilitate entry of these cells into the CNS as part of normal function. This mechanism for facilitating peripheral immune cell entry to the CNS, however, appears to be up-regulated in cases of neuroinflammation (e.g., during stroke and multiple sclerosis), and is associated with deleterious effects on pathology (Vexler et al., 2006; De Vries et al., 2012). There is, therefore, a fine line between a "helpful" inflammatory response to injury in the CNS and pathological neuroinflammatory state.

There is still controversy in the field whether or not resident perivascular cells are able to remove themselves from the CNS to instigate a peripheral immune reaction following failure of the CNS to mount an adequate immune response (Matyszak and Perry, 1998). It has been demonstrated that CNS-derived antigens can leave the CNS via drainage through lymph nodes—potentially making CNS antigens available to the periphery without the need for the movement of cells across the blood-brain barrier (Harling-Berg et al., 1989). An acute phase response can be initiated in the liver to induced cerebral inflammation, as a direct result of this type of signaling (Anthony et al., 2011). From recent evidence it seems more likely that perivascular cells contribute to multiple sclerosis disease progression (or EAE in animal models) by reactivation of encephalitogenic T-cells (Mcmahon et al., 2005). Multiple sclerosis is characterized by substantial diapedesis of T-cells across the blood-brain barrier, contributing to demyelinating plaques associated with the disease (Engelhardt and Coisne, 2011; Zaguia et al., 2013). It is currently unclear whether this is a normal immune/blood-brain barrier response to myelin antigen presentation (even if the presence of these antigens themselves is abnormal), or whether during diseases like multiple sclerosis they are exacerbated as a result of an abnormal and exaggerated response from the cerebral vasculature. Activated T-cells leave lymph nodes and are likely able to gain additional entry to the CNS across the choroid plexuses into CSF where they are re-stimulated by meningeal and choroid plexus antigen presenting cells (Kolmer's epiplexus macrophage cells) and produce cytokines. The mechanism for the entry of these cells across the choroid plexus is unclear, evidence supporting it has been recently reviewed by Engelhardt and Sorokin (2009). Choroid plexus epithelial cells constitutively express ICAM-1 and VCAM-2, and MADCAM1 (mucosal vascular addressin cell adhesion molecule 1) during inflammation on their apical surfaces. This localization means therefore, that they are not available for the basolateral to apical migration of the immune cells (blood-to-CSF direction; Wolburg et al., 1999). This is counterintuitive, considering the high number of leukocytes frequently observed in the CSF under neuroinflammatory conditions. A recent study has suggested $CCR6^+$ T-cells may use chemokine CC ligand 20 (CCL20) expressed by the choroid plexus epithelial cells to migrate across the CNS (Reboldi et al., 2009) rather than more traditional adhesion molecules, though P-selectin has also been identified at the choroid plexus epithelial barrier (Wolburg et al., 1999). Following entry of activated T-cells into the CNS, subpial vessels are activated and expression of adhesion molecules and chemokines increases, facilitating the process of T cell entry into the CNS. Typically in a disease such as multiple sclerosis, these cells will remain in the abluminal perivascular space unless further activated by interactions with perivascular macrophages and microglia, allowing them to invade the parenchyma (Ransohoff and Engelhardt, 2012). An additional new finding proposes that the choroid plexus epithelial tight junctions lack *Claudin3* (Liddelow et al., 2013), which when knocked out in mice blood-brain barrier endothelial cells causes increased peripheral immune cell diapedesis (Wolburg et al., 2003) suggesting that the choroid plexus has the junctional make-up to allow infiltration of peripherally derived immune cells and monitoring, without a destruction of other barrier mechanisms—important for the maintenance of the CNS internal milieu.

Immune surveillance, in the absence of specific inflammatory signals is therefore likely to occur primarily through the blood-CSF barrier, facilitated by the specific composition of the junctions between epithelial cells. Importantly, junctional rearrangement appears to be an essential element of inflammation-induced cellular recruitment to the brain.

The passage of immune cells through the blood-brain barrier is not a simple process and requires a sequential interaction between different type of molecules on the surfaces of immune cells and endothelial cells. This process has been well described and extensively reviewed (see Banks and Erickson, 2010; Engelhardt and Coisne, 2011; Greenwood et al., 2011). Briefly, adhesion molecules including VCAM-1 on endothelial cells bind to leukocyte integrins, initiating a number of signaling processes that ultimately combine with a number of adhesion molecules allowing a firm attachment between the cell types, and reorganization of the endothelial cytoskeleton to allow diapedisis to occur. This may include changing the interactions between the paracellular tight junctions (Greenwood et al., 2003; Carman and Springer, 2004) facilitating diapedisis via a paracellular route, as well as transcellular route, and potentially causing increased permeability to the solutes in plasma as well as the white blood cells. To facilitate this process, inflammatory stimuli can induce redistribution of junctional adhesion molecule A (JAM-A/*F11r*) away from the cell-to-cell junctions between endothelial cells. Inhibition of this redistribution can reduce the migration of monocytes and neutrophils (Stamatovic et al., 2012), supporting the concept that rearrangement of tight junction proteins facilitates infiltration of leukocytes into the brain via a paracellular route during inflammation. The earlier onset of EAE in *Pecam1* knock-out mice also suggests that weak junctional attachments between cells may sensitize the barrier to injury (Graesser et al., 2002). However, in this instance only increased white blood cell infiltration has been reported rather than increased permeability to solutes (discussed below). Peripheral cytokines stimulate this whole process by causing endothelial cells to increase expression of cell adhesion molecules on their surface, including selectins, ICAM-1, and VCAM-1 (Meager, 1999), in turn causing alterations in the effectiveness of immune cell penetration to the CNS. It has been shown that during the induction of EAE, endothelial cells forming the blood-brain barrier display an increase in the level of CCL2 proteins (also known as monocyte chemoattractant protein 1, MCP-1). CCL2 is the ligand of the activated mononuclear immune cell

receptor, CCR2, effectively increasing the number/rate entry of these peripheral immune cells across the blood-brain barrier (Sagar et al., 2012).

Upon entry to the CNS, immune cells can either set up residence in the glia limitans and monitor the health of CNS cells (as is the case with perivascular cells—see above) or they can immediately move further into the nervous tissue to stage further immune responses. If no inflammatory mediator/antigen is present upon entry to the glia limitans, these cells return to the periphery (Hickey et al., 1991). An equivalent phenomenon has been recognized in systemic vascular beds (Proebstl et al., 2012; Alon and Nourshargh, 2013).

CHANGES IN BARRIER PERMEABILITY

Besides the active role of the brain barriers in immune cell infiltration into the brain, it is thought that inflammation causes pathological changes, which result in increased passive permeability of brain barrier to solutes, contributing to exacerbation of the neuro-inflammatory response. Even in multiple sclerosis, where a focus is normally placed on cellular infiltration, the disruption of the blood-brain barrier tight junctions precedes the formation of sclerotic lesions (Minagar and Alexander, 2003; De Vries et al., 2012). Altered zonular occludin 1 (ZO-1) presence in cerebral microvessels in multiple sclerosis affected brains has been observed in both normal-appearing white matter and inactive lesions, in addition to areas with active lesions (Plumb et al., 2002; Kirk et al., 2003). Altered ZO-1 presence was associated with fibrinogen entry into the brain parenchyma even in the absence of leukocyte recruitment in active lesions (Kirk et al., 2003). Altered vessel distribution of tight junction proteins has also been observed in cases of cerebral amyloid angiopathy (Carrano et al., 2012). No white blood cell infiltration has been reported associated with vessels lacking tight junction proteins, though clumps of microglia in the nearby tissue support the inflammatory nature of these changes. Increased fibrinogen in the brain parenchyma suggests, as described above for multiple sclerosis that the loss or altered distribution of tight junction proteins can be associated with increased permeability to solutes from the blood, without associated cellular infiltration (Carrano et al., 2012). The blood-CSF barrier may also be affected by inflammation in this manner, though this barrier has been less extensively studied (in terms of both solute permeability studies and assessment of tight junction integrity) compared to the blood-brain barrier. In *in vitro* experiments, choroid plexus epithelial cell monolayers showed altered tight junction protein distribution for CLAUDIN-2 and OCCLUDIN when the cells were exposed to retrovirally-activated T-cells (Khuth et al., 2005). This cellular interaction or direct administration of pro-inflammatory cytokines also caused deficits in both active influx transport and efflux systems in the choroid plexus epithelial cells. *In vivo* peripheral inflammation caused by administration of lipopolysaccharide reduced expression of *Claudin3, 5* and *11* in the adult mouse choroid plexus epithelium (Marques et al., 2009). These *in vivo* results are very useful, though it should be noted that other authors (e.g., Liddelow et al., 2013) when using next generation RNA sequencing reported no expression of *Claudin3* or *5* in the choroid plexus epithelial cells.

The mechanism by which inflammation causes this change in tight junction integrity, particularly in the absence of leukocyte infiltration, is not completely understood. A study from Rigor et al. (2012) demonstrated protein kinase C-θ (PKC-θ) mediated barrier dysfunction via IL-1β treatment. The authors report increased PKC-θ activation in an *in vitro* model of the blood-brain barrier, followed by decreased transendothelial electrical resistance. It was proposed that ZO-1 protein phosphorylation and consequent tight junction disorganization might explain the low transendothelial electrical resistance after IL-1β exposure (Rigor et al., 2012). Although suggestive, these *in vitro* results require *in vivo* substantiation of this mechanism. PKC isozymes are capable of phosphorylating a variety of proteins that regulate diverse cellular signaling pathways: some proteins related with cytoskeleton rearrangement, which can affect tight junctions organization, may be activated as shown via TNFα modulated of p115RhoGEF phosphorylation (via PKC-α) and consequently RhoA activation. This activation promotes F-actin rearrangement and increased endothelial cell permeability *in vitro* (Peng et al., 2011), but has not been demonstrated *in vivo*.

In addition to increased permeability to solutes through the paracellular pathway following tight junction disruption, new evidence suggests that up-regulation of pinocytotic activity may contribute to increased solutes of permeability via a trancellular route, with no alteration in tight junction morphology (Armulik et al., 2010). This phenomenon is a well-recognized transport mechanism in the other biological systems and appears to be regulated by inflammation [as described, for example by Chidlow and Sessa (2010)], and needs to be investigated further at the brain barriers.

INFLAMMATION INDUCED CHANGES IN TRANSPORTERS AT THE BLOOD-BRAIN BARRIERS

The endothelial cells of the blood-brain barrier and epithelial cells of the choroid plexus blood-CSF barrier are studded with many influx and efflux transporters (for review see Saunders et al., 2013) Transport across the blood–brain barrier and blood–CSF barrier is directional, with different classes of transporters involved in movement into (e.g., most SLC transporters) and out of (e.g., ABC efflux pumps) the brain. Together, these combined transporters have different effects, ranging from removing solutes from the brain, preventing their entry into the brain (efflux mechanisms), setting up ion gradients or delivering specific nutrients, ions and other required molecules to the brain cells (influx mechanisms). These transporters function normally to maintain the internal homeostatic milieu of the CNS, however, many transporters at the barrier are altered during inflammation and can contribute to the overall inflammatory response (Khuth et al., 2005; Von Wedel-Parlow et al., 2009; Erickson et al., 2012).

Alteration in the expression of barrier transporters is not consistent across diseases or disease models. In multiple sclerosis, endothelial cells display reduced expression of the efflux transporter P-glycoprotein (PGP; Kooij et al., 2010), while in the SOD1 mouse model of amyotrophic lateral sclerosis, PGP is enriched on vessels within neurodegenerative regions (Jablonski et al., 2012). In isolated rat brain capillaries, increases of both PGP activity and protein levels were observed 6 h after TNFα and

endothelin-1 exposure (Bauer et al., 2007), while *in vivo* experiments in adult rats evidence suggests that inflammation after endothelin-1 intrathecal injection increases PGP and BCRP activity only, without changing the protein levels (Harati et al., 2012). In contrast, Parkinson's disease patients present with alterations in one of the active transport processes at the blood-brain barrier that associate with disease etiology. Polymorphisms in the *Abcb1* gene have been recognized in Parkinson's disease patients (Westerlund et al., 2009), and PET studies have shown decreased function of PGP in Parkinson's disease (Kortekaas et al., 2005; Bartels et al., 2008). It is hypothesized that this may contribute to pathology by increasing the cerebral burden of iron, as well as sensitizing the brain to damage following pesticide exposure (Bartels, 2011). Developmentally, there are changes in both gene expression and protein presence of many of these transporters at both the blood-CSF and blood-brain barriers. Ek and colleagues (2010) showed in the rat that PGP (*Abcb1*) expression in both the forebrain and brainstem increased between E13 embryos and adults. MRP1 (*Abcc1*) expression peaked at birth and MRP4 (*Abcc4*) at 1 week postnatally, while BCRP (*Abcg2*) levels remained constant through development. This was in contrast to the choroid plexus epithelium, which displayed a large decrease (20-fold) in *Abcg2* (BCRP) expression, and increases in *Abcc1* (MRP1) and *Abcc4* (MRP4) levels. The authors were able to also show these changes using immunohistochemistry of protein levels, suggesting the transcript was active. More recent studies show that the absolute levels of these transcripts are much higher at the choroid plexus when compared with the blood vessel endothelium (Daneman et al., 2010; Liddelow et al., 2012, 2013).

No study to date has investigated the alterations in transcript or protein following inflammation in developing brain barriers. Increased efflux activity following inflammation, for example by up-regulation of ABC transporters may be an important protective mechanism. However, increased expression does not necessarily equate to increased function. Thus, these data highlight the importance of not only looking at gene expression and protein levels, but also transporter activity to try to unveil the contribution of efflux pumps to inflammation pathophysiology. Therefore, it is important to keep in mind the different steps in the inflammatory response and the different inflammation scenarios that can modulate how brain barriers may contribute in protective or harmful ways. Thus, due to the effects of inflammation on various transporters, brain barriers can contribute to changes in the CNS environment independently, beyond the changes produced as an associated effect of the activation of immune cells within the brain.

INFLAMMATORY SIGNALING IN CEREBRAL ENDOTHELIAL AND EPITHELIAL CELLS

In ischemic stroke, compromised endothelial cells produce inflammatory cytokines and chemokines including the interleukins IL-1β, IL-8, and CCL2. These cytokines induce expression of cell adhesion molecules by endothelial cells, facilitating the movement of peripheral immune cells into the CNS (Stanimirovic and Satoh, 2000) and potentially contributing to the initiation of cellular responses to inflammation by microglia and astrocytes within the brain parenchyma.

Daneman et al. (2010) investigated the transcriptome of purified blood-brain barrier endothelial cells in postnatal mice and reported up-regulation of the LPS/IL-1 mediated inhibition of retinoic x receptor (RXR) pathway in brain endothelial cells when compared to lung and liver endothelium. Downstream genes of RXRα were also enriched in cerebral endothelium, while inhibitory molecules of the receptor were reported only in peripheral blood vessels. RXRα nuclear receptor can activate the transcription of numerous molecules and can be inhibited by a kinase cascade initiated by LPS, IL-1, or TNFα signals. These data suggest that the RXR pathway may play an important role in the maintenance of blood-brain barrier immunogenic properties. As outlined above, however, this pathway has not been investigated in CNS inflammation to confirm its role in blood-brain barrier function and dysfunction.

The choroid plexus blood-CSF barrier may also respond to inflammation by producing different inflammatory mediators, activating different pathways. Microarray analysis was able to identify a high number of genes that were up-regulated after peripheral injection of LPS in adult mice (Marques et al., 2009). Chemokines, including *Ccl4, Ccl5, Ccl7*, and *Cxcl1* as well as interleukins, *Il1*β, *Il6,* and *Il15*, cell adhesion molecules and many transporter molecules were enriched. Moreover, genes of the MAPK, NFκB, interferon signaling, and IL-10 pathways were also identified.

There is increasing evidence that the brain barriers are able to mount, at least an initial response to peripheral inflammation—either in reaction to infiltration of inflammatory mediators to the CNS, or due to the effects of infiltration of activated peripheral immune cells, and that this vascular inflammatory response may in itself contribute significantly to neuroinflammatory disease.

DEVELOPMENTAL INFLAMMATORY MEDIATORS AT THE BRAIN BARRIERS

Recent reports on the transcriptome of the blood-brain (Daneman et al., 2010) and blood-CSF (Liddelow et al., 2012, 2013; Kratzer et al., 2013) barriers during early development highlight the alterations in expression levels of a range of transcripts that are involved in the inflammatory response. Combined with studies looking at brain barrier cells following inflammatory insults (Marques et al., 2009) it is apparent that the brain barriers are able to take a more active role in responding to both peripheral and central immune responses than previously believed. Perinatal brain injury frequently complicates preterm birth and can lead to significant long-term morbidity. Cytokines and inflammatory cells are mediators in the common pathways associated with perinatal brain injury induced by a variety of insults, such as hypoxic-ischemic injury, reperfusion injury, toxin-mediated injury, and infection—all of which cause a rapid and sometimes sustained immune response. In addition to movement of peripherally produced inflammatory mediators across the brain barriers, the infiltration of peripheral immune cells can also alter throughout development. The differential expression of *Icam1* (intercellular adhesion molecule 1) is higher at the blood-brain barrier than at the blood-CSF barrier (Daneman et al., 2010; Liddelow et al., 2012; Saunders et al., 2013). The developmental changes in expression of *Icam1* are also different between the two

main brain barriers—with no developmental change in expression in the cerebral vasculature, but a slight increase in expression in the adult choroid plexus epithelium. Another transcript with product likely to be involved in the extravasations of peripheral cells, integrin α6 (*Itga6*) was also expressed at a higher level at the blood-brain barrier and was enriched in the adult when compared to postnatal mice (Daneman et al., 2010). In the choroid plexus *Itga6* transcript, though with lower expression than the cerebral vasculature, was enriched in the embryonic mouse over 7-fold (Liddelow et al., 2013) highlighting the potential developmental difference in the role of the choroid plexus and blood vessels in the contribution to immune surveillance of the brain and the response to inflammation.

The level of peripherally-derived, blood-borne cytokines entering the brain is low, however, it is comparable to other water-soluble molecules that are known to cross the brain barriers to a degree sufficient to affect brain function (e.g., morphine, Banks et al., 1995). There are a large number of transport systems for common inflammatory mediators that are present on both the blood-brain and blood-CSF barriers. IL-1, a pro-inflammatory cytokine, is able to exert a range of effects on the brain, including mediating key host defenses in response to many chronic CNS diseases. The functional family of IL-1 contains the agonists (IL-1α and IL-1β), the receptors (IL-1RT1 and IL-1RT2) and a naturally occurring antagonist molecule (IL-1RN). At the blood-brain barrier, endothelial cells contain measurable levels of IL-1β and IL-1RT2 (the receptor with a higher affinity for IL-1β) while levels of transcript for IL-1α and the type 1 receptor (IL-1RT1) fall below levels of detection (Daneman et al., 2010). Levels of transcript do not appear to change through development, at least in the mouse. In contrast choroid plexus epithelial cell expression of IL-1 members shows the predominant receptor transcript that is detected is IL-1RT1, with over a 10-fold increase in expression between the embryo and the adult (Liddelow et al., 2012).

Similar to IL-1, IL-6 signals through a cell-surface type I cytokine receptor complex. It is made up of the ligand-binding IL-6RA segment (*Il6r*) and the signal-transduction IL-6RB component (*Il6st*). It should be noted that IL-6RB is also a common signal-transducer for other cytokines (e.g., LIF, CNTF, IL-11, among others). *Il6* ligand transcript is low in endothelial cells, however, *Il6r* and *Il6st* are high from very early in development and do not change into adulthood. A similar lack of developmental expression changes was seen in the choroid plexus with low *Il6* and *Il6st* expression in both embryonic and adult mice (Liddelow et al., 2012), however, no expression for *Il6r* was detected in this study. A more recent RNA sequencing study by these authors, however, reports expression of *Il6r* in choroid plexus epithelium (Liddelow et al., 2013), highlighting the importance of validation of microarray genechip experiments to ensure no false positive or negative results.

The levels of transcript for TNFα by barrier cells (both cerebral endothelium and plexus epithelium) are extremely low, suggesting the majority of TNFα in the CNS is provided by local production from other cells types (e.g., microglia—though it is likely they only produce measurable levels of TNFα following injury), or by transport from the periphery. Having said this, following induction of a peripheral inflammatory response, levels of TNFα (as well as IL-1β) transcript in choroid plexus epithelial cells and meningeal endothelium increased (Quan et al., 1999). Knock-out animal models for TNFα receptors *Tnfrsf1a* (TNFR1/p55 receptor) and *Tnfrsf1b* (TNFR2/p75 receptor) have shown a reduction of the ligand penetrating the blood-brain barrier into the spinal cord, but not into the brain of single knock-out animals (Pan and Kastin, 2002). Double knock-out animals of both *Tnfrsf1a* and *Tnfrsf1b* showed a complete abolition of TNFα penetration—suggesting that both receptors are necessary for transporting the ligand into the CNS (Pan and Kastin, 2002). Genechip data from the blood-brain barrier (Daneman et al., 2010) show a high expression of both *Tnfrsf1a* and *Tnfrsf1b*, as well as several other TNFα receptor family members (*Tnfrs11a*, *12a, 19*, and 21) with no change in expression between early postnatal and adult mice. At the blood-CSF barrier, plexus epithelial expression of TNFα receptor family transcripts is low, however, there is embryonic enrichment of *Tnfsf1b* and *Tnfrsf21*.

It therefore appears that while the barrier systems may not produce a vast array of cytokines under resting conditions, both in development and adulthood, they express many receptors for inflammatory mediators and signal amplifiers, indicating the importance of an early vascular response to inflammatory signaling. Barrier cells also appear able to rapidly up-regulate the expression, and likely release, of some cytokines following an inflammatory insult in as little as a few short hours. The capacity of the barrier cells to respond to inflammatory signaling may be an important confounding factor in the developmental response of the brain to inflammation. While it is beyond the scope of this review, it is important to note that the systemic immune response is also changing over this time, and may contribute to the differences observed in the CNS response to inflammation/injury during development.

DEVELOPMENTAL INFLAMMATORY RESPONSE

It is now clear that the vasculature in the developing brain is primed to respond to inflammatory stimuli. Despite this, little work has been done to investigate the blood-brain barrier response to inflammation throughout CNS development. This is presumably partly due to the historical misconception that the blood-brain barrier is functionally immature in the developing brain. However, it has been well-established (as described above) that the structural and functional mechanism that contribute to the blood-brain barrier are present from very early in embryogenesis. Work from the last 10 years also suggests that the response of the blood-brain barrier to inflammation is selective and specific depending on the age at the time of insult and the location of the inflammatory signals (discussed below).

Work from our laboratories has shown that systemic inflammation causes a specific increase in the permeability of the blood-brain barrier in vessels in the periventricular white matter tract in neonatal rats (Stolp et al., 2005a). The reasons for the increased permeability in these blood vessels is not yet clear, though numerous explanations have been presented, including a developmental delay in the maturity of these vessels or a specific susceptibility to increased vascular flow. Regarding potential immaturity of cerebral vessels, work from Virgintino et al. (2004) and Anstrom et al. (2007) have clearly shown variation in the

complexity of tight junction proteins in the microvasculature of the human brain with different developmental ages and brain regions. While the complexity of the tight junctions has been much discussed in the context of brain development, it is not yet clear how well this correlates with barrier permeability (Møllgård et al., 1979). It is suggested that a delayed maturation of the vessel structure in the germinal matrix and periventricular white matter may lead to increased susceptibility of these brain regions to damage during premature birth, hypoxia, or inflammatory insults (Anstrom et al., 2007). This is an appealing hypothesis, which recognizes a maturation process that may be sufficient, for example in the controlled intrauterine environment, for normal function but which could be easily damaged by changes in blood pressure or some other environmental challenge. It has been established, however, that changes in cerebral blood flow are in themselves insufficient to account for damage in these brain regions following hypoxia-ischemia (Mcclure et al., 2008). Additionally, the age specific increase in blood-brain barrier permeability reported by Stolp et al. (2005a) is not easily explained if the complexity of the tight junction structure is the only contributing factor in the vascular response to insult. When a marsupial species was used to repeat experiments studying the age-specific response to inflammation, so that a longer developmental period could be assessed in a postnatal systemic inflammation paradigm, it was determined that the increased permeability of the periventricular white matter vessels was limited to a specific stage of development, rather than a general response of the developing brain (Stolp et al., 2005a). There are two potential explanations for this: the first that the inflammatory response at the earliest times is not sufficiently developed to stimulate the signaling pathway responsible for the increased permeability; or secondly, that there is a specific combination of factors that occur at the equivalent of the first post-natal week in the rat which combine to produce the susceptibility of the barrier in these specific vessels. There is certainly a substantial increase in the number of activated and migrating microglia and astrocytes in the white matter at this stage of development (Stolp et al., 2009; Verney et al., 2010, 2012), which may contribute to the central inflammatory response and increase the sensitivity of the nearby vessels to the inflammatory signals. The transcriptome of astrocytes activated following peripheral LPS inflammation in adults show a marked increase in the expression of many receptors to cytokines such as TNFα and TGFβ, however, there is not the same increase in the expression of the ligands themselves (Zamanian et al., 2012). There is, however, a relatively high expression of the lipocalin 2 receptor, *Slc22a17*, which is not present on choroid plexus epithelium (Liddelow et al., 2013), but is on cerebral endothelium (Daneman et al., 2010) in close association with astrocytic endfeet. Lipocalin 2 is involved in the innate immune response by sequestrating iron, in turn limiting bacterial growth (Yang et al., 2002), and has recently been shown to be the highest enriched transcript in reactive astrocytes (Zamanian et al., 2012), suggesting an astrocytic role in the innate immune system and the acute phase response to infection in the CNS, and therefore a potential for an atypical cerebral inflammatory response when astrocytes are apparently activated by migration during development.

Interestingly, different developmental barrier susceptibility has been identified in response to directly induced intracerebral inflammation. Injection of IL-1β into the striatum of postnatal day 2 (P2), P21, and adult rats produced a substantial difference in the inflammatory response (Anthony et al., 1997). A small increase in neutrophil accumulation was observed at P2 and in adult animals and a small increase in permeability of vessels to horseradish peroxidase within the injection site, as well as in the meningeal vessels. However, in P21 animals there was a significant increase in permeability of all the vessels in the injected hemisphere associated with a substantial increase in neutrophil extravasation into the brain. Subsequent experiments showed that the changes in permeability were neutrophil dependent, as neutrophil depletion by x-irradiation of the bone marrow prevented this response (Anthony et al., 1997). A neutrophil specific alteration in blood-brain barrier permeability has also been described in a model of stroke (Fernandez-Lopez et al., 2012). However, in this case the early postnatal brain appeared to be protected against altered blood-brain barrier permeability and neutrophil infiltration, compared to the adult. While various small changes in vascular structure (e.g., high basal levels of basement membrane proteins) and activation processes (variable adhesion molecule expression following stroke) were recognized, Fernandez-Lopez and colleagues (2012) determined that the reduced response in neonates was not due to a lack of capacity for neutrophil migration in early development, but instead may be due to altered ratios of chemoattractant molecules between the systemic and central systems. This highlights important differences between models of developmental brain injuries and the etiological mechanisms involved. There is a clear need for further research in this area to tease apart specific signaling systems. Particularly given the completely different response to that seen in the neonatal rat following systemic inflammation, where no neutrophil infiltration has been reported in relation to an age and location specific change in barrier permeability (Stolp et al., 2005a).

The observed developmental differences in the CNS response to inflammation are likely to reflect a combination of many aspects of brain development as well as maturation of the systemic inflammatory response. Specific studies are still lacking on the interactions between these two systems in development, as has been done in adult neuroinflammatory disease (see Anthony et al., 2011).

CONSEQUENCES FOR DISEASE AND AGEING

The consequence of the inflammatory signaling process and the potential association of changes in blood-brain barrier permeability may be widespread in the developing brain. The specific changes within the developing brain appear to vary depending on the timing of insult and reflect a mixture of the developmental stage of the CNS, as well as the specialities of the immune signaling response of the barrier systems at the time of insult.

In the second half of gestation in the rodent, equivalent to the 1st—2nd trimester in humans (Clancy et al., 2001), there is no evidence of blood-brain barrier disruption associated with experimentally induced inflammation. However, there is substantial evidence for changes to the developing brain, which reflect changes in immune signaling. There is a reported decrease

in (VZ) proliferation, but not the subventricular zone (SVZ), in response to low dose LPS-induced inflammation in mice at E13.5 of gestation (Stolp et al., 2011). The change in proliferation in the VZ but not the SVZ implies a variable contribution of the vasculature and the CSF for central immune signaling following induction of the systemic maternal inflammatory response (discussed further below), and indicate that the progenitor cells in the VZ and SVZ are in different environmental niches (**Figure 3**). Additional studies confirm the sensitivity of the VZ cell population to immune signaling, indicating presence of receptors to specific cytokines (e.g., IL-1β) or pathogen associated molecules (including TLR2 and 3) and stimulation of these receptors decrease neurogenesis and may alter cellular differentiation (Lathia et al., 2008; Okun et al., 2010; Crampton et al., 2012).

The change in permeability of the ventricular surface in concert with the decreased proliferation in the VZ (Stolp et al., 2011) but an absence of altered permeability at the blood-brain barrier supports the idea of a CSF-brain specific signaling mechanism regulating the proliferation of cells in the VZ in early development. Recent work by Lehtinen et al. (2011) shows that insulin-like growth factor 1 produced by the choroid plexus in late gestation in the mouse modulates proliferation of the VZ progenitors. Additionally, a selective fourth ventricle OTX-induced choroid plexus deletion, which significantly changes the composition of the CSF, also modifies proliferation in the cortical VZ throughout gestation (Johansson et al., 2013). Cunningham et al. (2013) hypothesize that microglia in the developing brain may be integral to the modulation of proliferation in the progenitor zones of the developing brain, however, their observations are true for both the VZ and the SVZ and may reflect an additional level of control, separate to CSF-specific inflammatory signaling pathways. Substantial changes in the number of F4/80 positive monocytes/microglia within the developing brain were not observed in a study of low-dose maternal immune regulation (Stolp HB, unpublished data). The presence of strap junctions between the neuroependymal cells in early fetal development but not in the adult (Møllgård et al., 1987), suggest a developmentally important role of junctions between the progenitor cells in the VZ. It has been suggested that junctions between these cells are important for regulation of polarity and therefore proliferation in the VZ (Huttner and Brand, 1997). It is possible that these junctions are modified in response to inflammation in a similar manner to that described for adult barrier junctions. Although the presence of strap junctions forming the inner CSF-brain barrier is only present early in development, there is still specific uptake of proteins by these neuroependymal VZ cells (**Figure 2**)—reiterating the importance of protein-cargo trafficking into the CSF and thence the brain during development (Knott et al., 1997; Liddelow et al., 2012).

A different response is seen slightly later in the process of brain development. In early postnatal rodents [approximately P1–7, equivalent to the 2nd—3rd trimester of human pregnancy, (Clancy et al., 2001)] increased permeability of the blood-brain barrier is observed specifically in the periventricular white matter and associated with damage (Stolp et al., 2005a,b, 2009). It is currently unclear how much the damage in this area of the brain is directly related to increased barrier permeability or other associated phenomenon. Large quantities of plasma proteins in the brain, as occurs with blood-brain barrier breakdown, have been associated with increased cell death (Nordborg et al., 1991; Wagner et al., 2002) and altered neuronal function, potentially leading to epileptic-type activity (Friedman, 2011; Tomkins et al., 2011). It is suggested, however, that changes in blood-brain barrier permeability associated with systemic inflammation in postnatal animals is not enough to account for the white matter damage alone, as it requires increased microglial activation (Stolp et al., 2009). Increased numbers of microglia, particularly with the morphological appearance of activation, have been

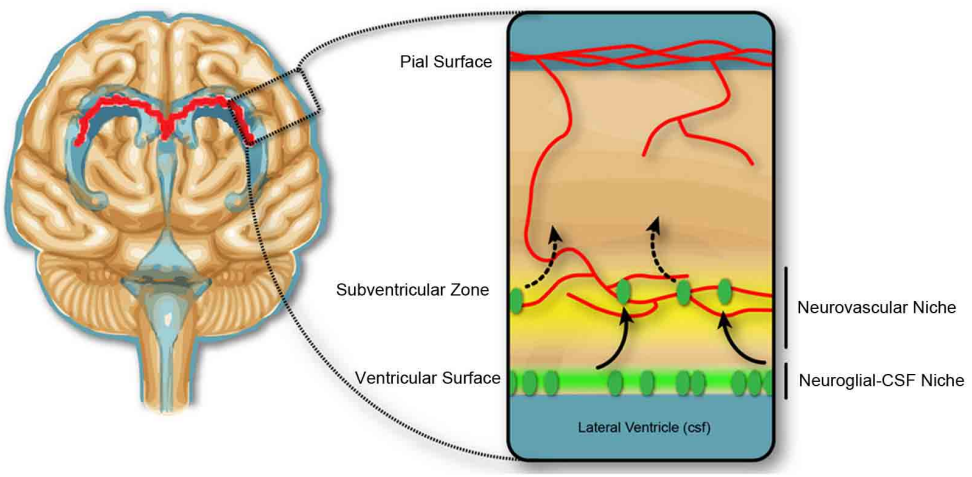

FIGURE 3 | Neurogenic niches in the developing brain. Dividing cells in the subventricular zone are closely associated with blood vessels, and the concept of a neurovascular niche (yellow), which reflects a zone of influence of vascular factors on neural progenitor cells, suggested in the adult and developmental subventricular zone. In early development, the dividing cells in the ventricular zone are not closely associated with the blood vasculature, but may be affected by trophic factors produced in the CSF, and therefore exist in a neuroglial-CSF niche. Cells from the ventricular zone migrate toward the neurovascular niche, before differentiating and migrating to outer cortical layers of the brain.

associated with the peak periods of white matter damage (Verney et al., 2010, 2012; Supramaniam et al., 2013). It is possible that the large number of migrating, activated microglia within the white matter tracts are primed to respond to inflammatory signaling transferred through blood vessels, or to the presence of systemic proteins following blood-brain barrier breakdown, and it is these immune cells that interact with oligodendrocytes in the developing white matter to cause injury.

These two scenarios indicate changes to the brain that are an immediate cause in systemic inflammation and inflammatory signaling into the developing brain. There are likely to be many more examples like this, as numerous immune mediators are important for the regulation of maturation processes in the brain (e.g., CXCR4 and CXCL7 as key migration cues). It is, however, necessary to also consider subtle changes that may alter the response of the maturing/ageing brain to insults later in life. One example of this is a long-term alteration in blood-brain barrier function that occurs following systemic inflammation early in life. In this case, the magnitude/prolonged nature of the inflammatory response is key—and long-term changes in barrier function only occur after prolonged exposure to systemic inflammatory (Stolp et al., 2005b).

Given the contribution of the blood-brain barrier to adult neuroinflammatory diseases (as discussed above), any structural deficits within the barrier junctions that exist as a result of injury in early life may increase the risk of early or delayed onset of neurodegenerative conditions [reviewed by Stolp and Dziegielewska (2009)].

SUMMARY REMARKS

(1) The brain, both in the adult and in development, is surrounded by a complex array of barrier mechanisms comprised of morphological (tight junctions), biochemical, and physiological (influx and efflux transporters) components that control and determine its internal environment. There is increasing evidence that one important function is an interaction with the immune system. Any structural deficits within the barrier junctions that exist as a result of injury in early life may increase the risk of early onset of neurodegenerative conditions.

(2) Evidence suggests that normal immune surveillance, which is likely to occur primarily through the blood-CSF barrier, is facilitated by the specific composition of the junctions between epithelial cells. Junctional rearrangement appears to be an essential element of inflammation-induced cellular recruitment to the brain.

(3) Transporters constitutively present at brain barriers can be affected by inflammation, therefore contributing to changes in the CNS environment alone or in association with the changes produced by the activation of immune cells.

(4) There is increasing evidence that the brain barriers are able to mount a response to peripheral inflammation and that this vascular inflammatory response may in itself contribute significantly to neuroinflammatory disease.

(5) The developmentally controlled CNS response to inflammation is a combination of many aspects of maturation processes of both the brain and the systemic inflammatory response itself. Specific study of the interactions between these two systems in development, as has been done in adult disease, is very important for proper understanding of normal and pathological mechanisms involved.

CONCLUSION

The brain barrier systems provide an essential interface between the periphery and the brain, which is intrinsically involved in the communication of inflammatory signals between these two compartments. Though very little is known about the responses of individual cells forming these barriers during inflammation, especially during development and ageing, it is apparent that they respond differentially to disease. We can say with confidence therefore that immunity is an active and fluid component of normal brain-barrier function. What we cannot say with a similar level of confidence, however, is how this function is altered under stress, or how one should approach these alterations from a clinical setting. There is still a substantial amount of work required before specific aspects of changes in the plethora of barrier mechanisms contributing to neuropathological conditions arising during development and in old age can be defined. More attention needs to be paid to changes in cellular-based barrier mechanisms, rather than focus on the integrity of tight junctions, which has been the emphasis of much of the research effort in this field so far.

ACKNOWLEDGMENTS

The authors would like to acknowledge the support on this project from the European Union Seventh Framework Programme [FP7/2007–2013] under grant agreement n° HEALTH-F2-2009-241778.

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Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Received: 05 June 2013; paper pending published: 24 June 2013; accepted: 31 July 2013; published online: 23 August 2013.

Citation: Stolp HB, Liddelow SA, Sá-Pereira I, Dziegielewska KM and Saunders NR (2013) Immune responses at brain barriers and implications for brain development and neurological function in later life. Front. Integr. Neurosci. 7:61. doi: 10.3389/fnint.2013.00061

This article was submitted to the journal Frontiers in Integrative Neuroscience.

frontiers in
INTEGRATIVE NEUROSCIENCE

REVIEW ARTICLE
published: 10 January 2014
doi: 10.3389/fnint.2013.00080

Pericytes: brain-immune interface modulators

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The premise that the central nervous system is immune-privileged arose from the fact that direct contact between immune and nervous cells is hindered by the blood–brain barrier. However, the blood–brain barrier also comprises the interface between the immune and nervous systems by secreting chemo-attractant molecules and by modulating immune cell entry into the brain. The majority of published studies on the blood–brain barrier focus on endothelial cells (ECs), which are a critical component, but not the only one; other cellular components include astroglia, microglia, and pericytes. Pericytes are poorly studied in comparison with astrocytes or ECs; they are mesenchymal cells that can modify their ultrastructure and gene expression in response to changes in the central nervous system microenvironment. Pericytes have a unique synergistic relationship with brain ECs in the regulation of capillary permeability through secretion of cytokines, chemokines, nitric oxide, matrix metalloproteinases, and by means of capillary contraction. Those pericyte manifestations are related to changes in blood–brain barrier permeability by an increase in endocytosis-mediated transport and by tight junction disruption. In addition, recent reports demonstrate that pericytes control the migration of leukocytes in response to inflammatory mediators by up-regulating the expression of adhesion molecules and releasing chemo-attractants; however, under physiological conditions they appear to be immune-suppressors. Better understanding of the immune properties of pericytes and their participation in the effects of brain infections, neurodegenerative diseases, and sleep loss will be achieved by analyzing pericyte ultrastructure, capillary coverage, and protein expression. That knowledge may provide a mechanism by which pericytes participate in the maintenance of the proper function of the brain-immune interface.

Keywords: pericytes, blood–brain barrier, immune response, inflammation, cytokines, REM sleep loss, brain endothelial cell, tight junction disruption

INTRODUCTION

The brain must respond to blood-borne signals but has no direct access to them (Persidsky et al., 2006; Saper, 2010). Likewise, the immune system does not contact directly the brain *milieu*; they interact through the brain-immune interface, the blood–brain barrier. The interface is comprised by endothelial cells (ECs), astrocytes, microglia, pericytes, and extracellular matrix components (basal lamina and glycocalyx; Risau, 1991; Ballabh et al., 2004; Ueno, 2007; Gómez-González et al., 2012). ECs limit blood-borne macromolecules or cells from crossing into the brain through junction complexes that fasten together adjacent cell membranes. In addition, transcellular trafficking of molecules is limited by the minimal expression of endocytosis and the presence of specialized carrier systems (Zlokovic, 2008; Abbott et al., 2010). Although ECs provide the physical and chemical barrier function *per se*, all elements are crucial for the development and maintenance of the blood–brain barrier, allowing it to be the interface between peripheral systems and the brain (Zlokovic, 2008).

Pericytes have been increasingly implicated in the regulation of local blood-flow in brain regions with increased synaptic activity, a phenomenon known as neurovascular coupling (reviewed in Hamilton et al., 2010); furthermore, they have also been involved in the regulation of the blood–brain barrier permeability to circulating molecules (Armulik et al., 2010). Better understanding of the immune properties of pericytes and their participation in the changes observed during brain infections and neurodegenerative diseases will provide a mechanism by which pericytes participate in the maintenance of the proper function of the brain-immune interface, the blood–brain barrier. Here we present recent evidence depicting the new roles of pericytes in regulating blood–brain barrier function under normal and pathological conditions and hypothesize its potential role in the regulation of the blood–brain barrier after chronic sleep loss.

PERICYTES AS BLOOD–BRAIN BARRIER COMPONENTS

Pericytes are smooth muscle-derived cells that play a crucial role in keeping brain homeostasis given their presence at the blood–brain barrier and particularly their active role in what is known as the neurovascular unit (Zlokovic, 2008; Gómez-González et al., 2012). Rouget (1874), for the first time, described a population of branched cells with contractile properties that surrounded ECs. Fifty years later, these mesenchymal cells were renamed "pericytes" by Zimmerman in concordance with their anatomical location: abluminal to ECs and luminal to parenchymal cells (Kim et al., 2006; Sá-Pereira et al., 2012). Anatomically, pericytes have projections that wrap around capillaries and are embedded within the basal lamina. The diversity in pericyte

marker expression may be related to vessel size or embryonic origin; the main markers are α-smooth muscle actin (αSMA), desmin, the regulator of G-protein signaling 5 (RGS-5), neuron-glial antigen 2 (NG2), platelet-derived growth factor receptor (PDGFRα and PDGFRβ), and amino-peptidase-N (CD13; Ozerdem et al., 2002; Bergers and Song, 2005). These proteins show different expression patterns under physiological and pathological states (see **Table 1**). Furthermore, pericytes express numerous macrophage markers, namely CD4, CD11b, CD146, and proteins related to immune function such as the fragment crystallizable receptor (FcR) and the major histocompatibility complex (MHC) classes I and II (Bergers and Song, 2005; Kamouchi et al., 2011). Differences in the expression of those markers are based on the local environmental influences on pericytes. For example, it has been reported that CD146 is expressed during embryonic development but not in all freshly isolated pericytes in adulthood. Also, RGS-5 protein expresses during embryonic development, but decreases after birth and is absent in pericytes of the normal adult central nervous system (Dore-Duffy, 2008; Sá-Pereira et al., 2012).

Although pericyte identification is rather difficult owing to the lack of one specific marker (Özen et al., 2012), its ultrastructure was described (Nag, 2003; Sá-Pereira et al., 2012). Two classes of pericytes exist in the brain: granular and agranular; this classification arises from the presence or absence of lysosome-like granules in the cytoplasm (Farrell et al., 1987). In humans, less than 5% of the pericyte population is agranular (Farrell et al., 1987; Nag, 2003). Both, granular and agranular pericytes exhibit an oval cell body and a prominent round nucleus that is different from the elongated nucleus of ECs. Each pericyte may cover 100 μm of capillary length with up to 90 ramifications 300–800 nm wide (Nag, 2003; Sá-Pereira et al., 2012). Pericyte distribution is intermittent along the walls of arterioles, venules and, particularly, in capillaries (Dore-Duffy, 2003). They are crucial for the development and maintenance of the main nervous system barriers, namely, blood–spinal cord barrier, blood–retinal barrier, blood–nerve barrier and blood–brain barrier. In fact, pericyte coverage of brain ECs *in vitro* is approximately 80%, in the capillaries of the retina it is 90%, and in the microvessels of the spinal cord it is less than 60%. Pericyte coverage and

Table 1 | Pericyte markers in health and disease.

Pericyte marker/ location	Main function	Main physiological role	Health	Disease	Reference
PDGFRβ/cell surface protein	Tyrosine-protein kinase; Kinase receptor	Embryonic development, proliferation, chemotaxis, host-virus interaction	+	+/− Fibrosis Tumor Blood–brain barrier disruption	Song et al. (2005), Armulik et al. (2010), Dore-Duffy and Cleary (2011)
αSMA/Filament protein	Contractility	Regulation of blood flow and motility	−	++ Fibrosis Tumor Blood–brain barrier disruption	Song et al. (2005), Dore-Duffy and Cleary (2011)
NG2/cell surface protein	Cell adhesion protein	Vasculo-genesis	+	+ Fibrosis Tumor Blood–brain barrier disruption	Ozerdem et al. (2002), Dore-Duffy and Cleary (2011)
RGS-5/intracellular protein	GTPase-activating protein	Cell motility	+	++ Fibrosis Tumor Blood–brain barrier disruption	Song et al. (2005), Dore-Duffy and Cleary (2011)
Desmin/filament protein	Contractility	Regulation of blood flow and motility	+	+ Fibrosis Tumor Blood–brain barrier disruption	Dore-Duffy and Cleary (2011), Kamouchi et al. (2011)
CD13/cell surface protein	Ectopeptidase	Pericyte differentiation	+	++ Fibrosis Tumor Blood–brain barrier disruption	Armulik et al. (2010), Kamouchi et al. (2011)

Symbols are as follow: (+) Indicates that the marker is present; (−) indicates that the marker is absent; (+/−) indicates a decrease in marker expression and; (++) indicates that the marker is over expressed.

number is related to the permeability of the biological-barriers, higher coverage correlates with lower permeability (Winkler et al., 2012). Specifically, it has been shown that pericytes contribute to regulate capillary structure and diameter (Peppiatt et al., 2006; Armulik et al., 2010; Bell et al., 2010; Daneman et al., 2010). Pericytes express junctional complexes that include gap junctions, tight junctions (Tjs), and focal adhesions with ECs (Zlokovic, 2008). These associations lead to the maintenance of low permeability of the cerebral endothelium (Lai and Kuo, 2005; Nakagawa et al., 2007). Brain pericytes promote a reduction in vesicular transport, (Daneman et al., 2010), and promote endothelial Tj protein expression (Zonula occludens, ZO-1, claudin-5, occludin; **Figure 1**; Armulik et al., 2005, 2010; Daneman et al., 2010). In addition, the morphological pattern of pericyte projections around brain capillaries is linked to their function and intimately correlates with brain health state (normal, angiogenic, or injured; Dore-Duffy and Cleary, 2011). The classic wrapping pattern consists of broad processes with a large continuous surface in the external wall of brain microvessels (Dore-Duffy, 2003; Nag, 2003; Dore-Duffy and Cleary, 2011). Under normal conditions, the wrapping pattern predominates, but in pathological conditions detachment and migrating patterns can be observed with the formation of finger-like projections followed by retraction of projections (**Figure 1**; Dore-Duffy and Cleary, 2011). Different morphological patterns in pericyte processes may appear in response to changes in the microenvironment. For example, the migrating pattern is associated to up-regulation of cell surface proteases in aversive conditions, and also with early stages of angiogenesis, in contrast with the wrapping pattern that predominates in normal capillaries (Dore-Duffy, 2003; Sá-Pereira et al., 2012).

Morphological changes in pericytes vary as a function of exposure to soluble molecules released by blood–brain barrier components such as ECs, neurons, microglia or astrocytes; pericytes can differentiate into fibroblasts, smooth muscle cells or macrophages, depending on the stimulus received (**Figure 1**). The molecules released to the basal lamina that can promote pericyte morphological changes include neurotransmitters, neurohormones and inflammatory mediators (Özen et al., 2012). To illustrate this, it has been shown that adenosine and adenosine triphosphate (ATP) released by neurons and glial cells may modify pericyte status by activating purinergic receptors; in addition, rat brain pericytes express ecto-nucleotidase 1 and 2 (Ceruti et al., 2011; Lecca et al., 2012). After immune challenges such as lipopolysaccharide (LPS) administration, hippocampal brain pericytes present increased ecto-nucleotidase expression and function and also morphological changes (Kittel et al., 2007). Activation of purinergic receptor P2X7 initiates an inflammatory response by inducing interleukin (IL) 1β secretion from ECs, astrocytes, microglia, and also pericytes (Derks and Beaman, 2004; Lecca et al., 2012).

Pericyte versatility is, for the most part, unexplored, but several studies suggest that pericytes may play potential roles in brain repair through contractile, migratory, pro-angiogenic and phagocytic functions but they can also promote brain impairment by uncontrolled immune response (Dore-Duffy et al., 2000; Dore-Duffy et al., 2006; Özen et al., 2012; Sá-Pereira et al., 2012).

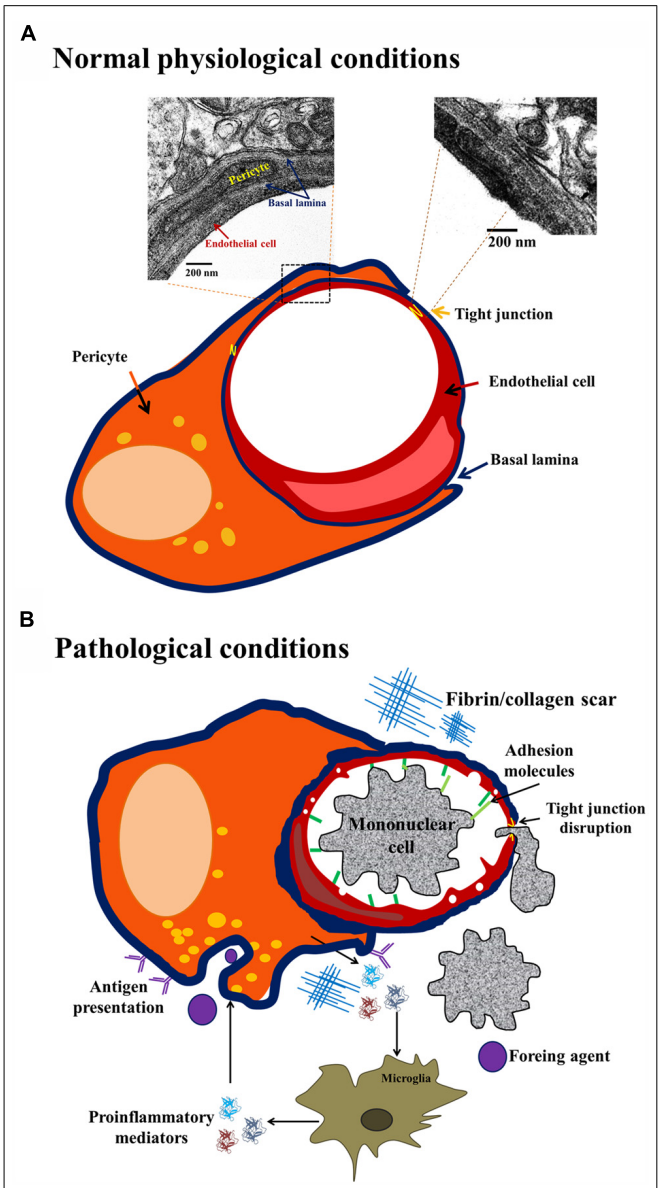

FIGURE 1 | Brain pericyte phenotype in normal and pathological conditions. Under normal physiological conditions **(A)** brain pericytes exhibit tight junctions (Tjs) with endothelial cells (ECs), and are embedded in the basal lamina. Under pathological conditions, such as injury, infection or neurodegeneration **(B)**, pericytes present a migrating phenotype with up-regulation of ICAM expression, pro-inflammatory cytokine release with ensuing recruitment of peripheral mononuclear cells. Additionally, under pathological conditions, the continuity of basal lamina is lost and the existence of fibrin scars contributes to blood–brain barrier impairment.

IMMUNE PROPERTIES OF BRAIN PERICYTES

Mesodermal or neural crest origins of pericytes are generally accepted. Pericytes are considered as "brain macrophages". In fact, for some authors, they represent the first line of defense in the central nervous system due to their antigen presentation properties and because they are directly associated with the microvasculature, in contrast to microglia (**Figure 1**; Balabanov et al., 1999; Guillemin and Brew, 2004). Thomas (1999)

reported pericytes leaving the basal lamina and migrating to the perivascular space where they are indistinguishable from perivascular macrophages and reactive microglia (Guillemin and Brew, 2004). Pericyte de-differentiation into cells presenting antigens may initiate a local pro-inflammatory response. Immune response in the brain induces monocyte and lymphocyte recruitment; this process is mediated by the increased expression of adhesion molecules (e.g., intracellular adhesion molecule 1, ICAM-1) in the luminal region of ECs that correlates with decreases in the number of Tjs (**Figure 1**; Guillemin and Brew, 2004). In addition, pericytes are able to produce chemo-attractants and promote transmigration to the brain of circulating immune cells, starting an inflammatory process. Pericytes may also release inflammatory mediators, such as IL-1β, IL-6, tumor necrosis factor (TNF) α, reactive oxygen species, nitric oxide (NO), and matrix metalloproteinases (MMP-2 and MMP-9), all of which contribute to pericyte detachment and blood–brain barrier disruption (Kovac et al., 2011).

These immunoactive properties of pericytes suggest mechanisms by which they can act as an integral part of the blood–brain barrier during brain inflammatory processes. A pro-inflammatory component is the hallmark of several brain diseases. Vascular damage associated to pericyte deficiency may precede neurodegeneration in brain infections, Alzheimer's or Parkinson's disease, diabetes (Özen et al., 2012), and perhaps in less-explored phenomena that exhibit considerable cognitive impairments, such as sleep loss.

PERICYTES AND BRAIN INFECTIONS

The blood–brain barrier provides a shield against foreign agents that initiate inflammatory responses (Al-Ghananeem et al., 2013). The structural variability and the nature of biotic/abiotic inflammatory agents that may promote neuropathology are reflected in the mechanisms used to access the brain. These mechanisms include receptor-mediated endocytosis, unspecific transport by pinocytotic vesicles, paracellular diffusion, transmigration through infected leukocytes, and crossing after blood–brain barrier breakdown (Alcendor et al., 2012; Nakagawa et al., 2012; Pulzova et al., 2012).

The inflammatory response to a foreign agent may cause irreversible brain damage by continuous exposure to pathogen-derived toxic molecules and immune mediators (Kumar et al., 2009; Hirooka and Kaji, 2012). Factors that promote a pro-inflammatory state in the brain include abiotic agents such as heavy metal ions or viruses, and biotic factors such as bacteria, fungi, and parasites (Gasque et al., 1998; Liou and Hsu, 1998; Alvarez and Teale, 2007; Hirooka and Kaji, 2012; Nakagawa et al., 2012). There is scant knowledge of pericyte function and structure under inflammatory response induced by foreign agents.

Heavy metal ions, such as methyl-mercury, cadmium and inorganic mercury induce a potent inflammatory response in the brain. These metal ions have high affinity to sulfhydryl groups favoring the formation of a methionine-like complex that easily crosses the blood–brain barrier. The methionine-like complex enters the brain by the large neutral amino acid transporter (LAT-1); once inside, the heavy metal ions induce cytokine and growth factor release by blood–brain barrier components. Heavy metal ions associate with the fibroblast growth factor type 2 (FGF-2); this union may cause cell damage because FGF-2 is unable to repair endothelial damage; therefore, heavy metal ions promote less auto-regulatory signaling inhibition of EC proliferation (Hirooka and Kaji, 2012).

In the case of viral and bacterial infections, such as congenital human cytomegalovirus (HCMV), human immunodeficiency virus type 1 (HIV-1), Japanese encephalitis (JE) virus and bacterial meningitis, the main transport routes through the blood–brain barrier include endocytosis of blood-circulating vesicles, microvessel wall degradation, and indirect crossing via previous blood–brain barrier disruption. When infectious agents are detected, pericytes begin an inflammatory response through increased expression of pro-inflammatory cytokines, such as IL-1β, IL-6, and TNF-α (Liou and Hsu, 1998; Alcendor et al., 2012; Nakagawa et al., 2012). In HIV-1 infection, pericytes express the chemokine receptors CXCR4 and CCR5 that are used by infected cells to contribute to the formation of viral reservoirs in the brain (Nakagawa et al., 2012). It is known that 80% of cultured pericytes infected by HCMV generate an inflammatory response; in fact, only 72 h after infection, a huge rise in IL-1β, a medium increase in IL-6, and a minimal increase in TNF-α concentration are observed. However, later on those pro-inflammatory cytokine profiles are reversed by the compensatory effect of anti-inflammatory cytokines (Alcendor et al., 2012). In contrast, bacterial meningitis infection increases expression of receptors C5a and C3a in brain pericytes. These complement molecules are powerful chemo-attractants to recruit polimorfonuclear cells and macrophages to the inflammation site causing cell activation (Gasque et al., 1998). On the other hand, it has been reported that *Taenia solium* infiltrates cause brain inflammation by pericyte release of pro-inflammatory cytokines and MMP-2 and MMP-9, which are associated to blood–brain barrier disruption. Blood–brain barrier breakdown allows infiltration of antigen-presenting cells and specialized immune cells (B cells and T cells), exacerbating the inflammatory condition (Alvarez and Teale, 2007).

These studies illustrate that although each pathogen exhibits a characteristic pathway, the same inflammatory mediators participate in the orchestration of the brain immune response (**Figure 2**). It is known that rises in pro-inflammatory cytokines, particularly IL-1 β, IL-6, and TNF-α, disrupt Tjs by down-regulating occludin and ZO-1 expression (Liou and Hsu, 1998; Alcendor et al., 2012; Nakagawa et al., 2012). Pro-inflammatory cytokines alter Tj integrity by promoting an increase in prostaglandin-E (PGE) receptors in pericytes, which leads to MMP overproduction and release, causing pericyte uncoupling with ECs (Alvarez and Teale, 2007). In fact, ECs are the unique brain cell type that expresses PGE-2 synthase (Yamagata et al., 2001); PGE-2 is produced in response to immune challenges (e.g., IL-1 or LPS administration; Cao et al., 1997; Laflamme et al., 1999) suggesting a relevant role of perivascular cells (astrocytes, interneurons and particularly pericytes) in the response to low doses of immune stimulators (Schiltz and Sawchenko, 2002). Interestingly, perivascular cell response is different for each type of molecule; e.g., pericytes elicit cyclooxygenases in brain ECs in response to low doses of IL-1, but with low doses of LPS perivascular cells

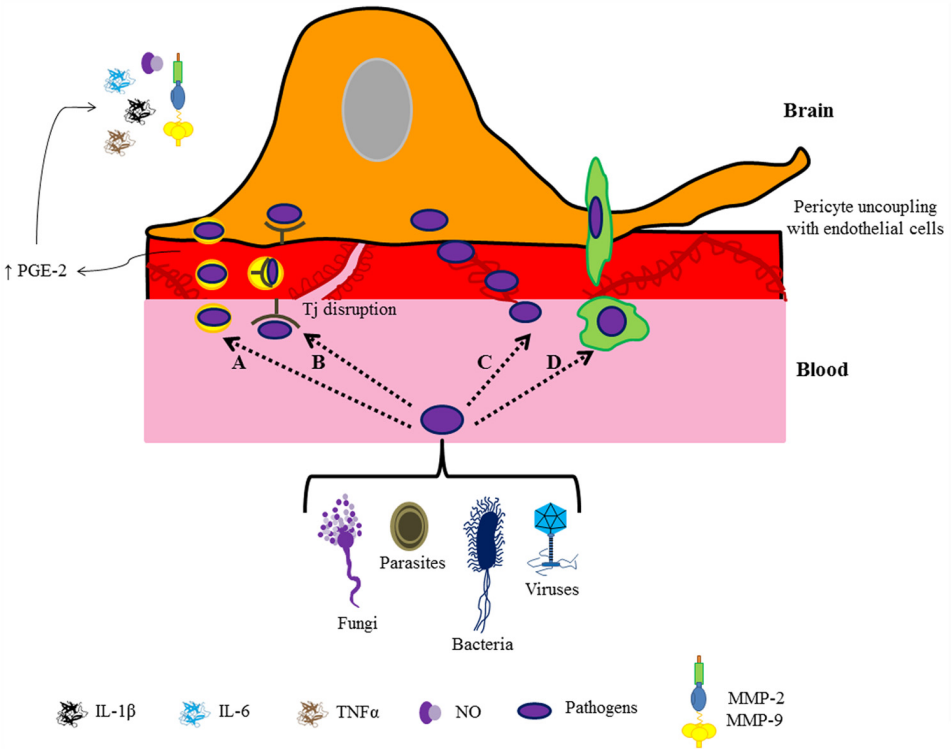

FIGURE 2 | Pathogen entry to the brain. The cartoon depicts different routes for pathogen entry to the brain: caveolae **(A)**, receptor-mediated transport **(B)**, paracellular diffusion **(C)**, and transmigration through infected leukocytes **(D)**. In all cases a pericyte-mediated immune response followed by blood–brain barrier breakdown is observed.

apparently have an inhibitory effect on cyclooxygenase production (Schiltz and Sawchenko, 2002). Some neuro-infections are associated with neurodegenerative diseases, for example, the bacteria *Borrelia burgdorferi* in Alzheimer's disease (Miklossy et al., 2004). So, is the pathogenic action on pericytes a promoter of neurodegenerative disease? Undoubtedly, pericyte function has an important role in the progression of brain pathologies. Although several studies provide relevant information on the immune role of pericytes in the protection of the brain against an infectious threat, the molecular and cellular mechanisms involved in blood–brain barrier disruption are poorly understood.

ROLE OF PERICYTES IN NEURODEGENERATIVE DISORDERS

Similar to infectious processes, during neurodegenerative and cerebrovascular diseases inflammatory phenomena occur, which are characterized by increased release of pro-inflammatory cytokines (IL-1β, IL-6, and TNF-α), subsequent hyperthermia, and mononuclear cell infiltration (Bleys and Cowen, 2001). In both, neurodegenerative and cerebrovascular diseases, pericyte detachment of ECs and differentiation into fibroblasts or phagocytes correlates with an increase in vesicle number in ECs, Tj disruption and immune cell recruitment (Özen et al., 2012). Additionally, fibrosis-like pathophysiological changes are described (**Figure 3**); pericyte-derived fibrin and collagen form scars, which are involved in cell death by neurotoxicity (Armulik et al., 2010; Fernández et al., 2013). Deposits of extracellular matrix components and organ failure are common after prolonged exposure to pro-inflammatory cytokines, suggesting that the first step leading to cell death relates to the immune response (Lin et al., 2008; Armulik et al., 2010). Furthermore, cytokine production is accompanied by oxidative stress; both, inflammatory mediators and oxidative stress are directly involved in increased blood–brain barrier permeability through the same signaling pathways.

Recent studies revealed the role of NO released by microglia and pericytes in neurodegenerative diseases and neuro-immune interactions; it was shown that amyloid β deposits in Alzheimer's disease promote pericyte constriction despite NO over-production. The role of NO in blood–brain barrier disruption is also related to its high ability to form free radicals such as peroxynitrite (ONOO-•), which may induce cell death (Hamilton et al., 2010; Kovac et al., 2011). In addition, it has been reported that amyloid β deposits promote over-production of reactive oxygen species in pericytes, endothelial, and glial cells (Veszelka et al., 2013). Blood–brain barrier disruption promotes lymphocyte recruitment in neurodegenerative diseases and stroke; hence, after cerebral ischemia, polymorphonuclear leukocytes impede reperfusion leading to generation of free radicals, and promoting pericyte constriction. Indeed, pericyte detachment from the vessel wall occurs following ischemia and reperfusion (Takahashi et al., 1997). Recently, Tigges et al. (2013) reported an increase in fibronectin and collagen I deposits in animal models of Alzheimer's disease, these deposits are related to pericyte

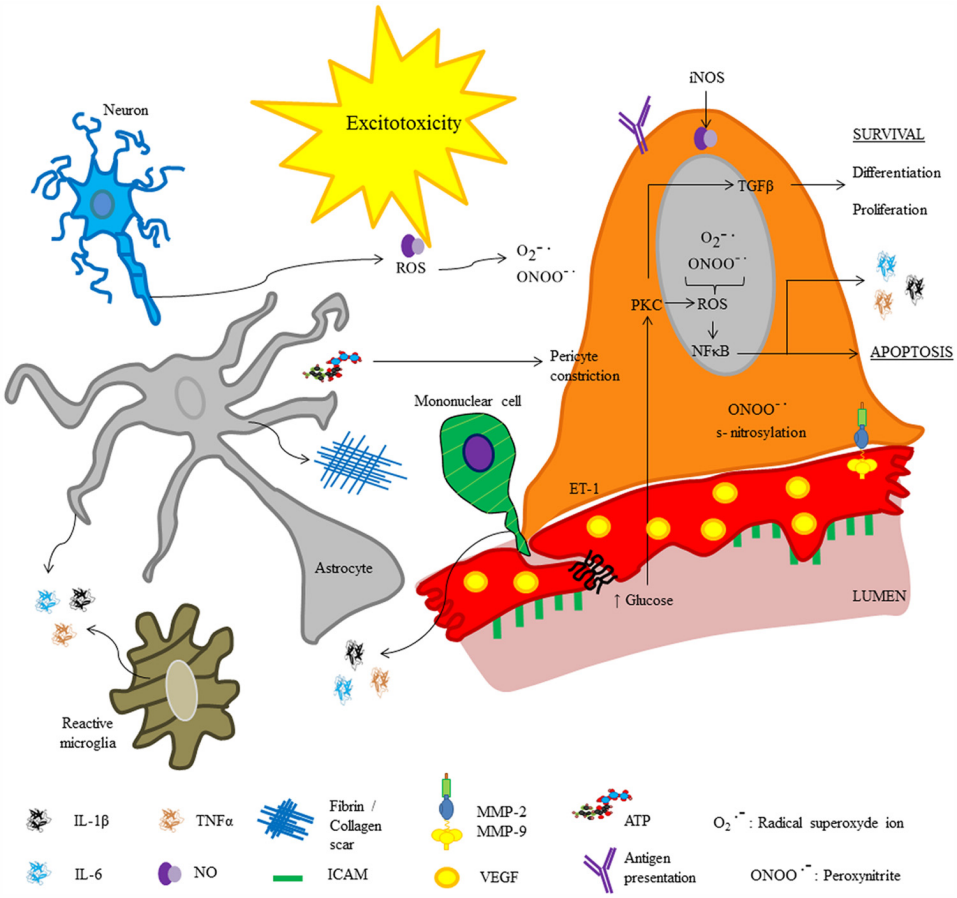

FIGURE 3 | Molecular inflammatory changes in brain pericytes in pathophysiological conditions. The cartoon depicts molecules released by pericytes under altered physiological conditions (e.g., neurodegeneration, infections or brain injury). Excitotoxicity may occur secondary to blood–brain barrier disruption.

differentiation and migration. Tigges et al. (2013) showed that under normal conditions, brain pericytes express high levels of α5 integrin and lower levels of α1, α2, and α6 integrins. This expression pattern has a crucial role in the attachment of pericytes to the vessel wall; in fact, an *in vivo* study shows that TNF-α promotes pericyte proliferation and detachment as well as a switch in integrin expression pattern, with predominance of α2 integrin (Tigges et al., 2013). Interestingly, Tigges et al. (2013) also found that α2 integrin expression strongly correlated with brain vessel remodeling in experimental autoimmune encephalomyelitis. Similarly, in Alzheimer's disease it is reported that fibrin deposition and increased extravascular immunoglobulin G (IgG) correlate with a reduction in pericyte coverage of ECs (Sengillo et al., 2013).

Fibrin deposits are a signal of fibroblast activity and probably represent an index of de-differentiation from pericytes to fibroblasts. Transforming growth factor-β (TGF-β) is the most potent known growth inhibitor for ECs, fibroblasts, neurons, and lymphoid cells. TGF-β inhibits proliferation of T-lymphocytes by down-regulating pro-inflammatory cytokines, e.g., IL-2-mediated proliferative signals (Dohgu et al., 2005). Under diabetic conditions, pericytes release TGF-β, which increases fibronectin levels (Shimizu et al., 2013). Shimizu et al. (2013) suggest that advanced glycation end-products (AGEs) induce blood–brain barrier disruption in diabetic conditions by stimulation of autocrine TGF-β signaling in pericytes, and up-regulation of vascular endothelial growth factor (VEGF) and MMP-2. Both, VEGF and MMP-2 modify trans-endothelial electric resistance (TEER) leading to Tj disruption and increased vesicular transport (Thanabalasundaram et al., 2011). Pericyte deficiency reported in diabetes is attributed to raises in glucose concentration, and production of reactive oxygen species through the NFκB pathway (Hamilton et al., 2010). Interestingly, in diabetic animal models, pericytes are highly immunosuppressive; under early hyperglycemic conditions retinal-derived pericytes inhibit T cell proliferation and protect ECs from inflammation-induced apoptosis (Tu et al., 2011). In addition, it is known that pericytes are especially susceptible to oxidative stress; for example, high glucose levels cause oxidative stress and apoptosis (Shah et al., 2013). In addition to the reactive oxygen species effect, the production of large amounts of NO by inducible-nitric oxide synthase (iNOS) can lead to changes in cerebral blood-flow, nitrosative stress, and subsequent cell death of pericytes, ECs and neurons through toxicity caused by excitatory amino acids and massive entry of toxic

molecules to the brain (Kischer, 1992; Li et al., 1997; Tu et al., 2011; Baloyannis and Baloyannis, 2012). A decrease in pericyte capillary coverage and cell number has been reported in hyperglycemia, early diabetes retinopathy, brain tumors, and Alzheimer's disease. Therefore, brain microvascular alterations seem to reciprocally interact with underlying neurodegeneration in inducing cognitive impairments (Pimentel-Coelho and Rivest, 2012). The role of pericytes in the genesis of neurodegenerative diseases and in brain regeneration is poorly studied; however, pericytes undoubtedly, cause alterations in brain physiology.

PERICYTES AND SLEEP LOSS: AN IMMUNOLOGICAL PERSPECTIVE

Sleep loss is a common problem in modern society (Mills et al., 2007; Yehuda et al., 2009) and a risk factor for the development of obesity, metabolic syndrome, diabetes, and neurodegenerative diseases (Tasali et al., 2009; van Leeuwen et al., 2009; Reynolds et al., 2012). Similar to infections and neurodegenerative diseases, sleep loss has an important pro-inflammatory component (Mills et al., 2007; Zager et al., 2007). Specific sleep function is yet unclear; but it has been proposed that sleep is associated with changes in parameters of host defense (Benca and Quintas, 1997). Sleep is divided into two distinct stages namely; slow wave sleep and rapid eye movement (REM) sleep (Siegel, 2010). Particularly, REM sleep has an important role in biological processes; REM sleep loss decreases neurogenesis in the hippocampus (Guzman-Marin et al., 2008; Mueller et al., 2008), alters the brain neurochemical content (Mohammed et al., 2011), and impairs learning and memory in both rodents and humans (Meerlo et al., 2008). Prolonged wakefulness promotes an increase of inflammatory mediators such as adenosine and NO (Kalinchuk et al., 2011; Cespuglio et al., 2012; Raymond et al., 2012), and increases plasma levels of IL-1β, IL-6, IL-17A, TNF-α (Yehuda et al., 2009), and endothelin-1 (ET-1; Mills et al., 2007). These changes may act directly on the blood–brain barrier components; for example, IL-1, IL-17, and ET-1 disrupt the blood–brain barrier (Banks et al., 1995; Blamire et al., 2000; Didier et al., 2003; Huppert et al., 2010). REM sleep deprivation also increases body temperature (Jaiswal and Mallick, 2009), which also disrupts the blood–brain barrier (Kiyatkin and Sharma, 2009).

Our research group recently found that chronic REM sleep restriction induces a generalized blood–brain barrier breakdown, and subsequent sleep opportunity is capable of restoring blood–brain barrier integrity. In addition, we studied EC ultrastructure and observed alterations in vesicle trafficking (Gómez-González et al., 2013). It is highly likely that pericyte dysfunction may contribute to increases in blood–brain barrier permeability secondary to sleep loss because ultrastructural changes in ECs are similar to those reported in pericyte-deficient mice, e.g., increased caveolae density, and endothelial derangement (Armulik et al., 2010). Chronic exposure to pro-inflammatory cytokines, NO and other inflammatory mediators released during sleep restriction may directly induce pericyte detachment from the vessel wall and subsequent differentiation into migratory and phagocytic phenotypes, mediating blood–brain barrier disruption. It is likely that the synthesis of antioxidants and anti-inflammatory molecules during sleep recovery may restore normal blood–brain barrier permeability through neutralization of free radicals (**Figure 4**).

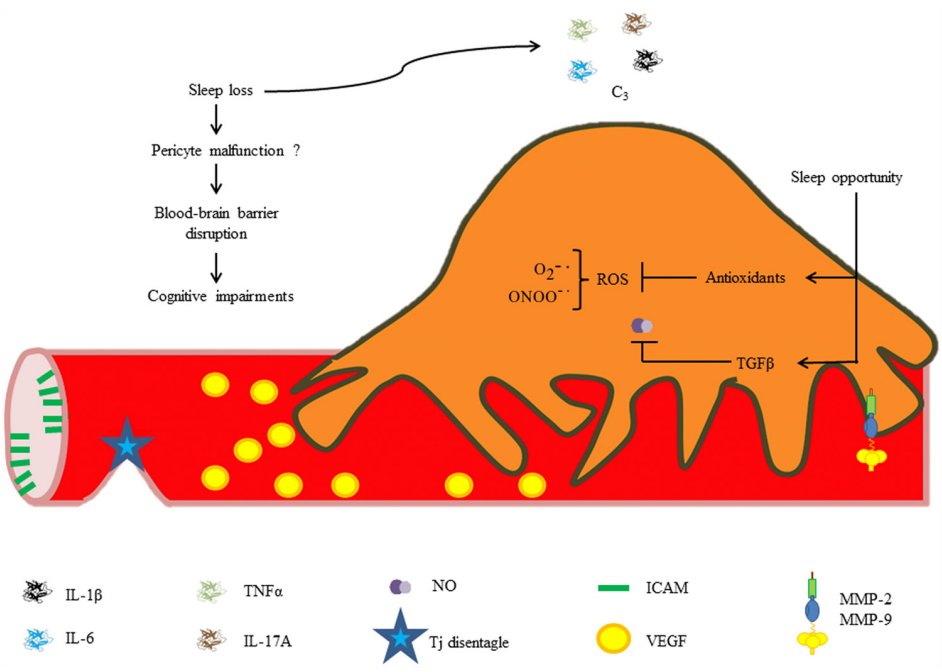

FIGURE 4 | Hypothetical roles of pericytes in REM sleep loss-induced blood–brain barrier disruption. Increased cytokine and chemokine concentrations during sleep loss may disrupt the blood–brain barrier. Brief periods of sleep opportunity may reestablish the blood–brain barrier integrity through synthesis of antioxidants and anti-inflammatory cytokines (e.g., TGF-β).

CONCLUSION

Classically, pericytes have been considered a cell population involved mainly in microvessel contractility. New research on pericyte contribution to optimal blood–brain barrier function and neural pathogenesis shows that they have a substantial influence on the neuro-immune response. The immunoactive properties of pericytes suggest mechanisms by which they could act as an integral component of the blood–brain barrier during inflammatory processes, such as during brain infections, neurodegenerative diseases or sleep loss. Future studies are needed to elucidate pericyte role under inflammatory conditions. Knowledge on pericyte contribution to disease pathogenesis will allow more specific treatment of brain pathologies and perhaps the development of better diagnostic markers. The field study of pericytes is generating frontier knowledge and may be exploited as an example of neuro-integration. Certainly, pericytes are crucial cells in optimal brain function, but their deficit results from molecular interactions between all brain cells.

ACKNOWLEDGMENTS

The authors express their gratitude to Dr. Edith Monroy for the correction of the English language. Supported by Grant 14412025 from CONACyT to B Gómez-González.

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Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Received: 15 May 2013; paper pending published: 10 July 2013; accepted: 26 December 2013; published online: 10 January 2014.

Citation: Hurtado-Alvarado G, Cabañas-Morales AM and Gómez-Gónzalez B (2014) Pericytes: brain-immune interface modulators. Front. Integr. Neurosci. 7:80. doi: 10.3389/fnint.2013.00080

This article was submitted to the journal Frontiers in Integrative Neuroscience.

frontiers in
INTEGRATIVE NEUROSCIENCE

MINI REVIEW ARTICLE
published: 02 September 2013
doi: 10.3389/fnint.2013.00064

Neuronal influence behind the central nervous system regulation of the immune cells

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Central nervous system (CNS) has a highly specialized microenvironment, and despite being initially considered an immune privileged site, this immune status is far from absolute because it varies with age and brain topography. The brain monitors immune responses by several means that act in parallel; one pathway involves afferent nerves (vagal nerve) and the other resident cells (neurons and glia). These cell populations exert a strong role in the regulation of the immune system, favoring an immune-modulatory environment in the CNS. Neurons control glial cell and infiltrated T-cells by contact-dependent and -independent mechanisms. Contact-dependent mechanisms are provided by several membrane immune modulating molecules such as Sema-7A, CD95L, CD22, CD200, CD47, NCAM, ICAM-5, and cadherins; which can inhibit the expression of microglial inflammatory cytokines, induce apoptosis or inactivate infiltrated T-cells. On the other hand, soluble neuronal factors like Sema-3A, cytokines, neurotrophins, neuropeptides, and neurotransmitters attenuate microglial and/or T-cell activation. In this review, we focused on all known mechanism driven only by neurons in order to control the local immune cells.

Keywords: neuronal immune modulation, central nervous system, neuron-microglia interaction, neuron-T cell interaction, CD200, neurotrophins, neurotransmitters, semaphorins

INTRODUCTION

The central nervous system (CNS) has a highly specialized immune-modulatory microenvironment, which has developed several mechanisms to protect itself from immune-mediated inflammation. This microenvironment is sustained by existing physiological and anatomical elements such as the blood-brain barrier (BBB) that limits peripheral immune cells and molecules entry; the afferent nerves of the autonomic nervous system with anti-inflammatory properties; and finally, the resident cells like astrocytes and neurons, which also contribute to the local immune privilege through the expression of anti-inflammatory suppressive factors and cell surface molecules (Carson et al., 2006).

The ability of neurons to sense changes in the brain and the body is a key factor in maintaining CNS-homeostasis. There is a large body of evidence that immune and neuronal systems communicate with each other by soluble factors as neurotransmitters, neuromodulators, and neuropeptides, or through cell-cell contact by neuroimmune regulatory molecules that can reduce or inhibit any exacerbated inflammatory response (Tian et al., 2009).

In this review, we focus on the general neuron-cell contact-dependent and contact-independent mechanisms involved in the immune modulation in order to maintain CNS immune privilege, even though microglia and astrocytes constitute the first line of defense.

CONTACT-DEPENDENT MECHANISM FOR IMMUNE MODULATION

Neurons can display an array of membrane molecules in order to control local immune functions; these molecules can target local immune cells like microglia and astrocytes or peripheral immune cells present in the CNS. When BBB is ruptured, immune privilege is lost and neurons may come in contact with T or mononuclear cells, endangering their survival. However, neurons might modulate these immune cells by several strategies, either indirectly suppressing T-cell activation by restriction of antigen presenting properties of glial cells, directly suppressing T-cell activation, favoring a Th2 profile or promoting apoptosis of activated microglia and T-cells (Tian et al., 2009).

MOLECULES INHIBITING GLIAL ACTIVATION

The neuronal cell adhesion molecule (NCAM/CD56) is expressed on the surface of neurons, astrocytes and microglia (Sporns et al., 1995; Krushel et al., 1998; Chang et al., 2000a,b), and has a critical role in cell-cell adhesion, synaptic plasticity, neurite outgrowth, among other processes (Tian et al., 2009). Astrocyte-neuron interactions via NCAM lead to modulate glial scar formation by the inhibition of astrocyte proliferation *in vitro* and *in vivo* after performed stab lesions in the striatum, cerebral cortex, or hippocampus (Krushel et al., 1995, 1998). NCAM requires the activation of the glucocorticoid receptor to inhibit growth

factor-induced mitogen activated protein kinase (MAPK) activity and therefore preventing astrocytic proliferation (Krushel et al., 1998). NCAM also modulates microglial activation, decreases the production of TNFα and nitric oxide (NO) after glial stimulation with lipopolysaccharide (LPS) by reducing the expression of transcription factors like c-Jun, among others (Chang et al., 2000a,b). For the mediation of glial immune responses the homophilic binding of third Ig domain of NCAM is crucial (Sporns et al., 1995; Krushel et al., 1998).

Another important molecule thought to contribute to the constitutive anti-inflammatory and regulatory environment of the brain is CD200, a highly expressed glycoprotein in the CNS, mainly in neurons (Chitnis et al., 2007; Koning et al., 2009). Neuronal CD200 down-modulates the activation state of perivascular macrophages and microglia trough the CD200 receptor (Hoek et al., 2000). Upon binding to its ligand, the tyrosine residues on the cytoplasmic tail of CD200R are phosphorylated and the downstream signaling leads to inhibition of p38 MAPK, c-Jun N-terminal kinase (JNK), and extracellular-signal-regulated kinases (ERK; Zhang et al., 2004), interfering with the activation of macrophages and microglia. Moreover, IL4 mediated neuronal CD200 expression maintains microglia in a quiescent state and anti-inflammatory/neuroprotective profile (Lyons et al., 2009). Additionally, aging leads to a depressed CD200 expression and microglial activation, favoring a pro-neurodegenerative disease environment (Cox et al., 2012). Also, defects in CD200-CD200R pathway play a critical role in neurodegenerative disease development such as multiple sclerosis (MS), Parkinson's and Alzheimer's diseases (Koning et al., 2007; Walker et al., 2009; Zhang et al., 2011).

CD22 is a regulatory sialic-acid-binding molecule that mediates neuron binding to microglia through CD45, inhibiting CD40L-induced microglial activation by suppression of the p38 and p44/42 MAPK signaling pathway and preventing microglial TNFα production after LPS stimulation (Tan et al., 2000; Mott et al., 2004; Zhu et al., 2008).

Neuronal membrane integrin-associated protein (CD47) is specially concentrated on synapses and exerts its neuroimmune functions mainly via two receptors (Tian et al., 2009). CD172 (SIRPα) ligation results in phosphatidylinositide 3-kinase (PI3K) signaling cascade activation, and reduces inflammation severity by increasing TGFβ levels, diminishing phagocytosis TNFα and INFα levels (Reinhold et al., 1995; Smith et al., 2003). Furthermore, decreased levels of CD47 are found in chronic active and inactive MS lesions, possibly favoring persistence of damage by the lack of regulation of activated microglia and macrophages (Koning et al., 2007). CD47 interaction with thrombospondin TSP, a further receptor, leads to T-cell and microglia apoptosis via CD95/CD95L pathway also reducing inflammation (Lamy et al., 2007).

Residential brain cells express CD95L (FasL) constitutively to limit possible damaging inflammatory responses. Neuronal CD95L expression induces apoptosis of infiltrating and autoreactive T-cells (Flügel et al., 2000), as well of activated microglia (Choi and Benveniste, 2004). Additionally, CD95L protects neurons from perforin-mediated T-cell cytotoxicity (Medana et al., 2001).

The expression of chemokine CX3CL1 (fractalkine) and its receptor CX3CR1 is limited to neurons and microglia, respectively (Hughes et al., 2002). CX3CL1 can be found membrane-anchored or secreted both in physiological and pathological conditions such as facial motor nerve axotomy or a toxic model of Parkinson's disease (Harrison et al., 1998; Cardona et al., 2006). CX3CL1-CX3CR1 interactions lead to the JNK MAPK pathway activation and Nrf2 recruitment suppressing the neurotoxic microglia activity and reducing neuronal death due to inflammation (Zujovic et al., 2000; Mizuno et al., 2003; Cardona et al., 2006; Noda et al., 2011).

MOLECULES INHIBITING IMMUNE CELLS

Plexin and semaphorin signaling has revealed that several members of this family are involved in immune cell processes. Among these semaphorins are Sema-3A, Sema-3E, Sema-4D, Sema-4A, Sema-6D, and Sema-7A (Roney et al., 2013). However, only Sema-3A and Sema-7A are expressed by neurons, respectively either as secreted or membrane-bound regulatory proteins that attenuate T-cell activation, proliferation, and function through T-cell receptor (TCR) signaling (Czopik et al., 2006; Lepelletier et al., 2006). Sema-3A exerts its action forming a complex with neuropilin-1 and plexin-A1 that leads to the prevention of immune response over-activation and the inhibition of human monocytes migration through the blockage of actin cytoskeleton reorganization, interfering with TCR polarization and signal transduction events by down-modulation of MAPK signaling cascades (Lepelletier et al., 2006). Also stressed neurons may induce apoptosis of INFγ or LPS activated microglia through Sema-3A secretion recruiting CD95 to lipid rafts next to neuropilin-1 (Majed et al., 2006; Moretti et al., 2008). Sema-7A, a glycosylphosphatidylinositol-linked semaphorin, negatively regulates TCR signaling and avoids activation of the ERK-MAPK pathway decreasing T-cell

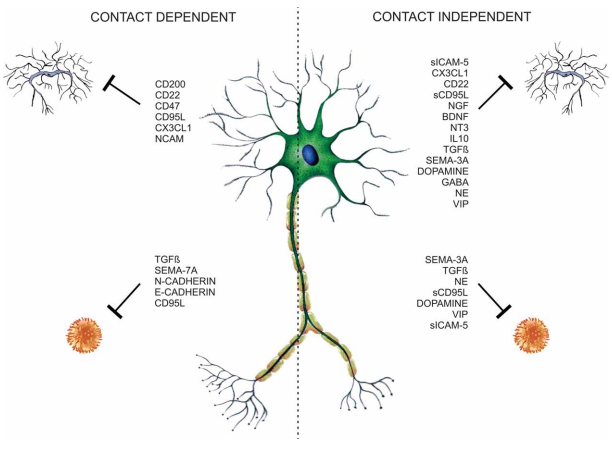

FIGURE 1 | Neuronal microglia and T-cell regulation. Neurons control T-cell and glia functions through neuronal membrane molecules or constitutive-secreted molecules like neurotrophins, neurotransmitters, neuropeptides, semaphorins, and cytokines, constituting contact-dependent and -independent regulatory mechanisms.

proliferation. Sema-7A deficient mice present T-cell hyperresponsiveness and hyperproliferation with severe experimental autoimmune encephalomyelitis pathology (Czopik et al., 2006).

Additionally, N- and E-cadherins are highly expressed in the CNS and bind to the killer cell lectin-like receptor G1 (KLRG1) on NK- and T-cells, preventing NK lysis of neurons and suppressing CD8 + T-cells antigenic proliferation and cytolytic activity (Gründemann et al., 2006; Ito et al., 2006).

Only soma and dendrites of neurons express the intercellular adhesion molecule-5 (ICAM-5/telencephalin; Tian et al., 2000). Neurons bind to T-cell through the ICAM-5-CD11a/Cd18 (LFA-1) interaction diminishing TCR dependent T-cell activation and enhancing TGFβ and INFγ expression in naïve T-cells (Tian et al., 2008). Additionally, ICAM-5 can be cleaved by activated T-cell or microglial-secreted matrix metalloproteinases-2 and -9, soluble ICAM-5 may compete with ICAM-1 costimulatory signal necessary for T-cell activation (Tian et al., 2008). Also, soluble ICAM-5 is present in blood and cerebrospinal fluid after hypoxia due to carotid artery ligation in mice and acute encephalitis in humans (Guo et al., 2000; Lindsberg et al., 2002). Moreover, ICAM-5 regulates microglia morphology and function by facilitating cell spreading and increasing CD11a/Cd18 expression (Mizuno et al., 1999).

NEURON-MEDIATED GENERATION OF REGULATORY T-CELLS

Regulatory T-cells (Tregs) are important in keeping CNS homeostasis in healthy and pathological conditions, and are also locally induced by glia cells and neurons (Liu et al., 2006; Saenz et al., 2010). Encephalitogenic T-cell production of IFNγ and TNFα leads to neuronal expression of TGFβ1, CD80, and CD86, which induce encephalitogenic CD4 + T-cells to become Tregs, in a cell-to-cell dependent and antigen independent way through the TGF-β1–TGF-βR and TCR signaling pathway (Issazadeh et al., 1998; Liu et al., 2006). Neuron-induced Tregs are able to inhibit progression of experimental autoimmune encephalomyelitis by suppression of encephalitogenic CD4 + T-cells proliferation (Liu et al., 2006).

Table 1 | Main neuronal immune regulatory molecules, their receptors and target cells in the CNS.

Neuronal molecule	Target cell	Receptor	References
CADHERIN SUPERFAMILY			
E-cadherin	NK-cell, T-cell	KLRG1	Gründemann et al., 2006; Ito et al., 2006
N-cadherin	NK-cell, T-cell	KLRG1	Ito et al., 2006
IMMUNOGLOBULIN SUPERFAMILY MOLECULES			
CD22	Microglia	CD45	Mott et al., 2004
CD47	Microglia	CD172a, TSP	Smith et al., 2003; Lamy et al., 2007
CD200	Microglia	CD200R	Hoek et al., 2000; Rijkers et al., 2008
ICAM-5	T-cell	CD11a/Cd18	Mizuno et al., 1999; Tian et al., 2000, 2008
NCAM	Microglia, Astrocyte	NCAM	Sporns et al., 1995; Krushel et al., 1998; Chang et al., 2000a
TUMOR NECROSIS FACTOR FAMILY			
CD95L	Microglia, T-cell	CD95	Choi and Benveniste, 2004
CYTOKINES AND CHEMOKINES			
IL10	Microglia, T-cell	IL10R	Strle et al., 2001
TGFβ	Microglia, T-cell	TGFβR	Pratt and McPherson, 1997; Liu et al., 2006
CX3CL1	Microglia	CX3CR1	Hughes et al., 2002
NEUROTRANSMITTERS AND NEUROPEPTIDES			
GABA	Microglia	$GABA_A$, $GABA_B$	Färber and Kettenmann, 2005
Dopamine	Microglia, T-cell	D_1, D_2, D_3, D_4, D_5	Färber et al., 2005
NE	Microglia, Astrocyte, T-cell	α_{1A}, α_{2A}, β_1, β_2	Färber et al., 2005; Gyoneva and Traynelis, 2013
VIP	Astrocyte, T-cell	$VPAC_1$, $VPAC_2$	Delgado et al., 2004, 2008
NEUROTROPHINS			
NGF	Microglia, Astrocyte	p75, NTR, TrkA	Neumann et al., 1998; Althaus and Richter-Landsberg, 2000; Cragnolini et al., 2012
BDNF	Microglia, Astrocyte	p75, NTR, TrkB	Neumann et al., 1998; Althaus and Richter-Landsberg, 2000
NT-3	Microglia	p75, NTR, TrkB, TrkC	Neumann et al., 1998; Althaus and Richter-Landsberg, 2000; Tzeng and Huang, 2003
SEMAPHORINS			
Sema-3A	Microglia, T-cell	Neuropilin-1 and plexin-A1	Lepelletier et al., 2006
Sema-7A	T-cell	Plexin-C1, α1β1 integrin	Czopik et al., 2006

BDNF, brain-derived neurotrophic factor; ICAM-5, intercellular adhesion molecule-5; GABA, γ-aminobutyric acid; NCAM, neuronal cell adhesion molecule; NE, norepinephrine; NGF, nerve growth factor; NT-3, neurotrophin-3; Sema-3A, semaphorin-3A; Sema-7A, semaphorin-7A; VIP, vasoactive intestinal peptide.

CONTACT INDEPENDENT MECHANISMS FOR IMMUNE MODULATION

Constitutive-secreted neurotrophins, neurotransmitters, and neuropeptides, as well as cytokines provide contact-independent routes for neurons to control microglial and T-cell activities.

Neurotrophins play a critical role in the control of neuronal survival, migration, and differentiation and modulate immune cell functions (Tabakman et al., 2004). Nerve growth factor (NGF), brain-derived neurotrophic factor (BDNF), and neurotrophin-3 (NT-3) can inhibit MHCII expression in microglia in hippocampal slice cultures via the low affinity p75 neurotrophin receptor (Neumann et al., 1998). NGF also down-regulates the co-stimulatory molecules CD40 and CD86 in microglia (Wei and Jonakait, 1999), is increased in cerebral spinal fluid of MS patients (Laudiero et al., 1992), and NGF treatment delays EAE onset and clinical severity (Arredondo et al., 2001). In addition, NGF arrests astrocyte cell cycle possibly restricting glial scar formation after CNS injury via the p75 neurotrophin receptor, attenuating cyclins D1 and E and preventing the degradation of cyclin-dependent kinase inhibitors p15INK and p27kip1 (Cragnolini et al., 2012). Interestingly, NT-3 has anti-inflammatory properties by diminishing microglial inducible form of NO synthase, NO, IL1-β, and TNFα levels, and phagocytic activity after LPS stimulation. NT-3 exerts its effects mainly through the TrkC receptor leading to the activation of MAPK and PI3K cascades and decreasing the NFκB-p65 activity (Tzeng and Huang, 2003; Tzeng et al., 2005).

IL10 and TGFβ cytokines have anti-inflammatory and suppressive properties that importantly regulate CNS inflammatory responses and resident cells survival (Pratt and McPherson, 1997; Strle et al., 2001). Both cytokines and their receptors are expressed by neurons and glial cells throughout the CNS (Szelényi, 2001). These regulatory molecules down-regulate microglia inhibiting the expression of MHCII, pro-inflammatory cytokines such as TNFα and IL1β, as well as NO synthesis after LPS activation (Suzumura et al., 1993; Sawada et al., 1999; Heyen et al., 2000). Furthermore, IL10 and TGFβ have an important role on Tregs and keep autoimmune T-cells under the steady state (Saenz et al., 2010).

Among the neuropeptides and neurotransmitters with modulatory properties that inhibit microglial LPS-induced pro-inflammatory factors like IL1β, IL6, TNFα, and NO, are vasoactive intestinal peptide (VIP), dopamine, norepinephrine (NE), and γ-aminobutyric acid (GABA; Färber et al., 2005; Bjurström et al., 2008; Delgado et al., 2008). VIP exerts its anti-inflammatory effects through the $VPAC_1$ and $VPAC_2$ receptors inhibiting p38 and p42/p44 MAPK and NFκB signaling cascades (Delgado et al., 2008). Also, VIP treatment avoids beta-amyloid neurodegeneration and MPTP-induced dopaminergic neuronal loss (Delgado and Ganea, 2003; Delgado et al., 2008). Additionally; VIP induces protective TH2 cells by up-regulation of macrophage B7.2 expression and Tregs in a EAE model (Delgado et al., 2004; Fernandez-Martin et al., 2006). Physiological concentrations of GABA activate functional $GABA_A$ channels on encephalitogenic T-cell decreasing cell proliferation, while $GABA_B$ channels activation on microglia attenuates IL6 and IL 12p40 levels after LPS stimulation (Kuhn et al., 2004; Bjurström et al., 2008). Functional dopamine receptors D_1 and D_2 are expressed by microglia and their activation lead to attenuate NO production after LPS stimulation (Kuhn et al., 2004). CNS NE levels are relevant in order to maintain tissue homeostasis since NE loss contributes to neuroinflammatory processes that lead to neurodegenerative diseases, for instance depressed mice with low NE levels respond with higher TNFα production after LPS stimulation while increasing NE levels are necessary to reduce EAE severity (Szelényi and Vizi, 2007; Simonini et al., 2010). Moreover, NE regulates microglia morphology and motility by microglial processes retraction; in this dynamic process the β2 and α2A receptors are involved in resting cells and activated microglia cells, respectively (Gyoneva and Traynelis, 2013).

CONCLUSIONS

Traditionally glial cells are considered to be responsible for the regulation of immune processes in the CNS. Nevertheless neurons contribute to immune modulation through contact-dependent and -independent mechanisms (**Figure 1**). Several neuronal secreted as well-membrane associated molecules (**Table 1**) are implicated in the control of glial and T-cell functions, thus contributing to CNS immune privilege.

ACKNOWLEDGMENTS

Funding was provided by the grant IN217612 from Dirección General de Asuntos del Personal Académico (DGAPA), National Autonomous University of Mexico. The authors wish to acknowledge Bernardo Pohlenz for his artwork assistance.

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Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Received: 07 May 2013; accepted: 07 August 2013; published online: 02 September 2013.

*Citation: Chavarría A and Cárdenas G (2013) Neuronal influence behind the central nervous system regulation of the immune cells. Front. Integr. Neurosci. **7**:64. doi: 10.3389/fnint.2013.00064*

This article was submitted to the journal Frontiers in Integrative Neuroscience.

frontiers in
INTEGRATIVE NEUROSCIENCE

REVIEW ARTICLE
published: 13 August 2013
doi: 10.3389/fnint.2013.00056

Hypothalamic neurohormones and immune responses

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The aim of this review is to provide a comprehensive examination of the current literature describing the neural-immune interactions, with emphasis on the most recent findings of the effects of neurohormones on immune system. Particularly, the role of hypothalamic hormones such as Thyrotropin-releasing hormone (TRH), Corticotropin-releasing hormone (CRH) and Gonadotropin-releasing hormone (GnRH). In the past few years, interest has been raised in extrapituitary actions of these neurohormones due to their receptors have been found in many non-pituitary tissues. Also, the receptors are present in immune cells, suggesting an autocrine or paracrine role within the immune system. In general, these neurohormones have been reported to exert immunomodulatory effects on cell proliferation, immune mediators release and cell function. The implications of these findings in understanding the network of hypothalamic neuropeptides and immune system are discussed.

Keywords: TRH, CRH, CRF, GnRH, LHRH, immune system, extrapituitary, receptors

INTRODUCTION

The neurosecretion in the hypothalamus can be traced back to the work of Scharrer and Scharrer (1940). Posterior studies by Harris specified that the hypothalamic substances secreted into the portal vessels were pituitary specific and propose the concept of "releasing factors" whose purpose was to initiate a cascade of events resulting in the release of peripherally active hormones (Harris, 1948).

The discovery and chemical characterization of the first identified hypothalamic releasing factor by Burgus et al. (1969) and Boler et al. (1969) provided ultimate confirmation for the founding principles of neuroendocrinology and was followed by the discovery of other peptide-releasing factors (Nillni and Sevarino, 1999).

THYROTROPIN-RELEASING HORMONE (TRH)

TRH, the smallest known peptide hypophyseotropic hormone, is a tripeptide (pGlu-His-Pro-NH_2; where pGlu stands for pyroglutamic acid) that is released from the hypothalamus and transported via the portal vascular system to the anterior pituitary where it stimulates the release of thyroid-stimulating hormone (TSH) and prolactin from the anterior pituitary (Matre et al., 2003).

TRH it is also known as thyrotropin-releasing factor (TRF), thyroliberin or protirelin (Khomane et al., 2011) and was the first chemically defined hypophyseotropic hormone (Bílek et al., 2011).

SYNTHESIS

This small tripeptide (362.42 Da) is indeed processed from a larger precursor protein, prepro-TRH (ppTRH), through now classic cleavage mechanisms, C-terminal amidation, and cyclization of the N-terminal Gln to pGlu (Guillemin, 2005).

The rat ppTRH is a 29 kDa polypeptide composed of 255 amino acids. The rat precursor contains an N-terminal 25-amino-acid leader sequence, five copies of the TRH progenitor sequence Gln-His-Pro-Gly flanked by paired basic amino acids (Lys-Arg or Arg-Arg), four non-TRH peptides lying between the TRH progenitors, an N-terminal flanking peptide, and a C-terminal flanking peptide. Rats and mice have five Gln-His-Pro-Gly TRH progenitor sequences, whereas humans have six TRH sequences (Chiamolera and Wondisford, 2009).

TRH EXPRESSION REGULATION

The maintenance of euthyroidism is dependent on a highly regulated balance of neuropeptides and neurotransmitters, where the dominant positive hypothalamic control for TSH is TRH, and the principal feedback control is through thyroid hormones (THs) (Nillni and Sevarino, 1999).

THs exert powerful feedback inhibition over the TRH response system by inhibiting TRH synthesis and processing in TRH neurons in the paraventricular region of the hypothalamus and decreasing TRH receptors (TRHR) and responses in the pituitary gland (Hinkle et al., 2012).

TRH gene expression is also regulated by temperature, food intake, and stress. Thus the TRH neuron is well positioned to integrate information about the environment as well as circulating TH levels and ultimately affect metabolism in response to these physiological changes (Chiamolera and Wondisford, 2009).

HALF-LIFE

The short half-life (4–5 mins) of TRH is most likely due to rapid degradation of the TRH at both the carboxy (COOH) and

amino (NH_2) termini. Cleavage of the pyroglutamyl moiety of TRH by enzymes like aminopeptidases and deamidases causes formation of the metabolite histidyl proline diketopiperazine (ClycoHistine-Proline, CHP) while its deamidation results in the formation of the free acid, that is, TRH-OH (Khomane et al., 2011).

TRHR

The TRHR is a member of a large family of transmembrane proteins (G-protein-coupled receptors, GPCRs) for which an interaction with an intracellular G protein is a critical part of the signal transduction pathway mediated by the receptor. It is thought that all GPCRs have a common tertiary structure composed of seven transmembrane helices. The topology of these membrane-bound proteins is defined by an extracellular amino terminus and an intracellular COOH terminus. Consequently, the helices are connected by intracellular-extracellular loops. Colson et al. (1998) suggested that TRH initially interacts with residues within the extracellular loops and then moves into the transmembrane binding pocket, aided by the motion of the loops.

A single TRHR gene has been found in humans and higher mammals and two genes encoding homologous receptors, TRHR1 and TRHR2, in rodents (Sun et al., 2003). TRHR1 predominates in the anterior pituitary gland while both TRHR1 and TRHR2 are found in rodent central nervous system (CNS) (Hinkle et al., 2012).

EXTRAHYPOTHALAMIC AND EXTRAPITUITARY LOCATION OF TRH AND TRHR RESPECTIVELY

Originally discovered in the hypothalamus, consistent with its classical role as a hypothalamic hypophysiotrophic factor, TRH and TRHR are now known to be distributed throughout the central and peripheral nervous system (PNS) and are found extensively in extrahypothalamic brain structures and in other organs and tissues (Sun et al., 2003; Kamath et al., 2009).

TRH OUTSIDE THE PITUITARY ZONE

TRH has been located in several brain areas other than the paraventricular nucleus as well as in non-CNS tissues:

- Pancreatic β-cells (Engler et al., 1981; Kawano et al., 1983).
- The whole gastrointestinal tract where it may modulate contractions (Morley, 1979; Engler et al., 1981).
- The genitourinary system. TRH is found in rat, canine and human prostate, seminal vesicles, ovary, testis, Leydig cells and epididymis (Pekary et al., 1980, 1983a,b; Feng et al., 1992).
- The presence of TRH in rat, human, porcine and bovine retina has been reported (Schaeffer et al., 1977; Martino et al., 1980a,b).
- Shambaugh et al. (1979) identified TRH activity in placental extracts by specific radioimmunoassay test (RIA).
- TRH has been found as part of immune system tissues and blood elements (Matre et al., 2003). This topic will be discussed in depth in the following sections.

TRHR OUTSIDE THE HYPOTHALAMIC ZONE

Besides the well-known TRHR location in the pituitary zone, TRH binding sites have been described throughout the central and PNS and in non-CNS tissues including:

- Uterus, ovary and testis where TRHR mRNA levels appear to be relatively high (Fukusumi et al., 1995; Montagne et al., 1999).
- Wang et al. (1997) reported that small intestine epithelial cells were found to express receptors for TRH and to be a primary source of intestine-derived TSH.
- TRHR is also found in retina (Satoh et al., 1993) and adrenal gland (Montagne et al., 1999).

Furthermore, TRHR expression in organs and cells of the immune system has been confirmed:

- Expression of TRHR mRNA and TRHR in lymphoid tissues has been found: Thymus, mesenteric lymph nodes and spleen (Raiden et al., 1995; Mellado et al., 1999).
- Thymus expression of the TRHR has been reported by several authors (Fukusumi et al., 1995; Mellado et al., 1999; Montagne et al., 1999; Matre et al., 2003).
- Expression analysis in rat demonstrated the presence of TRHR mRNA in bone marrow (Montagne et al., 1999; Matre et al., 2003) and in the rat natural-killer cell line RNK-16 (Matre et al., 2003). This expression was shown to be functional as evidenced by TRH binding to surface receptors on thymocytes and RNK-16 cells.
- Analysis of human peripheral blood lymphocyte (Raiden et al., 1995; Mellado et al., 1999) and tonsil-derived leukocyte populations showed TRHR expression in non-activated and phytohemagglutinin-activated T and B cells (Mellado et al., 1999).

The widespread distribution of TRH and the TRHR within and outside the CNS supports a diverse range of roles for this molecule, roles likely to involve many functions outside of the traditional hypothalamic-pituitary-thyroid (HPT) axis (Nillni and Sevarino, 1999).

TRH FUNCTIONS

TRH indirectly stimulates TH biosynthesis and release. Thus, TRH is central in regulating the HPT axis. TRH influences the release of other hormones, including prolactin, growth hormone, vasopressin, insulin, and the classic neurotransmitters noradrenaline and adrenaline (Nillni and Sevarino, 1999).

EXTRAHYPOPHYSIOTROPIC FUNCTIONS OF TRH

More than two-thirds of immunoreactive-TRH in the brain is found outside of the traditional "thyrotrophic zone" of the hypothalamus. This extrahypophysiotropic TRH is believed to function as a neuromodulator of known neurotransmitters. Indeed, it might act as a neurotransmitter itself; it is present in secretory granules whose exocytosis is responsive to membrane depolarization, it acts through specific receptors that are widely distributed throughout the CNS, and it is rapidly cleared through specific catabolic pathways. In several areas of the brain, TRH is colocalized with other neurotransmitters and/or neuromodulators (Nillni and Sevarino, 1999).

These effects of TRH have been demonstrated using TRH and/or TRH analogs. Available TRH analogs have higher affinities for the TRHR, longer half-lives, etc. (Engel et al., 2006; Khomane et al., 2011; Monga et al., 2011).

Beside endocrine functions, TRH also possesses several neuropharmacological effects like cerebral nerve activation (stimulation of motor function), effects on sympathetic nervous system (elevation of blood pressure and stimulation of respiratory), effects on spinal function (stimulation of spinal motor neuron), effects on CNS (antidepressant activity) and peripheral actions (suppression of gastric acid secretion, stimulation of glucagon secretion). These CNS-mediated effects provide the rationale for the use of TRH in the treatment of brain/spinal injury and certain CNS disorders, including schizophrenia, Alzheimer's disease, epilepsy, amyotrophic lateral sclerosis, Parkinson's disease and depression (Khomane et al., 2011).

TRH AND IMMUNE SYSTEM

Two-way communication between the immune and neuroendocrine systems is now well recognized. These interactions are mediated by cytokines, neuropeptides, hormones, other soluble factors and their receptors (Grasso et al., 1998).

As mentioned above, cells of the immune system have been found to contain receptors for neuroendocrine hormones and can also be considered a source of pituitary and hypothalamic peptides (Matre et al., 2003).

IMMUNE RESPONSE TO TRH ADMINISTRATION

Proliferation

Induction. Acute and chronic administration of TRH enhances the proliferation of splenic and thymic lymphocytes in rats (Pawlikowski et al., 1992; Raiden et al., 1995; Winczyk and Pawlikowski, 2000). In fact, it has been reported that TRH promotes thymic reconstitution in mice with anterior hypothalamic area lesions (Lesnikov et al., 1991). According with these data, it was also reported that TRH enhances the *in vitro* plaque-forming cell response (Kruger et al., 1989), antagonizes the dexometasone-induced thymocyte and splenocyte suppression (Winczyk and Pawlikowski, 2000) and administration in humans led to increased secretion of IL-2 into the blood (Komorowski et al., 1993).

Suppression. It has also been reported that TRH significantly inhibits monocyte activity (Lersch et al., 1989). The experimental work of Kunert-Radek et al. (1991) addressed the proliferation of murine splenocytes *in vitro* considering the ^{3}H-thymidine incorporation into splenocyte DNA as an index of proliferation. They found that TRH suppressed the proliferation of splenocytes.

TRH has the capability to modulate the natural cell-mediated cytotoxicity. To try it out, TRH was added to the feed of White Leghorn male chicks and peripheral blood lymphocytes were cultured *in vitro* with or without different mitogens (Phytohaemagglutinin-A (PHA), ConA, or Lipopolysaccharide (LPS)), and the culture supernatants were tested for the presence of lymphokines. Results showed that the supernatant from *in vivo* 5 ppm TRH-treated lymphocytes significantly suppressed the natural cell-mediated cytotoxicity (Haddad and Mashaly, 1992).

Recently was published that oral administration of TRH in mice with experimental autoimmune encephalomyelitis (EAE), an animal model of multiple sclerosis, reduces the spinal cord inflammatory foci without increased frequency of regulatory T cells (Treg) in spleen (Brod and Bauer, 2013).

Induces TSH production by immune cells

At very low concentrations, TRH induces splenocyte production of TSH *in vitro* (Kruger et al., 1989).

Peripheral blood mononuclear cells (PBMC), rat splenocytes and transformed T cell lines produce TSH in response to TRH (Harbour et al., 1989; Raiden et al., 1995).

Induces immune mediators production

PBMC, rat splenocytes and transformed T cell lines can enhance or modulate the *in vitro* antibody response in response to TRH (Kruger et al., 1989; Hart et al., 1990).

Koshida and Kotake (1993) investigated the role of TRH on the superoxide anion (O_2^-) production of rabbit peritoneal macrophages. Their results showed that TRH acts on macrophages and suggest that TRH possesses the priming action of O_2^- release in response to the chemotactic peptide N-formyl-methionyl-leucyl-phenylalanine, and opsonized zymosan. Authors suggest that if this type of priming exists *in vivo* there should be a role to play in an inflammation process.

In vitro studies have shown that a TRH fixation occurs at the level of the human polymorphonuclear neutrophil, which suggests putative membrane receptor (s) for the hypothalamic hormone. The correspondent *in vivo* analyses demonstrated that after TRH administration, enzyme modifications (myeloperoxydase, alkaline phosphatase) and metabolism changes (PAS, Sudan black) happen, showing a functional activation of that blood cell (Blum et al., 1976). These *in vivo* effects could be due to TRH is being directly attached to its receptor on neutrophils or an indirect effect is being mediated by TSH or THs.

Intravenous bolus of TRH in normoprolactinemic women increases plasma gamma interferon (IFN-γ) levels 45 mins after administration. In order to investigate a possible direct action of TRH on immune cells, the authors also examined the effects of this hypothalamic peptide on IFN-γ production by PBMCs stimulated *in vitro* with suboptimal concentration of bacterial superantigen staphylococcal enterotoxin A or concanavalin A (ConA), collected before the intravenous administration of TRH. The *in vitro* findings showed a directly enhanced IFN-γ production by activated lymphocytes (Grasso et al., 1998).

Furthermore, splenic and CNS lymphocytes showed significant decrease in levels of profile 17 helper t cells (T_H17) and T_H1 cytokines: Interleukin-17 (IL-17), tumor necrosis factor (TNF-α) and IL-2 respectively, after oral administration of TRH in EAE mice. This treatment also increased CNS lymphocyte production of T_H2 cytokines, in particular IL-13 (Brod and Bauer, 2013). Thus, oral TRH shows a unique pattern of immunomodulation for an ingested neuropeptide although the poor oral bioavailability associated with TRH and some of its analogs (Khomane et al., 2011).

TRH expression response against immune system challenges

It is known that high concentrations of TRH seem to decrease the production of antibodies against sheep red blood cells (SRBC) (Kukain et al., 1982).

Pérez Castro et al. (1999) investigated whether TRH mRNA levels in the hypothalamus are altered after SRBC (a T cell-dependent antigen) immunization. An increased level of TRH mRNA detected by Northern blot autoradiography was observed at 4, 6, and 24 h post immunization in SRBC-injected animals.

The SRBC-induced effects on TRH mRNA levels are opposite to those induced by LPS, a T cell-independent response through macrophage and B lymphocyte activation. LPS induces a decrease in hypothalamic TRH mRNA levels that is observed at 24 h and more pronounced at 4 h (Pérez Castro et al., 1999).

As we can see, TRH effects could seem controversial but is important to consider that interactions between the CNS, the endocrine system and the immune system are mediated at multiple levels. These mediators include secreted chemical messengers such as hormones, cytokines, neurotransmitters and neuropeptides acting directly or via the nervous system.

Evidence indicates interactions at the level of receptors (e.g., the presence of neuroendocrine peptide receptors on immune cells), at the level of secretory function (e.g., the synthesis and secretion of neuroendocrine peptides by immune cells), and at the level of signal transduction (Kamath et al., 2009). Integration of these complex interactions in a single route would be inaccurate, for this reason, the most wise conclusion could be consider the TRH-immune system homeostatic hypothesis proposed by Gary et al. (2003) and Kamath et al. (2009), which states that TRH mediated mechanisms respond to many elements of the immune system and affect them in ways that tend to maintain or restore homeostasis.

The effects of TRH administration on immune system are summarized in **Figure 1**.

CLINICAL RELEVANCE AND FUTURE DIRECTIONS

Studies on the effects of TRH *in vitro* hardly can be extrapolated to *in vivo* conditions. The direct effect of TRH on the immune system provides a modulatory physiological role. However, pharmacological administration in humans and in experimental animals involves two pathways impact (1) through stimulation of TSH secretion and therefore of THs and (2) the direct effect on the immune system. TRH administration may affect these two ways and with addition or synergy effects. One possibility to explore the direct mode might be using hypophysectomized or thyroidectomized animals to avoid endocrine influence. According to the studies described above, it is possible to consider the possibility that clinical treatment with TRH may improve the immune response in patients with immune deficiencies or reduced response in autoimmune diseases such as multiple sclerosis or lupus erythematosus. Such expectations are a challenge to be explored in the future.

CORTICOTROPIN-RELEASING HORMONE (CRH)

CRH or Corticotropin-releasing factor (CRF) was characterized, purified and synthesized in 1981 by Vale. The composition of this 41 amino-acid peptide is different from one species to another. Its principal origin in the brain are the neurons from paraventricular nucleus of the hypothalamus. By the portal hypothalamic-pituitary vessels, it reaches the anterior pituitary lobe where it stimulates the corticotroph cells (Raux-Demay and Girard, 1985; Emeric-Sauval, 1986). The plasma half-life of CRH in humans is 4 mins (Schürmeyer et al., 1984). The action of CRH on adrenocorticotrophic hormone (ACTH) secretion is predominant, but it acts in relation with arginine-vasopressin and potentially with angiotensin and catecholamines. Adrenal steroids (glucocorticoids) act on feedback control of CRH secretion in the hypothalamus and also on pituitary corticotrophs. CRH has shown to be an important regulator of the endocrine stress response, and it is now known to play a role in diverse functions in the homeostatic balance, important in mobilization of resources and behaviors during stress (Bale and Vale, 2004), it is involved in the regulation of food intake and satiety, as well as gastrointestinal tract motility, vascular tone, and development, and also acoustic and cardiac function (Heinrichs et al., 1992; Koob and Heinrichs, 1999; Maillot et al., 2000; Inoue et al., 2003; Janssen and Kozicz, 2013).

CRH RECEPTOR (CRHR)

High-affinity CRHRs which mediate the actions of the hypothalamic peptide on ACTH release, have been principal identified in the anterior pituitary gland. Interaction of the pituitary receptor by CRH agonists stimulates ACTH release. CRH exerts its effects by binding to specific cell surface receptors, of which two receptor subtypes have been reported, CRH-R1 and CRH-R2. These receptors share high sequence homology and belong to the family of seven transmembrane GPCRs (Vaughan et al., 1995; Lewis et al., 2001).

Actions of this peptide are initiated by binding and activating GPCRs and subsequently the activation of adenylate cyclase and cyclic adenosine monophosphate dependent protein kinase (Grammatopoulos and Chrousos, 2002). In the regulation of ACTH secretion, the effects of CRH on the corticotroph are integrated with the stimulatory actions of cyclic adenosine monophosphate and the inhibitory effects of glucocorticoids. Following adrenalectomy, the progressive elevation of plasma ACTH levels is accompanied by a concomitant decrease in pituitary CRHRs (down-regulation) (Wynn et al., 1985; Aguilera et al., 1986).

Extrapituitary expression of CRHR

CRH-R1 is a 415 amino acid peptide and has a widespread expression in stress-related areas located in the CNS (Justice et al., 2008; Kuhne et al., 2012). Outside the CNS, the CRH-R1 is expressed in the adrenal gland, gastrointestinal tissue, ovary, testis, skin, thymus and spleen (Dufau et al., 1993; Nappi and Rivest, 1995; Slominski et al., 1995; Baigent and Lowry, 2000; Müller et al., 2001; Chatzaki et al., 2004). CRH-R2 is a 397–437 amino acid protein and it is abundantly expressed in the CNS (Korosi et al., 2007; Justice et al., 2008; Kuhne et al., 2012). In the periphery, the receptor has been identified in the retina, heart, skeletal muscle, vasculature, adrenal gland, and gastrointestinal tissue (Lovenberg et al., 1995; Müller et al., 2001; Chatzaki et al., 2004).

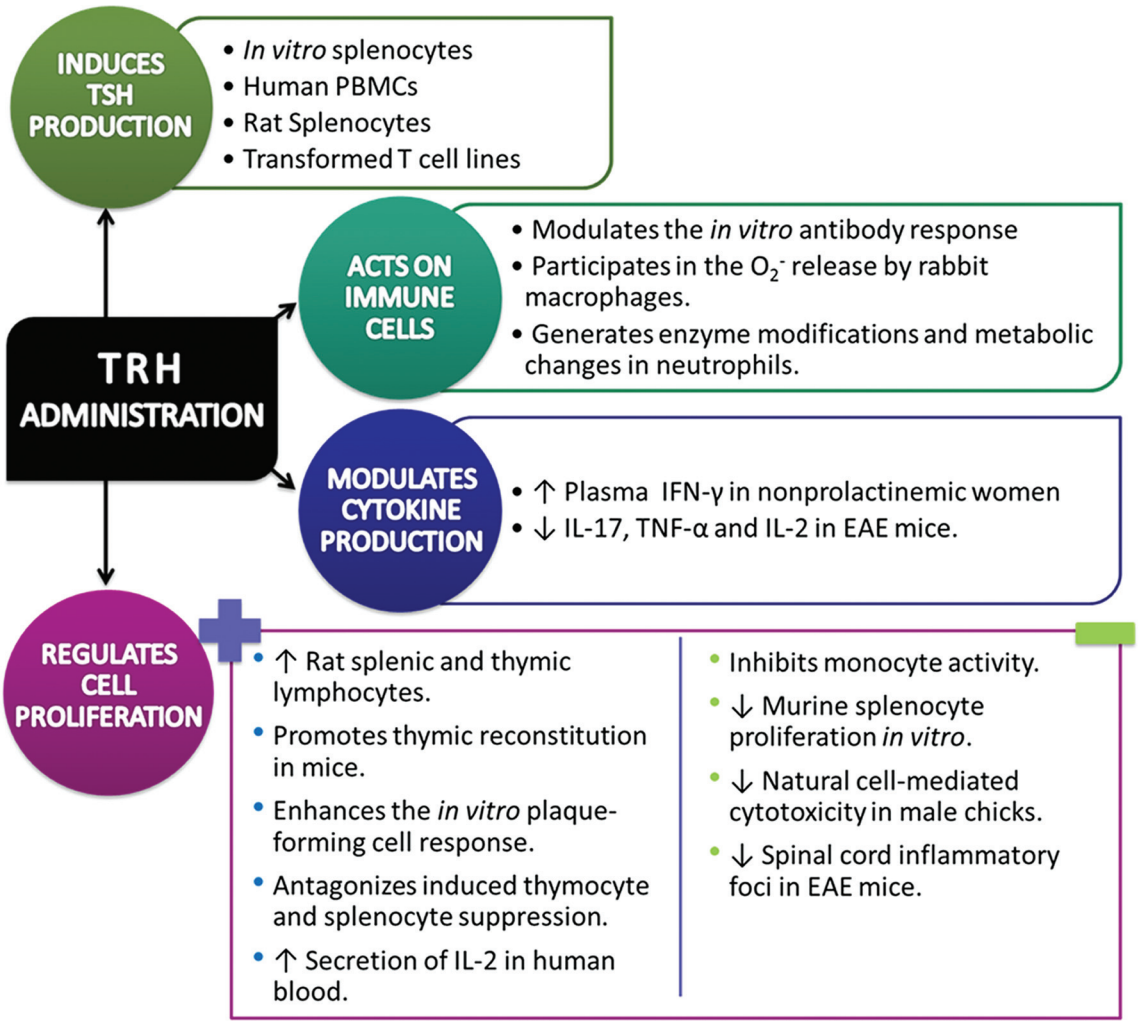

FIGURE 1 | Immune response against Thyrotropin-Releasing Hormone/Thyrotropin-Releasing Hormone agonist administration in a diversity of normal and pathological conditions (↑ = increase, ↓ = decrease).

Binding sites for CRH have been reported on adherent splenocytes (Webster et al., 1990), monocyte-macrophages (Audhya et al., 1991) and T lymphocytes (Singh and Fudenberg, 1988). CRH-R1 and CRH-R2 expression was described in granulocytes and lymphocytes in inflamed tissues (Mousa et al., 2003). CRH-R1 mRNA expression had been reported in the human leukemic mast cell line (Theoharides et al., 1998) and mast cells were reported to express immunoreactive CRH-R1 in inflamed human synovial tissue (McEvoy et al., 2001).

CRH EFFECTS ON CNS AND PNS

Independently of anterior pituitary, CRH has effects within of nervous system. The central administration of CRH produces hypertension, tachycardia and an elevated oxygen consumption. CRF acts within the brain to increase plasma concentrations of noradrenaline and adrenaline resulting in increased plasma concentrations of glucagon and in hyperglycemia. Likewise, also acts increasing the plasma concentrations of vasopressin. CRH induces a reduction in food intake and increases grooming behavior and locomotor activity. The physiological significance of the peripheral actions of CRH on various organ systems is unknown. Intravenous administration of CRH reduces gastric acid secretion, gastric emptying and blood pressure but increases heart rate, venous return to the heart, mesenteric and aortic blood flow and pancreatic bicarbonate and protein secretions (Lenz, 1987; Chen et al., 2007).

CRH AND IMMUNE SYSTEM

The cellular components of the immune-inflammatory response include leukocytes, such as monocytes–macrophages, polymorphonuclear neutrophils, eosinophils and basophils, platelets, dentritic cells, mast cells, epithelial and endothelial cells, and fibroblasts which belong to the innate immune system, as well as lymphocytes T, B and natural killer (NK) which are included in the adaptive immune system. These cells cooperate using molecular signals, including ILs, colony-stimulating factors, TNF, IFNs,

transforming growth factor, and chemokines, vasoactive amines (histamine and serotonine), plasma proteases (kinine and complement systems), arachidonic acid metabolites (prostaglandins, leukotrienes, and lipoxins), platelet-activating factor, nitric oxide, and neuropeptides (Mastorakos et al., 2006).

The majority of *in vivo* studies suggest that direct stimulation of hypothalamic CRH by cytokines is the principal route by which immune activation stimulates the hypothalamic–pituitary–adrenal (HPA) axis (Besedovsky et al., 1986; Harbuz et al., 1992) and this process is prostaglandin-dependent. Thus, experimental evidence suggests that CRH may modulate the immune and inflammatory responses via two pathways: an anti-inflammatory one operated by centrally released CRH, most likely through stimulation of glucocorticoid and catecholamine release, and one pro-inflammatory, through direct action of peripherally secreted CRH (Karalis et al., 1997).

CRH in immune cells

Mast cells. Mast cells are critical for allergic reactions due to their secretion of numerous vasoactive molecules and cytokines (Kobayashi et al., 2000). Evidence indicates that mast cells are also involved in innate and acquired immunity (Marone et al., 2002), as well as in neuroinflammatory conditions such as those affected by stress (Theoharides and Cochrane, 2004). Mast cells secrete numerous pro-inflammatory cytokines, such as IL-6, IL-8, and TNF-α (Kobayashi et al., 2000; Marone et al., 2002), and secretion of vascular endothelial growth factor (VEGF) by CRH stimulation (Cao et al., 2005). Interestingly, human mast cells were shown to synthesize and secrete large amounts of CRH, suggesting that this peptide could regulate mast cells through CRH receptor in an autocrine manner. Secretion of CRH from mast cells could also be triggered by pro-inflammatory molecules that are released during the initial phases of inflammation (Kempuraj et al., 2004). CRH causes mast cell degranulation in human skin, releasing great amounts of histamine, which appears to be the principal mediator of the vasodilatory effects of CRH (Wright, 2003).

Mononuclear cells. In mononuclear cells, it has been reported that CRH stimulates immune functions, proliferation of lymphocytes both in the absence and presence of T cell mitogens, and the expression of IL-2R antigen on T cells (Singh, 1989; Singh et al., 1990). It has also been reported the presence of binding sites for CRH on monocytes, T and B lymphocytes and thymus (Singh and Fudenberg, 1988). The CRH is also a stimulator of production of two interleukins, IL-1 and IL-2. IL-1 has been shown to stimulate the secretion of CRH by hypothalamic cells (Sapolsky et al., 1987) and CRH stimulates the production of IL-1 and IL-2, suggesting the presence of a complete network between the neuroendocrine system and the immune system (Singh and Leu, 1990).

The release of CRH plays a role in modulating NK activity following stress also implies that CRH in the brain may be involved in the functional population of lymphocytes. Studies have been conducted to analyze the effect of CRF on the reduction of several cellular immune measures such as splenic and peripheral blood NK activity and lymphocyte responses to mitogenic stimulation (Strausbaug and Irwin, 1992; Boyadjieva et al., 2006). Other observations also indicate that central doses of CRH slow the kinetics and reduce the level of IgG response to a specific T-cell-dependent antigen (Irwin, 1993). The administration of central CRH antagonist induces suppression of NK cytotoxicity. In contrast, peripheral application has no effect on CRH modulation of immunity (Irwin et al., 1987).

Other studies have shown CRH to inhibit IFN-γ secretion (Angioni et al., 1993). IL-2-induced splenocyte proliferation as well as LPS-mediated IL-1 and IL-6 production by peripheral blood monocytes (Hagan et al., 1992).

The effects of CRH were almost exclusively examined in the mononuclear cells, however in other studies has been reported that polymorphonuclear cells (neutrophils), express higher levels of CRH-R1 than mononuclear immune cells in mouse (Radulovic et al., 1999). It has been shown that CRH inhibits the secretion of IL-1β from mature neutrophils purified from spleens of mice injected with LPS (Radulovic and Spiess, 2001).

It has been described the CRH presence in macrophages (Brouxhon et al., 1998). Results showed that CRH could be detected in normal peritoneal macrophages and the levels increased significantly after stimulation with LPS (Wang et al., 2012). The role of CRH may be immunomodulatory, however the effects remains to be studied.

On other hand, studies shown that cluster of differentiation (CD14+) group of cell surface marker proteins, the CRH can activate CD14+ cells to produce TNF-α cells and compromise endothelial barrier function by inducing apoptosis of the endothelial cells (Song et al., 2013).

CRH and its relationship to immune therapies

Drugs have been proposed to either block CRHR-1 and CRHR-2 receptors as CRH inhibitors with therapeutic properties through modifying the immune response as tool for both CNS and inflammatory disorders associated with central and peripheral CRH. CRH-R1 antagonist could be considered for the treatment of allergic conditions, such as urticaria, asthma, eczema, or disorders that increase blood–brain barrier permeability (Theoharides et al., 1998; Esposito et al., 2002), chronic inflammatory bowel syndromes, irritable bowel disease, and ulcerative colitis and other pathologies (Kawahito et al., 1995; Gravanis and Margioris, 2005).

The effects of CRH administration on immune system are summarized in **Figure 2**.

Future directions

There are two major problems associated with the HPA axis. The inflammatory process and the reduction in the immune response. Chronic exposure to antiinflammatory steroids have effects such as immunosuppression which under certain conditions can cause undesired effect. Identifying specific receptor subtypes to CRH, or applying interleukin receptor blockers may provide a viable alternative to maintaining of the homeostatic mechanisms.

Emotional stress causes somatic alterations including the immune response and is perhaps, one of the most pressing problems in global public health that needs to be addressed.

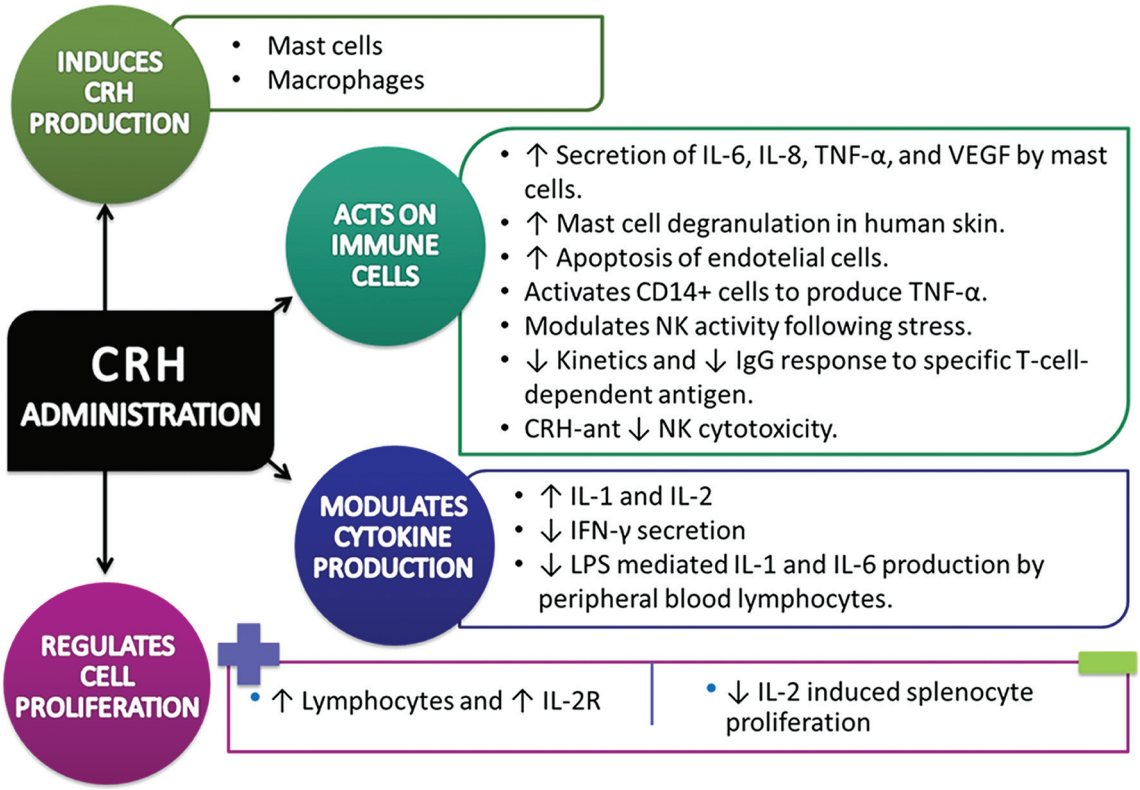

FIGURE 2 | Experimental data of Corticotropin-Releasing Hormone/Corticotropin-Releasing Hormone agonist impact on immune system cells and processes (↑ = increase, ↓ = decrease).

GONADOTROPIN-RELEASING HORMONE (GnRH)

GnRH, also known as luteinizing hormone releasing hormone (LHRH), is a small peptide hormone (pGlu-His-Trp-Ser-Tyr-Gly-Leu-Arg-Pro-Gly-NH_2) of 1.2 kDa, with an established role as central regulator of the neuroendocrine reproductive axis in both males and females (Cheung and Wong, 2008).

This neuropeptide is mainly synthesized in hypothalamic neurons of the medial preoptic area. It is well established that adenohypophysial gonadotropic cells stimulate the synthesis and secretion of gonadotropic hormones such as follicle stimulating hormone (FSH) and luteinizing hormone (LH) (Cheng and Leung, 2005). LH and FSH are released into the systemic circulation in a pulsatile pattern, which is dependent upon the GnRH pulse frequency, and act to affect gonadal steroidogenesis and gametogenesis (Sasaki and Norwitz, 2011).

GnRH ISOFORMS

At the present, it is known that, in addition to the hypothalamic GnRH, several other isoforms of the decapeptide exist in vertebrates. All these isoforms are characterized by the conservation of the length of the peptide as well as by the amino acid sequences of the N-terminal (Glp-His-Trp-Ser) and C-terminal (Pro-Gly-NH_2) domains, indicating that these molecular features are critical for the binding to, and the activation of, the receptor (Limonta and Manea, 2013). The classical hypophysiotropic GnRH located in the anterior hypothalamus/preoptic area is also designed as GnRH-I (Stevenson et al., 2012).

A second isoform of GnRH, originally isolated from the chicken hypothalamus and referred to as chicken GnRH or cGnRH, has been isolated in most vertebrate species—including humans—is now referred to as GnRH-II and is 70% homologous to GnRH-I (Sasaki and Norwitz, 2011). GnRH-II is expressed at a higher level outside of the brain (Tan and Bukulmez, 2011).

A third GnRH isoform, GnRH-III, was isolated from sea lamprey and was also detected in the brain of mammals (Tan and Bukulmez, 2011). This isoform has 60% homology with GnRH-I. In the lamprey, GnRH-III is localized in brain areas involved in the control of reproductive functions and it has been reported to stimulate steroidogenesis and gametogenesis, but it has 500–1000 times less potency in LH and FSH secretion both *in vitro* and *in vivo* (Limonta and Manea, 2013).

GnRH is small and has good thermal and chemical stability and no internal Cys bridges (minimal fixed structure). Its size and stability has led to the availability of thousands of analogs (Conn and Janovick, 2009).

SYNTHESIS AND SECRETION

GnRH is synthesized as a prohormone, human preproGnRH that contains 92 amino acids (Harrison et al., 2004). The gene encoding preproGnRH1 gene has been cloned in number of species and

is approximately 4300-bp with four relatively short exons separated by three large introns (Clarke and Pompolo, 2005).

Neurons that synthesize and release GnRH (GnRHergic neurons) have their origin in embryonic olfactory placodes. These embryonic cells, migrate and colonize the basal forebrain, preoptic area around the basal and medium hypothalamus. The median eminence in mammals contains a large amount of GnRH, so is the area in which the peptide is stored in neural terminals before release into the hypothalamus-pituitary portal system (Clarke and Pompolo, 2005).

GnRH is released as synchronized pulses from the nerve endings of about 1000 neurons into the hypophyseal portal system every 30–120 mins to stimulate the biosynthesis and secretion of LH and FSH from gonadotrophs (Millar, 2005). In adult rodents, GnRH is released from the median eminence at a frequency of about one pulse every 30 mins. A slightly slower frequency of release (approximately 50–60 mins intervals) is observed in primates (Yin and Gore, 2006). On the other hand, continuous (non-pulsatile) GnRH administration or long-lasting GnRH-a desensitize GnRHR, thereby decreasing/inhibiting the release of LH and FSH by the pituitary (Wilson et al., 2007).

HALF-LIFE

GnRH half-life normally is about 2–4 mins (Walters et al., 2008). Its short half-life is because of the rapid cleavage of the bonds between amino acids at positions 5–6, 6–7, and 9–10. Substitution at position 6 in GnRH agonists (GnRH-a) yields them resistant to degradation and increases its half-life and the time of receptor occupancy (Magon, 2011).

GnRH RECEPTOR (GnRHR)

The human pituitary GnRHR belongs to the family of GPCRs and is one of the smallest GPCRs (328 amino acid protein in the human); it may contain only the essentials required for ligand binding and signal transduction (Conn and Janovick, 2009).

Distinct forms of the GnRHR can be broadly divided into two groups:

1. The type I GnRH-Rs have greater affinity for GnRH-I than for GnRH-II and lacks a typical intracellular COOH terminus; this group contains all known mammalian GnRH-Rs except for the recently described primate type II GnRH-R (McArdle et al., 2002; Ciechanowska et al., 2010).
2. Type II GnRH-Rs have greater affinity for the GnRH-II and possess C-terminal tails of varying length; this group contains all the known receptors from non-mammalian vertebrates (catfish, goldfish, chicken, goldfish, *Xenopus*, etc.) in addition to the type II primate GnRH-Rs (McArdle et al., 2002).

EXTRAHYPOTHALAMIC AND EXTRAPITUITARY LOCATION OF GnRH AND GnRHR RESPECTIVELY

GnRH outside the pituitary zone

GnRH has been located in several extrahypothalamic cells and tissues: GnRH-like substances have been reported to be secreted from tissues outside the CNS of rats and humans. Particularly, a (GnRH)-like protein has been detected in human ovaries (Aten et al., 1987; Dong et al., 1993; Peng et al., 1994). Also, Oikawa demonstrated by PCR amplification of cDNA from rat hypothalamus, granulosa cells, and whole ovary, a 241-bp product identical to the authentic GnRH sequence based on analysis on both strands (Oikawa et al., 1990).

RT-PCR analysis together with Southern blot analysis demonstrated the presence of GnRH mRNA in human testes and mammary gland (Dong et al., 1993).

Furthermore, the gene coding for GnRH is expressed in immune tissue and immunoreactive GnRH is measurable in immune cells. Analysis of mononuclear cells by RT-PCR coupled to Southern hybridization of total RNA confirmed that GnRH genes are expressed in human peripheral blood lymphocytes. The GnRH transcripts in mononuclear cells were identical to the hypothalamic GnRH and PRL release inhibiting factor cDNA, at least within this 380-bp fragment (Chen et al., 1999).

Rat splenocytes contain an immunoactive and bioactive GnRH (Emanuele et al., 1990; Azad et al., 1991) that has also been found in lymphocytes from rat thymus (Marchetti et al., 1990; Maier et al., 1992). Moreover, it has been reported that normal and leukemic human T cells produce GnRH-II and GnRH-I and that the incubation of those cells with these neuropeptides triggered *de novo* gene transcription and cell-surface expression of a 67-kDa non-integrin laminin receptor that is involved in cellular adhesion and migration and in tumor invasion and metastasis (Chen et al., 2002).

The fact that recent evidence shows the existence of diverse isoforms of GnRH, with high sequence homology and a core variable region raises the issue that previous GnRH distribution studies may have identified a variety of isoforms. To confirm the distribution of GnRH-I only, Khan et al. (2003), reported that intense GnRH-I immunoreactivity was found in Kupffer cells, and less intense in spleen lymphocytes and follicular dendritic cells in rat.

Other cells that have been reported to express GnRH and its mRNA are mast cells from the nervous system and the peritoneal cavity, as detected by immunohistochemistry and RT-PCR, respectively (Silverman et al., 1994; Gill and Rissman, 1998; Khalil et al., 2003).

Extrapituitary GnRHR

GnRH binding sites have been described throughout the CNS and PNS as in several non-CNS cells and tissues:

Human ovarian granulosa-luteal cells also express the GnRHR mRNA (Chen et al., 1999).

GnRHR expression in organs and cells of the immune system has been confirmed: GnRH binding sites have been reported in rat spleen (Batticane et al., 1991) and thymus, thus potentially enabling GnRH-a to have direct stimulatory effects that might contribute to improved T-cell function (Marchetti et al., 1989; Batticane et al., 1991; Morale et al., 1991).

RT-PCR coupled to Southern hybridization of total RNA obtained from mononuclear cells confirmed that the GnRHR is expressed in human peripheral blood lymphocytes (Chen et al., 1999) as in porcine lymphocytes (Standaert et al., 1992).

Chen and colleagues reported that the GnRHR transcript expressed in mononuclear cells has an identical nucleotide sequence as the pituitary GnRHR cDNA. In this work, a time-course study of the effect of GnRH on mononuclear cells was performed and it was found that GnRHR mRNA increased gradually after *in vitro* culture, and this increase was further augmented by treatment with GnRH for up to 24h. In contrast,

the expression of GnRH mRNA was decreased by GnRH treatment in a dose-dependent manner and the IL-2Rγ subunit gene was highly expressed in mononuclear cells. At this point we can speculate that these GnRH-binding sites in lymphocytes are probably functional, as many studies have demonstrated that GnRH-a caused a dose-dependent increase in LH production, whereas GnRHR antibodies blocked this action (Chen et al., 1999).

It has also been shown that blockade of central and peripheral GnRHR with a potent GnRH antagonist (GnRH-ant) during a critical period of maturation can impair the morphological development of thymus and the cellular and humoral immune responses in rats (Morale et al., 1991) and monkeys (Mann et al., 1994).

Particularly, neonatal exposure of male primates to a GnRH-ant alters early postnatal programming of immune function by reducing the number of B cells and T cells in the thymic medulla and T cells in the periarterial lymphatic sheaths of the spleen. GnRH-ant treatment increased the frequency of clinical problems, lowered circulating levels of lymphocytes, total T cells, CD8+ T cells and B cells, and altered the proliferative index of lymphocytes to mitogens. As adults, the cell- and humorally-mediated immune responses remained impaired (Mann et al., 2000).

To evaluate regions of GnRH-I binding activity, a biotinylated GnRH-I sequence was used with avidin-labelled HRP, and results showed GnRH-I binding activity present in Kupffer cells, spleen lymphocytes and follicular dendritic cells, as well as in thymocytes and peripheral blood lymphocytes and neutrophils (Khan et al., 2003).

GnRH AND IMMUNE SYSTEM

GnRH and sex steroids seem to be important components of immune system modulation and may play a role in the regulation of immune mediated diseases. A comprehensive description of such interactions has been reviewed by Tanriverdi et al. (2003) and Quintanar (2011).

Immune response to GnRH administration

Proliferation

Induction. When splenocytes and thymocytes are preincubated with GnRH-a or GnRH peptide, an augmentation of the proliferative response is obtained. Marchetti et al. (1989) showed a significant dose-dependent increase on proliferative response to the mitogen ConA in rat thymocytes. This response was abolished with simultaneous addition of a GnRH-ant. Also, splenocytes and thymocytes from proestrus female rats incubated with GnRH or its analogs, significantly increased the basal proliferative activity which was accompanied by a specific increase in IL-2 receptor-positive cells (Batticane et al., 1991).

Furthermore, *in vivo* GnRH-a treatment prevents thymus atrophy and markedly stimulates thymocyte blastogenic activity in hypophysectomized rats, thus suggesting that GnRH and its agonistic analogs are may capable to exert a direct modulation of immune system function (Marchetti et al., 1989).

Injection of GnRH-a increases CD8+ T-cell levels and proliferation to a variety of mitogens (Mann et al., 1994). In another study, it has been reported that GnRH immunization in rats induced the presence of eosinophils in intertubular tissue of testes whereas in unimmunized controls these cells were not present (Duckett et al., 1997).

Moreover, in a rat model of pregnancy-induced thymic involution, GnRH-a infusions markedly attenuated pregnancy-induced thymic involution resulting in significant increases in thymic weight and thymocyte numbers (Dixit et al., 2003a).

In the same way, Sutherland et al. (2008) made a pilot study in patients, where a GnRH-a (goserelin) was given prior to allogeneic or autologous hematopoietic stem cell transplantation. GnRH-a administration significantly increased neutrophil and lymphocyte numbers within the first month of posttransplantation indicating that either directly or indirectly, GnRH induced an increase in circulating neutrophils.

It has been reported that treatment with the GnRH-a, leuprolide acetate (50 μg/mouse, s.c.) prior to restraint stress, significantly prevented its effect on cell-mediated immunity, antibody titer levels, total leukocyte count and relative thymus weight, showing a significant increase of these immunological parameters (Umathe et al., 2008).

Supression. To test whether a GnRH-a could alter *in vivo* human immune cells, Ho et al. (1995) treated a group of infertile patients under a protocol of buserelin acetate administration and peripheral B cells, NK cells, CD4+ and CD8+ T cells, and the expression of CD69, CD25, human leucocyte antigen-DR (HLA-DR), and CD71 antigens on the T cells were serially examined by dual-color flow cytometry. They found that the GnRH-a had a transiently immunosuppressive effect on CD4+ and CD25+ T cells, but CD69+, CD25+, and HLA-DR+ T cells were activated during and after successful implantation. Regarding to the B cells, NK cells, CD8+ T cells, and CD71+ T lymphocyte subpopulations there were no changes throughout the whole course of treatment.

On the other hand, several groups have provided evidence that the *in vitro* proliferation of a variety of human ovarian, endometrial, and breast cancer cell lines can be inhibited by agonistic and/or antagonistic analogs of GnRH in a dose- and time-dependent manner (Eidne et al., 1987; Thompson et al., 1991; Emons et al., 1993a,b; Emons and Schally, 1994).

In ovarian cancer cells, GnRH seems to have two opposite activities:

1. The antimitotic activity through inhibition of signal transduction of mitogens such as epidermal growth factor, and
2. The inhibition of Doxorubicin-induced apoptosis via activation of transcription nuclear factor kappa B (NFkB) as shown by Gründker et al. (2000). In this report is clearly suggested that GnRH-a Triptorelin inhibits Doxorubicin-induced apoptosis via activation of NFkB.

Furthermore, biopsy specimens collected from lesions, myometria and corresponding endometria of women with ovarian endometrioma, adenomyosis and uterine myoma treated with the GnRH-a, leuprolide acetate, for a variable period of 3–6 months before surgery, showed a significantly decrease in the infiltration of CD68-positive Mϕ, micro-vessel density and tissue levels of monocyte chemotactic protein I (MCP-I). Furthermore, when compared with the non-treated group, a significant increase in apoptotic index and quantitative-histogram scores of activated

caspase-3 after GnRH-a therapy were observed in the eutopic endometria, lesions and myometria of these diseases (Khan et al., 2010).

Tanriverdi et al. (2005) demonstrated that GnRH-I and GnRH-II have differential modulatory effects on human PBMC proliferation and IL-2Rγ-chain mRNA expression in healthy males. Treatment of *in vitro* PMBCs with GnRH-I and with IL-2 resulted in a significant increase in cell proliferation as well as in an increased expression of IL-2Rγ mRNA in a dose dependent manner compared with the untreated control. In contrast, GnRH-II administration did not affect the proliferation of PMBCs alone, and IL-2Rγ expression was significantly decreased in all concentrations. This study clearly indicates the potential stimulatory effects of GnRH-I in PBMCs and reduction of these effects by GnRH-II.

Induces GnRH production by immune cells. Numerous interesting and solid studies show that T-cells (and other immune cells) can in fact produce and secrete various endogenous neurotransmitters, either spontaneously or after induction by external stimuli. Among neurotransmitters produced by T-cells are GnRH-I and GnRH-II (Levite, 2008).

Immunoactive and bioactive GnRH peptide is present and the GnRH message is expressed in human peripheral blood T-cells. Both $CD4^+$ and $CD8^+$ subsets of T-cells produce GnRH and it is significantly increased when cells are activated by either phytohemagglutinin or anti-CD3 antibody. This GnRH production in activated T-cells is an early activation event that is independent of IL-2 system activation and DNA synthesis (Azad et al., 1993).

Induces immune mediators production. To investigate the role of GnRH in the modulation of T helper cytokines in pregnant rats undergoing termination of pregnancy, Dixit et al. (2003b) administrated a GnRH-a to evaluate effects on Th-1 and Th-2 cytokines. A marked increase in IFN-γ, inhibition of IL-4 production and an IL-2 and IL-10 absent response was observed. It is possible that IL-2 levels are unchanged because it might has been consumed by activated T cells (Quintanar, 2011).

Recently, Goldberg et al. (2009) demonstrated in a clinically relevant model of allogeneic bone marrow transplantation, that after leuprolide acetate treatment, the enhanced peripheral T cell reconstitution is due to both increases in lymphoid-committed precursors as well as enhanced thymic regeneration.

GnRH has also been reported to regulate four angiogenic chemokines expression (CXCL2, CXCL3, CXCL6, and CXCL8) in human placenta, where trophoblasts were subsequently shown to recruit lymphocytes. Additionally, in Jurkat T cells and primary peripheral blood T and uterine NK cells; chemokine release was detected in chemotaxis assays and this was shown to be GnRH dependent (Cavanagh et al., 2009).

There are two works evaluating the efficacy of the GnRH-a, leuprolide acetate, on NK cells activity in endometriosis. Results are contradictory since the first one suggests an increased NK cell activity in peripheral blood samples determined by 51Cr release assay (Umesaki et al., 1999); and the second one reported that NK cell cytotoxicity from control and patients was significantly decreased with leuprolide acetate (Wong and Simon, 2004). These findings suggest a direct immunomodulatory role of GnRH on NK cell activity.

In an attempt to know the possible immunomodulatory effects of GnRH on LPS activated human peripheral blood monocytes cultured *in vitro*, Komorowski and Stepien (1995) found that GnRH did not affect the secretion of the cytokines IL-1β and IL-6.

On the other hand, there is a report where the immunomodulation exerted by GnRH on freshly isolated primary peritoneal macrophages is clearly observed. In this work the authors found that the production of nitric oxide, co-stimulated with LPS and IFNγ, and the activity of NF-kB was suppressed by GnRH exposure. Taken together, these results demonstrate that GnRH participates in macrophage function and indicate that the NF-kB signaling pathway may be responsible for GnRH-mediated immune modulation (Min et al., 2009).

GnRH expression response against immune system challenges

There is a work that reported that when lymphocyte lysates were applied to rat anterior pituitary cells in monolayer culture, significant stimulation of GnRH secretion was seen. This response evoked by lymphocyte lysates was found to be dose dependent and could be significantly inhibited by a GnRH-ant (Emanuele et al., 1990).

Otherwise, TNF has a number of regulatory actions on gonadotrophic functions: TNF and other cytokines, notably IL-1β, have powerful LH suppressing abilities on pituitary activity (Kalra et al., 1998).

GnRH and autoimmunity

Given that GnRH possesses direct immunostimulatory actions, it has been hypothesized that GnRH might play a role in the development of autoimmune disease.

Jacobson et al. (1994) demonstrated that the administration of GnRH antagonists in a mouse model of systemic lupus erythematosus (SLE), led to a reduction in autoantibody levels, total immunoglobulin levels, renal disease, and significantly improved survival, independently of gonadal steroids.

Based on the above mentioned findings and considering that (1) in the nonobese mouse model of autoimmune diabetes (NOD mouse), castration of male NOD mice leads to increased incidence of diabetes, and that (2) castration of male mice leads to an increase in GnRH action, Ansari et al. (2004) determined the effect of GnRH agonists and antagonists on expression of autoimmune diabetes in this mouse model. Their results showed that Antide administration prevented the increased incidence of diabetes, reduced total serum IgG levels, IL-6 cytokine expression in cultured splenocytes, and the lymphocytic infiltration of islets. GnRH administration exerted reciprocal effects, leading to earlier timing of onset of diabetes and increased serum total IgG levels.

In addition, due to that has been reported that motoneurons of spinal cord of rat from embryonic until adult stage, express GnRHRs which respond to GnRH (Quintanar et al., 2009) a possible clinical use in spinal cord injury (Calderón-Vallejo and Quintanar, 2012) and CNS autoimmune diseases has been proposed. Specifically, treatment with GnRH (Quintanar, 2011) or a synthetic analog (Guzmán-Soto et al., 2012) induces an improvement of clinical signs in the EAE

model, where they remained significantly lower in EAE rats with GnRH administration compared to animals without treatment. In these experiments, also a significantly increased expression of neurofilaments (NFs) and myelin basic protein as well as more axons of larger areas in the spinal cord of EAE animals were found.

A great advantage of GnRH and its analog leuprolide acetate, is that they are capable to cross the blood brain barrier (Kastin et al., 1980; Barrera et al., 1991) and reach their targets into the CNS. Taking into account this advantage of leuprolide acetate and its effect on recovery in locomotion and increased expression of biological markers, the use of this agonist as a potential therapeutic approach for traumatic or neurodegenerative diseases such as multiple sclerosis should be considered.

The effects of GnRH on immune system are summarized in **Figure 3**.

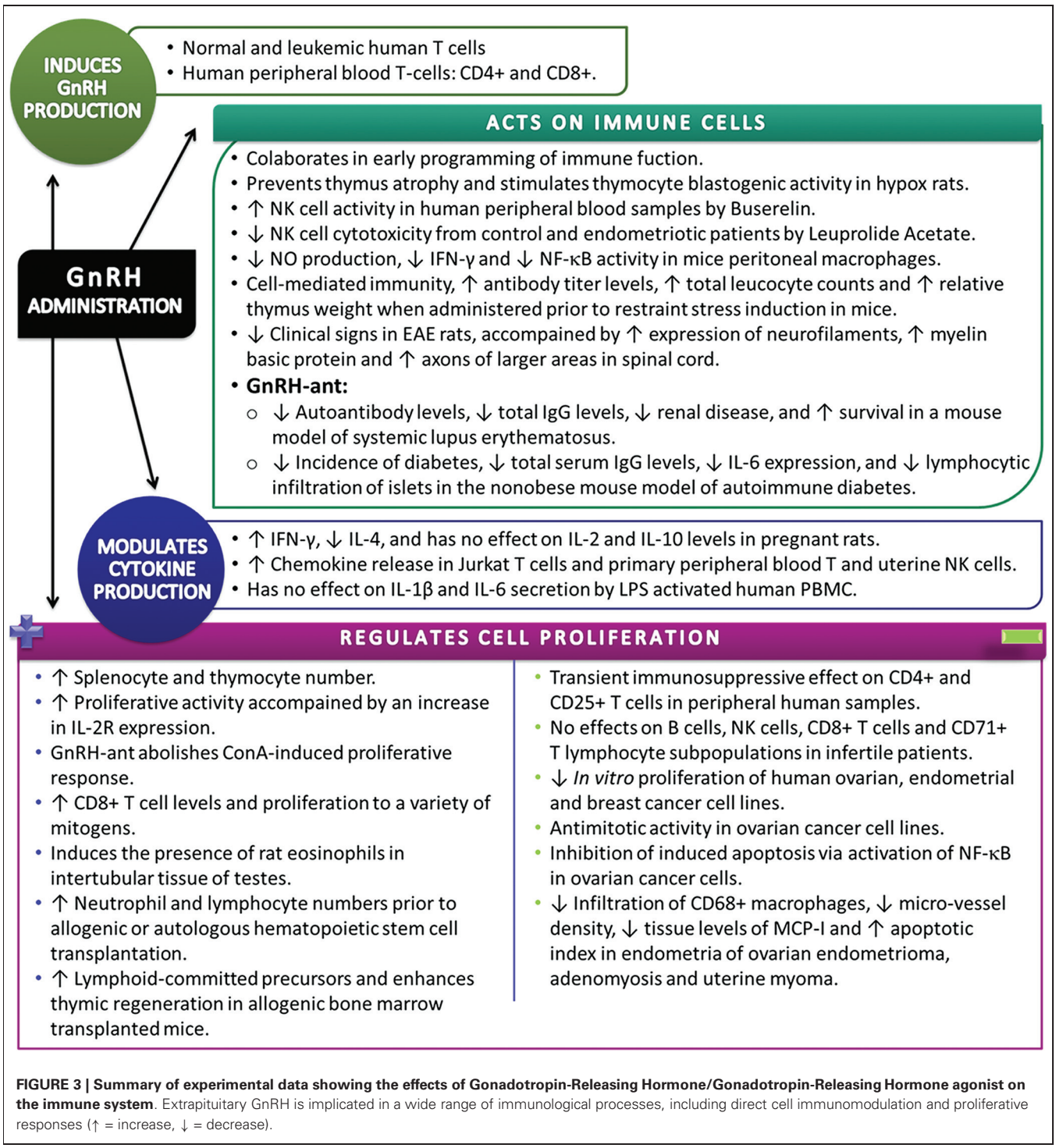

FIGURE 3 | Summary of experimental data showing the effects of Gonadotropin-Releasing Hormone/Gonadotropin-Releasing Hormone agonist on the immune system. Extrapituitary GnRH is implicated in a wide range of immunological processes, including direct cell immunomodulation and proliferative responses (↑ = increase, ↓ = decrease).

Clinical relevance and future directions

GnRH induces the secretion of FSH-LH and consequently of sex hormones, which are involved in the immune response. Controversial is the idea that sex hormones are immunoprotective because depending on the type of hormone and experimental conditions can be obtained opposite effects.

The presence of GnRH receptors and GnRH secretion by cells of the immune system, opening the spectrum of possibilities for interaction of autocrine or paracrine regulation. However, the pharmacological use of GnRH or its analogues, independently of the direct effect of sex hormones, raise the possibility that can be used as a inflammatory regulator in autoimmune diseases. Retrospective studies can be analyzed to establish whether treatment with GnRH analogues have improved immunological conditions of patients with autoimmune pathology. This option can be explored in future works.

CONCLUSION

Hypothalamic hormones are closely related to the immune system. Their direct and indirect effects establish a bidirectional network to establish the required mechanisms to maintain homeostasis.

TRH, CRH and GnRH exhibit common patterns of specific receptors in immune cells and tissues. It is hard to think in the action of a single independent factor within the neuroimmunoendocrine network. When any of these elements destabilize, the system is altered and is difficult to define which factor of all may have been responsible for the alteration. Perhaps because of the complexity of the network, experiments to demonstrate such events become complicated to study. Using *in vitro* models allow clarify in a specific manner some of the elements involved in the homeostasis, however, extrapolate it to *in vivo* models could lead to unreliable interpretations.

In the evolution of the knowledge of the involvement of neurohormones on the immune system, we can find that many of the investigations are related to their pharmacological application, however, few of them concern to the physiological mechanism of its immunomodulation either autocrine or paracrine. If more is known about the physiology of these neurohormones, expectations about their applications at therapeutic level or to the understanding of our complex responses to internal or external challenges to maintain the survival of the individual could be proposed.

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Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Received: 31 May 2013; paper pending published: 20 June 2013; accepted: 16 July 2013; published online: 13 August 2013.

*Citation: Quintanar JL and Guzmán-Soto I (2013) Hypothalamic neurohormones and immune responses. Front. Integr. Neurosci. **7**:56. doi: 10.3389/fnint.2013.00056*

frontiers in
INTEGRATIVE NEUROSCIENCE

MINI REVIEW ARTICLE
published: 17 January 2014
doi: 10.3389/fnint.2013.00110

Hormonal influences on neuroimmune responses in the CNS of females

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Particular reproductive stages such as lactation impose demands on the female. To cope with these demands, her physiology goes through numerous adaptations, for example, attenuation of immune and stress responses. Hormonal fluctuation during lactation exerts a strong influence, inducing neuroplasticity in the hypothalamus and extrahypothalamic regions, and diminishing the stress and inflammatory responses. Thus, hormones confer decreased vulnerability to the female brain. This mini-review focuses on the adaptations of the immune and stress response during maternity, and on the neuroprotective actions of progesterone and prolactin and their effects on inflammation. The importance of pregnancy and lactation as experimental models to study immune responses and disease is also highlighted.

Keywords: stress, progesterone, prolactin, lactation, neuroimmune, HPA axis, female

INTRODUCTION

Reproduction is one of the most significant events in the life of a mammalian female. It can be described as a rich social and hormonal experience that begins by interacting and mating with a male, followed by pregnancy, parturition, and ultimately lactation, interaction with pups, and weaning (Mann and Bridges, 2001; Serafim and Felicio, 2002). Striking behavioral and neuroendocrine alterations due to motherhood have been reported in several mammalian species including mice, rats, rabbits, sheep, and humans. They are reflected by changes at almost all brain levels and are essential to protect the developing embryo, and for successful delivery, maternal behavior, and pup survival (Kinsley and Lambert, 2008). These neuroanatomical and functional changes observed during pregnancy and lactation are necessary to cope with the demands of reproduction and to protect the maternal organism against dramatic hormonal variations (Mann and Bridges, 2001; Kinsley and Lambert, 2008). They include a marked adaptation of the hypothalamus-pituitary-adrenal (HPA) axis, which results in hypo-responsiveness to stress, diminished inflammatory responses (Walker et al., 1992; Windle et al., 1997a; Lightman et al., 1998; Tilbrook and Clarke, 2006), and diminished sensitivity of the brain of the mother against excitotoxic damage (Morales, 2011).

MECHANISMS GOVERNING THE HYPO-RESPONSIVENESS TO STRESS DURING PREGNANCY AND LACTATION

One of the best-known examples of naturally attenuated stress response is seen in the female rat during late pregnancy and lactation (Tilbrook and Clarke, 2006). During these reproductive phases, the HPA axis maintains minimal responses necessary to cope with any situation that may threaten the homeostasis of the female. This axis comprises corticotropin-releasing hormone (CRH)- and vasopressin- neurons in the paraventricular nucleus of the hypothalamus (PVN), which when stimulated, release these peptides into the median eminence to stimulate pituitary cell production of adrenocorticotropic hormone (ACTH) that reaches the adrenal cortex to release cortisol or corticosterone. HPA axis activity is regulated by glucocorticoid negative feedback on the pituitary, the PVN, and higher brain centers.

The attenuation of the stress response during pregnancy and lactation has been documented in various different stress models (Lightman et al., 2001; Russell et al., 2008). Pregnancy in the rat is accompanied by a progressive decrease in HPA axis responses to a range of psychological (Neumann et al., 1998) and physical (Brunton et al., 2005; Douglas et al., 2005) stressors particularly in the last week of pregnancy, reflected by reduced ACTH and corticosterone secretion. This hypo-responsiveness persists through parturition (Wigger et al., 1999; Neumann et al., 2003) and lactation until weaning (Windle et al., 1997b). Suppressed responses to stress in pregnancy can be explained by adaptations in both the anterior pituitary and the hypothalamus (Brunton et al., 2005; Russell et al., 2008). Corticotrophs in the pituitary are less sensitive to secretagogs (Shanks et al., 1997; Neumann et al., 1998), and CRH mRNA expression induced by stress is attenuated (Brunton et al., 2005). Moreover, HPA axis responses to immune stress in early mid pregnancy are strong and similar to that in virgins, although activation of hypothalamic vasopressin neurons, rather than CRH neurons, may be more important in

the stress response in pregnancy (Ma et al., 2005; Parker et al., 2011).

Studies in lactating rats have shown a flattening of the diurnal rhythm of corticosterone secretion (Walker et al., 1992; Atkinson and Waddell, 1995) during this phase, such that the nadir levels of corticosterone rise (Stern et al., 1973; Fischer et al., 1995) and the peak evening levels decrease (Windle et al., 2013). During lactation, basal plasma concentrations of both ACTH and corticosterone increased in lactating animals compared with those in virgin rats (Shanks et al., 1997, 1999). There is also an increased basal expression of CRH mRNA in the PVN in early lactation (day 3; da Costa et al., 1996), but low basal expression of CRH mRNA by the middle phase of the lactation period (days 7–10; Windle et al., 1997a; Walker et al., 2001). However, CRH expression in response to stress is diminished at either stage (Lightman and Young, 1989; Windle et al., 1997b), similar to findings in late pregnancy (Douglas et al., 2005).

The mechanisms for this altered neuroendocrine responses include diminished CNS excitatory signaling within stress-responsive systems like the catecholaminergic brainstem and limbic circuitry (Hoffman et al., 1994; da Costa et al., 1996; Wintrip et al., 1997) as well as an altered hypothalamic and adrenomedullary catecholamine function (Patel et al., 1993; Toufexis and Walker, 1996; Windle et al., 1997a; Toufexis et al., 1998). Attenuated noradrenergic tone also underlies stress hyporesponsiveness in lactation, and is clearly dependent upon suckling (Hoffman et al., 1994; Toufexis and Walker, 1996; Toufexis et al., 1998). Moreover, opioids switch to having a net inhibitory effect on HPA activity such that naloxone treatment enhances the ACTH response to IL-1β in late pregnant rats (Brunton et al., 2009). Growing evidence for an integrated participation of many other factors besides noradrenaline and opioids that underlie altered responses to stress, include CRH (Johnstone et al., 2000; da Costa et al., 2001), oxytocin (Neumann et al., 2003; Windle et al., 2004), prolactin (PRL; Torner and Neumann, 2002), steroids and neurosteroids (Douglas et al., 2000; Brunton and Russell, 2008, 2011; **Figure 1**).

Another mechanism for the attenuation of the stress response involves nitric oxide (NO) regulation of CRH expression within the PVN. NO is present in the PVN of female rats during lactation (Popeski et al., 1999; Otukonyong et al., 2000; Monasterio and Morales, 2011), as indicated by the NO markers NADPH-diaphorase and neuronal synthase (nNOS), and its synthesis dependent on suckling (Otukonyong et al., 2000) and PRL (Vega et al., 2010). These markers increase within the PVN in response to stress in male rats, but paradoxically, basal level of NO markers are present in the PVN of lactating females whose response to stress is attenuated. The activational c-fos response and an increase in NADPH-diaphorase- and nNOS-positive cells are clearly detected in the PVN of diestrus rats after an immune challenge, but not in lactating rats. Furthermore, the total concentration of nitrates in the hypothalamus and the circulating level of corticosterone and IL-6 increase significantly after stress in diestrus, but not in lactating rats, compared to their corresponding controls. Intracerebral administration of L-NAME, a general NOS inhibitor, reverses the attenuation of the activational response to stress in the lactating rats and increases CRH expression, suggesting that sustained NO production in the PVN during lactation contributes to attenuate the neuroendocrine response to stress (Monasterio and Morales, 2011; **Figure 1**).

HPA AXIS HORMONES, PROGESTERONE, AND PROLACTIN IN THE REGULATION OF THE IMMUNE RESPONSES DURING PREGNANCY AND LACTATION

HPA AXIS AND IMMUNE RESPONSES

Cytokines, peptide hormones, neurotransmitters, and their receptors are endogenous elements of the nervous, endocrine, and immune systems. These systems share ligands and receptors that serve to communicate (Haddad et al., 2002; Elenkov and

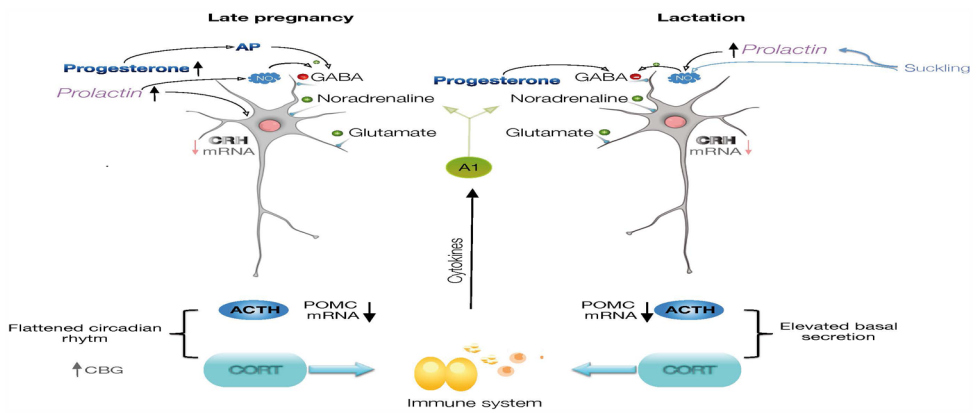

FIGURE 1 | Diagram illustrating the regulation of CRH expression in the parvocellular neurons of paraventricular nucleus of the hypothalamus. During late pregnancy and lactation, there is a diminished noradrenergic tone reaching CRH neurons, and progesterone, allopregnanolone, prolactin, and nitric oxide will contribute to the inhibitory regulation of the CRH neurons. The cascade of neuroendocrine responses, synthesis of ACTH and corticosteroids will also be modified. Neuroimmune interactions are represented by effects of glucocorticoids on cells of the immune system, and which molecules will affect CNS functions. ACTH, adrenocorticotropic hormone; AP, allopregnanolone; CRH, corticotropin-releasing hormone; CBG, corticosteroid-binding globulin; POMC, proopiomelanocortin.

Chrousos, 2006). In addition to altered endocrine responses during lactation, stress also changes immune function in comparison to non-lactating animals (Monasterio and Morales, 2011), suggesting that bi-directional communication between the immune and endocrine systems is also altered during lactation. Pregnancy and lactation represent interesting experimental models that illustrate how the elevated basal plasma level of corticosterone and the diminished stress responses, affect the immune system. It is established that dampened HPA responses are associated with increased vulnerability to inflammation (Shanks et al., 1997, 1999) and therefore, alterations in endocrine regulation of immune responses during lactation may predispose the animal to inflammatory disease. However, not all of the immune responses are dampened during pregnancy and lactation (Jaedicke et al., 2009).

Several reports have documented motherhood-induced adaptations of the immune system in female rats, showing that this is a not always an immune suppression condition (Jaedicke et al., 2009). Early pregnancy has been associated with an increased cell density in the thymus and spleen, whereas late pregnancy and lactation have been associated with a decreased cellularity despite the expansion of the thymus medulla (Kendall and Clarke, 2000). Lactation delays the return of the thymus medulla to the original pre-pregnancy state. In addition, the changes in the thymus can be attributed to the adaptation of the maternal immune system to the semi-allogeneic fetus (Kendall and Clarke, 2000). Hormonal and neural changes can partially explain these modifications. Progesterone, PRL, and estradiol levels in plasma fluctuate in early, mid, and late pregnancy (Neville et al., 2002; Brusco et al., 2008). Nerve growth factor, nearly absent in the medulla of the adult thymus, is high in the medulla of the thymus during late pregnancy (Aloe et al., 1997). Also, lactation is associated with an increased susceptibility to parasitic infections (Barger, 1993), but much controversy exists in the effects of pregnancy and lactation on the progression of autoimmune diseases as documented by studies in rodents and in humans (Elenkov et al., 2001; Buchel et al., 2002; Vukusic et al., 2004; Gregg, 2009).

The immune system of lactating rodents has been the focus of only a few studies, which have shown that some immune functions become suppressed in this phase, while others remain unaffected or are enhanced (Jaedicke et al., 2009). For example, antibody production after immunization, and IL-6 (Monasterio and Morales, 2011) and IL-2 production in the spleen (Shanks et al., 1997) were suppressed during lactation in rodents. Conversely, evidence of increased concentrations of plasma IL-6 or an enhanced proliferative response of lymphocytes from mesenteric lymph nodes suggests activation of other immune responses (Shanks et al., 1997). In male rats, activation of the HPA axis and glucocorticoid release occurs during bacterial lipopolysaccharide (LPS) exposure, triggering an elaborate inflammatory response that involves the release of the pro-inflammatory mediators IL-1β, IL-6, and tumor necrosis factor (TNF)-α (Grinevich et al., 2001), which stimulate cells to upregulate the inflammatory reaction, and systemically they activate the HPA axis (Chrousos, 1995). However, in lactating rats, LPS significantly elevates circulating levels of ACTH and corticosterone, but the magnitude of hormonal and hypothalamic responses to LPS are significantly reduced in lactating animals relative to virgin controls (Shanks et al., 1999). Despite this attenuated stress response, systemic immune responses to stressors are modulated during lactation indicating that the immune system is not generally suppressed but rather adjusted in this stage (Jaedicke et al., 2009).

In humans, the early postpartum period has been associated with up-regulated inflammatory responses and a relapse of autoimmune disorders such as rheumatoid arthritis and multiple sclerosis, often interpreted as a flare-up due to the rebound of the immune system after pregnancy (Elenkov et al., 2001; Buchel et al., 2002). However, long term-studies have shown that relapse rate of multiple sclerosis remains similar to pre-pregnancy level, after an increase in the first trimester postpartum (Vukusic et al., 2004). A broad state of immune activation is also characteristic of the early postpartum period, as measured by levels of neopterin, soluble IL-2 receptor, and soluble CD8 antigen (Burns et al., 1999). This may help women recover from the biological stress of parturition, but more studies about the magnitude and length of such a state are necessary. Immune and inflammatory activation in postpartum women may be factors leading to anxiety and depression in the early days after delivery (Maes et al., 2000). CD4 cell counts are reported to rise during postpartum, primarily due to γδ T cells (Watanabe et al., 1996). Natural killer (NK) subsets with weak cytotoxic activity (CD16+, CD57+) were found to increase during months 1–4 postpartum (Watanabe et al., 1997), and lymphocyte proliferation was higher than non-postpartum controls. Furthermore, the postpartum period is also associated with the onset of autoimmune thyroid syndrome (Muller et al., 2001). Thus, immune responses linked to reproduction can either be dampened or enhanced depending on the stimulus and hormonal status (De Bellis et al., 2005; Hughes, 2012; Shelly et al., 2012).

In summary, during both pregnancy and lactation the immune responses are altered, but instead of considering them to be immune-suppressed stages, they represent reproductive conditions in which either stress or immune responses can vary depending on the hormonal status (Mor and Cardenas, 2010). The immunology of reproduction is the result of the combination of signals and responses originating from the fetal-placental and the maternal immune systems. This last is under the regulation of the CNS through the hormonal (glucocorticoids) stress response, pituitary responses, and the autonomic nervous system.

PROGESTERONE ACTIONS ON CNS IMMUNE RESPONSES

Among the hormones that have been considered candidate inducers of pregnancy- and lactation-related adaptations in HPA axis and immune responses is progesterone. Progesterone is a steroid hormone synthesized by the corpus luteum in cycling females and by the placenta during pregnancy; it can enter the brain from the circulation and can also be synthesized in the brain by oligodendrocytes and excitatory neurons (Stein, 2011). During pregnancy, progesterone levels in plasma and brain are increased and are accompanied by elevated levels of its metabolite allopregnanolone (Brunton and Russell, 2008; Mostallino et al., 2009).

Progesterone (and its metabolite allopregnanolone) has important actions in the female's brain, such as the expression of

maternal behavior (Bridges, 1984). During pregnancy, high levels of allopregnanolone suppress HPA -axis responses to stress: allopregnanolone may enhance the action of GABA in the PVN or on afferent inputs to the CRH neurons to suppress stress responses, and it also induces and maintains the endogenous inhibitory opioid mechanism in the nucleus of the solitary tract (Brunton et al., 2009). Blocking allopregnanolone restores HPA axis responses to systemically administered IL-1β in late pregnant rats (Brunton and Russell, 2008). Allopregnanolone acts as an agonist on the GABA receptor, exerting anxiolytic, sedative, and antiepileptic effects, and it enhances the myelination/remyelination process in the central and peripheral nervous system (Wang et al., 2008; Gangisetty and Reddy, 2010). These steroid hormones have been described as potent regulators of growth factor expression during pregnancy: epidermal growth factor (EGF), insulin-like growth factor (IGF), and transforming growth factor (TGF-β1) in particular are all up-regulated, promoting neural proliferation (Wang et al., 2008). Moreover, they increase neurogenesis within the subgranular zone of the dentate gyrus and subventricular zone (Pawluski et al., 2009), and induce regenerative responses in a mouse model of Alzheimer's disease (Pike et al., 2009; Borowicz et al., 2011). During lactation, progesterone participates in glial changes in brain areas such as the cingulate cortex (Salmaso et al., 2009) and the dentate gyrus of the hippocampus (Cabrera et al., 2013), and this steroid is part of the hormonal cocktail responsible for diminished responses of astrocyte and microglial cells in the hippocampus of lactating rats to damage induced by excitotoxic insults (Cabrera et al., 2013).

Progesterone and allopregnanolone attenuate traumatic brain injury, and diminish the elevation of pro-inflammatory cytokines in a time-dependent manner, suggesting that the protection occurs by limiting the overexpression of cytokines, when they peak at 3 h after a brain injury, rather than inhibiting their expression later in the post-injury cascade of toxic events (He et al., 2004). Both steroids prevent breakdown of the blood–brain barrier in edema and stroke (Ishrat et al., 2010). Progesterone exerts some of its actions through the intracellular, membrane-bound progesterone receptor, while allopregnanolone does not bind to the progesterone receptor (Ishrat et al., 2010). Also, progesterone can reduce the excessive excitotoxicity and inflammation by stimulating activation of the neuroprotective mitogen-activated protein kinase (MAPK) and PI3-K pathways (Kaur et al., 2007). Through use of calcium imaging, electrophysiology, and the measurement of changes in activity-dependent gene expression, progesterone was found to block calcium entry through voltage-gated calcium channels, leading to alterations in the signaling of the activity-dependent transcription factors nuclear factor of activated T-cells (NFAT) and cAMP response element-binding protein (CREB; Luoma et al., 2012). This effect of progesterone on calcium signaling provides a putative mechanism for its neuroprotective actions (Luoma et al., 2012). Furthermore, allopregnanolone is an allosteric modifier of the GABAA receptors expressed by oxytocin neurons (Koksma et al., 2003). Electrophysiological studies of hypothalamic oxytocin neurons showed that allopregnanolone acts via a G-protein mechanism involving protein kinase C to delay the closure of the Cl^- channel after activation (Brussaard and Koksma, 2003), enhancing both the tonic and phasic actions of GABA in oxytocin neurons. This modulatory action on GABA transmission represents an alternative candidate pathway for the protective actions of this steroid.

Progesterone also plays an important role in the periphery. Progesterone-dependent immunomodulation is one of the mechanisms that enable pregnancy to proceed to term, because it protects the fetus from immunological rejection. Recent evidence suggests that autocrine/paracrine factors such as cytokines play a crucial role, possible as effectors of steroid hormones (Agrawal et al., 2011). A growing body of evidence implicates progesterone in the establishment of an adequate immune response during pregnancy (Hirsch and Muhle, 2002; Aisemberg et al., 2013).

Thus, progesterone and its metabolite allopregnanolone are important modulators of the immune responses in the periphery and within the CNS. Such effects are aimed to diminish the impact of an acute lesion or degenerative process in the nervous system while in the periphery contributes to the regulation of immune response against the fetus (Aisemberg et al., 2013).

PROLACTIN ACTIONS ON CNS AND IMMUNE SYSTEM

Prolactin is a peptide hormone secreted from the anterior pituitary into the circulation; it is thought to cross the blood–brain barrier and is known to regulate a wide variety of physiological process (Bole-feysot et al., 1998; Grattan and Kokay, 2008). PRL is also produced in a broad spectrum of extrapituitary sites including cells of the nervous and the immune system (Montgomery, 2001; Torner et al., 2002; Ignacak et al., 2012), and it is an important mediator of the immunoneuroendocrine network. However, effects of PRL on the immune system are complex. Removal of PRL by hypophysectomy impairs thymus growth (Nagy and Berczi, 1978) and immune reaction to immunogenic factors (Bernton et al., 1988) in rats. On the other hand, hyperprolactinemia (HP), in mice injected with *Listeria monocytogenes* increases mortality associated with impaired lymphocyte proliferation and decreased macrophage-activating factor production by T lymphocytes (Bernton et al., 1988). Furthermore, a PRL-like mRNA and a secreted product have been detected in human B-lymphoblastoid cell lines (Bernton et al., 1988; Baglia et al., 1991). Peripheral blood mononuclear cells also secrete a PRL-like protein, suggesting that it binds to PRL receptors and migrates to the nucleus, where it serves as a co-mitogen and autocrine regulator of cell growth. But, there are several reports showing that PRL is not essential for the proper development and function of the mouse immune system. Using PRL-deficient animals it was shown that PRL is not required for normal hematopoiesis (Horseman et al., 1997), and that PRL receptor signaling is not required for normal immunity (Bouchard et al., 1999). PRL is known to have other contradictory actions on the immune system that depends upon the concentration: it inhibits lymphocyte proliferation at high concentrations, while having enhancing effects at lower concentrations (Matera et al., 1992).

During pregnancy in rats, PRL plasma levels are high in the first half, they decrease until term, and then rise again at postpartum and throughout lactation (Neville et al., 2002). PRL actions in early gestation are crucial to prepare the mammary gland for lactation and in the CNS to establish the appropriate adaptive behavioral responses of the mother (Grattan and Kokay, 2008)

toward the pups. PRL has been related to behavioral and neuronal effects, such as maternal behavior (Mann and Bridges, 2001), attenuation of anxiety and hormonal and neuronal responses to various stressors (Lightman et al., 2001; Torner and Neumann, 2002; Donner et al., 2007), neurogenesis (Shingo et al., 2003; Torner et al., 2009; Larsen and Grattan, 2010; Walker et al., 2012), and neuroprotection (Torner et al., 2009; Tejadilla et al., 2010).

Inflammatory response after a brain injury, such as proliferation and activation of glia is enhanced by PRL (Möderscheim et al., 2007), and elevated levels of PRL stimulate mitogenesis in astrocyte and oligodendrocyte populations of the subventricular zone (Larsen and Grattan, 2010). PRL also increases oligodendrocyte precursor cell proliferation, which in turn enhances the capacity to generate new oligodendrocytes and myelination. This process is associated with the capacity to repair white matter damage in the maternal CNS by increasing myelin basic protein expression (Gregg, 2009). During lactation, a physiological hyperprolactinemic state, there is a reduced sensitivity to kainic acid-induced cell damage in the dorsal hippocampus of the dam, showing that lactation is a natural model of neuroprotection (Vanoye-Carlo et al., 2008; Cabrera et al., 2009) in which PRL can participate. PRL systemic administration has been reported to protect the dorsal hippocampus of female rats against excitotoxicity induced by kainic acid administration, blocking cell loss and neurodegeneration, and diminishing the progression of kainate-generated behavioral manifestation of seizures (Tejadilla et al., 2010). Overall, these studies on protective effects of PRL within the CNS draw attention to the importance of studying females, and the relevance of neuroendocrine–immune interactions when investigating effects of this hormone.

An interesting example of the neuroendocrine–immune interactions is seen in the HP occurring in patients with autoimmune diseases, such as rheumatoid arthritis and systemic lupus erythematosus, and with organ-specific autoimmune diseases, as celiac disease, type 1 diabetes mellitus, Addison's disease, autoimmune thyroid diseases (reviewed by, De Bellis et al., 2005). In these diseases PRL increases the synthesis of IFNγ and IL-2 by Th1 lymphocytes. Moreover, PRL activates Th2 lymphocytes with autoantibody production. The inhibitory effects of IL-1ß on the tuberoinfundibular dopaminergic neurons that inhibit PRL secretion could explain this HP. González et al. (2004), showed that i.c.v. injection of LPS in rats, produces a decrease in tyrosine hydroxylase (TH) activity in the medial eminence, an increase in the serum levels of PRL, and a decrease in the number of TH- and TH mRNA-positive cells in the arcuate nucleus, indicating that dopamine neurons of the hypothalamus are functionally susceptible to local inflammatory stimuli. Additionally, treatment with the dopamine-agonist, bromocriptine, inhibits both PRL secretion and the severity of acute experimental encephalomyelitis (Riskind et al., 1991). This is an example of how molecules of the immune system could affect the neurons in the hypothalamus increasing the secretion of PRL, which in its turn will enhance peripheral inflammatory responses.

CONCLUSION

In summary, during pregnancy and lactation, responses of the HPA axis and the immune system are altered and clearly regulated by suckling and hormone fluctuation. Pregnancy and lactation are the most important periods for the conservation of the species, and they represent fundamental stages at which both mother and offspring must be protected. The immune system is crucial to protect the mother and the product against the environment and there is evidence supporting the notion that immunity is suppressed during motherhood. In this sense, the immune responses of the mother should be adjusted to conserve defenses, but should be tuned to preserve the developing offspring (Mor and Cardenas, 2010). Therefore, pregnancy and lactation are unique conditions in which the immune system is modulated or adjusted, but not fully suppressed.

AUTHOR CONTRIBUTIONS

Nela Monasterio conceived the main premises that are the focus of this review and wrote the largest portion of the paper; Edgar Vergara wrote important subsections of the paper; and Teresa Morales conceived the main premises that are the focus of this review, designed the structure of the paper, wrote a large portion of the paper, and edited the final version.

ACKNOWLEDGMENTS

The authors thank Dr. Dorothy Pless for grammatical review. Cited work from our group was supported by PAPIIT-DGAPA, UNAM, and CONACYT, México. We are grateful to the reviewers whose comments help to improve this work.

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Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Received: 30 May 2013; paper pending published: 19 August 2013; accepted: 25 December 2013; published online: 17 January 2014.

*Citation: Monasterio N, Vergara E and Morales T (2014) Hormonal influences on neuroimmune responses in the CNS of females. Front. Integr. Neurosci. **7**:110. doi: 10.3389/fnint.2013.00110*

This article was submitted to the journal Frontiers in Integrative Neuroscience.

frontiers in
INTEGRATIVE NEUROSCIENCE

REVIEW ARTICLE
published: 07 October 2013
doi: 10.3389/fnint.2013.00070

The microbiota–gut–brain axis: neurobehavioral correlates, health and sociality

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Recent data suggest that the human body is not such a neatly self-sufficient island after all. It is more like a super-complex ecosystem containing trillions of bacteria and other microorganisms that inhabit all our surfaces; skin, mouth, sexual organs, and specially intestines. It has recently become evident that such microbiota, specifically within the gut, can greatly influence many physiological parameters, including cognitive functions, such as learning, memory and decision making processes. Human microbiota is a diverse and dynamic ecosystem, which has evolved in a mutualistic relationship with its host. Ontogenetically, it is vertically inoculated from the mother during birth, established during the first year of life and during lifespan, horizontally transferred among relatives, mates or close community members. This micro-ecosystem serves the host by protecting it against pathogens, metabolizing complex lipids and polysaccharides that otherwise would be inaccessible nutrients, neutralizing drugs and carcinogens, modulating intestinal motility, and making visceral perception possible. It is now evident that the bidirectional signaling between the gastrointestinal tract and the brain, mainly through the vagus nerve, the so called "microbiota–gut–vagus–brain axis," is vital for maintaining homeostasis and it may be also involved in the etiology of several metabolic and mental dysfunctions/disorders. Here we review evidence on the ability of the gut microbiota to communicate with the brain and thus modulate behavior, and also elaborate on the ethological and cultural strategies of human and non-human primates to select, transfer and eliminate microorganisms for selecting the commensal profile.

Keywords: microbiota–gut–brain axis, neurobiology, psychoneuroimmunology, evolutionary psychology, social bonds, kissing

INTRODUCTION

There is hardly a place on earth without bacteria. They are found in every habitat imaginable: in every leaf in the lush Amazon forests; below scorching deserts' sands; within the coldest ice of the Antarctica; and even in the inhospitable environment of the ocean depths, under crushing pressures and in streams of boiling water. Not surprisingly, their realm includes the body surfaces and interior of animals, from minute crawlers to those with exceptional cognitive capacities like the human being. Recent genome sequencing projects suggest that most life forms share up to a third of their genes, and that those in humans show up to a 37% homology with those found in Bacteria and Archaea (McFall-Ngai et al., 2013). The sheer quantity of microorganisms inhabiting human bodies is enormous: more than 1000 different species have been found in a single sample (Relman, 2012). Such data have impacted our self-perception; From a viewpoint of the human body as a self-sufficient individual, to a perception of our bodies as super-complex ecosystems. This change of perspective has included a reappraisal of the role of microorganisms within our bodies (i.e., *endosymbionts*). While the popularly-held belief is that any microorganism found within the human body must have a detrimental effect on its health, emerging research has renewed an emphasis on the fact that many microorganisms have mutually beneficial relationships with their hosts (Archie and Theis, 2011), acting as a *probiotic*: a live microbe with a beneficial effect on the host via modifications of host-associated microbial communities, enhancing the host's response toward disease, its nutrient-exploitation capacity, or improving its environment (Verschuere et al., 2000). Recent research suggests how *microbiota*, i.e., a microbial community occupying a particular habitat (e.g., the *gut* microbiota), can serve its host by protecting it against pathogens, metabolizing complex lipids and polysaccharides that otherwise would be inaccessible nutrients, neutralizing drugs and carcinogens, modulating intestinal motility, and affecting visceral perception. Across evolution, endosymbionts have established important feedback channels with the central nervous system (CNS), some of which are crucial for maintaining homeostasis. For example, as microbial life was increasingly tolerated across generations of organisms, its presence has shaped the evolution of the immune system (Kelly and Mulder, 2012). The recognition that the gut microbiota influences several signaling pathways led to the suggestion of the concept of a microbiota–gut–brain

(MGB) axis, a topic covered by extensive reviews (Rhee et al., 2009; Cryan and Dinan, 2012; Forsythe et al., 2012). The proposal of a MGB axis suggests that through a dynamic alignment, microbiota inhabiting the intestinal lumen affects its host's CNS activity (including vegetative and cognitive functions), and *vice versa* brain activity impacts microbiota development and composition. Clinical and experimental evidence indicate that this is also the case of human subjects, with such relationship playing a pivotal role in the development of metabolic and mental diseases. According to the World Health Organization, metabolic and mental disorders lead the global burden of disease, urging researchers and clinicians to set research priorities, and to governments, public agencies and private funds to apply urgent actions and investment (Mathers et al., 2008). In this regard, understanding the bidirectional signaling between the microbiota, gut and brain, underlie potential and significant impacts on global health, opening new preventive and therapeutic opportunities. Based on the above, the first section of our work provides an overview of the neurobiology supporting such interactions, focusing on key experimental and clinical data of the MGB axis and its potential impact on relevant metabolic and mental human disorders.

While recent years have witnessed an increasing interest in proximate questions regarding different aspects of microbiota, the coevolutionary interactions of animals and bacteria have been relatively unattended. This, in spite of the possibility that a focus on the evolution of the MGB axis could provide new theoretical frameworks for understanding complex evolutionary relationships involved in mutualisms between hosts and commensal bacteria. This includes the possibility that such mutualisms could influence the evolution of immunological systems, shape higher cognitive functions at the individual level, and work as a selective force promoting socialization and social structures, with an influence on the psychobiological basis of gregariousness, social perception, mate choice, and sexual behavior (Archie and Theis, 2011; Neuberg et al., 2011; Schaller, 2011). Therefore, our review is also aimed at understanding the relationship between the exchange of microbial-life among individuals and sociality. Clear suggestions in this regard have been advanced by Troyer (1984a) and Lombardo (2008). They have championed the hypothesis that social interactions may "tip" the precarious but crucial balance between the costs and benefits of group-living by providing an important but surreptitious benefit in the form of an exchange of mutualistic endosymbiotic microbes (e.g., as a defense against pathogens: Dillon et al., 2005). Thus, the next section, on the relationship between microbial-life and sociality, assumes a comparative viewpoint for evaluating such hypothesis. First, it begins by providing an overview of the evidence of symbioses across different animal taxa. It includes studies that either: (a) focus on the social aspect of endosymbiont-transmission or (b) describe whether an experimental intervention was used to clarify the degree by which the impairment of the vertical or horizontal transmission of endosymbionts may affect an organism's survival and/or reproductive success. Second, we suggest that the premises of Lombardo (2008) and Troyer (1984a) can be tested by means of the hypothesis that *the similarity of microbial communities across individuals is an index of the strength of their social bonds*. In our opinion, testing this hypothesis may add an important analytical tool to research focused on how social bonds (a social relationship defined by the degree upon which the exchange of any kind of information -or lack of it- has the potential to affect the survival and/or reproductive success of the individuals involved) translate into cooperation and cohesion at the group-level, an approach that could ultimately shed light on the origin and evolution of sociality (Dunbar and Shultz, 2010). We do this via a focus on the association between sociality and direct and indirect means of microbial-transmission in primates, with particular attention to mouth-to-mouth interactions. Finally, by means of an integrative perspective, the last section provides an overview of the different topics covered.

MICROBIOTA–GUT–BRAIN AXIS AND ITS HEALTH IMPACT

Multiple direct and indirect pathways maintain intensive and extensive bidirectional interactions between the gut microbiota and the CNS; involving endocrine, immune and neural pathways (Grenham et al., 2011), and form the basis of the so called MGB axis (**Figure 1**). For instance, under stress, the brain may influence the composition of the gut microbiota (Bailey and Coe, 1999) via the hypothalamus–pituitary–adrenal (HPA) axis, which regulates cortisol secretion, affecting immune cells activity; both locally in the gut and systemically. When the organism suffers an injury, the first immunological reaction is characterized by redness, pain and heat. These responses are constrained by neuronal regulation of inflammation process, carried out by the HPA axis via catecholamine (Sternberg, 2006) production. The necessary communication processes are based on neurotransmitters, neuropeptides, cytokines, hormones, growth factors (among others), which mediate the relationship between the immune system and the CNS. A feedback process leading to homeostasis (Downing and Miyan, 2000). Yet, disorders like stress (Glaser and Kiecolt-Glaser, 2005)can impact such equilibrium, leading to disease, allergic reactions, inflammatory disease and predisposition to infection. Additionally, cortisol can alter gut permeability and barrier function, and thus contribute to variations in gut microbiota composition (O'Mahony et al., 2011). Vice versa, experimental evidence indicates that the gut microbiota, and pre- and probiotic agents can alter the levels of circulating cytokines, which in turn can have a marked effect on several brain functions (Duerkop et al., 2009; Forsythe and Bienenstock, 2010). Additionally, both the afferent branch of the vagus nerve (Bercik et al., 2011a; Bravo et al., 2011) and modulation of systemic tryptophan, precursor of the neurotransmitter serotonin (Desbonnet et al., 2009), are strongly implicated in relaying the influence of the gut microbiota to the brain.

Experimental approaches on elucidating the MGB axis have so far included, the use of germ-free animals, animals with pathogenic bacterial infections, and animals exposed to probiotic agents or to antibiotics. For instance, germ-free mice have been used to assess neurodevelopmental effects of microbiota loss. Additionally, the administration of probiotic bacteria strains in adult animals or humans has been used to assess the effects of these bacteria on the host. On the other hand, infection studies have been used to assess the effects of pathogenic bacteria on brain and behavior, which are mediated largely through activation of the immune system. Finally, administration of antibiotics can disturb

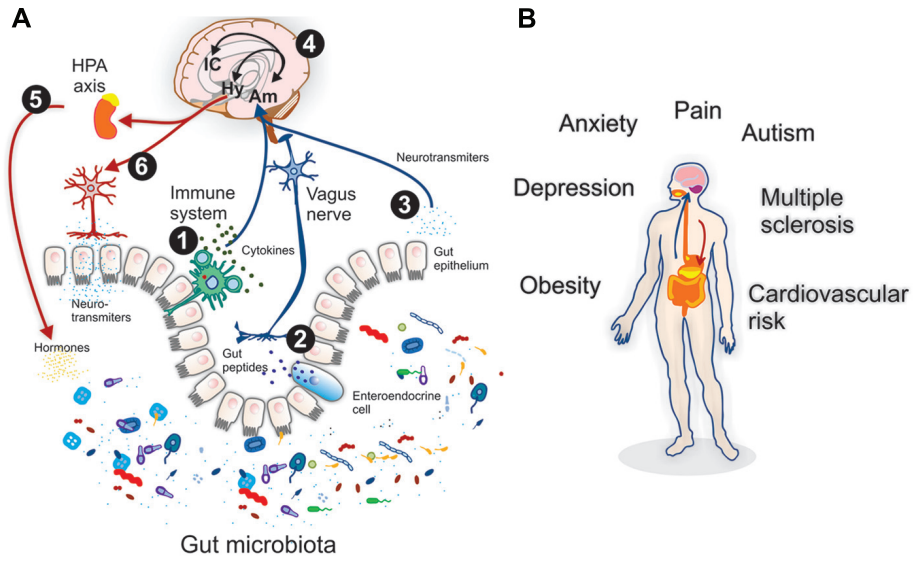

FIGURE 1 | (A) Microbiota–gut–brain (MGB) axis. Direct and indirect pathways support the bidirectional interactions between the gut microbiota and the central nervous system (CNS); involving endocrine, immune and neural pathways. On the afferent arm (blue arrows): (1) lymphocytes may sense the gut lumen and internally release cytokines which can have endocrine or paracrine actions, (2) Sensory neuronal terminals, such as on the vagus nerve may be activated by gut peptides released by enteroendocrine cells, (3) Neurotransmitters or its precursors produced as microbiota metabolites may reach the gut epithelium having endocrine or paracrine effects. (4) Centrally, after brainstem relays (e.g., nucleus *tractus solitarii*) a discrete neural network has been described consistently involving the amygdala (Am) and the insular cortex (IC) as main integrators of visceral inputs. Consistently hypothalamic (Hy) activation initiates the efferent arm (red arrows): (5) corticosteroids, release as results of the hypothalamic–pituitary–adrenal (HPA) axis activation, modulates gut microbiota composition. (6) Neuronal efferent activation may include the so called "anti-inflamatory cholinergic reflex" and/or sympathetic activation, both liberating classical neurotransmitters that may affect directly the gut microbiota composition. **(B)** Health conditions affected by the MGB axis. Recent and growing evidence suggests that several health conditions may be affected by intestinal microbiota, including: visceral pain (McKernan et al., 2010; Wang et al., 2010; Clarke et al., 2012), autism spectrum disorders (Adams et al., 2011; de Theije et al., 2011; Thomas et al., 2012; Wang et al., 2012), obesity (Turnbaugh and Gordon, 2009; Davey et al., 2012; Manco, 2012), cardiovascular risk (Tang et al., 2013), anxiety/depression (Bravo et al., 2011; Heijtz et al., 2011; Foster and McVey Neufeld, 2013), and multiple sclerosis (Berer et al., 2011; Lee et al., 2011).

microbiota composition in a temporally controlled and clinically realistic manner and has therefore been a powerful tool to assess the role of the gut microbiota on behavior.

To date, studies investigating the effects of intestinal microbiota composition on brain function predominantly involve animal models of behavioral disorders such as anxiety, depression and cognitive dysfunction; however, accumulating evidence suggests that the composition of the gut microbiota may also have a role in several other metabolic conditions that involve the CNS (**Figure 1**). In addition, recent data have revealed that MGB axis has multiple effects on emotions, motivation and other higher and complex cognitive functions; reviewed elsewhere (Mayer, 2011). Such evidence suggests that various forms of subliminal interoceptive inputs from the gut, including those generated by intestinal microbiota, may even influence memory formation, emotional arousal, affective behaviors and decision making processes (Craig, 2002; Berntson et al., 2003). The human insular cortex and related brain networks (including the anterior cingulate cortex, orbitofrontal cortex and amygdala), have emerged as the most plausible brain regions to support this integration (Craig, 2009).

MICROBIAL LIFE AND SOCIALITY

SYMBIOSES ACROSS ANIMAL TAXA

Symbioses have played a substantial role in the development of animal life. In their interaction with the geosphere, they shaped the ancient biosphere in which multicellularity and animal life emerged (Hickman, 2005). Multicellular organisms may have evolved as new conflict-mediation mechanisms (i.e., genetic codes) restricted lower-level individual fitness, increasing that of new individuals at higher levels of biological organization (Michod, 2003). From such humble beginnings and over extended periods of time, a diverse array of cooperative and organized groups of individuals (i.e., societies), have evolved. Since transmission vectors may originate across both the physical and the social environment, microbial communities found in an organism are dependent on its geoecology, physiology, and genotype, but also on the intensity of its social relationships (Archie and Theis, 2011). The following paragraphs provide an overview of selected research describing symbioses between microorganisms and different animal taxa.

Microbial endosymbionts have played an important role in the evolutionary and developmental modification of tissues and organs of several marine or *aquatic invertebrates*, for example in the construction of mineralized exoskeletons (Hickman, 2005). Hickman (2005) points out the example of sponges. This group acquires large symbiotic and diverse bacterial communities through vertical transmission, which perform functions like nutrient acquisition, stabilization of the sponge's skeleton, processing of metabolic waste, and production of important secondary metabolites. The phyla with the largest biomass (e.g., arthropods) are

also those with more symbioses reported (McFall-Ngai, 2005). McFall-Ngai (2005) suggests that a crucial difference between vertebrates and invertebrates is based on the relationship between the immune system and its association with microbial life. On the one hand, invertebrates rely on an "innate immune system" consisting of a germline-encoded receptor system associated to cells like macrophages or epithelia. On the other hand, in addition to the innate immune system, vertebrates possess a "combinatorial" immune response (using T-cells and a major histocompatibility complex) which may have evolved as a more "permissive" form of association with long-term endosymbiont microbial consortia, but also as an improved capacity for distinguishing "friend from foe," leading to the identification of *specific* pathogens (McFall-Ngai, 2005). However, some recent studies suggest that these differences may be less clear-cut than previously thought. For instance, a study in *Daphnia magna* (an aquatic crustacean), found that, compared to those experimentally challenged with a different strain, individuals exposed to the specific strain of the pathogen to which their mothers had been exposed had better fitness, suggesting that some invertebrates may have some kind of specific adaptive immunity (Little et al., 2003). One of the best examples of a beneficial symbiotic-relationship in an aquatic invertebrate is the case of the mutualism between the squid *Euprymna scolopes* and the luminous, symbiotic bacteria *Vibrio fischeri*. This symbiosis is maintained by means of cyclic transmission, where the bacterial symbionts must be acquired from the environment each generation (McFall-Ngai, 1998). *V. fischeri* is first acquired directly from the environment, but then, the light organ of *E. scolopes* undergoes specific metamorphic changes that maintain the symbiosis (McFall-Ngai, 1994). Such relationship helps *E. scolopes* generating bioluminescence, camouflaging it from both prey and predators by eliminating the projection of its shadow (Ruby and McFall-Ngai, 1992). Finally, a study by Verschuere et al. (2000) suggests that *Artemia spp.* is protected from the pathogenic effects of *Vibrio proteolyticus* by specific bacterial strains.

Perhaps the best-studied examples of symbioses and transmission of microbiota is found among social *insects*. The phenomenon received close attention as a model for the study of the origins of sociality, permeating both scientific and popular accounts of the human society during the nineteenth and twentieth centuries (Sleigh, 2002). The niche of the hymenoptera, including a subterranean way of life, large biomass density at the nest, and frequent direct individual contact, can all make them particularly vulnerable to pathogens via a fast-spread of disease among conspecifics (Kaltenpoth and Engl, 2013). Therefore, based on selection at the individual and colony-level, social insects have developed different forms of prophylactic and active responses against some parasite-related costs of social-living, such as: environmental parasite uptake, parasite intrusion (i.e., into a colony), parasite establishment and spread within the colony, and transmission between colonies (Cremer et al., 2007). These responses are referred to as "collective" or "social immunity" by Cremer et al. (2007). Among them is also the possibility for the social-transmission of beneficial microbiota. Recently, Koch and Schmid-Hempel (2011) suggested that both honey and bumble bees present bacterial communities not found among solitary species which, importantly, protect them against a virulent and naturally occurring parasite (*Crithidia bombi*). In an experimental setting, they demonstrate that in order to observe the protective effect of microbiota, individuals had to be exposed to feces from nest mates after pupal eclosion, providing strong evidence for an important benefit of the transmission of microbiota between individuals. On the other hand, Evans and Lopez (2004) suggest that nonpathogenic bacteria may have a positive effect on honey bee immunity, helping them to survive pathogen-infection across different life-stages. In turn, McFrederick et al. (2012) suggested that beneficial *Lactobacillus* found in bees were acquired by both vertical transmission or by contact with flowers, and that the *Lactobacillus* strains associated to Sweet bees could suppress mold-growth and other spoilage organisms at the nest. Other species of eusocial insects feed each other by regurgitation of liquid secretions originating in the crop or alimentary tract (Wilson, 2000), a phenomenon named *trophallaxis* by Wheeler (1918). In termites, trophallaxis allows for the social transmission of protozooans, which they lose after periodic molting but are crucial for the digestion of cellulose (Wilson, 2000).

Experimental studies focused on the transmission of microbiota have been practiced in a few non-eusocial insects. For example, germ-free desert locusts (*Schistocerca gregaria*) were associated with up to three species of locust gut bacteria and then fed with a pathogen (*Serratia marcescens*) by Dillon et al. (2005). Results of this study showed a negative relationship between the density of *Serratia marcescens* and the number of gut bacterial species present, as well as a negative relationship between bacterial community-diversity and the proportion of locusts harboring *Serratia*. A more recent study (Wang and Aksoy, 2012) investigated the role of *Wigglesworthia glossinidia* as an endosymbiont associated to the nutrition, fecundity, and development of the immune system in Tsetse flies (Diptera: Glossinidae). The study describes how the peptidoglycan recognition protein (PGRP-LB) is found only in adult flies (as an important component of the milk that nourishes developing progeny), and how the experimental reduction of PGRP-LB decreases female fecundity by damaging the transmission of *Wigglesworthia* through induction of an antimicrobial peptide (Attacin). The conclusion of Wang and Aksoy (2012) is that the transmission of PGRP-LB has a major role in the fitness of Tsetse flies by means of protecting such symbiosis.

There are fewer but equally interesting studies on this subject in fishes, amphibians, and reptiles. Coldwater *fish* appear to acquire their microbiota from the environment after hatching (Hansen and Olafsen, 1999), and there is some indication that different types of probiotic bacteria may have beneficial effects as biological control agents in aquaculture, including immune system improvements (Gómez and Balcázar, 2008) or enhancement of water quality (Verschuere et al., 2000). In the freshwater zebrafish (*Danio rerio*), an experimentally induced lack of microbiota arrests the development of the species' gut at specific points of differentiation, an effect than can, nevertheless, be reversed by the introduction of bacteria (Bates et al., 2006). In the case of *amphibians*, Walke et al. (2011) found that innate immune defenses with a beneficial effect on the inhibition of the fungal pathogen *Batrachochytrium dendrobatidis* can be vertically transmitted. Their work found that both antimicrobial skin peptides

and mutualistic microbiota found in the adult Panamanian "glass-frogs" of the species *Hyalinobatrachium colymbiphyllum*, could be transmitted to embryos, with a possible role of different types of physical contact as a means for this transmission, including female-eggs contact during deposition, and/or male urination on the egg-clutch. Another study by Troyer (1984b) focused on the horizontal acquisition of microbiota in *reptiles*. She investigated how green iguana (*Iguana iguana*) hatchlings employ a significant amount of time in acquiring microbiota before fully exploiting the food resources in their habitat. They do this in a three-step process. First, before having a fully functional digestive activity, they consume soil from within the nest chamber, by which they increase their hindgut microbial populations. Then, up to a week after hatching, they leave the nest and begin eating both plants and soil. Finally, between 2 and 3 weeks after hatching they leave the area around the nest, associate with other conspecifics and eat the feces of older individuals, gaining access to more complex microbial communities.

In comparison, Kohl (2012) presents a thorough review of the many aspects by which microbial communities influence nutrition, development, immunity, and processing of toxins in many species of *birds*. One interesting aspect observed by Kohl is that the symbiotic relationships between birds and microbiota can be, on some occasions, extraordinarily similar to those found in the relationship between mammals and their endosymbionts, while on other instances, they are just slightly distinguishable, by a few, nevertheless remarkable, aspects. A particularly interesting study (Kyle and Kyle, 1993) described by Lombardo (2008) observed that food-provisioning by itself was insufficient for enhancing the survival of orphan chimney swifts nestlings. To achieve this objective, food needed to be coated with the saliva of adults. While all nestlings younger than 6 days receiving food that was not covered by an adult's saliva died, a high proportion of those that received the saliva-covered food survived.

There is also a vast literature describing the influence of microbiota on a variety of *mammals* (a portion of it reviewed by Lombardo, 2008). Therefore, as a preface for the following subsection, next we focus solely on aspects of the relationship between kinship, lactation and microbiota in human and non-human primates. Several studies have examined the bacterial microbiota of breast-fed and bottle-fed human infants using both conventional plating and molecular techniques. These studies have shown that the large gut microbiota of breast-fed infants is generally dominated by bifidobacteria and lactic acid bacteria, both considered beneficial (Penders et al., 2006). In contrast, the gut microbiota of formula-fed infants is more diverse, but less stable, often containing more *Bacteroides*, *Clostridium* and *Enterobacteriaceae*. Early start of feeding formula milk changes the composition of the intestinal-microbiota, promoting colonization by obligate anaerobes such as the *Clostridium coccoides* group, the *Clostridium leptum* subgroup, *Prevotella* and *Atopobium* cluster during the 3 months after birth (Tsuji et al., 2012). Human milk is a complex bio-fluid containing mainly lactose, lipids, and protein. However, it is not widely recognized that, after lactose and lipids, oligosaccharides are the third largest solid component of human milk. The majority of this type of sugars is not digestible by human infants, instead, their main function may be related to their interaction or support of intestinal microbiota. Oligosaccharides in human milk encourage the growth of beneficial bifidobacteria in the colon, while they also bind competitively to cell adhesion receptors. This binding may prevent pathogen-binding to intestinal epithelial cells and thus pathogenesis. Analysis of the oligosaccharides in human milk resulted in 200 different molecules ranging in size from disaccharides up to approximately 22 residues (Ninonuevo et al., 2006). In this sense, through lactation, mothers provide food for both their infants and the bacteria helping assimilating milk's nutrients, allowing for the continued inoculation and establishment of infant's microbiota (Hinde and German, 2012). The importance of this "triangled" relationship is highlighted by the seminal work of Bailey and Coe (1999), who observed that due to strong emotional stress, the disruption of the mother-infant bond (i.e., by separation from the mother) in rhesus monkeys (*Macaca mulatta*) altered the composition of infants' gut microbiota, increasing their vulnerability to disease. The study of Bailey and Coe (1999) represents an important developmental piece of evidence of a health-related *cost* (i.e., increased vulnerability to disease) observed when the composition of microbiota is altered due to the interruption of microbial-transmission (i.e., by a disruption of the mother-infant bond).

Turnbaugh et al. (2009) found no significant correlations between separation of family members and the degree of similarity between their gut microbiota, as well as no significant differences in the composition of gut microbiota between monozygotic and dizygotic twins, suggesting a likely genetic factor underlying such commonalities. On the other hand, Yatsunenko et al. (2012), characterized bacterial species in fecal samples from 531 individuals from different nationalities. Groups included healthy children and adults from the Venezuelan Amazonas, rural areas of Malawi and US metropolitan areas, and included mono and dizygotic twins. An effect of kinship on gut microbiota was found across countries, focusing the discussion on how differences in social structures may influence the extent of vertical transmission of the microbiota and the flow of microbes among members of a group of people or family. Importantly, their results showed that the phylogeny of fecal microbiota of monozygotic twins was no more similar than the microbiota of dizygotic twins in all age groups tested. Likewise, there were no significant differences in the degree of similarity between the fecal microbiota of mothers and their teenage offspring, nor between teens and their biological fathers. Moreover, the microbiota of co-habiting couples was more similar to each other than to members of other households (Yatsunenko et al., 2012). These results, consistent across the populations studied, suggest that, as in other species, endosymbionts may have an important role in kin-recognition in humans.

THE ASSOCIATION BETWEEN MICROBIAL TRANSMISSION AND SOCIAL BONDING IN PRIMATES

In the case of primates, a behaviorally and cognitively complex social life has evolved across a range of group sizes and social structures as a prerequisite for individual's development, survival, and reproduction (Mitani et al., 2012). Either in the form of predator deterrence (e.g., Zuberbühler et al., 1997), cooperative breeding (e.g., Burkart and van Shaik, 2010) or group hunting (e.g., Watts and Mitani, 2002), primates use group-level cooperative strategies

that in general enhance individual fitness. However, sociality also involves important costs, such as within-group competition for resources (van Schaik and van Noordwijk, 1986) or reduction of reproductive output due to socially induced stress (Dunbar, 1980; Altmann et al., 1988). Thus, a balance between the costs and benefits of social-life is by no means an easy task for primates. By increasing the frequency of contact and proximity between individuals, species living at higher densities, in larger groups, or with promiscuous mating are thought to be the most vulnerable to infection (Altizer et al., 2003). Nonetheless, primates exhibit cohesive and sometimes large groups with strong and long-lasting social bonds (Mitani, 2009) of a kind that, in other animal orders, are almost exclusively found among pair-bonded species (Shultz and Dunbar, 2007). These two observations are somewhat incompatible. However, this apparent paradox could be solved by recognizing the role of partner-choice mechanisms in the structuring of primate societies (Hinde, 1983a). Research suggests that endosymbionts are at the base of different mechanisms for individual recognition and partner selection (Archie and Theis, 2011) underlying social-bonding and the formation of cliques within larger social structures. When partner choice is exerted, not all subjects interact with all other group members, and thus, social bonds (or its absence) may act as a social barrier to pathogen transmission (Loehle, 1995). Hence, if endosymbionts' transmission is of any benefit to sociality, transmission should be facilitated as social bonds between individuals are stronger, and made more difficult as species use more frequent or more complex partner-selection mechanisms. As partner-choice limits the size of each individual's social network (Kudo and Dunbar, 2001), the increasing risk of pathogen transmission associated to large groups may set an upper limit to total group size (Freeland, 1976, 1979; Coté and Poulin, 1995; Bonds et al., 2005), leaving the beneficial link between endosymbionts and complex sociality (*sensu* Lombardo, 2008) intact in the form of bonded relationships. For example, the large social groups of the Hamadryas baboon (*Papio hamadryas*: Schreier and Swedell, 2009), classified as a multi-layered, fission-fusion society (Mitani et al., 2012), is a good example of the way social contact is less intense the larger a social-unit is, providing "borders" to microbial-transmission (**Figure 2**). Considering the role of social structure as a barrier against parasite transmission may explain why larger but more subdivided groups, tend to slow the spread of infectious diseases (Griffin and Nunn, 2011; Nunn, 2012), and connect the evolution of the MGB axis to primate sociality. This suggests that the *quality* of social relationships between subjects, but not necessarily the size of their social groups, should be associated to more frequent or direct mechanisms underlying endosymbionts' transmission between individuals.

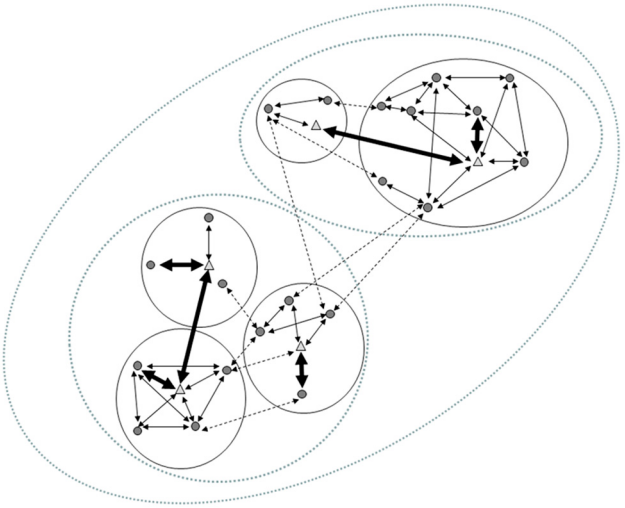

FIGURE 2 | The typical structure of the multi-layered Hamadryas baboon society. The smaller social unit (a "one-male-unit", OMU) in the Hamadryas society is that formed by an adult male (*triangles*), adult females (*circles*) and their offspring. In these units social relationships tend to be circumscribed to members of the same unit (expressed here by arrows, representing social relationships -within bold circles). These units are often formed when larger OMUs fission or when young bachelors sequester peripheral (usually young) females from a large OMU; retaining them in close proximity by force and aggression. Adult females in the same OMU are seldom kin. This produces that strong social relationships (*bold arrows*) are usually established between a female and her unit's male, not among females in the same OMU. While both sexes can have relatives in other OMUs, social contacts among females from different units are less common (*dotted arrows*), whereas adult males may in fact establish strong alliances with males from other units (which may actually be their kin) forming "clans" (*bold arrows across different* OMUs). Spatial association between individuals of different OMUs (e.g., foraging in relative proximity) can result in the third level of the Hamadryas society: a "band" (*medium-sized dotted ovals*). Finally, different bands congregate in the same cliffs to sleep, forming the largest identifiable group: the "troop" (*largest dotted oval*).

Indirect mechanisms

In indirect mechanisms of microbial transmission, inoculation is somehow mediated (e.g., by either a free-living pathogen, inanimate environmental features or by another infected host species; Cortez and Weitz, 2013). *Coprophagy* and *urine ingestion* are good examples of indirect mechanisms: often considered abnormal behaviors appearing due to stress (e.g., Baker and Easley, 1996; Nash et al., 1999), they promote beneficial mutualisms required for digestion of plant materials (as in colobus, howler monkeys and gorillas: Milton, 1981; Lambert, 1998; Graczyk and Cranfield, 2003). Even if they exacerbate exposure to parasites, risks can be circumvented by selectively ingesting excretes of relatives while avoiding those of parasitized conspecifics (Lombardo, 2008); two strategies made possible by the widespread individual-recognition capacities of primates (Langergraber, 2012).

Social *traditions* are behaviors maintained and transmitted by social learning that can distinguish between lineages, groups or populations (Avital and Jablonka, 2000). These traditions may represent an important horizontal mechanism for the transfer of microbiota in primates: on the one hand, they can help distinguishing between different chimpanzee communities (Whiten et al., 2001), while on the other, members of contiguous chimpanzee communities can be distinguished based on the contents of their gut microbiota (Degnan et al., 2012). Several social traditions involve the transfer of objects between individuals, such as that observed in *tool-sharing*. For instance, Pruetz and Lindshield (2012) describe how female chimpanzees retrieved tools directly

from the mouth of other adults and later used them for their own termite-fishing. In another example, after naïve subjects or infants (both their offspring and other unrelated subjects) approached their tools with their mouths, adult chimpanzees offered them their *previously licked* tools, which were again used and licked (Hirata and Celli, 2003; Hirata, 2006).

Food itself can be another indirect vehicle for microbial transfer. In primates, *food sharing* occurs both among related and non-related individuals (Schaub, 1996; Stevens and Gilby, 2004). However, compared to social insects, food sharing among primates can be a highly selective process of partner choice. In a meta-analysis, Jaeggi and van Schaik (2011) recorded which variables predicted food-sharing among subjects of different primate species. Their results suggested, first, that as greater efforts were required to exploit a particular food-source (i.e., as in tool-use), food sharing between parents and offspring was more common; second, in species with a tendency to share food with infants, sharing between adults was also predicted, although diet-characteristics did not explain food sharing patterns among adults; third, instead, food sharing among *unrelated* adults was predicted by their propensity to exert partner choice, and patterns of *reciprocity* explained interchanges such as "food-for-sex" or "food-for-coalitionary-support." Particularly relevant for our argument is their observation of an indication that, within single-male species, food sharing between sexes was more common in monogamous species.

Direct mechanisms

Direct mechanisms of transmission of microbial life depend on physical contact between conspecifics (Cortez and Weitz, 2013). Among primates, social *grooming* is the most widespread example (**Figure 3**). During grooming, primates explore their own or other individual's body surface while removing ectoparasites or debris (e.g., food; Dunbar, 1991), which they often ingest. Primates select their social-partners carefully (Dunbar, 1998). They may exhibit "levels" (Zhou et al., 2005) of acquaintanceship, where those most closely bonded form each other's immediate "support clique" (Dunbar and Spoors, 1995; Crockford et al., 2008). However, when time available for sociality is scarce or during social instability, they can save social-time by reducing overall sociality, focusing grooming on a few primary partners (Dunbar and Dunbar, 1988; Wittig et al., 2008). Perhaps based on saliva's healing properties (Gröschl, 2009), different species practice *preening* or *licking* as another form of grooming (Mooring et al., 2004). In primates, this behavior is commonly observed between primate females and their offspring (e.g., apes: Lindburg and Hazell, 1972; *Colobus* sp.: Horwich and Manski, 1975; *Lemur catta*: Nakamichi and Koyama, 2000; *Alouatta palliata*: Duarte-Dias, 2005; *Macaca fuscata*: Turner et al., 2009). While other types of relationship develop through a mutually regulated process of acquaintanceship, as we have described above in detail, the strong bond between primate mothers and their infants is based on important physiological events like lactation (Hinde, 1983b; **Figure 3**).

FIGURE 3 | Behaviors supporting an association between microbial-transmission and social bonding in primates. The intense sociality of primates provides several different *direct* (i.e., with contact between individuals) and *indirect* (i.e., mediated by any environmental feature) opportunities for the transmission of microbial-life associated to a social-bonding mechanism. Important examples (described in more detail in main text) include: (*upper-left*) mouth-to-mouth contact (in chimpanzees, *Pan troglodytes*): where microbial-life may be directly transmitted in saliva between individuals; (*upper-right*) social grooming (in savannah baboons, *Papio cynocephalus*): where groomers may feed on ectoparasites or food-debris, allowing for the transmission of microbiota; (*bottom-right*) lactation (in vervet monkeys, *Chlorocebus aethiops*): where microbiota directly acquired from the mother helps feeding bacterial communities which in turn help offspring assimilating milk's nutrients; and (*bottom-left*), indirect transmission of microbiota mediated by a social tradition (i.e., touching religious objects), a possible pathway for the homogenization of microbial-life across individuals of a culturally defined human group (all photos by Augusto J. Montiel-Castro).

Mouth-to-mouth interactions

Another means of direct microbial exchange is mouth-to-mouth contact (**Figure 3**). While this behavior has been reported for many primates (e.g.,: *Papio anubis*: Bolwig, 1978; *Pan paniscus*: Kuroda, 1980; *Cebus capuchinus*: Manson et al., 1997; *Cebus apella*: de Waal, 2000a; *Pan troglodytes*: Wittig and Boesch, 2003; *Callithrix jacchus:* Kasper et al., 2008; *Homosapiens*: Hughes et al., 2007; *Pongo abelii*: Hardus et al., 2012), different species perform it at different rates and in different contexts. For instance, the possible relationship between mouth-to-mouth exchange of microbiota and affiliative behavior can also be inferred from the *sociosexual* behavior of bonobos (*Pan paniscus*). Compared to that of common chimpanzees (*Pan troglodytes*) bonobo society is less aggressive, more relaxed or friendlier; sex can be used to repair social relationships, and among bonobos but not chimpanzees, reconciliation often involves sexual contact (Wrangham, 1993). Bonobos can mate several times per day; they manipulate other individuals' genitals with hands or mouth and have more varied forms of copulatory behavior than chimpanzees, including ventro-ventral copulation (i.e., partners facing each other), a sexual position used by bonobo females with their most-trusted partners (Wrangham, 1993). Moreover, bonobos use their tongues intensely (de Waal, 1989), in a way similar to humans, during mouth-to-mouth *kissing*. Being two concomitant forms of "face-to-face" interaction, ventro-ventral copulation and mouth-to-mouth contacts are particularly relevant for supporting the hypothesis that similarities in the composition of microbiota between individuals could be used as indices of bond-strength. This, given that: (1) they allow for the possibility that facial expression may act as a means for the communication of emotional states (Dobson, 2012), while (2) mutual body-contact can stimulate the production of oxytocin, vasopressin or endorphines: neuropeptide mechanisms underlying social-bonding processes in different species (Young and Wang, 2004; Dunbar, 2010); last but not least, (3) during ventro-ventral contact, mouth-to-mouth kissing can act as a means for the *concurrent* reciprocal exchange of microbiota between partners. Altogether, the process could provide the basis for the association of conditioned (e.g., mouth-to-mouth contact) and unconditioned stimuli (e.g., production of oxytocin), reinforcing such behavior through associative learning and thus producing a conditioned response (i.e., oxytocin release) even in the absence of copulation.

Kissing: differences across cultures

In humans, mouth-to-mouth kissing is frequently interpreted as the archetypal sign of a strong bond, an index of intimacy and relationship-satisfaction (Gulledge et al., 2003). Eibl-Eibesfeldt (1989, p.138) describes the behavior as follows: "the initiator presses his lips against the partner's and, when the behavior is fully executed, pushes his tongue between the partner's lips, while the recipient opens his lips and (in complete execution) begins suckling." From this archetypal form, some variations can be encountered. While it can be considered a (human) universal sign of affection, there are cultures where, when practiced *in public*, mouth-to-mouth kissing is considered a taboo (Eibl-Eibesfeldt, 1977). Yet, in the opinion of Eibl-Eibesfeldt (1989), as humans are capable of suppressing innate behaviors, this fact does not weaken the argument of its universality. Given that Eibl-Eibesfeldt (1975, 1989) offers an illustrative review of the homogeneity of this behavior across traditional cultures, the focus of the following paragraphs is on aspects of its cross-cultural variation.

Mouth-to-mouth kissing is certainly not found across all human cultures, nor it follows the same behavioral sequence where it is recorded (Eibl-Eibesfeldt, 1975). One occurs in some European traditional villages, where young men chew pine-resin and leave some pieces of it protruding from their mouths. Then, with this resin in their teeth, they playfully "dare" the girl of their romantic-interest to approach and try to pick it with their mouths (Eibl-Eibesfeldt, 1975). Another frequent variation is that observed among groups like Amerindians, Polynesians and Japanese, where, instead of a concurrent touching of lips, people smell (Mykytowycz, 1972) one another. Chamberlain (1906) recorded the observations of D'Enjoy (1897), who suggested that European kisses could be distinguished from Mongolian and Malayan ones given that the latter were forms of "sniffs" and "nose-rubbing," respectively. Similar observations on human groups from the artic regions (i.e., "Eskimos"), have been interpreted as indications that they sniff each other to check their health: not as a sign of affection, but as a preventive gesture against disease (Washburn Hopkins, 1907). Indeed, the term "Eskimo kiss" is still known and used in English, referring to a "touch of noses" but not of lips. Washburn Hopkins (1907) makes an encyclopedic argument with a detailed analysis of ancient Indian literature, based on which he suggests that ancient Indian words for "kissing" were functionally equivalent to those for "smelling," and thus that behaviors were similarly used. Another important source of cultural variation of the behavior revolves around who is, and who is not meant to be kissed. In a contemporary and empirical, cross-cultural study of jealousy, women reported being upset when their partner kissed someone else but not when they danced or hugged others, while men reported greater jealousy when their partners had sexual fantasies about other people, compared to when they hugged or danced with others (Buunk and Hupka, 1987). In a more recent study, significant ethnic differences were found among data on the age at which teenagers of different ethnic groups kissed for the first time. Compared to Asian Americans, more African Americans, Caucasians, and Latino/Hispanic subjects had kissed for the first time at earlier ages (Regan et al., 2004). Another cross-cultural study found no differences in the way Asians, versus American students expressed love: intimate behavior, including kissing, was an indicator of love in marriages but not in friendships (Kline et al., 2008). For Frijhoff (1991), regardless of intense historical and contemporary western influence, and the fact that the gesture *is* observed in these cultures, Africans and Asians consider the public performance of mouth-to-mouth kissing as disgusting or immoral, relegating it to the sphere of *intimacy*; not because the gesture is absent, but because it is not recognized as a "legitimate public rite." It is in these cultures, Frijhoff (1991) suggests, where greetings commonly involve the use of the "sniff-kiss" or/and a bow of the body, or a hand gesture. Chamberlain (1906) and Frijhoff (1991) also refer to contemporary cultural differences related to the greeting aspect of kissing: common (even among males) in Russia or France (where this gesture's underlying degree of affection may be

indexed by its loudness), but rarer in England, the Netherlands or the USA.

The socio-religious aspect of kissing has been thoroughly analyzed by Frijhoff (1991), who makes interesting conclusions distinguishing the public and private aspects of kissing. On the one hand, he suggests, rituals may have adapted kissing and/or embracing as signals of group-membership, a sign of association and/or fraternity, ruled by cultural standards of public expression that provide a sense of group-identity (e.g., the rite of publicly kissing the feet of the statue of St. Peter in the Vatican, or touching a religious icon: **Figure 3**). On the other hand, Frijhoff (1991) suggests that, in the private sphere, both the experience of a religious person (e.g., when kissing a religious icon), and that of lovers during mouth-to-mouth kissing, appear to separate them from the group, creating a sense of psychological intimacy; a likely reason for why, in some cultures, kissing (i.e., mouth-to-mouth) in public is not considered polite.

Kissing: evolutionary perspective

In view of the previous analysis, it is somewhat surprising that while kissing is found across several cultures and is a topic of strong popular concern (Walter, 2008), its evolution is not yet fully understood. Hypotheses have certainly been suggested. For example, kissing is one of the many different behaviors observed during reconciliation in non-human primates (de Waal, 1989) suggesting its role as a means for appeasement. However, in spite of substantial evidence highlighting the role of kissing in the context of reconciliation (de Waal, 1989, 2000b), one could ask: if grooming can itself produce intrinsic positive reinforcement (i.e., beta-endorphins) in a primate's nervous system (Keverne et al., 1989), why would an additional behavior, one increasing the probability of pathogen-transmission, be used during reconciliation? This is an important consideration. Mouth-to-mouth contact and direct exchange of saliva expose individuals to pathogen transmission. In view of this probable cost and its role as an index of reconciliatory tendencies, one would expect it to provide some intrinsic benefit (Hendrie and Brewer, 2010) and to be a highly selective form of inter-individual interaction. Comparative evidence of mouth-to-mouth feeding in parent-offspring dyads across birds and mammals suggests that kissing could evolve as a form of mouth-to-mouth food exchange between offspring and progenitors (Eibl-Eibesfeldt, 1975). However, common marmosets (*Callithrix jacchus*: a new world monkey that exchanges food often) do not restrict these interactions to matting partners or offspring, and tolerate mouth-to-mouth exchanges with both dominants and subordinates (Kasper et al., 2008). Instead, since tolerating or rejecting a stressful event such as the transgression of personal space may provide information about the quality of a relationship, Kasper et al. (2008) suggest that these "up-close" exchanges may serve as tests of the quality of a relationship. For Nicholson (1984), kissing involves some form of social-bonding by means of semiochemical addiction: a direct and continued exchange of sebum and pheromones facilitating bonding and love. The possibility that chemo-signals have a role in communication, via body-secretions has been recently confirmed in humans by de Groot et al. (2012), observing that chemo-signals of fear and disgust can produce multimodal emotional synchronization between sender and receiver, and thus, that communication of emotional states is not restricted to language and visual stimuli. However, results showing that women prefer the scent of males' t-shirts with whom they have greater immunological dissimilarity (Wedekind et al., 1995), highlight the role of smell (Penn and Potts, 1998) as an alternative route for the development of a purported semiochemical-addiction.

The research of Hughes et al. (2007) on the relationship between pair bonding strategies and kissing in humans, points out other important aspects of this behavior. While men may use kissing for increasing the likelihood of sexual intercourse, women use it as a form of mate-assessment and a behavioral-monitor of the quality of long-term relationships (Hughes et al., 2007). This affiliative aspect of kissing may also be interpreted as a willingness to sustain close social bonds *at the risk of* contracting an illness (Hughes et al., 2007). Therefore, kissing has also been suggested as a strategy aimed at avoiding contagion by pathogens such as the human cytomegalovirus during infant's gestation, for which testing for such possibility before conception or before the onset of vulnerable periods of fetal development would be highly advantageous (Hendrie and Brewer, 2010). Moreover, since couples sharing previously used, food-related items "contaminated" by their partner (e.g., a licked spoon) are perceived as "more intimate" by third-parties (Alley, 2012), kissing may also function as a group-oriented "advert" or proxy of the strength of the bond between two individuals.

FINAL COMMENTS: INTEGRATIVE PERSPECTIVES

THE MICROBIOTA–GUT–BRAIN AXIS AND ITS IMPACT ON HEALTH

The excitement of emotion, the state of alertness and enhanced activation linking the viscera, in particular heart and gut, to the human mind, as well as the mechanisms for bidirectional signaling between these organs, was among the topics by which Charles Darwin himself advocated evolutionary continuity in *The Expression of Emotions in Man and Animals* (Darwin, 1872). With similar intentions, the present review has suggested how mutualistic endosymbionts may have a crucial role in these processes. Thus, the first integrating ideas emerging from our review are focused on the relevance of the different communication channels between the gut microbiota and the brain. For instance, the crucial relationship described in sections above, that between microbiota, cytokines, short-chain fatty acids, systemic tryptophan and their effect on brain function, signals interesting possibilities for fruitful research. For example, the recognition of the role of microbiota in the modulation of tryptophan and thus serotonin, could complement insights on the social and evolutionary basis of schizophrenia (Burns, 2004), while other fertile approaches should focus on the importance of probiotics as modifiers of health, behavior and mood. Such attempts should provide alternatives in clinical settings, and preventive aspects of some of the most prevalent mental and metabolic disorders of modern human societies, such as depression and obesity.

The reports on the role of gut microbiota as an influence in the formation of memories and emotional arousal suggest the existence of a crucial relationship between interoceptive stimuli and the evolution of higher cognitive processes, one that may be based on a system supporting *empathy*, or a capacity for understanding the feelings of other individuals. On the one hand, results

showing how the anterior insular cortex can be activated by the images of other humans experiencing disgust (Wicker et al., 2003) suggest the action of a mechanism homologous to *mirror neurons* (Gallese, 1998). These neurons, first located in the ventral premotor cortex of macaque brains, activate both when subjects perform a particular action and when they observe similar actions performed by other individuals (Rizzolatti and Fogassi, 2007). Mirror neurons may be crucial for understanding the underlying intentions of actions, which has led some to suggest that they are part of the system upon which empathy is constructed (Ferrari et al., 2003). On the other hand, evidence suggests that another kind of cells, "*Von economo*" neurons or "spindle cells" in layer five of the anterior cingulate cortex are particularly involved in processes of self-experience, empathy and social bonding (Parr et al., 2005). For Parr et al. (2005), there are at least two important characteristics suggesting the role of such neurons as a neurological basis of sociality. First, these cells have been identified in humans and apes but not in monkeys, suggesting a recent evolutionary origin associated to higher cognition; second, since they seem to be reduced and abnormally located in autistic individuals, they may underlie the lack of empathy characterizing autism. Finally, such system of representation of emotions of others, may be complemented by the action of the vagus (i.e., the "pneumogastric" nerve, according to Darwin, 1872), as a means for activating responses and control of the metabolic output necessary for social interaction. In this regard, the Polyvagal theory (Porges, 2003) suggests that the myelienated branch of the vagus, found only in mammals, is a key for understanding the non-endocrine bases of social behavior. Given that this branch of the parasympathetic system controls facial expression, swallowing, breathing and vocalizing, and has an inhibitory effect upon the sympathetic system innerving the heart, it promotes the calmness and autonomic substrate of effective social interaction (Porges, 1997). Nonetheless, several details regarding such a system remain to be determined. For example, whether intuitive decision making is based on an interoceptive map of gut responses enabling the brain to make gut-based decisions based on interoceptive stimuli (Preuschoff et al., 2008).

MICROBIAL LIFE AND SOCIALITY

Lombardo (2008) has provided suggestions as to how to distinguish between the transmission of microorganisms as a causal benefit of social interactions versus a mere correlate, byproduct or cost of sociality. His first suggestion, primarily revised in the first section of this review, involves using antibiotics to modify microbial communities in an organism and then observing the effects of such intervention. Evidence across our review suggests that type of interventions can result in significant fitness effects in the experimental subjects. The second set of tests suggested by Lombardo (2008) are those impairing group-living individuals from horizontal transmission of microbial life but not from social contact. He suggests that by means of such intervention we would gain knowledge on how hosts may fail to thrive, not because of lack of social contact *per se*, but because of the impairment of endosymbiont-acquisition from conspecifics. In this sense, our review of the variety of symbioses found across different animal taxa was aimed at providing evidence describing the varied benefits due to such symbioses, and thus suggesting how an interruption of either vertical or horizontal transmission of microbial life could result in significant costs in terms of fitness. Then, based on evidence supporting the idea that "social immunity" is found among several animal taxa, the relationship between intense sociality and the exchange of microbiota was approached by examining the association between direct and indirect patterns of transmission of microbial-life and the intensity of social-partner selection in primates. For this purpose, we suggest that the hypothesis that more similar microbial communities would be found among subjects with stronger social bonds could be used for testing whether primates also obtain benefits associated to microbial transmission. Ultimately, as Dunbar and Shultz (2010) suggest, adding another operational index of sociality to previous, more orthodox, measures of bondedness (e.g., grooming or inter-individual distances), could help expanding our understanding of the evolution social complexity. Thus, we argue that, if endosymbionts' transmission is of any benefit to primate sociality, direct transmission of microbial life should be associated to stronger social bonds, but not to large group sizes. Such possibility, we suggest, allows for the beneficial exchange of endosymbionts across individuals, while at the same time, permits the necessary partner-choice mechanisms (associated to social-bonding processes) restricting group-size, leading to the formation of social-borders which limit the extent of microbial transmission. Indeed, research on primates suggests that greater modularity or greater structuring of social groups reduces parasite success (Griffin and Nunn, 2011). Moreover, indirect mechanisms of transmission allow subjects to exert at least three strategies for selection of the microbial load transmitted: first, one focused on the conditions or characteristics of the objects exchanged (i.e., the spoilage of food) allows subjects to decide whether a particular item deserves further processing or not (Laska et al., 2007); second, subjects may directly assess the phenotypical-characteristics of the interacting subjects, deciding whether to engage in social interaction or not. For example, by stressing immigrants in order to "test" whether they carry pathogens before allowing their full integration into a group (Freeland, 1976); third, certain species may apply *a posteriori* mechanisms for the elimination of pathogens like zoopharmacognosy (Huffman, 1997). In contrast, direct interaction restricts those mechanisms to the second and third strategies. Hence, individuals with stronger social bonds incur greater risks when interacting with their close associates compared to cases when they interact with "mere acquaintances," likely leading to an easier transmission of microbial life among strongly bonded subjects. In this sense, another suggestion stated above was that the transmission of microbial life would be increasingly difficult as primates would employ more complex partner selection mechanisms. From this perspective, since the Social Brain Hypothesis (Dunbar, 1998) is focused on the evolution of both sociality and primate neocortex by means of intense partner-selection due to increasing cognitive capacities, we can relate this hypothesis to the ideas of both Troyer (1984a) and Lombardo (2008) and hypothesize that, in primates: (i) other measures of the strength of social bonds will be positively correlated to the similarity of microbial species shared by members of a social group or reproductive pair; (ii) the number of microbial species shared by any two members of a social group will be positively correlated to that species relative

neocortex size; (iii) the number of microbial species common to any two members of a social group and group size will be negatively correlated.

A particular point of concern emerging from this section is the apparent opposition of reports describing the relationship between microbiota and kinship in humans and apes. While both genetically and socially related humans show similar compositions of their gut microbiota (Degnan et al., 2012), the chimpanzees of Gombe showed similarities within sexes, as well as between members of different communities but not between individuals of the same community. This is remarkable, especially when taking into account evidence suggesting that primate females transmit necessary microbiota to their offspring. Such opposing results could imply that, while similarities in microbiota could be a useful index of the strength of social bonds for humans, the index would not be and adequate measure of prosocial tendencies in chimpanzees. In turn, this possibility would reduce the usefulness of the similarity of gut microbiota as a comparative index for examining the strength of social bonds in other species. However, these variations may be explained by pointing out important differences between the social behavior of apes and humans. Despite the fact that the social system of both chimpanzees and humans has been characterized as *fission-fusion* (Aureli et al., 2008), just like the slight differences observed between the socio-sexual behavior of *Pan troglodytes* and *Pan paniscus*, there are also important differences in the kind and degree of direct social contact observed among *Homo sapiens* and *Pan troglodytes*. The fission-fusion pattern of chimpanzee societies is substantially more fluid than that of humans: chimpanzee individuals within the same community can be alternatively found interacting within groups composed by different subjects at different points in time and in different locations (Aureli et al., 2008), whereas in humans, as bonds are stronger, they are generally more spatially, and socially stable. This observation, could explain why, compared to humans, most members of a chimpanzee community would show a similar composition of their gut microbiota (i.e., based on an incapacity to detect differences between its strongly interacting composing members). The second aspect of the discrepancy may be based on the fact that, while males remain within the same community, forming and strengthening their bonds with other relatives, females migrate between communities (Robinson and Janson, 1987).

Interesting lines of research relating social structure and composition of microbiota could develop from comparisons between the degree of similarity in the microbiota of humans (grouped, e.g., by pattern of social organization), and different non-human primate species (e.g., across different reproductive systems). If more frequent and direct contact promotes greater similarities in the composition of microbiota between individuals, we would expect that the most heavily bonded species (e.g., pair-bonded species), would show greater similarity to humans with stronger social bonds (e.g., couples), followed by the results of non-human primates across distinct reproductive patterns and humans in different types of groups. In this regard, Fincher et al. (2008), provide an important study suggesting an association between human group-level cohesion and pathogenicity. In this study, even after controlling for other confounding variables, human groups with a higher historical pathogen prevalence were also those with the strongest evidence of collectivism-prone *cultural* values, whereas those with a lower historical pathogen prevalence showed stronger cultural values supporting individualism. Therefore, we suggest that in the context of our proposal, this study provides strong evidence suggesting that, in humans, the more cohesive groups (e.g., indexed by more frequent cultural values favoring collectivism, and thus stronger social bonds between subjects) will show stronger similarities in the microbial communities of their individual members.

MOUTH-TO-MOUTH INTERACTIONS

Costly Signaling theory suggests that a possible function of seemingly "pointless" behaviors or traits, may be the conveyance of honest information that can benefit different interactants; both signalers and observers (Smith and Bird, 2005). In this context, direct means of microbial transmission have higher probabilities of involving potentially costly behaviors that could, nevertheless, provide valuable information to interactants, for example, in the case of mouth-to-mouth kissing (Hughes et al., 2007). In turn, these considerations suggest that the above accounts of the possible function of kissing may be reduced to a single one, in the form of kissing as a social signal or a means of communication. Here, communication may be defined as an action that alters the probability pattern of the behavior of another organism in a way adaptive to either one or both interactants (Wilson, 2000). Thus, either in the form of reconciliation, as a derivative of food sharing, and as a test of the quality of a relationship, kissing can be interpreted as a behavioral signal aimed at increasing the probability of future cooperation. In turn, in the case of the exchange of microbiota via mouth-to-mouth contact, or as a test for the risk of illness, kissing would represent an exchange of potentially costly information (*sensu* Smith and Bird, 2005), again, relating it to communication. The behavior may also be used by group members as an index of the strength of the relationship between two individuals: a group-oriented signal based upon which they can adjust their responses toward the individuals performing it. In the end, perhaps only cross-cultural research investigating the effects of kiss frequency relative to the composition of gut microbiota (to the best of our knowledge, not yet attempted), could bring significant light unto the matter. A comparative approach could focus in the sociosexual behavior of *Pan paniscus*, testing whether partners more often engaged in ventro-ventral copulation, mouth-to-mouth contact, and/or face-to-face interactions, compared to those engaging in other kinds of affiliative behavior, show greater similarities in their gut microbiota.

We cannot be blind to another alternative that may, nevertheless, still lend support to the hypothesis of an association between similar microbial communities and strong social bonds between individuals. Its main distinction lying in the suggestion that sociality does not represent the primary "medium" through which microbial communities across individuals are transmitted or homogenized. From this perspective, instead, given the beneficial effect of focused social contact upon glucocorticoid production (Crockford et al., 2008; Wittig et al., 2008), close social interaction will have a reducing effect on glucocorticoid production, which in turn will improve individual's capacity for sustaining an effective

immune response. That "improved" resistance (e.g., byproduct of intense social support), would allow subjects to remain in close proximity without turning increasingly susceptible to the pathogens transmitted by the social-partner. Again, this possibility would still result in similarities in the microbial communities of closely bonded individuals. Further research should be focused into determining the effects of impairing microbial transmission between subjects while still allowing normal social interactions. For example, this suggestion could be approached by creating social groups where all but one of its members are kept under germ-free conditions by means of antibiotics and then evaluating the survival and reproductive success of the "non-germ free" individuals across groups. While such research design may be difficult, ethically and pragmatically, it could provide a way of discriminating between the alternatives at hand.

AUTHOR CONTRIBUTIONS

Augusto J. Montiel-Castro, conceived the main premises and relationships that are the focus of this review; wrote the largest portion of the paper; identified the theoretical relevance of the paper; edited the final version of the paper; designed **Figures 2** and **3**. Rina M. González-Cervantes, conceived some of the relationships that are discussed in the review; helped in the theoretical analysis of the premises of the review; wrote one important subsection of the paper; Gabriela Bravo-Ruiseco, wrote specific portions of the review; helped in the suggestion of specific relationships reviewed. Gustavo Pacheco-López, conceived the main premises and relationships that are the focus of this review; identified the theoretical relevance of the paper; designed the structure of the paper; wrote a large portion of the paper; edited the final version of the paper; designed **Figure 1**.

ACKNOWLEDGMENTS

This work was possible thanks to a postdoctoral scholarship, granted to Augusto J. Montiel-Castro by Iztapalapa Campus, Universidad Autonoma Metropolitana (UAM-I). Gustavo Pacheco-López was funded by a SEP-PROMEP grant No. 54510017 and by a Lerma Campus, Universidad Autonoma Metropolitana (UAM-L) institutional funding for publication. Authors would also like to thank the Centro Darwin de Pensamiento Evolucionista (CEDAR) for the support and efforts during the organization of the series of conferences and seminars between UAM-I and UAM-L that made this work possible. The suggestions of referees resulted in substantial improvements in the contents and clarity of the final version.

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Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Received: 31 May 2013; paper pending published: 26 June 2013; accepted: 04 September 2013; published online: 07 October 2013.

*Citation: Montiel-Castro AJ, González-Cervantes RM, Bravo-Ruiseco G, and Pacheco-López G (2013) The microbiota–gut–brain axis: neurobehavioral correlates, health and sociality. Front. Integr. Neurosci. **7**:70. doi: 10.3389/fnint.2013. 00070*

This article was submitted to the journal Frontiers in Integrative Neuroscience.

frontiers in
INTEGRATIVE NEUROSCIENCE

REVIEW ARTICLE
published: 02 December 2013
doi: 10.3389/fnint.2013.00086

Stress modulates intestinal secretory immunoglobulin A

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Stress is a response of the central nervous system to environmental stimuli perceived as a threat to homeostasis. The stress response triggers the generation of neurotransmitters and hormones from the hypothalamus pituitary adrenal axis, sympathetic axis and brain gut axis, and in this way modulates the intestinal immune system. The effects of psychological stress on intestinal immunity have been investigated mostly with the restraint/immobilization rodent model, resulting in an up or down modulation of SIgA levels depending on the intensity and time of exposure to stress. SIgA is a protein complex formed by dimeric (dIgA) or polymeric IgA (pIgA) and the secretory component (SC), a peptide derived from the polymeric immunoglobulin receptor (pIgR). The latter receptor is a transmembrane protein expressed on the basolateral side of gut epithelial cells, where it uptakes dIgA or pIgA released by plasma cells in the lamina propria. As a result, the IgA-pIgR complex is formed and transported by vesicles to the apical side of epithelial cells. pIgR is then cleaved to release SIgA into the luminal secretions of gut. Down modulation of SIgA associated with stress can have negative repercussions on intestinal function and integrity. This can take the form of increased adhesion of pathogenic agents to the intestinal epithelium and/or an altered balance of inflammation leading to greater intestinal permeability. Most studies on the molecular and biochemical mechanisms involved in the stress response have focused on systemic immunity. The present review analyzes the impact of stress (mostly by restraint/immobilization, but also with mention of other models) on the generation of SIgA, pIgR and other humoral and cellular components involved in the intestinal immune response. Insights into these mechanisms could lead to better therapies for protecting against pathogenic agents and avoiding epithelial tissue damage by modulating intestinal inflammation.

Keywords: SIgA, pIgR, intestinal mucosa, restraint-stress, glucocorticoids, brain-gut axis

INTRODUCTION

Stress is a response of the central nervous system (CNS) to environmental stimuli perceived as a threat to homeostasis. The stress response involves a complex network of mechanisms essential for survival, mediated by neurotransmitters, peptidic hormones and endocrine hormones from the enteric nervous system (ENS), a branch of the autonomic nervous system that among other functions affects the production of interleukins (ILs). These molecules in turn modulate the humoral and cellular components of the intestinal immune system. The ENS contains both vagal and spinal sensory neurons, which play an essential role in the transference of information from the CNS to ENS and vice versa (de Jonge, 2013).

Experimental assays have evidenced that the stress modulates the generation of secretory immunoglobulin A (SIgA; Jarillo-Luna et al., 2007; Martínez-Carrillo et al., 2011) and the expression of pIgR (Reyna-Garfias et al., 2010). Through transcytosis this receptor transports immunoglobin-pIgR complexes (dIgA-pIgR and pIgA-pIgR) across gut epithelial cells. Upon reaching the apical side of these cells, pIgR is cleaved to release SIgA or SC, a pIgR derived peptide into the intestinal lumen (Brandtzaeg, 2009). Along with the gut microflora, both SIgA and pIgR have an essential role in two important intestinal processes. They protect against pathogenic agents that colonize and/or invade the intestinal epithelium, and modulate the gut inflammatory response to maintain homeostasis (Michetti et al., 1992; Uren et al., 2003; Sait et al., 2007; Bruno et al., 2010; Drago-Serrano et al., 2010).

Few studies have explored the effect of stress on SIgA and pIgR, or the capacity of these molecules to maintain homeostasis in the intestine. Most studies involving molecular and biochemical mechanisms of the stress response have focused on systemic rather than intestinal immunity.

The aim of the present review is to explore the impact of stress on the humoral and cellular components of the intestinal immune response, especially IgA, SIgA and pIgR. Although most of the evidence is from studies involving rodent models of restraint/immobilization, other stress-producing protocols affecting SIgA are mentioned, including loud noise, alternating home and metabolic cages, exposure to heat, repeated electric foot shock, and the mixing of newborns from distinct litters (**Table 1**).

Table 1 | Impact of stress on intestinal immunity.

Animal model	Effect	Reference
Alternating home/metabolic cages (male rats)	↓SIgA; fecal/urine corticosterone excretion unchanged	Eriksson et al. (2004)
Heat stress (rats)	↓SIgA; ↓ IL-2,-4, and -10 in small gut; ↑CD8+ T cells in MLN	Liu et al. (2012)
Repeated electric foot shock (mice)	↓IFN-γ released by gut IEL and α/β TCR+ cells; ↑glucocorticoids	Zhang et al. (2005)
Electric foot shock (EFS) and psychological stress (PS) (rats)	↓SIgA (PS); ↑IgA (EFS) in MLN; ↑corticosterone (EFS)	Yamamoto et al. (2009)
Repeated restraint stress (mice)	↓SIgA; lamina propria IgA+ plasm a cell levels unchanged; ↓gut intraepithelial lymphocytes via adrenal hormones	Jarillo-Luna et al. (2007, 2008)
Restraint stress (mice)	↓T cell and B cells; ↑apoptosis in PP; ↑glucocorticoids	Sudo et al. (2001)
Chronic restraint stress (mice)	↓SIgA+ plasma cells, CD8+T and B cells in PP	Martínez-Carrillo et al. (2011)
Immobilization and acoustic stress (CB1R ko mice)	↓SIgA; ↑ bacterial translocation	Zoppi et al. (2012)
Repeated immobilization (rats with MCAO)	↓SIgA; ↑ bacterial translocation, colon inflammation	Caso et al. (2009)
Acute immobilization stress (rats)	↓SIgA; ↑colon inflammation	Ponferrada et al. (2007)
Repeated restraint stress (rats)	↑SIgA and α- chain mRNA in proximal and distal gut	Reyna-Garfias et al. (2010)
Weaning, cold stress, mix of piglets infected with ETEC	↑SIgA and ETEC fecal shedding	Jones et al. (2001)

↓ down modulation; ↑ up modulation; EFS, electric foot shock; IgA, immunoglobulin A; IFN-γ, interferon-γ; IL, interleukin; IEL, intraepithelial lymphocytes; MCAO, middle cerebral artery occlusion; mRNA messenger ribonucleic acid; MLN, mesenteric lymph node; PP, Peyer's patches; SIgA, secretory IgA; TCR, T cell receptor; TNF, tumor necrosis factor; CB1R ko, cannabinoid 1 receptor knock out; Mix, mixing newborns from distinct litters; ETEC, enterotoxigenic E. coli.

THE NERVOUS SYSTEM AND THE INTESTINAL IMMUNE RESPONSE

The mutual influence of the nervous system and the intestinal immune response has been widely studied using experimental models of stress. ILs and microbiota from the gut can modulate the nervous system. On the other hand, the nervous system can modulate intestinal immunity by several pathways (de Jonge, 2013).

For instance, the nervous system regulates immune function through the hypothalamic pituitary adrenal axis (HPA). In response to stress, the hypothalamus releases the corticotrophin releasing factor (CRF) into the anterior pituitary, causing the release of adrenocorticotropic hormone (ACTH) into the blood flow. ACTH stimulates the generation of glucocorticoids (cortisol in humans and corticosterone in mice) in the cortex of the adrenal medulla (McClennen et al., 1998; Jezova et al., 1999), which are then released into the blood. Additionally, the nervous system modulates intestinal immunity through the sympathetic autonomic nervous system by triggering the release of catecholamines (adrenaline and noradrenaline) from the adrenal gland medulla (Kvetnanský et al., 1995; Jezova et al., 1999; **Figure 1**).

Stress also triggers the response of a plethora of peptide hormones and neurotransmitters by intrinsic innervation of the ENS. The ENS is connected bidirectionally to the CNS through sympathetic and parasympathetic nerve pathways forming the brain-gut axis (BGA; de Jonge, 2013). The autonomic ENS comprises sympathetic (noradrenergic) and parasympathetic (cholinergic) fibers that interact directly with the CNS through parasympathetic (vagal) and sympathetic splanchnic fibers. Within the ENS the intrinsic nerve fibers are organized in myenteric (Auerbach's plexus), submucosal (Meissner's plexus) and mucosal plexus. The latter contain nerve endings that can make contact with antigen presenting cells (APCs) to control gut immune responses (de Jonge, 2013). Regulation of the BGA is accomplished by the integration of four control levels. Control level one is accomplished by the ENS endowed with local innervations that functionally are independent of the extrinsic nervous connections. Level two entails the prevertebral sympathetic ganglia where peripheral reflex pathways are influenced by preganglionic sympathetic fibers from the spinal cord. Levels three and four are within the CNS. In the level three sympathetic and parasympathetic fibers outflow to the gut is determined in part by reflex with sensory fibers that travel with autonomic nerves. The level four includes higher nerve centers that supply descending signals that are integrated with incoming sensory signals at the level three (**Figure 2**).

At the intestinal level the bilateral communication between the nervous system and intestinal immune response occurs through sympathetic innervations, which influence (i) the differential distribution of immunocytes in different regions of the small intestine (Ke et al., 2011), (ii) the migration of lymphocytes towards Peyer's patches and mesenteric lymphoid nodules (MLN; Gonzalez-Ariki and Husband, 1998), and (iii) the ontogeny of IgA+ B cells populating the intestinal lamina propria (Gonzalez-Ariki and Husband, 2000).

Mouse gut is innervated by fibers expressing adrenergic receptors (Hirafuji et al., 2001; Nasser et al., 2006). Upon interacting with an agonist, these receptors enable enterochromaffin cells to release neuropeptides that affect IgA levels. The secretion of IgA is also influenced by the interaction of peptidic innervations inside Peyer's patches with immunocytes, and of nerve fibers from the

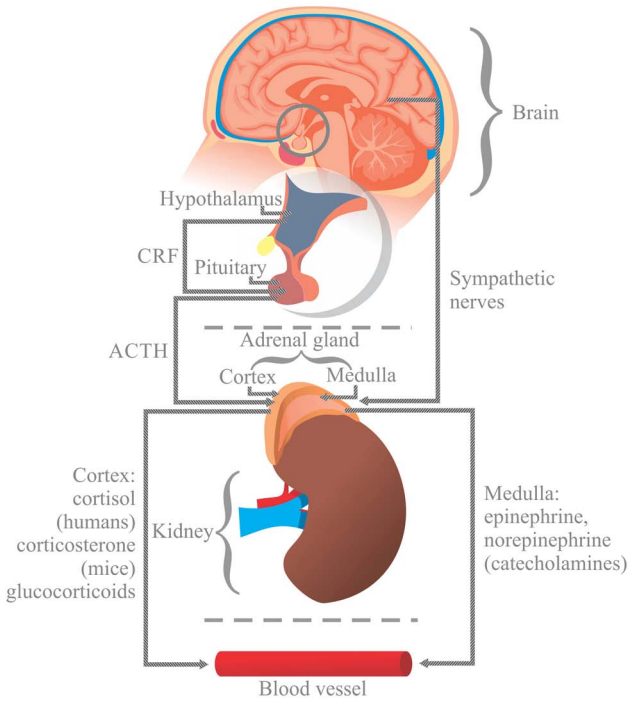

FIGURE 1 | In response to stress, the hypothalamus (H) releases the corticotrophin releasing factor (CRF) into the anterior pituitary (P), causing the release of adrenocorticotropic hormone (ACTH) into the blood flow. ACTH stimulates the generation of glucocorticoids (cortisol in humans and corticosterone in mice) in the cortex of the adrenal gland (A), which are then released into the blood. Stress also activates the autonomic sympathetic nerves in the medulla of the adrenal gland to elicit the production of catecholamines, norepinephrine and epinephrine, which are then released into the blood. Glucocorticoids and catecholamines influence the generation of interleukins, which are involved in the viability and proliferation of immunocompetent gut cells via receptors.

gut basement membrane with IgA+ cells (Hisajima et al., 2005; Vulchanova et al., 2007). Moreover, the release of norepinephrine (Schmidt et al., 2007), acetylcholine (Wilson et al., 1982; Schmidt et al., 2007) and neuropeptides (Schmidt et al., 1999) by gut nerve fibers modulates the secretion of intestinal IgA and the expression of pIgR (Cox et al., 2007). Additionally, gut nerve fibers release the vasoactive intestinal peptide, neuropeptide Y (Mongardi Fantaguzzi et al., 2009) and somatostatin (Schmidt et al., 1999), which all help modulate the intestinal production of SIgA (Shibata et al., 2008).

THE GENERATION OF INTESTINAL IgA

From an immunological point of view, intestinal SIgA is produced by a multistage process modulated by ILs. This process involves the activation and class switch recombination of IgM+ to IgA+ B cells, the latter of which are committed to IgA synthesis either by a T-cell dependent or T-cell independent pathway (Cerutti, 2008).

The T-dependent pathway is induced in follicular areas of Peyer's patches after the interaction between the APCs and helper Th2 lymphocytes (**Figure 3**). On their surface, APCs (like dendritic cells) express the CD40 antigen and a peptide-derived antigen associated with the major histocompatibility class II molecule (MHC-II). The CD40 antigen interacts with CD40L on Th2 cells, and the peptide-MHC-II complex with the Th2 cell receptor (TCR). In either case, immunological synapses lead to the release of the transforming growth factor (TGF)-β1 by Th2 cells, which is an essential step for the activation and class switch recombination of IgM+ B cells to IgA+ B lymphocytes.

Other Th2-derived ILs, including IL-4,-5,-6, and -10, promote the proliferation of IgA+ B cells and their differentiation into IgA secreting plasma cells. In the presence of retinoic acid, IgA+ B cells express gut-homing receptors, such as α4β7 integrin, CCR9 and CCR10, and cause these cells to migrate from Peyer's patches to the MLN via the circulation of efferent lymphatic vessels. From the MLN these cells go to the thoracic duct, enter the bloodstream, and finally home to the lamina propria, the effector site of the gut immune system.

Epithelial cells that line the lamina propria express mucosal address in cell adhesion molecule 1 (MadCAM1) and the chemokines CCL25 and CCL28, which are the ligands for α4β7 integrin, CCR9 and CCR10, respectively, on B cells. In the lamina propria IgA+ B cells mature to plasma cells capable of releasing dimers or polymers of IgA joined to J-chain (Brandtzaeg, 2009; **Figure 3**).

The T-independent pathway for the production of intestinal IgA occurs in extra-follicular structures, including isolated lymphoid follicles and lamina propria. The class switch recombination of IgM+ B cells to IgA+ B lymphocytes takes place through two pathways, and both involve a T-independent antigen. IgM+ B lymphocytes express B cell receptors (BCRs) and Toll-like receptors (TLRs). Polysaccharides interact with BCRs and bacterial lipopolysaccharide (LPS) and/or nucleic acids with TLRs (Cerutti, 2008). In the lamina propria IgA+ B cells further differentiate into IgA+ plasma cells.

THE TRANSPORT OF INTESTINAL IgA

The expression of pIgR is necessary for the transport (transcytosis) of dIgA or pIgA across the epithelial layer. pIgR is a 120 kDa transmembrane protein consisting of five extracellular immunoglobulin (Ig) homology domains, a transmembrane region and a cytoplasmic domain. The amino (NH_2) terminal of this protein chain is oriented to the extracellular space, while the carboxyl (COOH) terminal has an intracellular orientation and contains signals for intracellular sorting and endocytosis (Asano and Komiyama, 2011; Johansen and Kaetzel, 2011).

pIgR is expressed on the basolateral surface of epithelial cells. Its expression can be constitutive or regulated at a transcriptional level by IL-4 and pro-inflammatory cytokines, the latter including tumor necrosis factor α (TNF-α) and interferon γ (IFN-γ; Johansen and Kaetzel, 2011). IgA transcytosis begins when pIgR uptakes dIgA or pIgA released in the lamina propria by plasma cells (Cerutti, 2008). The dIgA-pIgR or pIgA-pIgR complex is transported by vesicles across the epithelial cell, and upon reaching the apical side pIgR is cleaved to render SC bound to dIgA/pIgA. The resulting SIgA is released into the intestinal lumen (Asano and Komiyama, 2011).

The cleavage of pIgR to yield SC occurs at the linker that connects domain 5 to the transmembrane region (**Figure 4**).

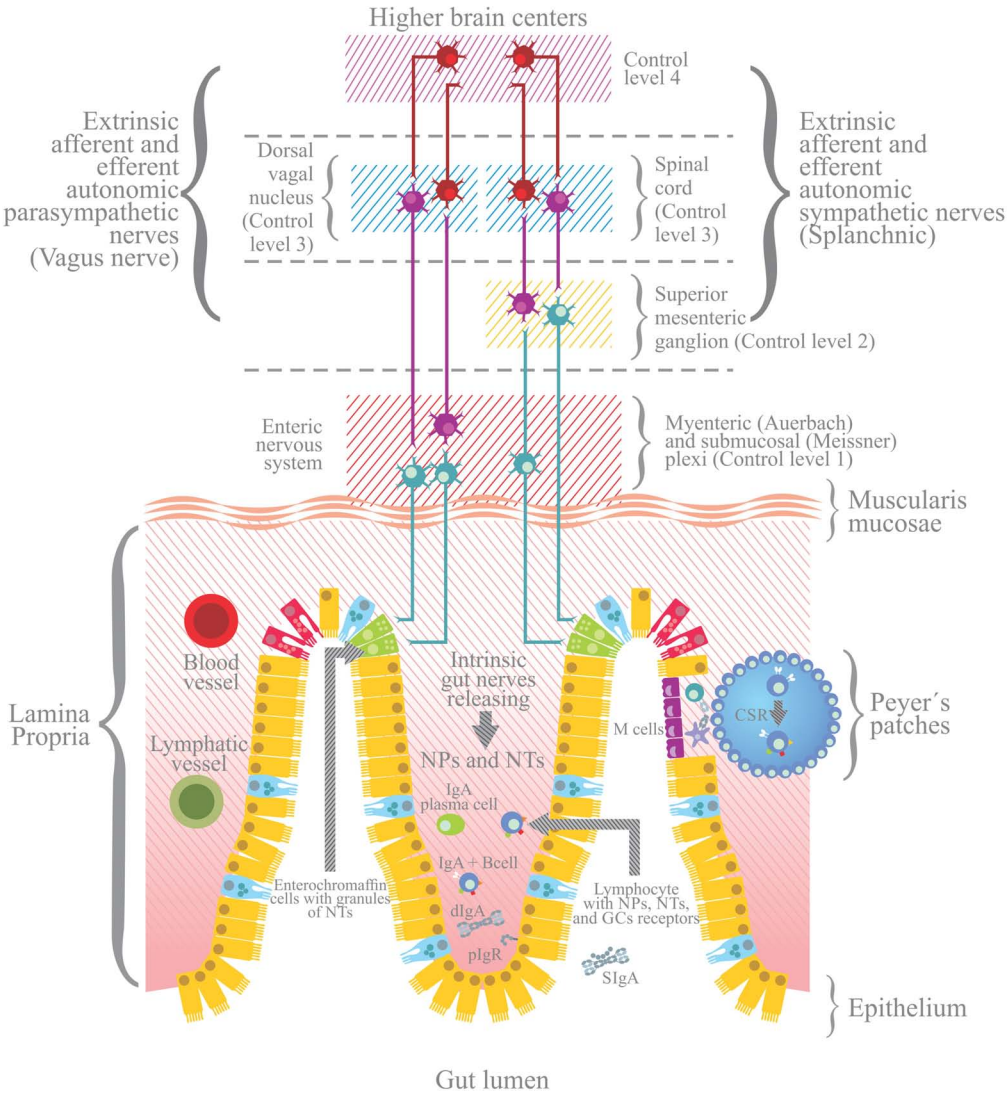

FIGURE 2 | Stress also triggers the activation of the enteric nervous system, including afferent and efferent intrinsic intestinal nerves (afferent nerves send signals from periphery to the brain; efferent nerves from the brain to the periphery) and extrinsic innervations, whether sympathetic (splanchnic) or parasympathetic (Vagus nerve). The enteric nervous system is connected to the CNS via sympathetic and parasympathetic pathways, forming the brain-gut axis (BGA). Four levels for the control of BGA are shown (Wood et al., 1999). The stress response of the BGA influences the generation of dIgA and/or the pIgR mediated trancytosis. NTs, neurotransmitters; NPs, neuropeptides; GCs, glucocorticoids.

Biochemically and morphologically, trancytosis involves: (i) the endocytosis of dIgA from clathrin coated pits and its delivery to basolateral endosomes, (ii) microtubule dependent translocation to apical recycling endosomes, and (iii) delivery of the plasma membrane to apical endosomes (Johansen and Kaetzel, 2011).

THE ROLE OF SIgA AND pIgR

Both SIgA and pIgR have an essential role in immune exclusion, which protects against infections caused by enteropathogens. This role has been explored in the murine model of *Salmonella typhimurium* infection (Michetti et al., 1992; Drago-Serrano et al., 2010).

SIgA helps to limit the adhesion of luminal antigens to the epithelium. These antigens, if not excluded in gut secretions, are able to elicit the release of cell derived inflammatory cytokines, which can enhance permeability and disrupt the functional integrity of the gut. As a result of increased gut permeability, penetration of luminal antigens into the systemic compartment may cause a strong and even life-threatening systemic inflammatory response (Brandtzaeg, 2009; Corthésy, 2007).

pIgA and the different forms of IgA also have an anti-inflammatory role. For instance, pIgA and dIgA protect host tissue by neutralizing pro-inflammatory antigens inside and below the

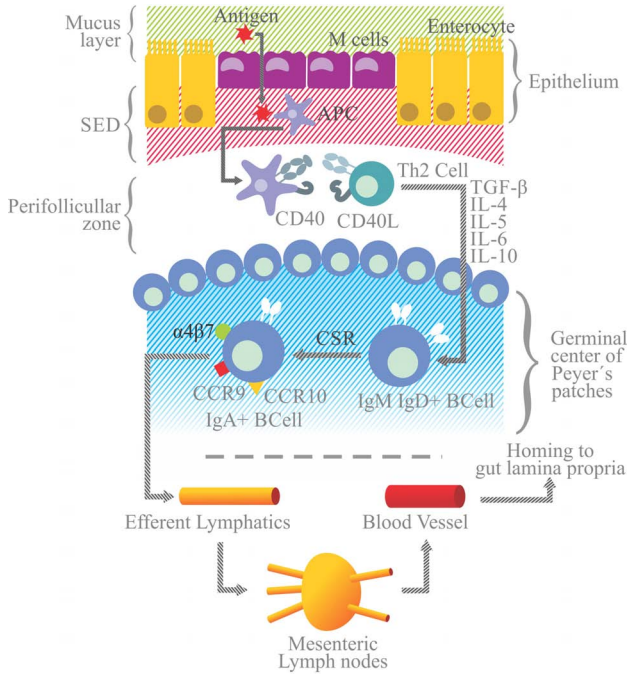

FIGURE 3 | Simplified model of IgA generation. Antigen presenting cells and dendritic cells are located in the subepithelial dome (SED) beneath M cells. After sampling, luminal antigen is processed and expressed on the surface as an antigen-derived peptide associated with the MHC-II molecule. Dendritic cells with the MCH II peptide and the CD40 antigen on the surface migrate to the perifollicular zone of Peyer's patches to interact with Th2 lymphocytes endowed with TCRs and CD40 ligand (CD40L) on their surface. Immunological synapsis between APC and Th2 cells via the binding of the MHC-II peptide to TCR and of CD40 to CD40L leads to the activation of Th2 and the release of TGF-β. Along with signals elicited by the CD40/CD40L binding, TGF-β is a Th2-derived IL essential for the class switch recombination of IgM+ B cells to generate IgA+ B cells, occurring in the germinal center of Peyer's patches (highlighted in blue). Other Th2 ILs (e.g., IL-4, -5, -6, and -10) favor the differentiation and maturation of IgA+ B cells into IgA+ plasma cells. IgA+ B lymphocytes acquire the expression of gut-homing receptors, such as α4β7, CCR9 and CCR10, and migrate toward efferent lymphatic vessels and mesenteric lymphoid nodules. From there they can reach the blood stream and migrate to the lamina propria (the gut effector site). Gut epithelial cells express the MadCAM1 ligand for α4β7, and release CCL25 and CCL28, chemokines that are ligands for the CCR9 and CCR10 receptors, respectively.

gut epithelium layer. On the other hand, dIgA and pIgA are unable to elicit the production of pro-inflammatory cytokines on cells by binding with receptors specific for the Fc α-domain (Corthésy, 2007). Furthermore, SIgA and pIgR, along with the intestinal microflora, contribute to gut homeostasis by maintaining the intestinal inflammatory response within the normal physiological limit (Uren et al., 2003; Sait et al., 2007; Bruno et al., 2010). Since the gut microbiota and SIgA are bilaterally modulated, an alteration in one may affect intestinal homeostasis and lead to intestinal inflammation (Suzuki et al., 2004; Bruno et al., 2010).

Hence, the generation of IgA+ B cells in Peyer's patches, the homing of IgA+ B cells to the gut lamina propria, and the transcytosis of dIgA/pIgA via pIgR are all potential targets of stress-related effects that can alter SIgA levels.

THE RESTRAINT MODEL AND THE INFLUENCE OF STRESS IN THE INTESTINE

Assays based on the restraint model have provided important insights into the influence of stress on the humoral and cellular components involved in the intestinal immune response via neuroendocrine pathways. In the restraint procedure, a rodent is placed (without forced squeezing) inside a cylindrical plastic tube. This represents mainly psychological stress, as the perception of confinement mimics a collapsed tunnel for these burrow-dwelling animals (Dhabhar and Viswanathan, 2005). Another restraint procedure, known as immobilization, involves adhering outstretched rodent limbs on a board with tape. Compared to restraint in a plastic tube, this model has elicited a much more robust stress response through the generation of neuroendocrine mediators (e.g., glucocorticoids and catecholamines; Glavin et al., 1994).

The restraint stress model has provided evidence of intricate neurological pathways underlying the regulation of SIgA. Such pathways involve neurotransmitters and endocrine hormones released from the blood flow or produced locally (e.g., glucocorticoids released by intestinal epithelial cells), and their interaction with the receptors of target cells (Cima et al., 2004).

It is now known that modulation of SIgA production is influenced by the duration (acute or chronic) and intensity of stress. Generally at a systemic level, acute stress (represented by a single session lasting a few minutes to a few hours) tends to upregulate the number of immune cells. Contrarily, the multiple sessions over a period of several days, weeks or months that represent chronic stress (Dhabhar, 2009) tend to downregulate the systemic immune response. Experimental assays with skin delay type hypersensitivity response in rats corroborate this same general pattern of acute and chronic stress (Dhabhar and McEwen, 1997).

Intensity of stress is another factor that can influence the result on the immune response. It is measured by the increase in levels of adrenal hormones, neurotransmitters and physiological parameters (e.g., heart rate and blood pressure), as well as the period of time that these changes persist (during and after the stress-inducing event; Dhabhar, 2009). This assays on mice have shown that (i) the acute stress response elicited by intense running promotes the accumulation of T lymphocytes in Peyer's patches via adrenergic mechanisms, evidenced by the fact that this exercise-dependent increase was blocked by α- (phentolamine) or β- (nadolol) adrenoceptor antagonists (Krüger et al., 2008), and (ii) repeated sessions of chronic restraint stress have a negative influence on intestinal levels of SIgA, which may be due to the capability of corticosteroids to decrease the trancytosis of SIgA via pIgR (Jarillo-Luna et al., 2007). Another study on mice reported that corticosteroids decrease pIgA levels in mucosal secretions and increase such levels in serum. This effect is due in part to the greater production in serum of SC, which is derived from hepatic pIgR. The binding of SC to pIgA retards the clearance of the latter from the blood by the liver (Wira et al., 1990).

In addition to reducing intestinal SIgA levels, repeated stress has a negative influence on the number of lymphoid cells in Peyer's patches and the intestinal intraepithelial compartment. These suppressive effects of restraint stress were mimicked by the administration of pharmacological dose of catecholamines and glucocorticoids, results suggested the activation of two

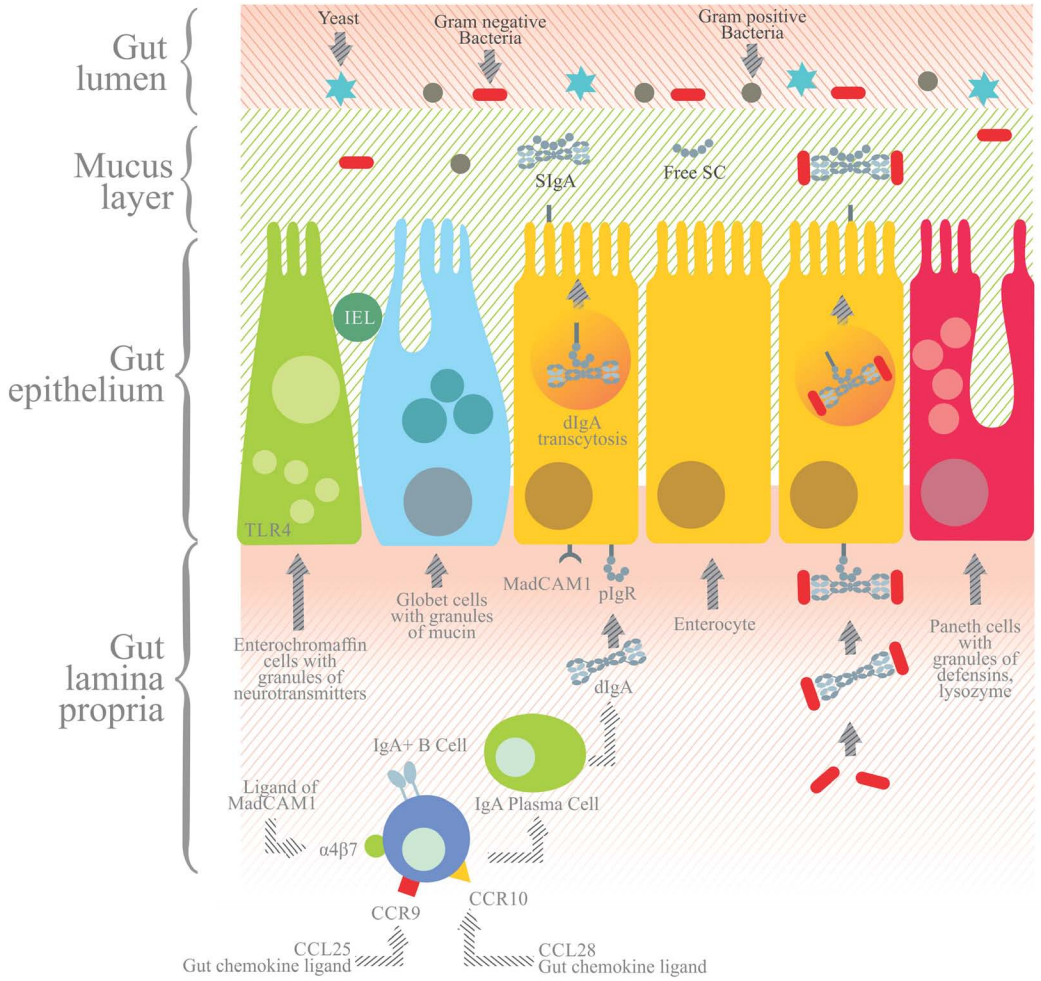

FIGURE 4 | In the gut lamina propria, IgA+ B cells further differentiate into IgA+ plasma cells that secrete dIgA or pIgA antibodies joined by the J chain. dIgA or pIgA are captured by the polymeric immunoglobulin receptor (pIgR), a transmembrane receptor with five extracellular domains and an intracellular tail expressed at the basolateral side of the enterocytes. The dIgA-pIgR complex is internalized and transported by transcytosis to the apical side, and the extracellular portion of pIgR with five domains is proteolytically cleaved from the transmembrane region. The former is then released to the gut lumen as secretory component (SC) bound to dIgA to yield secretory IgA (SIgA). SIgA and SC secreted into the mucus layer prevent the direct adhesion to the epithelium of pathogenic agents, which are eventually cleared from the lumen. Apart from enterocytes, other cell components of the gut epithelium include enterochromaffin cells with granules of neurotransmitters, Paneth cells with granules containing defensins and lysozyme, and goblet cells with mucin granules and IEL.

pathways— the sympathetic autonomic nervous system and the HPA axis (Jarillo-Luna et al., 2007, 2008; Martínez-Carrillo et al., 2011). In another study, the endogenous production of glucocorticoids, triggered by a continuous 12-h period of restraint stress, decreased the number of T and B cells in Peyer's patches, which is in line with a reduction in the levels of intestinal SIgA and lymphocytes (Sudo et al., 2001).

Other experimental stress protocols have been reported to have a negative influence on the gut immune response (**Table 1**). The stress produced in rats by exposure to heat negatively affected some intestinal parameters, including the levels of CD3+ and CD4+ T lymphocytes, the expression of TLR-2 and TLR-4, as well as the transcriptional mRNA expression of IFN-γ and IL-2, -4, and -10 (Liu et al., 2012). Assays on rats with an electric shock protocol showed that stress suppressed the production of IFN-γ through T cells with TCRαβ in the intraepithelial compartment, while at the same time elevating the level of endogenous glucocorticoids (Zhang et al., 2005). Studies on rodents under psychological stress have also reported a decrease in levels of intestinal SIgA, caused by: (i) an expectation of electric foot shock (Yamamoto et al., 2009), (ii) a continuous back and forth transference from housing cages to metabolic cages (Eriksson et al., 2004), and (iii) immobilization, in some cases combined with exposure to loud noise (Ponferrada et al., 2007; Caso et al., 2009; Zoppi et al., 2012).

THE IMMUNOMODULATORY MECHANISMS OF STRESS (TABLE 2)

CELL MIGRATION

The aforementioned stress-related changes in the levels of IgA, IgA secreting cells and IgA producing ILs may be related to cell

Table 2 | Mechanisms of immune modulation by stress.

Effect	Mechanism	Reference
↓SIgA levels by acute immobilization stress	↓ SIgA attenuated by peroxisome proliferator-activated receptor- γ (PPAR)-γ activation	Ponferrada et al. (2007)
↓SIgA levels by immobili-zation and acoustic stress	↓ SIgA attenuated by cannabinoid 1 receptor (CB1R) activation	Zoppi et al. (2012)
↓ activation/migration of T cells induced by restraint stress	Alterations of cytoskeletal actin and plasma membrane factors by stress hormones	Flint et al. (2011)
↓ number of lymphocytes in spleen by restraint stress	Apoptosis through p53 and PI3K/NF-κB pathways	Zhang et al. (2008a)
↓ number of T lymphocytes in Peyer's patches by exercise associated stress	Fas/FasL apoptosis pathway	Krüger et al. (2009)
↓ number of lymphocytes in spleen by chronic restraint stress	μ-receptor mediated apoptosis, dependent on endogenous opioids and independent of glucocorticoids from activation of HPA axis	Wang et al. (2002)
↓ number of splenocytes by chronic restraint stress	CD95 (Fas/APO-1) mediated apoptosis, dependent on endogenous opioids but independent of the activation of HPA axis	Yin et al. (2000)
↑Immunosuppression by chronic restraint stress	Apoptosis via TLR4/PI3K signaling	Zhang et al. (2008b)
↑Immunosupression by restraint stress	↓ MHC-II expression in peritoneal macrophages along with ↑corticosterone levels	Zwilling et al. (1990)
↑Immunosuppression by restraint stress	↓ MHC-II expression influenced by corticosterone and some hormones not associated with the activation of HPA axis	Zwilling et al. (1993)
↑ T cell proliferation or apoptosis by restraint stress	Activation of *GADD45g* and *pura* genes, responsible for apoptosis and proliferation, respectively	Flint et al. (2005)
↑ SIgA levels and ETEC proliferation following stress by weaning and short term exposure to cold	Catecholamines enable iron acquisition that promotes bacterial proliferation	Jones et al. (2001), Lyte et al. (2011)

↓ down modulation; ↑up modulation; MHC-II, major histocompatibility complex class II; PI3K/NFκB, phosphoinositide 3 kinase/nuclear factor κB; TLR4, Toll-like receptor 4; HPA, hypothalamus-pituitary-adrenal axis; GADD45g, growth arrest and DNA-damage inducible gamma; pura, purine rich element binding protein A; ETEC, Enterotoxigenic Escherichia coli; SIgA, secretory immunoglobuline A.

migration, a phenomenon that has been studied mostly in the systemic response (Bauer et al., 2001). Acute restraint stress decreases the number of peripheral helper T lymphocytes, upregulates the expression of adhesion molecules (CD11a and CD11b) on T cells, and increases the levels of circulating NK cells and glucocorticoids. These changes were not found in mice previously exposed to chronic intermittent restraint stress, suggesting an adaptation response to prolonged stress.

One presumable mechanism entails greater restraint-induced levels of glucocorticoids and/or catecholamines, which influence lymphocyte trafficking through the expression of adhesion molecules on endothelial cells (Dietrich, 2004; Krüger and Mooren, 2007; Krüger et al., 2008). This effect was found in mice under stress caused by exercise (Krüger et al., 2008). It seems that stress hormones influence lymphocyte migration and function through specific alterations in the actin cytoskeleton, an effect also found in mice under restraint stress (Flint et al., 2011).

CELL VIABILITY AND T CELL ACTIVATION

Another downmodulatory mechanism related to restraint stress is a decrease in cell viability and/or T cell activation. For instance, at a systemic level the restraint stress protocol elicits a reduction in splenic lymphocytes by apoptosis through the activation of p53 and PI3K/NFκB pathways (Zhang et al., 2008a). p53 is a pro-apoptotic factor which upmodulates the expression of Fas. Phosphoinositide 3 kinases (PI3K) are signal transduction enzymes involved in regulating cell proliferation, and the nuclear factor κB (NFκB) modulates the expression of genes involved in the innate and adaptive immune responses, as well as in cell survival and death (Zhang et al., 2008a).

At the intestinal level, the decreased number of viable lymphocytes in Peyer's patches induced by restraint stress may also lead to apoptosis (Sudo et al., 2001), which seems to be dependent on the Fas/Fas ligand activation signal, as evidenced by T cells in Peyer's patches of mice under stress by intense exercise (Krüger et al., 2009). One report suggested that glucocorticoids are the main apoptotic inducers involved in the decreased number of intestinal intraepithelial lymphocytes (Brunner et al., 2001), which is in agreement with other studies. The molecular mechanisms of glucocorticoid-induced apoptosis are highly dependent on the binding of this ligand with its receptor (Schmidt et al., 2004), which is a cytosolic ligand-dependent transcription factor. After

binding to the ligand, the glucocorticoid receptor dissociates from the protein complex, dimerizes and translocates into the nucleus, where it then binds to regulate the transcription of apoptotic genes (Schmidt et al., 2004).

Another presumable mechanism of lymphocyte apoptosis induced by restraint stress relies on signals triggered by the interaction of endogenous opioids with μ-opioid (Wang et al., 2002) and CD95 receptors (Yin et al., 2000). Cell death caused by the binding of endogenous opioids with CD95 (also known as Fas or apo1) or μ-opioid seems to be independent of the HPA axis (Yin et al., 2000). The binding of CD95 with specific agonists induces the activation of a cascade of caspases, and ultimately nucleases, that results in apoptotic cell death (Yin et al., 2000). Endogenous opioid peptides (endorphins, enkephalins and endomorphins) play a critical modulatory role in emotional stress-induced changes in the immune system (Bodnar, 2012).

An additional mechanism by which restraint stress downmodulates lymphocytes is through the activation of TLR-4, which in turn inhibits the activation of the PI3K (Zhang et al., 2008b). While inhibition of the PI3K signaling pathway induces lymphocyte apoptosis (Yin et al., 2006), its activation inhibits the same (Wu et al., 2000). TLR-4 can also mediate signaling that leads to cell death through the interaction of the death domain of myeloid differentiation factor 88 (MyD88) with the Fas associated death domain (FADD; Haase et al., 2003).

THE INHIBITION OF MHC-II

Expression of the MHC-II glycoprotein by APC is essential for the initiation of the immune response by CD4+ T cells (Blum et al., 2013). A stress-induced decrease in MHC-II expression is carried out by elevated levels of corticosterone (Zwilling et al., 1990) and other hormones not associated with the HPA axis (Zwilling et al., 1993). It seems that a higher level of corticosterone triggered by restraint stress diminishes the number of IFN-γ receptors on macrophages. Since the binding of lFN-γ to its receptor triggers the signaling necessary for MHC-II expression (Zwilling et al., 1992), a reduction in the expression of this receptor decreases the expression of MHC-II.

ENDOGENOUS RECEPTORS CAN ATTENUATE THE STRESS-INDUCED DOWN REGULATION OF SIgA

Assays conducted on mice under a protocol of immobilization, in some cases with exposure to loud noise, evidenced that the immunosuppressive influence of stress on SIgA can be attenuated by the activation of the cannabinoid 1 receptor (CB1R; Zoppi et al., 2012) and the peroxisome proliferator-activated receptor (PPAR)-γ (Ponferrada et al., 2007). Both the CB1R (Hill and McEwen, 2010) and PPAR-γ nuclear receptors (Dubuquoy et al., 2006) have an essential role in the modulation of colon inflammation by stress.

Cannabinoid 1 receptor is one of the most prominent receptors for cannabinoids distributed in the CNS and peripherally in immune cells. It is a G-protein coupled receptor whose endogenous ligands are arachidonate derived lipophilic molecules, N-arachidonylethanolamine anandamide and 2- arachidonylglycerol, which affect emotional behavior (Hill and McEwen, 2010).

Peroxisome proliferator-activated receptor-γ is a nuclear receptor expressed in the colon that forms a heterodimer with the retinoid X receptor (RXR). It is activated by natural endogenous ligands, polyunsaturated fatty acids (PUFAs) and eicosanoids, allowing for its heterodimerization with RXR and its binding to the nuclear peroxisome proliferator response element (PPRE). PPAR-γ and RXR play a central role in the regulation of inflammatory signaling pathways by acting on kinases and transcription factors, such as NFkB, and by inhibiting mucosal production of inflammatory cytokines (Dubuquoy et al., 2006). When PPAR-γ expression in intestinal epithelial cells is induced by LPS-activated TLR-4, it leads to the regulation of NFkB and MAPK pathways and modulation of the inflammatory response. Up regulation of TLR-4 expression together with impaired expression of PPAR-γ in epithelial cells may lead to superficial colonic inflammation in patients with ulcerative colitis.

THE UPMODULATORY EFFECTS OF STRESS

Although stress has been regarded as immunosuppressive, it can enhance the levels of IgA (Yamamoto et al., 2009) and CD3+/CD8+ T lymphocytes in the MLN of rats under stress by electric foot shock or exposure to heat (Liu et al., 2012). The capacity of restraint stress to activate the gene expression of purine rich element binding protein A (*pura*) has been reported to be responsible for priming T cells to undergo proliferation (Flint et al., 2005). Thus, the upmodulatory effects of restraint stress hormones on SIgA levels and on mRNA expression of pIgR should not be surprising (Reyna-Garfias et al., 2010). Indeed, at the molecular level it has been reported that glucocorticoids upmodulate the transcriptional mRNA expression of pIgR via a glucocorticoid DNA response element located in the 5'upstream region of the pIgR gene in rat duodenum (Li et al., 1999).

Experimental studies have evidenced that stress can trigger SIgA secretion in response to an enhanced bacterial proliferation, as reported in feces from piglets infected with ETEC under protocols involving weaning and short-term exposure to cold (Jones et al., 2001). In this case, the presumable influence of stress in promoting bacterial proliferation may be related to catecholamines, which can make iron available to bacteria by removing it from host proteins like transferrin and lactoferrin (Lyte et al., 2011).

Although the context of the development of the immune response in the intestine and systemic compartments is different, the modulatory influence of the stress response may share some mechanisms in both cases.

CONCLUSION AND PERSPECTIVES

Gut homeostasis results from neuroimmune modulation by anti- and pro-inflammatory ILs, neurotransmitters and endocrine hormones, all of which influence the generation of intestinal SIgA. This immunoglobin in turn affects intestinal inflammation and permeability, which are essential factors in the functional integrity of the gut under stress conditions. Experimental studies with the restraint/immobilization rodent model have resulted in an up or down modulation of SIgA levels depending on the intensity and time of exposure to stress. In the case of down regulation, there is an increased susceptibility to infection and intestinal inflammation. Pharmacological modulation

of the cannabinoid system and the PPAR-γ may be therapeutically useful for intestinal dysfunctions resulting from a stress-induced decrease in SIgA levels. Future studies should explore the adaptation of experimental models for the evaluation of therapeutic and preventive strategies to control intestinal inflammation and/or infection in patients with high vulnerability to stress.

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Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Received: 05 May 2013; accepted: 12 November 2013; published online: 02 December 2013.

Citation: Campos-Rodríguez R, Godínez-Victoria M, Abarca-Rojano E, Pacheco-Yépez J, Reyna-Garfias H, Barbosa-Cabrera RE, Drago-Serrano ME (2013) Stress modulates intestinal secretory immunoglobulin A. Front. Integr. Neurosci. 7:86. doi: 10.3389/fnint.2013.00086

This article was submitted to the journal Frontiers in Integrative Neuroscience.

frontiers in
INTEGRATIVE NEUROSCIENCE

REVIEW ARTICLE
published: 08 August 2013
doi: 10.3389/fnint.2013.00057

Fetal cholinergic anti-inflammatory pathway and necrotizing enterocolitis: the brain-gut connection begins *in utero*

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Necrotizing enterocolitis (NEC) is an acute neonatal inflammatory disease that affects the intestine and may result in necrosis, systemic sepsis and multisystem organ failure. NEC affects 5–10% of all infants with birth weight ≤ 1500 g or gestational age less than 30 weeks. Chorioamnionitis (CA) is the main manifestation of pathological inflammation in the fetus and is strong associated with NEC. CA affects 20% of full-term pregnancies and upto 60% of preterm pregnancies and, notably, is often an occult finding. Intrauterine exposure to inflammatory stimuli may switch innate immunity cells such as macrophages to a reactive phenotype ("priming"). Confronted with renewed inflammatory stimuli during labour or postnatally, such sensitized cells can sustain a chronic or exaggerated production of proinflammatory cytokines associated with NEC (two-hit hypothesis). Via the cholinergic anti-inflammatory pathway, a neurally mediated innate anti-inflammatory mechanism, higher levels of vagal activity are associated with lower systemic levels of proinflammatory cytokines. This effect is mediated by the α7 subunit nicotinic acetylcholine receptor (α7nAChR) on macrophages. The gut is the most extensive organ innervated by the vagus nerve; it is also the primary site of innate immunity in the newborn. Here we review the mechanisms of possible neuroimmunological brain-gut interactions involved in the induction and control of antenatal intestinal inflammatory response and priming. We propose a neuroimmunological framework to (1) study the long-term effects of perinatal intestinal response to infection and (2) to uncover new targets for preventive and therapeutic intervention.

Keywords: necrotizing enterocolitis, chorioamnionitis, preterm birth, inflammation, neuroimmunology, vagus nerve, intestines, prevention

INTRODUCTION

In this review we summarize the emerging understanding of the mechanisms of necrotizing enterocolitis (NEC; **Table 1**) of the neonate and the clinical significance of the cholinergic anti-inflammatory pathway (CAP) as a neuroimmunological mechanism to prevent NEC. We propose how this emerging understanding of neuroimmunological basis for the NEC etiology may lead to new avenues of clinical research that could result in low cost widely available treatment strategies of NEC across the world. Overall, this review provides a comprehensive, up-to-date review of the literature about the role of CAP in the pathogenesis of NEC as a neuroimmunological modulation system and the emerging therapeutic strategies.

THE BURDEN OF NEC

NEC is an acute neonatal inflammatory disease that affects the intestine and may result in necrosis, systemic sepsis and multisystem organ failure. The incidence varies from 0.3 to 2.4 infants per 1000 live births, with nearly 90% of cases occurring in infants born at less than 36 weeks' gestation. NEC affects 5–10% of all infants with birth weight ≤ 1500 g or gestational age less than 30 weeks, and 2–5% of all preterm neonates (Lin and Stoll, 2006). NEC accounts for up to 8% of all admissions to the neonatal intensive care unit. Moreover, with the increased survival of very preterm infants with birth weights ≤ 800 g, the incidence of NEC has increased, despite surfactant replacement therapy (Lin et al., 2008). NEC is the leading cause of death and long-term disability from gastrointestinal disease in preterm infants. Overall mortality ranges from 10–50%, approaching 100% in infants with the most severe form of the disease, which is characterized by full-thickness destruction of the intestine leading to intestinal perforation, peritonitis, bacterial invasion, sepsis and multiorgan failure.

A NEC diagnosis in the very low birth weight infant poses a significant burden to the individual family and the neonatal community, as well as a serious financial burden to society as a whole. NEC is not only one of the most serious clinical problems in neonates, but also one of the most challenging to treat.

Table 1 | Abbreviations.

CAP	Cholinergic anti-inflammatory pathway
CNS	Central nervous system
ENS	Enteric nervous system
LPS	Lipopolysaccharide
α7nAChR	α7 subunit nicotinic acetylcholine receptor
NEC	Necrotizing enterocolitis
TNF-α	Tumor necrosis factor alpha

Because the etiology and underlying mechanisms are unknown, treatment is symptomatic. Minor cases and early stages are managed with antibiotics and cessation of oral feeding; advanced cases, marked by intestinal necrosis, may require intestinal resection.

Despite several decades of research on the pathogenesis of NEC (Hunter et al., 2008), the overall mortality rate remains high and our overall understanding of its causes remains low. Clearly, a more complete understanding of the causes of NEC is required to design more effective and widely affordable preventive strategies aimed at reducing the incidence of NEC as well as therapies (Bisquera et al., 2002; Ganapathy et al., 2012).

CHORIOAMNIONITIS: PATHOLOGICAL FETAL INFLAMMATION IS A RISK FACTOR

The events leading to NEC are complex and multifactorial, including preterm birth, complicated early neonatal trajectory, adverse intrauterine environment and poor perinatal transition. The most important ones are preterm birth and a history of enteral feeding (Lin and Stoll, 2006). Chorioamnionitis is the main manifestation of pathological inflammation in the fetus and affects 20% of term pregnancies and up to 60% of preterm pregnancies and is commonly an occult finding (Lahra and Jeffery, 2004; Gotsch et al., 2007). Chorioamnionitis is histologically defined by the presence of polymorphonuclear infiltrates in the placenta and its membranes. Even silent, asymptomatic inflammation may inhibit placental angiogenesis and thus modulate the course of the pregnancy (Garnier et al., 2008). Thus, a significant number of fetuses are exposed to various degrees of inflammation, which impacts on their intestinal development. Chorioamnionitis associated with maternal infection has been strongly implicated in fetal intracerebral hemorrhage (Andrews et al., 2006, 2008; Aziz et al., 2009). However, an increased incidence of NEC has also been reported in neonates of mothers presenting with chorioamnionitis, in several independent studies (Andrews et al., 2006; Aziz et al., 2009; Been et al., 2009) as well as a recent meta-analysis (Been et al., 2013), where clinical chorioamnionitis (OR 1.24; 95%, CI 1.02–1.52) and histological chorioamnionitis with fetal involvement (CI 3.29; 95%, OR 1.87–5.78) were significantly associated with NEC. However, the association of histological chorioamnionitis with NEC was not statistically significant. The role of prenatal infection in the development of NEC is most significant in very preterm infants (24–26 weeks' gestational age) (Aziz et al., 2009), but is also apparent in preterm births < 32 weeks (Andrews et al., 2006; Been et al., 2009) and in full-term births (Martinez-Tallo et al., 1997).

TIGHT JUNCTIONS: INTESTINAL PERMEABILITY AND INTEGRITY

After birth, the intestinal lumen is subject to external environmental influences, including bacterial colonization of the gastrointestinal tract (Gronlund et al., 1999). Cells that cover the intestinal surface must form a barrier to protect the "*milieu intérieur*" from the external world and prevent unrestricted exchange of materials. The intestinal epithelial barrier needs to allow the passage of water and nutrients but prevent microbial contamination and the invasion of interstitial tissues by foreign antigens (**Figure 1**) (Turner, 2009). Tight junctions, essential to the paracellular pathway, are the primary determinants of barrier function. Tight junctions are situated at the apical pole of epithelial cells, and comprise over 50 associated proteins. The first group includes claudins (a family of at least 24 members) and occludin. These proteins span the plasma membrane and are attached to a second group of proteins including zonula occludens (ZO) -1, -2 and -3, which link them to actin and myosin in the cytoskeleton (Turner, 2009).

The properties of tight junctions differ from one tissue to another, as evidenced by the 1000-fold variation in electrical resistance between different epithelia. Barrier properties are not fixed: they can be modulated and regulated on a short- or long-term basis. Short-term regulation is achieved by deformation of the cytoskeleton: myosin light chain phosphorylation permits interaction with actin and contraction of the actin filaments in the perijunctional ring. Contraction of the actin filaments favours the passage of macromolecules by opening the pores. Myosin light chain kinase-1 (MLCK-1) controls the phosphorylation/dephosphorylation of the myosin light chains in the villus enterocytes and surface colonocytes (Clayburgh et al., 2004). Further downstream, during alterations of the tight junction permeability, the transmembrane protein occludin is subject to endocytosis (Schwarz et al., 2007; Turner, 2009). Long-term regulation of intestinal permeability depends on the synthesis and trafficking of claudin-2, a molecule that is overexpressed in intestinal cells of animal models of colitis and in human ulcerative colitis specimens (Heller et al., 2005).

It has been suggested that abnormalities in intestinal permeability may be key to the facilitation of intestinal inflammation leading to NEC (Petrosyan et al., 2009). Intestinal barrier integrity and its appropriate regulation are essential to the prevention of antigen diffusion and bacterial contamination into the lamina propria and interstitial tissue. McElroy et al. (2012) however, have recently suggested an alternate "bottom up" hypothesis whereby NEC may be caused by injury to the Paneth cells located in the crypts of Lieberkühn (McElroy et al., 2012).

INTESTINAL PERMEABILITY IS INFLUENCED BY PATHOGENS AND INFLAMMATION

Intestinal barrier dysfunction in newborns may be triggered by exogenous agents. Such enteric pathogens trigger local inflammatory responses through specific receptors (e.g., toll-like receptor 4 (TLR4) for lipopolysaccharide (LPS) recognition) or by proinflammatory cytokines (e.g., tumour necrosis factor alpha

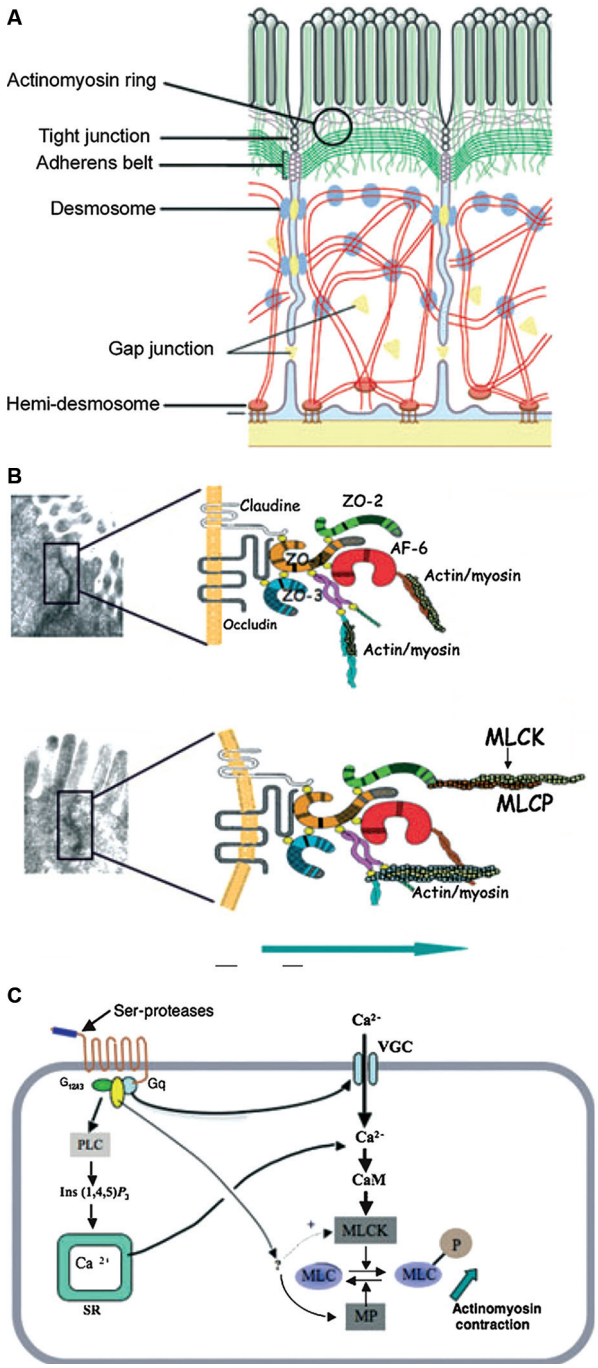

FIGURE 1 | Structures and factors controlling paracellular permeability. Tight junctions are luminal structures that filter the passage of ions and macromolecules **(A)**. The intercellular structural proteins, claudins and occludins, are anchored to the cytoskeleton actinomyosin apical ring through intermediary proteins (ZO-1, -2, -3, AF6). The phosphorylation of myosin light chain causing contraction is associated with opening of tight junctions and junction protein deformations that favour macromolecule passage and bacterial translocation **(B)**. The phosphorylation/dephosphorylation of myosin light chain is controlled by myosin light chain kinase (MLCK) and myosin phosphatase (MP). PAR-2 activation increases MLCK activity through external and internal calcium (Ca++) mobilization and calmodulin binding **(C)**. (With permission from John Wiley and Sons) (Bueno and Fioramonti, 2008).

(TNF-α) (Clayburgh et al., 2005), interferon-γ (Wang et al., 2005), interleukin (IL) 1-β (Al-Sadi et al., 2008) and high-mobility group box 1 (HMGB1) protein (Sappington et al., 2002; Liu et al., 2006; Raman et al., 2006). TNF-α-induced barrier loss is associated with increased transcription and translation of MLCK (Clayburgh et al., 2005). In vivo, intestinal epithelial MLCK is induced by TNF-α. In the rat model of sepsis, an intraperitoneal injection of LPS demonstrably resulted in a rapid rise of TNF-α in the colonic mucosa followed by an increase in myosin light chain phosphorylation and colonic permeability (Moriez et al., 2005). Interestingly, studies in the fetal rat model of NEC have also shown that prenatal bacterial LPS exposure alters the development and permeability of intestinal epithelium (Giannone et al., 2006) and increases ileal injury (Giannone et al., 2009). Similarly, in fetal sheep, preterm intra-amniotic LPS exposure induces abnormal expression of ZO-1 in the ileum (Wolfs et al., 2009).

ENS CONTROLS EPITHELIAL BARRIER FUNCTION

The enteric nervous system (ENS) is defined as the arrangement of neurons and supporting cells throughout the gastrointestinal tract, from the esophagus to the anus (Goyal and Hirano, 1996). The ENS is organized in ganglia that contain neurons, glial cells and interstitial cells. Each enteric ganglion contains many different neuron types (Furness, 2000). The ENS consists of some one hundred million neurons, or about one-tenth of the number of neurons of the spinal cord. Glial cells in the ENS have similar properties to those in the central nervous system (CNS) (Gershon et al., 1993). The numbers and types of neurotransmitters expressed by enteric neurons are comparable to those found in the CNS. The ENS is capable of autonomy with elicitation of reflexes (complete reflex circuitry in the intestinal wall comprises intrinsic sensory neurons, interneurons and intrinsic motor neurons) after total extrinsic denervation of the gut (Furness et al., 1995). However, the ENS is under physiological influence of the sympathetic and vagus nerves. The ENS controls intestinal motility, modulates visceral sensation and plays a role in the regulation of the intestinal blood supply and the secretion of digestive hormones (Costa and Brookes, 1994; Kunze and Furness, 1999; Boeckxstaens, 2002). It also plays a major role in water and electrolyte transport. As a consequence, intestinal permeability is under neural control (Keita and Soderholm, 2010). The ENS should thus be considered, along with the microflora, immune system and fibroblasts, as a major player in the maintenance of intestinal homeostasis and integrity. The ENS has the ability to fine-tune intestinal barrier function via the release of mediators such as acetylcholine that enhance—via muscarinic receptors—(Cameron and Perdue, 2007) and vasoactive intestinal peptide (VIP) that constrict (Neunlist et al., 2003) intestinal permeability over short-term or long-term periods (Neunlist et al., 2012). Similarly, the ENS can modulate the proliferation and differentiation of the intestinal epithelial barrier via the secretion of distinct neuromediators such as VIP (Neunlist et al., 2012). VIP exerts antiproliferative effects, while mediators such as acetylcholine, glucagon-like peptide 2 (GLP-2) or substance P stimulate intestinal epithelial cell proliferation (Neunlist et al., 2012).

NEC: IMMATURE IMMUNE RESPONSE

Local intestinal immune response is normally tightly controlled (Su et al., 2009).

The premature gastrointestinal tract has increased permeability, low levels of protective mucus and secretory immunoglobuline A, higher risk of bacterial overgrowth caused by dysmotility due to ENS immaturity and decreased regenerative capabilities (Neu, 2007). Uncontrolled intestinal inflammation may result from immaturity of the innate immune system of the developing gut (Lin and Stoll, 2006; Lin et al., 2008). Immature regulation could lead to an exaggerated inflammatory response, leading to greater injury and increased intestinal barrier damage. Alternatively, immature regulation could result in minimal immune response due to insufficient inflammatory signalling, thus contributing to bacterial overgrowth and invasion of interstitial tissue. The uncontrolled intestinal inflammation observed in NEC may also depend on dysregulation of intestinal permeability in relation to localized immune response (Turner, 2009). In most individuals and specifically in healthy full-term newborns, a localized break in the intestinal barrier induces a localized immune response that is finely tuned and controlled to avoid over-inflammation and a subsequent increase of intestinal permeability. This normal immunoregulatory response is the result of a delicate balance between pro-inflammatory (TNF-α, IL-1β) and anti-inflammatory (IL-10) processes. If even small anomalies occurred in any of the components of the system (tight junction dysregulation, immune regulatory response), the inflammatory response would be amplified and would result in intestinal injury. Such anomalies may occur secondary to immaturity in preterm babies.

Furthermore immunomodulatory nutrients such as glutamine, arginine, nucleotides, omega-3 polyunsaturated fatty acids and lactoferrin are provided with enteral nutrition and prevent diseases such as NEC. Difficulties with enteral feeding in the first weeks of life predispose premature infants to sepsis and NEC (Neu et al., 2013).

DEVELOPMENT AND MONITORING OF THE AUTONOMIC NERVOUS SYSTEM ACTIVITY

The autonomic nervous system (ANS) plays a predominant role in complex coordinated control of multiple vitally important physiological subsystems in the organism and is part of the neuroimmunological response to pathogens via CAP (Fairchild et al., 2011). Since ANS development and activity are reflected in the heart rate patterns, an appropriate analysis of the fetal heart rate variability (fHRV) may provide information regarding the individual fetal development (Hoyer et al., 2013; Van Leeuwen et al., 1999). fHRV is a non-invasively obtainable marker of changes in vagal (parasympathetic) and sympathetic activity (Frank et al., 2006). Increase in fHRV is associated with fetal growth in general and with the increase in neural integration in particular (Van Leeuwen et al., 2013). Understanding of the dynamics of fHRV in human and ovine fetuses during physiologic (e.g., sleep states) and pathophysiologic (e.g., asphyxia) conditions has evolved over the past two decades (Karin et al., 1993; Frank et al., 2006; Shapiro et al., 2013). Fetal heart rate (FHR) and fHRV are regulated by a complex interplay of the parasympathetic and sympathetic nervous systems accounting for the baseline FHR as well as short-term and long-term variability and nonlinear properties (Frasch et al., 2009; Gieraltowski et al., 2013). Nonlinear properties of fHRV in late gestation fetuses are present and a higher vagal tone is associated with more efficient regulation of homeostasis (Groome et al., 1999).

CAP CONTROLS IMMUNE HOMEOSTASIS

CAP has been implicated in the regulation of the inflammatory reflex in adult organisms including humans (Tracey, 2002, 2007, 2009; Cailotto et al., 2012; Olofsson et al., 2012). CAP is a neural mechanism that influences the magnitude of the innate immune response and maintains homeostasis. As part of CAP, increased vagal activity inhibits the release of proinflammatory cytokines (**Figures 2**,**3**). Vagal nerve stimulation decreases LPS-induced systemic TNF-α release in adult rats (Borovikova et al., 2000). Suppression of proinflammatory cytokine expression via agonistic action on the α7 subunit nicotinic acetylcholine receptor (α7nAChR) was confirmed in innate immune cells such as macrophages (reviewed in Tracey, 2007) (**Figures 2**,**3**). Systemically via CAP, this is mediated via the spleen: Adrenergic nerve fibres in the spleen activate acetylcholine-producing T-lymphocytes, thereby inhibiting systemic cytokine production (Huston et al., 2007; Rosas-Ballina et al., 2011; Olofsson et al., 2012).

Vagal nerve activity in basal conditions provides inhibitory input that dampens innate immune response (Haensel et al., 2008; Thayer and Fischer, 2009). The CAP influences the magnitude of the innate immune response and maintains homeostasis (Tracey, 2009). Depressed vagal nerve activity is associated with an exaggerated proinflammatory response and increased morbidity and mortality in various contexts such as acute or stable coronary heart disease, metabolic syndrome or impaired glucose tolerance and kidney failure (Haensel et al., 2008; Thayer and Fischer, 2009). The inhibitory role of CAP on innate immune function can be thought of as analogous to the inhibitory role of the vagus nerve on the resting heart rate (Tracey, 2009).

Under resting conditions, the inflammatory reflex helps establish the set point for the magnitude of the innate immune response to molecules arising from infection, injury or ischemia. Vagus nerve output maintains homeostasis by limiting proinflammatory response to the healthy, protective and non-toxic range (**Figure 4**). However, when vagal activity is absent or diminished, the set point increases; exposure to pathogens then results in an exaggerated proinflammatory response and eventual tissue damage as demonstrated in different experiments with murine models (Ghia et al., 2006, 2007a,b, 2008).

Numerous factors can experimentally or clinically impair the CAP, each resulting in an exaggerated innate immune response. For instance, in animal models with vagotomy or deficient in α7nAChR, the magnitude of the proinflammatory cytokine response and the extent of tissue damage increase during infection, haemorrhagic shock and stroke (Guarini et al., 2003; van Westerloo et al., 2005; Ghia et al., 2008; Ottani et al., 2009; Tracey, 2009). The observation that vagal nerve activity influences circulating TNF-α amounts and the shock response to endotoxemia has widespread implications. It is a previously

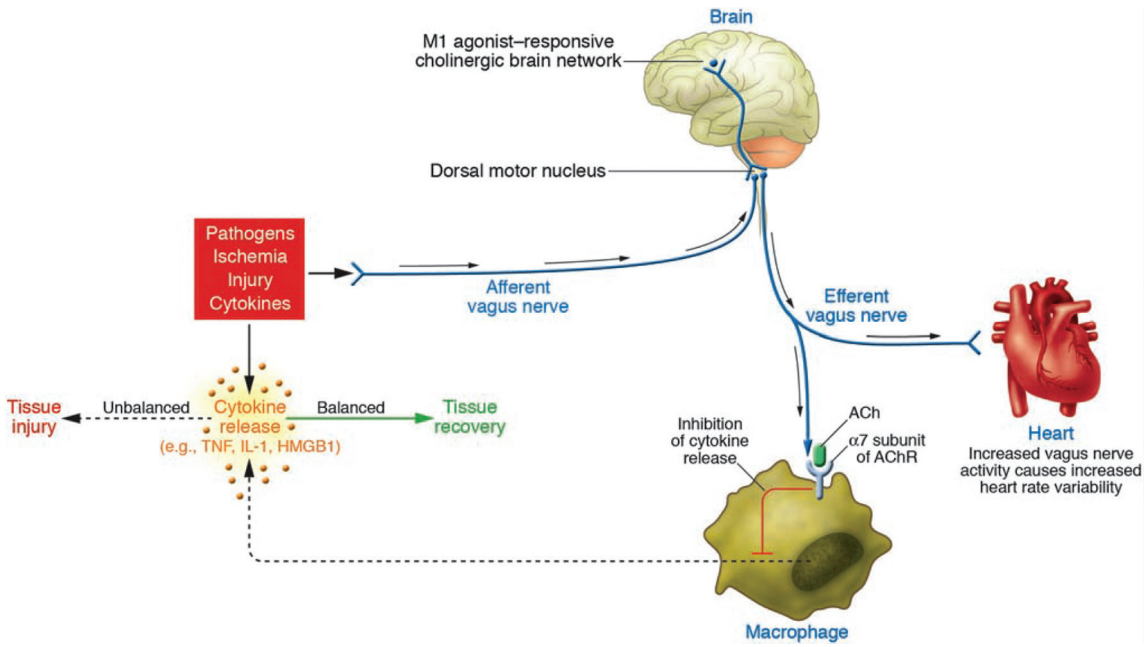

FIGURE 2 | Wiring of the cholinergic anti-inflammatory pathway (CAP), which balances cytokine production. Pathogens as well as ischemia and other forms of injury activate cytokine production, which normally restores health. If the cytokine response is excessive, however, then these same mediators can cause disease. Efferent signals from the vagus nerve inhibit cytokine production via pathways dependent on the *α7 subunit* of the acetylcholine receptor (*AChR*) on macrophages and other cells. Efferent vagus nerve activity also increases instantaneous heart rate variability (HRV). A cholinergic brain network that is responsive to M1 agonists can increase the activity of CAP and also increase instantaneous HRV. Afferent signals carried in the vagus nerve can activate an efferent response that inhibits cytokine release, termed the *inflammatory reflex* (With permission from American Society for Clinical Investigation) (Tracey, 2007).

unrecognized, direct and rapid endogenous mechanism that can suppress the lethal effects of biological toxins. The CAP has much shorter response times than humoral anti-inflammatory pathways. Electrical stimulation of the vagus nerve or administration of α7nAChR agonists reduces the magnitude of proinflammatory cytokine production by 50–75% but does not eliminate cytokine activity (Borovikova et al., 2000; Tracey, 2009). Activation of CAP has not been observed to cause immunosuppression because maximal suppression only reduces proinflammatory cytokine levels from the toxic to the healthy range (**Figure 4**). This concept has been studied as a potential treatment for a range of inflammatory diseases, including infection (reviewed in Tracey, 2009). The role of CAP in the perinatal inflammatory response and the priming of subsequent innate immune response require elucidation.

Adult clinical data indicate that loss of inhibition from the CAP unleashes innate immunity, produces higher levels of proinflammatory mediators, and exacerbates damage to the organism (Lindgren et al., 1993; Lanza et al., 2006; Thayer and Sternberg, 2006; Marsland et al., 2007; Sloan et al., 2007; Tateishi et al., 2007; Haensel et al., 2008; Holman and Ng, 2008; von Kanel et al., 2008) and exacerbation of tissue damage. Studies are needed to explore the impact of CAP activity on chorioamnionitis (Shapiro et al., 2013). Such studies could lead to the development of novel prognostic markers to better identify fetuses at risk of NEC. We hypothesize that increased CAP activity would inhibit the activation of intestinal innate immune cells such as macrophages and thus suppress the inflammatory response to bacterial infection. Effectively, pathological inflammation and intestinal permeability as a *locus minoris resistentiae* of incipient NEC would be decreased or normalized.

VAGAL NERVE STIMULATION AND THE GUT INFLAMMATION

As vagal nerve stimulation stimulates CAP activity without causing immunosuppression (Borovikova et al., 2000), it has been shown to improve intestinal inflammation in murine models of experimental colitis (Ghia et al., 2006, 2007a,b, 2008) and postoperative ileus (The et al., 2007; van Bree et al., 2012). The role of intestinal macrophages seems pivotal (The et al., 2007; Costantini et al., 2010a; Rosas-Ballina et al., 2011; van Bree et al., 2012). In a murine model of dextran sodium sulphate-induced colitis, Ghia et al. demonstrated an increased inflammatory response in the colonic mucosa of the vagotomy group as compared to control animals. They showed the protective effect of vagal activity in acute colitis (Ghia et al., 2006) and in acute relapses within a background of chronic inflammation (Ghia et al., 2007b).

In a murine model of postoperative ileus, the anti-inflammatory effect of intracerebroventricular injection of semapimod was abolished in the presence of vagotomy (The et al., 2011). Vagal nerve stimulation also modulates intestinal permeability and integrity. In a murine model of intestinal injury caused by severe burns, vagal nerve stimulation performed before injury improved intestinal barrier integrity through an efferent signalling pathway and was associated with improved tight junction

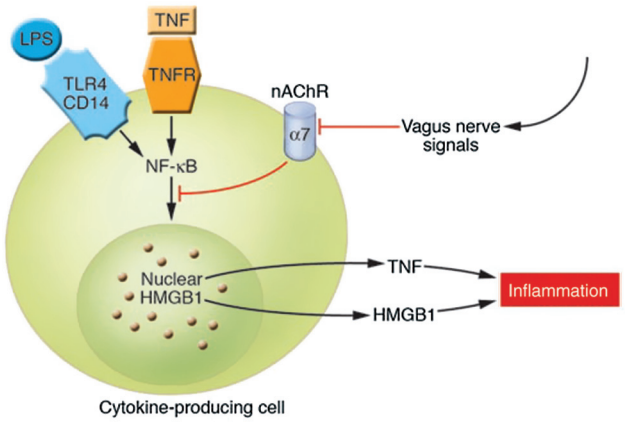

FIGURE 3 | Cholinergic signals derived from vagus nerve stimulation inhibit the release of TNF-α, IL-1, HMGB1, and other cytokines by transducing a cellular signal that inhibits the nuclear activity of NF-κB. TNFR stands for TNF receptor (Wang et al., 2004; Tracey, 2007). The cytokine producing cell can be a macrophage, among others. The nAChR family are ligand-gated ion channels that mediate diverse physiological functions and were originally identified in the nervous system. They consist of different subtypes formed by the specific assembly of five polypeptide subunits including α1-10, β1-4, γ, δ, and ϵ. The subunits fall into two groups: neuronal nicotinic receptors (consisting of α2–10 and β2–4) and muscle nicotinic receptors (consisting of α1, β1, γ, δ, and ϵ). Functional neuronal nAChR subtypes are either homomeric (consisting of 5 identical α-subunits, as in α7- or α9 nAChR) or heteromeric (consisting of combinations of the α- and β-subunits, such as α3β2nAChR) (Yeboah et al., 2008). Remarkably, it is specifically the α7nAChR that is required for CAP's effects on peripheral innate immune cells (**Figures 2,3**) (With permission from American Society for Clinical Investigation) (Tracey, 2007).

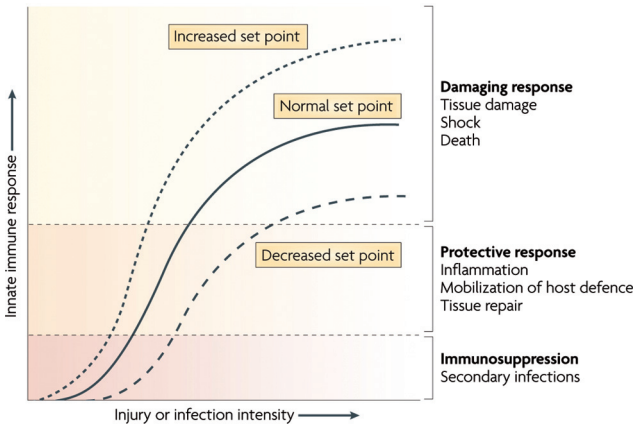

FIGURE 4 | The set point function of the immune response is defined by the magnitude of innate immune responses relative to the infection or injury stimulus. Increasing the set point or shifting the curve to the left increases the chance that tissue damage will occur from the response to infection or injury. Decreasing the set point or shifting the curve to the right reduces the probability that tissue damage will occur. CAP is the neural circuit that provides acute compensatory input to adjust the magnitude of the immune response relative to the set point (With permission from Nature Publishing Group) (Tracey, 2009).

protein expression (Costantini et al., 2010b). In the same model, stimulating the vagus nerve at the time of injury promoted enteric glial cell activation. Either method could prevent intestinal barrier injury (Costantini et al., 2010a). Activation of enteric glial cells has been reported in a rat model of burn-induced stress and gut injury (Costantini et al., 2010a).

The optimal choice of vagal nerve stimulation parameters in order to activate the CAP for therapeutic purposes remains challenging. Although the literature provides a spectrum of possibilities, further studies are needed. Huston et al., 2007 demonstrated improved survival in murine polymicrobial sepsis with transcutaneous vagal nerve stimulation (Huston et al., 2007), suggesting that this mode might be an effective therapy for sepsis generally and NEC specifically. Pharmacologically, cholinergic neuronal circuitry can be stimulated peripherally using α7nAChR agonists to act on macrophages; or centrally using intracerebroventricular injections of semapimod (The et al., 2011), muscarinic receptor agonist McN-A-343 (Pavlov et al., 2006), or an acetylcholinesterase inhibitor (galantamine) (Pavlov et al., 2009). Importantly, enteral feeding of lipid-rich nutrition may also be used to activate the CAP pathway (Luyer et al., 2005).

SIGNIFICANCE AND NOVELTY

Intrauterine infection leads to macrophage activation in LPS-induced chorioamnionitis in fetal sheep and in a rat model of postnatal sepsis. Intrauterine infection may prime innate immune cells for subsequent exacerbated response and increase intestinal permeability. However, the effects of CAP on this process have not been elucidated. The existence of CAP in adult mammals, including humans, is by now well established. CAP may play an important role in fetal and perinatal development by serving as a neuroimmunological network for "internal surveillance" linking the CNS and the vagus nerve to modulate systemic and intestinal inflammation. The presence of such a brain-gut network and its significance for perinatal health have not yet been fully studied. Further research on the physiology and pathophysiology of fetal and perinatal neuroimmunological interactions may open new avenues for diagnosis of fetuses at risk of intestinal injury, such that appropriate preventive or therapeutic interventions may be taken. Specifically, fetal CAP activation is likely to suppress the adverse effects of macrophage activation thus decreasing antenatal and perinatal intestinal injury. New pharmacologic targets or validated non-invasive methods of vagus nerve stimulation for manipulating the inflammatory response in compromised fetuses may emerge to improve perinatal and postnatal outcome.

ACKNOWLEDGMENTS

The authors are grateful to Danielle Buch, medical writer/editor at the Applied Clinical Research Unit of the CHU Sainte-Justine Research Centre, for a quick revision of the manuscript.

FUNDING SUPPORT ACKNOWLEDGMENT

L. Garzoni: Swiss National Science Foundation (SNSF). M.G. Frasch: Canadian Institutes of Health Research (CIHR), Fonds de Recherche Santé Québec (FRSQ) and Molly Towell Perinatal Research Foundation.

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Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Received: 20 May 2013; paper pending published: 03 June 2013; accepted: 18 July 2013; published online: 08 August 2013.
Citation: Garzoni L, Faure C and Frasch MG (2013) Fetal cholinergic anti-inflammatory pathway and necrotizing enterocolitis: the brain-gut connection begins in utero. Front. Integr. Neurosci. 7:57. doi: 10.3389/fnint.2013.00057

frontiers in
INTEGRATIVE NEUROSCIENCE

REVIEW ARTICLE
published: 13 August 2013
doi: 10.3389/fnint.2013.00059

Inflammatory process in Alzheimer's Disease

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Alzheimer Disease (AD) is a neurodegenerative disorder and the most common form of dementia. Histopathologically is characterized by the presence of two major hallmarks, the intracellular neurofibrillary tangles (NFTs) and extracellular neuritic plaques (NPs) surrounded by activated astrocytes and microglia. NFTs consist of paired helical filaments of truncated tau protein that is abnormally hyperphosphorylated. The main component in the NP is the amyloid-β peptide (Aβ), a small fragment of 40–42 amino acids with a molecular weight of 4 kD. It has been proposed that the amyloid aggregates and microglia activation are able to favor the neurodegenerative process observed in AD patients. However, the role of inflammation in AD is controversial, because in early stages the inflammation could have a beneficial role in the pathology, since it has been thought that the microglia and astrocytes activated could be involved in Aβ clearance. Nevertheless the chronic activation of the microglia has been related with an increase of Aβ and possibly with tau phosphorylation. Studies in AD brains have shown an upregulation of complement molecules, pro-inflammatory cytokines, acute phase reactants and other inflammatory mediators that could contribute with the neurodegenerative process. Clinical trials and animal models with non-steroidal anti-inflammatory drugs (NSAIDs) indicate that these drugs may decrease the risk of developing AD and apparently reduce Aβ deposition. Finally, further studies are needed to determine whether treatment with anti-inflammatory strategies, may decrease the neurodegenerative process that affects these patients.

Keywords: Alzheimer disease, amyloid-β, neurodegeneration, microglia, astrocyte, neuroinflammation, pro-inflammatory cytokine, anti-inflammatory strategies

INTRODUCTION

Alzheimer's disease (AD) is the most common cause of dementia in elderly adults. AD is clinically characterized by progressive loss of memory and other cognitive functions. Histopathologically is recognized by the presence of neuritic plaques (NPs) and neurofibrillary tangles (NFTs). The origin causes of AD can be classified as familial or sporadic. The incidence of familial cases is low (5–10%) and is related to the presence of mutations in three different genes: presenilin-1 (PS1), presenilin-2 (PS2), and amyloid-β precursor protein (APP-β) (Chartier-Harlin et al., 1991; Goate et al., 1991; Murrell et al., 1991; Levy-Lahad et al., 1995; Duff et al., 1996; Sisodia et al., 1999). Sporadic AD represents 90–95% of total cases, and although its etiology is multi-factorial, age is the main risk factor. Although there are different genetic and environmental causes, all patients have a similar clinical behavior and develop identical brain lesions: NFTs consisting of Tau (τ) protein and NPs consisting of amyloid-β (Aβ) peptides. These alterations are the final result of post-translational modifications and involve different genes and render AD as a complex multigenic neurodegenerative disorder.

In addition to this multi-genic complexity in AD, now we know that Aβ promotes an inflammatory response mediated by microglia and astrocytes, thus activating signaling pathways that could lead to neurodegeneration. It is currently unknown whether brain inflammation in AD patients is the cause of the disease or a secondary phenomenon. Although it was previously thought that the central nervous system (CNS) was an immune-privileged site, now is well known that certain features of inflammatory processes occur normally in response to an injury, infection or disease. The resident CNS cells generate inflammatory mediators, such as pro-inflammatory cytokines, prostaglandins (PGs), free radicals, complement factors, and simultaneously induce the production of adhesion molecules and chemokines, which could recruit peripheral immune cells. This review describes the cellular and molecular mediators involved in the inflammatory process associated with AD and several possible therapeutic approaches describe recently.

NPs AND Aβ IN THE NEURO-INFLAMMATORY PROCESS

NPs, are extracellular deposits structures that contain a highly insoluble fibrillar Aβ core formed by fragments of 39–42 amino

acids surrounded by microglia, reactive astrocytes, and dystrophic neurites produced from degenerating neuronal processes (Iversen et al., 1995). Aβ normally originates from APP-β (Kang et al., 1987) through the sequential action of beta secretase and the multi-protein gamma-secretase complex.

Aβ accumulation in the brain, in one of the main pathological processes of AD patients. The formation of these deposits initiates a series of cellular events which are able to elicit an immune response where resident cells such as microglia and astrocytes could participate. Aβ accumulation in parenchyma and blood vessels causes microglial migration and promotes acute and chronic inflammatory responses against the aggregates, thus inducing the production of nitric oxide (NO), reactive oxygen species (ROS), pro-inflammatory cytokines (TNFα, IL-1β and IL-6), and PGs (PGE2), which eventually could promote neuronal death (**Figure 1**) (Akiyama et al., 2000; Kitazawa et al., 2004).

CELLULAR MEDIATORS

MICROGLIA

Microglia are resident brain cells, derived from monocyte precursors cells during embryogenesis, and are able to provide the initial response against any injury that occurs in the CNS. Although these cell were observed by Nissl over 100 years ago, their definitive identification and characterization was performed by Mrak (2012). Activated microglial association around NPs suggested that microglia participate in the accumulation of the Aβ observed in AD patients (Glenner et al., 1984); this hypothesis was supported by several studies (Rozemuller et al., 1986; Dickson et al., 1988).

Normally, microglia exists in an inactive state; morphologically, these cells have a small soma with branching processes. When activated by pathogens or injury, these cells suffer visible morphological changes, including decreased branching and soma growth, the acquisition of an amoeboid form and display a wide variety of specific cellular surface markers (Town et al., 2005). NPs in AD patients' brain activate the inflammatory response mediated by microglia and cause pro-inflammatory cytokine secretion, which may directly cause neuronal injury.

Microglia *in vitro* cell cultures are able to phagocyte the amyloid peptide. However, ultrastructural analysis of tissues from AD patients demonstrated that there is no presence of amyloid fibrils in the lysosomal compartments of local microglia cells (Frackowiak et al., 1992). Whereas microglia has the ability to phagocyte Aβ *in vitro*, its phagocytosis capacity is limited. An important observed feature was the abnormal presence of macrophages infiltrating from the periphery, which showed amyloid fibers in their lysosomal compartments (Wisniewski et al., 1991; Akiyama et al., 1996). Currently, we know that there are two types of phagocytic cells within the CNS that are able to initiate the innate immune response: microglia and peripheral macrophages (Rezai-Zadeh et al., 2009; Gate et al., 2010). These macrophages are recruited into the CNS by specific cytokines and

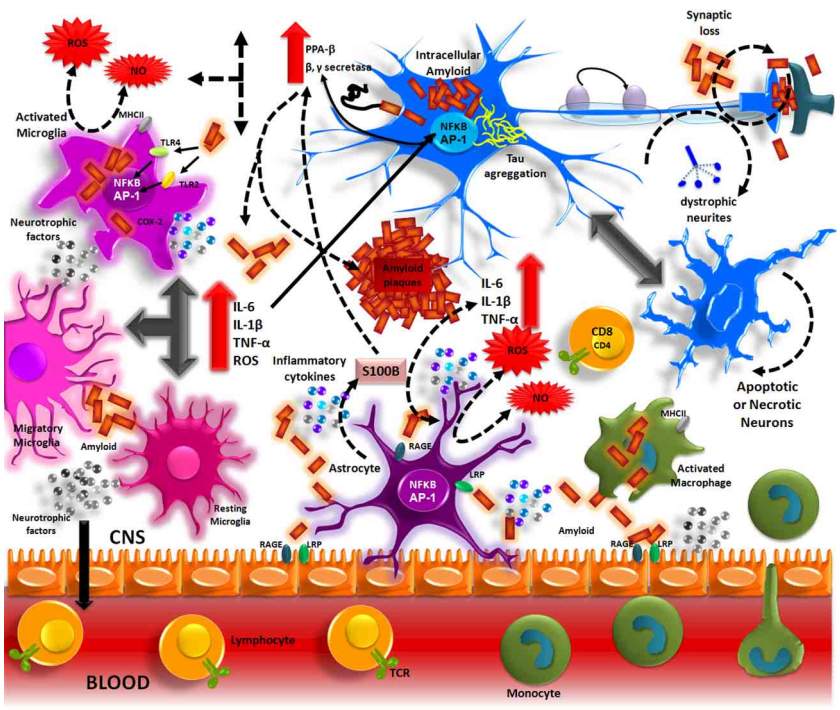

FIGURE 1 | Inflammation in Alzheimer's disease. The Aβ peptide produced by APP processing, form aggregates that activate microglia through TLRs and RAGE receptors. These receptors in turn, activate NF-κB and AP-1 transcription factors, which induce the reactive oxygen species (ROS) production and the expression of inflammatory cytokines (IL-1, IL-6, TNF). These inflammatory factors directly acting on the neurons and also stimulate the astrocytes, which amplify the pro-inflammatory signals, inducing a neurotoxic effects. The inflammatory mediators generate by resident CNS cells, induce the production of adhesion molecules and chemokines, which recruit peripheral immune cells.

chemokines, which are released during microglial and astrocytic activation and are able to cross the blood-brain barrier.

Similarly to macrophages, microglia recognizes pathogens through pattern recognition receptors (PRRs), which include specific toll-like receptors (TLRs), nucleotide-oligomerization binding domain (NOD) proteins, and C-type lectin receptors. These receptors interact with pathogen-associated molecular patterns (PAMPs) or damage-associated molecular patterns (DAMPs) to initiate the cellular defense mechanisms (Sterka and Marriott, 2006; Rubartelli and Lotze, 2007). Thus, the formation and release of ROS, pro-inflammatory cytokines (IL-1β, IL-6, TNF-α, and IFN-γ) (Lue et al., 2001), chemokines (MIP1α, MIP1β, RANTES, and MCP1), and growth factors such as macrophage colony-stimulating factor (MCSF) and complement factors (C1q, C3, C4, and C9) (Walker et al., 1995) begin. In addition to the before mentioned molecules, microglia is also able to express receptors for advanced glycosilation end products (RAGE), Fc receptors, CD40, FP receptors, and various scavenger-type receptors (El Khoury et al., 1998; Tan et al., 1999; Walker and Lue, 2005; Okun et al., 2010). Although microglia has the capacity to respond to various stimuli, the presence of Aβ is particularly important. Aβ induces a high accumulation of surface molecules of type I and II major histocompatibility complex (MHC) (McGeer et al., 1988).

Microglial cells primarily have an immunomodulatory role and express a large variety of immune response-related antigens and molecules; however, the specific role of microglia within the CNS remains controversial. Microglial activation is not a simple and unique phenomenon; instead, activation is a continuous series of events in which microglia awakens innate and adaptive phagocytic immune responses and consistently display activation through antigens on the cellular surface (Town et al., 2005). Under this principle, microglia displays a "variable" response, in which a mixture of the classical activation pathway and exacerbated increase of alternative activation can be observed in AD patients, which could leads to irreparable damage that result in persistent neurodegeneration.

ASTROCYTES

Astrocytes are the most abundant glial cells present in the CNS and have important functions in brain organization and maintenance (Sofroniew and Vinters, 2010). These cells interact with neurons and are involved in neurotransmitter secretion and recycling, ion homeostasis, energy metabolism regulation, synaptic remodeling and modulation of oxidative stress, information processing, and signal transmission (Halassa and Haydon, 2010; Henneberger et al., 2010).

In early stages of AD, activated astrocytes are located in two regions: the molecular layer of the cerebral cortex and near the amyloid deposits below the pyramidal cell layer. The mechanisms leading to the activation of these cells in response to the pathological changes produced by AD are not obvious, but it has been demonstrated that the presence of amyloid activates astrocytes. Activated astrocytes can phagocytose and degrade amyloid, which suggests that they importantly contribute to the removal of accumulated Aβ in parenchyma. Astrocytes and microglia are activated through TLRs and RAGE-dependent pathways, thus causing local inflammation that eventually could intensifies neuronal death.

Overall, the inflammatory process in AD patients is shown by changes in microglial morphology and astrogliosis, which is manifested by an increase in number, size and motility of astrocytes. Astrocyte activation is present in many neurodegenerative conditions, expressing high levels of glial fibrillary acidic protein (GFAP), vimentin, and nestin. Unfortunately, these changes cause a "disruption" of normal activities in astrocytes, which are essential for normal neuronal function. Maintenance of glutamate concentration in the extracellular space is among the normal physiological functions; altering homeostasis causes a local neuron depolarization, which leads to citotoxic damage. Thus, although astrocytic activation has a protective role in brain, intense activation exacerbates neuronal damage and accelerates disease progression.

Similarly to microglia, astrocytes quickly respond to injury; these cells are located near the fibrilar Aβ deposits, which are responsible for the astroglial activation observed in AD patients. Injection of Aβ oligomeric forms into the retrosplenial cortex of rats caused a significant astrocyte activation, as shown by transcription factor NF-κB activation and the presence of inflammatory mediators such as tumor necrosis factor (TNF-α), interleukin 1 (IL-1β), and cyclooxygenase-2 (COX-2)(Carrero et al., 2012). The activated astrocytes express on their cell surface, receptors that bind the Aβ peptides such as RAGE, receptor-like density lipoproteins, proteoglycans and various scavenger receptors (Wyss-Coray et al., 2003; Medeiros and Laferla, 2013). Thus, activated astrocytes are able to cause neurodegeneration and express inflammation-associated factors, such as S100β, consequently inducing neurite outgrowth. S100β expression correlates with the number of dystrophic neurites in AD patients (Mrak et al., 1996).

In astrocytes, NF-κB significantly controls chemokine and adhesion molecules secretion, favors peripheral lymphocyte infiltration and increases inflammatory response (Moynagh, 2005); this process becomes a self-regulating mechanism, which leads to neurodegeneration.

OLIGODENDROCYTES

Although oligodendroglia is important to maintain neuron morphology and function, little is known about how they are affected by Aβ deposits However, there are studies that indicate changes in white matter and abnormalities in myelin in AD patients (Kobayashi et al., 2002; Roth et al., 2005). Specifically, aberrations have been reported in the white matter of asymptomatic familial AD patients, particularly in patients with mutations in PS1 (Ringman et al., 2007). The presence of Aβ in oligodendrocyte cultures caused cell death. Although cell death can be prevented by the presence of anti-inflammatory agents, morphological alterations of the cells persist, suggesting that the damage cannot be completely reversed (Roth et al., 2005). Subsequently, oligodendrocyte differentiation and function is affected by the simultaneous presence of mutations in PS1 ($hPS1^{M146V}$) and Aβ accumulation, as demonstrated in a mouse model. These abnormalities can lead to the development of abnormal patterns of myelin basic protein (MBP) (Desai et al., 2011),

which completely alters oligodendrocyte homeostasis. Finally, the loss of trophic support provided by these cells might lead to increased neuronal vulnerability and inflammation, thus favoring neurodegeneration.

MOLECULAR MEDIATORS OF INFLAMMATION IN AD

The Aβ deposition activates the acute immune response of microglial cells and astrocytes. At the same time, amyloid plaques are responsible for the production and the activation of inflammation-related proteins such as complement factors, acute-phase proteins, chemokines and cytokines like interleukin 1 (IL-1), interleukin 6 (IL-6), tumor necrosis factor α (TNF-α), and transforming growth factor β (TGF-β). These molecular mediators of inflammation have been linked with a series of concomitant deleterious and beneficial effects (**Figure 2**).

THE COMPLEMENT SYSTEM

The complement system is an important innate and adaptive immune response effector. This system is composed of a number of proteins and proteases that are activated in cascade (Forneris et al., 2012), and it appears to have a fundamental role in neurodegenerative diseases. Overall, the complement system uses the C1q molecule, mannose-binding protein or the interaction with the C3 multifunctional protein to recognize certain molecular patterns on pathogens. C3 activation recruits cells with phagocytic activity, and Membrane Attack Complex (MAC) is formed by binding C5–C9 (Ricklin et al., 2010). In adaptive immune response, the complement system is involved in T-cell response regulation and T-helper lymphocyte differentiation (Pekkarinen et al., 2011).

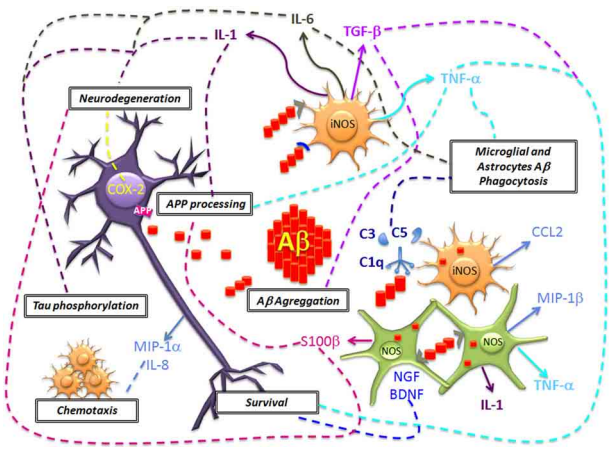

FIGURE 2 | Neuronal damage and Aβ deposition triggers microglial and astrocytes activation and the generation of inflammation molecular mediators. The acute production of molecules of the complement system (C1q, C3, and C5), pro-inflammatory cytokines (IL-1, IL-6, TNF-α), chemokines (CCL2, MIP-1α, MIP-1β, and IL-8) mediate the Aβ clearance. However, in a chronic stage these molecules could promote an increased expression and alteration of APP processing, Aβ deposition, Tau phosphorylation and neurodegeneration. Also, another effect of glial cells includes the generation of NO that promotes oxidative stress. The inflammatory microenvironment favors the production of COX-2 in neurons that leads to apoptosis. In contrast, it has been proposed that glial cells could mediate neuronal survival, by the production of TGF-β and neurotrophic factors (BDNF and NGF), but the disease progression results in failure to repair neurons.

In neurodegenerative diseases, there is a deregulation of the classical complement pathway. Studies in AD patient brains have revealed an increase in the immunoreactivity of C1q, C3b, C4d, C5b-9, and MAC surrounding senile plaques (McGeer et al., 1989; Rogers et al., 1992) and microglia surrounding the fibrillar aggregates of Aβ in the microvasculature (Fan et al., 2007). The co-existence of these molecules with the Aβ aggregates suggests a possible link between classical complement pathway activation, inflammation and pathological aggregation of Aβ. A decrease in C1q levels has been reported in cerebrospinal fluid (CSF) of AD patient and this result contrasts with an increase of C1q levels in the CNS (Smyth et al., 1994). *In vitro* studies revealed that C1q recognizes fibrillar and aggregated forms of Aβ1–42 and Aβ1–40, but not the monomeric forms. The C1q receptor is expressed in microglia, which contributed to the belief that the increase of this molecule in AD patient brains could affect the phagocytosis of Aβ. However, in primary microglia rat culture studies, exposure to Aβ1–42 and a nanomolar concentration of C1q caused a decrease in the phagocytosis of Aβ, compared to cultures exposed only to Aβ1–42 (Webster et al., 2000). A similar result was observed in a transgenic mouse model, which expressed mutated hAPP and an absence of C1q expression ($APPQ^{-/-}$). These mice showed no change in the amount of Aβ aggregates, but a decrease in glial cells activation was observed (Fonseca et al., 2004), thus establishing that C1q importantly contributes to microglial activation.

Microglia cells also produce C3. When these cells are exposed to Aβ synthetic peptides, their activation can be observed as well as an increase in C3 synthesis to 5-to 10 fold (Haga et al., 1993). When this molecule is inhibited, in the brain of an hAPP transgenic model, by the overexpression of the soluble form of the protein related to the complement receptor (sCrry), a 2- to 3-fold increase in Aβ deposits formation was observed in one-year-old hAPP/sCrry mice, which was accompanied by extensive neurodegeneration (Wyss-Coray et al., 2002). $APP/C3^{-/-}$ double transgenic generation confirmed that the molecule modulated the microglia phenotype, and promotes Aβ degradation (Maier et al., 2008).

Complement receptor 1 (CR1) is expressed in cells with phagocytic activity, C3b and C4b are CR1 ligands, and their binding facilitates phagocytosis. Genome-wide association studies (GWAS) in AD patients have shown a direct relationship between CR1 gene variants with cognitive impairment in patients and an increase in amyloid plaque formation (Chibnik et al., 2011).

The anaphylatoxin (C5) has been linked to excitotoxicity and apoptosis activation in the CNS, which is believed to play an important role in the development of neurodegenerative diseases. This molecule also promotes chemotaxis and glial cells activation. In AD animal models, the use of the C5a receptor antagonist (C5aR or CD88) reduces the amount of Aβ aggregates and hyperphosphorylated tau protein (Fonseca et al., 2009; Ager et al., 2010). It appears that the complement system activation in AD

might have beneficial effects for Aβ clearance; however, this activation could eventually become deregulated and favor neurotoxic effects by promoting unwanted inflammation. For these reasons, more studies are required to understand the changes affecting the complement system in AD development.

CYTOKINES

Immune system cells are able to produce cytokines, which are soluble proteins that mediate inflammation. In the CNS, cytokines are produced by microglial cells and astrocytes and play a key role in CNS development during the embryonic stages. Cytokines are involved in the inflammatory processes of neurodegenerative diseases. In AD, these proteins may be important in the development of the pathology. Studies of AD patient tissues and CSF have shown elevated levels of pro-inflammatory cytokines such as IL-1β, IL-6, IL-10, TNF-α, and TGF-β (Blum-Degen et al., 1995; Tarkowski et al., 2002; Mrak and Griffin, 2005; Jiang et al., 2011). The increase of these cytokines is strongly related to microglia activation by exposure to Aβ aggregates (Meda et al., 1999). Animal models that overexpress the mutant hAPP protein have shown a direct relationship between the amount of Aβ aggregates and elevated levels of TNF-α, IL-6, IL-12, IL-1β, and IL-1α (Patel et al., 2005). In particular, studies have been performed seeking the relationship between each cytokine with AD.

IL-1

IL-1 is known to be able to induce APP-β mRNA expression in endothelial cells (Goldgaber et al., 1989), which suggests that IL-1 increasing in AD patients could be linked to Aβ formation. In a study with AD patients, it was also proposed that IL-1 was produced by microglial cells surrounding NPs and this cytokine could promote S100β synthesis in astrocytes (Griffin et al., 1989). Subsequently, it was discovered that IL-1 was a contributing factor to initiate dystrophic neurite formation in Aβ diffuse deposits (Griffin et al., 1995). Similarly, the exposure of primary cultures of rat cortical neurons to S100β promotes dystrophic neurite formation and APP-β mRNA expression (Li et al., 1998). Thus, IL-1 might promote the Aβ formation and neuronal degeneration through S100β present in reactive astrocytes. Additionally, the increase in IL-1 in AD patients might promote an increase in p38-MAP kinase activity, which could lead to Tau hyperphosphorilation (Sheng et al., 2000; Li et al., 2003). IL-1β signaling blockade decreases GSK-3β activity and Tau phosphorylation and promotes neurogenesis through the Wnt/β-catenin pathway (Kitazawa et al., 2011). However, it has been recently suggested that IL-1β could promote Aβ removal (Matousek et al., 2012).

IL-6

IL-6 is produced by microglial cells and is involved in the immunoreactivity present in patient tissues with clinical dementia (Hull et al., 1996). Similarly to IL-1, IL-6 promotes APP-β expression (Ringheim et al., 1998) and could also contribute to NFT formation by inducing Tau phosphorylation through cdk5/p35 pathway deregulation (Quintanilla et al., 2004). However, IL-6 overexpression has been observed in the brain of two hAPP transgenic models (TgCRND8 and Tg2576). This overexpression resulted in significant gliosis, a decrease in Aβ deposits in TgCRND8 mice brains due to phagocytic marker upregulation in glial cells and increasing microglial Aβ phagocytosis. IL-6-induced neuroinflammation, and has not any effect on APP-β processing in TgCRND8 mice, which suggests that reactive gliosis, could have a beneficial effect in early stages of disease by promoting Aβ elimination (Chakrabarty et al., 2010).

TNF-α

TNF-α is a cytokine that can have beneficial or harmful effects on different neurons. This cytokine stimulates NF-κB transcription factor which, induces the pro-inflammatory molecules expression, and promote the synthesis of neuronal survival factors such as calbindin, manganese superoxide dismutase enzyme, and anti-apoptotic Bcl-2 protein (Wajant et al., 2003; Kamata et al., 2005). This cytokine can stimulate microglia glutaminase to release glutamate, thus generating excitotoxicity (Takeuchi et al., 2006) and promoting the development of neurodegenerative diseases. In AD, the direct role of this cytokine remains uncertain; however, TNF-α could be associated with an increased β- and γ–secretase enzyme expression (Blasko et al., 2000; Liao et al., 2004). *In vitro* models have shown that TNF-α directly stimulates BACE1 expression, thus favoring APP processing (Yamamoto et al., 2007). The use of soluble TNF-α inhibitors had a protective effect on APP deregulation by decreasing amyloid aggregate formation and attenuating the cognitive impairment observed in 3xTgAD mice exposed to chronic peripheral inflammation (McAlpine et al., 2009). However, a long-term suppression of the TNF-α receptor signaling pathway can suppress the microglial ability to efficiently remove Aβ aggregates, thus favoring its aggregation at early stages (Montgomery et al., 2011).

TGF-β

TGF-β; is another cytokine with pleiotropic functions, which has an anti-apoptotic function and promotes neuronal survival. It is believed that this cytokine is involved in neurodegenerative diseases (Krieglstein et al., 2002; Konig et al., 2005). It has been reported an increase in TGF-β1 levels in the brain of AD patients (Flanders et al., 1995). Animal models (hAPP/TGF-β1) have shown that TGF-β1 overexpression promotes amyloidogenesis in the meninges and cerebral vasculature at early stages (2–3 months) (Wyss-Coray et al., 1997). By contrast, in hAPP/TGF-β1 transgenic mice parenchyma, there is a decrease in amyloid plaque formation, which correlates with microglial activation (Wyss-Coray et al., 2001). In AD patients, although TGF levels are elevated, there is a significant decrease in the expression of the TGF-β receptor type II (TβRII); this decrease may suggest that the signaling pathway mediated by this receptor has significant neuroprotective functions and could be altered during the development of the disease (Tesseur et al., 2006).

In summary, the role played by cytokines remains controversial. On one hand, cytokines might favor microglial and astrocytic activation to promote Aβ phagocytosis and neuronal survival. On the other hand, chronic cytokine production has been described as contributors of neurodegeneration.

CHEMOKINES

Chemokines are small proteins whose principal function is to attract monocytes, macrophages, lymphocytes, neutrophils, basophils, eosinophils, and dendritic cells toward sites in which an inflammatory response is required. Chemokines function through the activation of their G protein-coupled receptors and are divided into four families, CXC, CC, C, and CX_3C (Wells et al., 1998; Cyster, 1999).

Astrocytes and microglial cells, are the main producer of chemokines in the CNS, and their receptors are observed to be present in neurons. Chemokines and their receptors also participate in the CNS immune response and promote lymphocyte migration from the lymphoid organs in order to establish the inflammatory process (Ransohoff et al., 1996).

Evidence of the participation of chemokines in AD is the presence of monocyte chemotactic proteins (MCP-1 or CCL2) and chemokine receptors CCR3 and CCR5 in reactive microglia that surround senile plaques of AD patients. By contrast, the macrophage inflammatory protein 1α (MIP-1α) is located mainly in neurons, and MIP-1β is located primarily in astrocytes that surround the plaques (Ishizuka et al., 1997; Xia et al., 1998). The differential expression of chemokines and their receptors promotes glia-neuron communication to establish a local inflammatory response, which could favor Aβ phagocytosis in early stages of AD. Similarly, it is known that this inflammation contributes to Tau pathology and thereby accelerates the disease progression (Zilka et al., 2012).

Chronic inflammation in AD promotes the increased chemotaxis of phagocytic cells, thus favoring microglial recruitment around Aβ aggregates (Yamamoto et al., 2005). A recent study of CSF from AD patients revealed increased CCL2 levels, which correlated with cognitive decline (Westin et al., 2012). Moreover, because of Aβ aggregation, an increase in IL-8 production (CXCL8) is generated in neurons, which correlates with an increase in the formation of brain-derived neurotrophic factor (BDNF) (Ashutosh et al., 2011).

The presence of Aβ, in the microvasculature, promotes the release of IL-8, MCP-1, MIP-1 MIP-1α, and MIP-1β chemokines, and thereby promotes monocyte differentiation into macrophages and their migration through the blood-brain barrier (Fiala et al., 1998). CSF analysis from AD patients also showed an increase in MCP-1 and IL-8 chemokines levels (Galimberti et al., 2003; Correa et al., 2011). Similarly, *in vitro* models have demonstrated the migration ability of lymphocytes ($CD4^+$ and $CD8^+$) through the blood-brain barrier (Man et al., 2007) because of an increased MIP-1α level.

In conclusion, chemokines in the CNS are able to promote local and peripheral immune system cell migration in order to establish an immune response. In AD, it has been proposed that the chronic production of chemokines contributes to disease progression.

CYCLOOXYGENASES

Cyclooxygenases (COX) are enzymes responsible for converting arachidonic acid to H2 prostaglandin, which is a PG precursor. In general, these lipidic molecules are involved in inflammation because they cause vasodilation, thus allowing the immune system cell transport to operate at the target site (Williams, 1978). COX-1 and COX-2 isoforms are expressed in mammalian brain. COX-1 is expressed in microglia and neurons located in the pons and spinal cord that perform autonomous and sensory functions. COX-2 occurs in glutamatergic neurons of hippocampus and cortex and is thought to be involved in the modulation of plasticity processes and long-term potentiation (Yamagata et al., 1993; Breder et al., 1995); in pathological conditions, the presence of COX-1 in the CNS has been linked to inflammation development, whereas COX-2 is associated with neurotoxicity. Microglia surrounding the NPs in AD patient shows a high expression of COX-1, which suggests inflammation (Yermakova et al., 1999). On the other hand, COX-2 expression in the hippocampal CA3 region appears to be related with the quantity of NPs and NFTs and the observed cognitive impairment (Ho et al., 2001). COX-2 overexpression in a triple transgenic model (hAPP/PS1/hCOX-2) has resulted in an increase in active Caspase-3 immuoreactivity and retinoblastoma protein phosphorylation (S^{795}-pRb). The Rb protein regulates the G1 phase of the cell cycle, but S^{725} phosphorylation suppresses cell growth. *In vitro* studies revealed that hCOX-2 overexpression in primary cultures of cortical and hippocampal neurons derived from transgenic mice, accelerates the apoptotic damage induced by Aβ, causing cell cycle abnormalities (Xiang et al., 2002a). COX-2 also promotes amyloid plaque formation in parenchyma and increases prostaglandin E2 production. This increased plaque formation, correlates with an increase in amyloid peptide formation (Aβ1–40 and Aβ1–42) through the γ–secretase activation without affecting the APP expression levels (Xiang et al., 2002b). Studies performed in a mouse model using $COX\text{-}2^{-/-}$ deficient mice demonstrated that the inflammatory response mediated by Aβ was diminished, which suggested that COX-2 inhibition could be an important target to be use for therapy (Choi and Bosetti, 2009).

The analysis of post-mortem brain tissue from AD patients demonstrated that the maximum immunoreactivity for COX-2 and ppRb occurred in the early stages of the disease (Braak), before occur the maximum astrocytic and microglial activation. Contrary to *in vitro* observations, the post-mortem tissue analysis did not support the direct relationship between the microglial and astrocytic activation with the neuronal COX-2 and ppRb expression in AD (Hoozemans et al., 2005).

Currently, the cause of alterations in COX-2 levels during different stages of AD, remains unknown. However, it has been suggested that inflammation in the early stages of AD could promote such alterations, and IL-1β could promote an increase in COX-2 expression and PGs production (Hoozemans et al., 2001).

NITRIC OXIDE

NO is a molecule that contributes importantly in cell signaling. In the presence of oxygen, L-arginine, is converted to L-citrulline and release NO. Nitric oxide synthase (NOS) is the enzyme responsible for catalyzing this reaction, and it occurs in three isoforms. In the CNS, the neuronal isoform (nNOS) is widely distributed in neurons, astrocytes and blood vessels.

The endothelial isoform (eNOS) is located in the hippocampal pyramidal neurons, endothelial cells and some astrocytes. The expression of the inducible isoform (iNOS) is typically low but is increased in microglia and astrocytes during neuroinflammation. Under physiological conditions, it is believed that NO regulates the release of neurotransmitters and hormones and promotes cell survival and long-term potentiation. However, high levels of NO are generated in inflammatory conditions, which might contribute to synaptic transmission dysfunction, protein and lipid oxidative damage, excitotoxicity, and neuronal death (Liu et al., 2002; Bishop and Anderson, 2005; Calabrese et al., 2007).

Tissue and neuronal analyses of AD patients have revealed that Aβ is able to promote NOS expression and NO production in microglia cells and reactive astrocytes (Goodwin et al., 1997; Wallace et al., 1997; Akama et al., 1998). Aβ also promotes the pro-inflammatory cytokines IL-1β and TNF-α liberation which contribute to NO and peroxynitrite formation (Rossi and Bianchini, 1996; Combs et al., 2001) and cause protein and lipid modifications, mitochondrial damage, apoptosis and promote Aβ formation, increasing the γ-secretase complex activity (Torreilles et al., 1999; Keil et al., 2004; Guix et al., 2012). Under normal conditions, NO is synthesized in the microvasculature and regulates β-secretase activity which participate in APP processing (Austin et al., 2010). NOS2 also favors Aβ elimination by regulating MMP-9/TIMP-1 expression, which is a key enzyme that degrades amyloid (Ridnour et al., 2012). In addition, it has been shown that chronic NO formation may alter insulin-degrading enzyme activity (Kummer et al., 2012). NO synthesis during AD development could also contribute to NFT formation. In co-cultures of astrocytes and hippocampal neurons of rats, exposure to Aβ25–35 was found to increase NO levels, which correlated with increased hyperphosphorylated Tau protein. Interestingly, the use of a NOS inhibitors reduced Tau phosphorylation levels (Saez et al., 2004).

INFLAMMATION MODULATOR TREATMENTS: THERAPEUTICS IN AD

In the early 1990s, studies began to emerge relating the inflammation process with AD. Studies such as those of Fillit et al. (1991) showed that certain pro-inflammatory cytokines were elevated in AD patients.

Epidemiological studies (Li et al., 1992) indicates that subjects with arthritis have a lower prevalence of AD, after which it was proposed that this negative association could be related to the chronic use of anti-inflammatory drugs (Dickson and Rogers, 1992). From these and other results, these drugs began to be proposed as a new therapeutic approach for AD treatment. Currently, numerous epidemiological studies, experimental *in vitro* and *in vivo* models and clinical trials have been designed to elucidate the relationship between modulators of inflammation and AD.

Typically, anti-inflammatory agents are divided into non-steroidal anti-inflammatory drugs (NSAIDs) and glucocorticoids. This section will review the mechanisms of action of both types of anti-inflammatory agents and their role in AD and the results of some of the most relevant epidemiological and experimental studies. This section will also analyse the use of active and passive immunotherapy as a therapeutic strategy for AD (**Table 1**).

ANTI-INFLAMMATORY DRUGS

NSAIDs are a heterogeneous group of drugs that share a common mechanism of action, which involves COX-1 and COX-2 enzymes inhibition either selectively or non-selectively (Vane and Botting, 1987). These enzymes catalyze arachidonic acid conversion to prostaglandin H_2 (PGH_2), which is subsequently converted into various PGs (PGE_2, PGD_2, $PGF_{2\alpha}$, and PGI_2) and thromboxane (TX) (Dubois et al., 1998).

PG and TX synthesis increases in occurrence of tissue damage, acting as inflammation mediators, increasing blood flow and vasodilation in the damaged tissue and increasing microvascular permeability (Flower et al., 1976).

The results of several epidemiological studies designed to identify AD risk factors have shown that prolonged use of NSAID could reduce AD risk. The following are the results from some of the most representative studies conducted on different worldwide populations.

The *Sydney Old Persons Study* showed that NSAID use, is significantly lower in subjects who developed AD than in those who did not develop any type of dementia during the study period (Broe et al., 2000). Notably, NSAID use is not associated with any other type of dementia except AD. This result could suggest that NSAID use in AD may act through a different mechanism from their properties as cyclooxygenase inhibitors. The Rotterdam study (In'T Veld et al., 2001), Multi-Institutional Research Alzheimer's Genetic Epidemiology Study (Yip et al., 2005) and Canadian Study of Health and Aging (CSHA) (Coté et al., 2012) also observed an association between the NSAID use and a reduced AD risk. Szekely et al. conducted a meta-analysis of six studies (the Baltimore Longitudinal Study of Aging, Cache County Study, CSHA, Cardiovascular Health Study, Framingham Heart Study and Monongahela Valley Independent Elders Study) and reiterated the association between the NSAID use and a reduced AD risk (Szekely et al., 2008). However, some studies have failed to replicate this association. The Longitudinal Aging Study Amsterdam observed a reduction in AD risk associated with the use of aspirin alone and observed no association between AD risk and other NSAIDs (Jonker et al., 2003). Bendlin et al. also observed no significant differences in the results of neuropsychological memory and learning tests between NSAIDs users and non-users (Bendlin et al., 2010).

Other studies have observed controversial results, such as Fourrier et al. who observed an association between NSAIDs use and a decrease in Mini-Mental State Examination (MMSE) scores (Fourrier et al., 1996). Similarly, Breitner et al. reported a greater incidence of AD among NSAIDs users compared with controls (Breitner et al., 2009).

Martin et al. obtained equally unfavorable results when testing the effect of naproxen and celecoxib in older adults with a family history of AD (Martin et al., 2008). In this study, subjects treated with naproxen and those treated with celecoxib scored lower on the MMSE than subjects treated with placebo, which suggested a detrimental effect of the use of these drugs on cognitive performance. Aisen et al. tested the effect of rofecoxib and naproxen

Table 1 | Inflammation modulator treatments and their effects in animal models and clinical trials.

Therapy	Drug	Effects in animal models and clinical trials
NSAIDs	Naproxen	No beneficial effects reported. High frequency of adverse effects in drug-treated group, in clinical trials
	Celecoxib	Drug-treated group was found to score lower than placebo-treated group in cognitive tests, in clinical trials
	Rofecoxib	No beneficial effects reported. High frequency of adverse effects in drug-treated group, in clinical trials
	Indomethacin	In mice treated with this drug, lower Aβ levels in cortex and hippocampus were observed
	Mefenamic acid	Mice treated with this drug performed as well as healthy controls in cognitive tests
	Triflusal	In mice treated with this drug, a decrease in microglial and astrocytic activation, and a decrease in cytokine levels were observed
	Ibuprofen	Mice treated with this drug performed as well as healthy controls in cognitive tests. However, an increase in soluble Aβ levels and some behavior disturbances were observed in the drug-treated group
Glucocorticoids	Corticosterone/ Dexamethasone	Cortex, cerebellum and brainstem from mice treated with this drug showed increased expression of APP and beta-secretase, Aβ deposition, tau aggregation and caspase 3, cytochrome c, IL-1 and TNF-α levels. Drug-treated mice were found to score lower than placebo-treated mice in memory tasks
	Prednisone	No beneficial effects reported. Higher frequency of behavior disturbances was observed in the drug-treated group, in clinical trials
Immunotherapy	Aβ peptides	In mice, this therapy was able to prevent synaptic loss. Clinical trials have shown some beneficial effects in subjects who formed antibodies, but the incidence of meningoencephalitis as a side effect is notably high
	Anti-Aβ antibodies	In treated mice, a reduction in synaptic loss and number of dystrophic neurites and an increase in the number of dendritic spines have been reported. In clinical trials, no beneficial effects have been reported
	Tau peptides	Prevention of cognitive decline and a decrease in the number of NFTs has been reported in mouse model studies
	Anti-tau antibodies	A decrease in the number of NFTs has been reported in treated mice

Although most of these treatments have been successful in animal models, these beneficial results have not been reproduced in clinical trials, and even have been cause of detrimental effects.

on patients with mild to moderate AD (Aisen et al., 2003). The study, which lasted one year, showed no significant differences in the performance of subjects treated with rofecoxib or naproxen or placebo. In addition, subjects in NSAID-treated groups reported a high frequency of adverse effects such as fatigue and dizziness and had a significantly higher incidence of hypertension. Another study with rofecoxib, conducted in patients with mild cognitive impairment (MCI), suggested a deleterious effect of rofecoxib (Thal et al., 2005).

The results of AD animal model studies appear to support the hypothesis that the use of NSAID is beneficial, not only as a method of preventing the disease, but also as a therapeutic strategy. This support was observed in Tg2576 (Swe-APP) transgenic mice treated with indomethacin, which showed a marked decrease in Aβ levels ($A\beta_{1-40}$ and $A\beta_{1-42}$) in both cortex and hippocampus (Sung et al., 2004).

Subsequently, Joo et al. showed that mice treated with mefenamic acid for 3 weeks after being treated with Aβ1–42 or expressing Swe-APP, re-established their performance in the Morris water maze test in a comparable way to the vehicle-treated group (Joo et al., 2006). Coma et al. obtained similar results in Tg2576 transgenic mice treated with Triflusal (a NSAID from the salicylate family but a non-aspirin derivative), thus re-establishing transgenic mice (Tg+) performance in Morris test and a conditioning test (Coma et al., 2010). Although Triflusal had no effect on reducing NP size (NPS) or number, it reduced the amount of activated astrocytes and microglia and IL-1β and TNF-α levels in the hippocampal CA1 region and entorhinal cortex. Van Dam et al. observed similar results in TgAPP23 mice after treatment with Ibuprofen (Van Dam et al., 2010).

In the animal model (5XFAD), which overexpressed the Swedish double mutation (K670N, M671L), Florida mutation (I716V), London mutation (V717I), and double mutation in PS1 (M146L and L286V), treatment with ibuprofen for 3 months resulted in a reduction of the inflammatory response. However, it is notable that there was an increase in soluble Aβ42 levels and there was some changes in behavior, thus questioning whether anti-inflammatory drug use may actually be beneficial for AD treatment (Hillmann et al., 2012).

Whereas inflammation is a neuropathological feature always present in AD, and inflammatory processes significantly contribute to cell damage in this disease, previous studies performed *in vivo* and *in vitro* suggest that NSAIDs participation in AD occurs not only through an anti-inflammatory mechanism but also by modulating Aβ synthesis and removal in the CNS. Several studies have shown that NSAIDs stimulate the

non-amyloidogenic APP-processing pathway (Avramovich et al., 2002), decrease β-secretase levels (Sastre et al., 2006), and reduce the Aβ formation (Hirohata et al., 2005).

Although animal models appear to show that NSAIDs are a promising therapeutic option, currently, no clinical trials conducted with these drugs have achieved favorable results in AD patients.

GLUCOCORTICOIDS

Similar to NSAIDs, glucocorticoids have potent anti-inflammatory effects, which have made them strong candidates for AD treatment. Basically, the mechanism of action of glucocorticoids is based on their ability to bind to its receptors (Glucocoticoid Receptor, GR), which is observed in the cytoplasm in its free form, which translocates into the nucleus after binding to one of its ligands. When GR locate at the nucleus, it binds to certain nucleotide sequences, glucocorticoid response elements (GREs), which are located in the promoter region of a variety of genes.

Depending on the type of GRE sequence to which it binds, GR will positively or negatively regulate gene expression (Beato et al., 1989). The importance of this binding in the inflammatory process is that some genes which GR binds to and activates, have anti-inflammatory effects such as annexin-1 (lipocortin-1), interleukin 10 and NF-κB inhibitor (IκB-α) (Barnes, 2006). In addition, it has also been suggested that glucocorticoids interact directly with Aβ.

The study of post-mortem AD brains showed a decrease in the presence of NPs in subjects under chronic treatment with corticosteroids. No significant differences were observed in the number of NFTs. This study also reported no association between chronic treatment with NSAIDs and the number of NPs (Beeri et al., 2012).

Despite its anti-inflammatory effect, the use of glucocorticoid as a therapeutic strategy in the treatment of AD is controversial, because numerous studies have associated increased levels of glucocorticoids (cortisol), to an increased AD risk. In 1994, Swaab et al. measured cortisol levels in post-mortem samples of CSF and observed an 83% increase in AD subjects compared to healthy controls (Swaab et al., 1994). These results coincide with the results of Laske who observed significantly higher levels of serum cortisol in AD patients compared with age-matched healthy controls (Laske et al., 2009).

The majority of animal model experiments does not support the use of corticosteroids as a therapeutic strategy for AD and suggests that these drugs promote the development of the neuropathological features of this disease.

The treatment of rats with glucocorticoids (corticosterone and dexamethasone) induces an increase in APP expression in cortex, cerebellum, and brain stem (Budas et al., 1999), thus suggesting an adverse effect. Additionally, there is an increase in Aβ formation because of the increased APP and β-secretase levels. These glucocorticoid levels also correlate with Tau accumulation (Green et al., 2006), memory, and learning impairment (Yao et al., 2007) and increased levels of caspase 3 and cytochrome c, which indicates the presence of a pro-apoptotic enviroment (Li et al., 2010). Finally, high levels of corticosterone in hippocampal cells have a pro-inflammatory effect, thus favoring IL-1 and TNF-α expression (Macpherson et al., 2005).

These studies indicates that the mechanisms that might associate glucocorticoids with an increase in AD risk are related with the increasing expression of proteins involved in Aβ synthesis (APP and β-secretase) or promoting apoptosis (Cotman and Anderson, 1995).

In humans, there is only one clinical trial investigating the effect of glucocorticoids in the treatment for AD, the trial investigate the used of a daily dose of prednisone or placebo for 1 year (Aisen et al., 2000). There were no significant differences in the cognitive performance of AD subjects treated with prednisone or placebo at the end of treatment; however, the prednisone-treated subjects showed a higher frequency of behavioral disturbances compared to controls.

In general, there is little evidence to suggest the neuroprotective or therapeutic role of glucocorticoids in AD; in contrast, there is extensive evidence of a possible neurotoxic role. Whereas it is possible that under certain conditions these substances are actually neuroprotective, details of their mechanism of action should be extensively evaluated in AD before a clinical testing protocol can be considered.

IMMUNOTHERAPY

Currently, there is no consensus among researchers regarding what triggers AD pathophysiology or which events are causes and which are bodily responses to counteract the damage caused by the disease. This problem has also been raised regarding inflammation.

Whereas therapies using NSAIDs and glucocorticoids were designed based on the idea that the immune response in AD has an adverse effect (Blasko et al., 2004) or may be a causal factor (McGeer and McGeer, 1995), there are other approaches which suggest that the immune response may be beneficial because it attempts to counteract the deleterious effects of Aβ oligomers and Tau aggregation.

Immunotherapy is one of the therapeutic strategies designed under these bases because it is underlying on promoting the immune response against the altered molecules that are present in the most distinctive histopathological lesions of the disease (NPs and NFTs) and destroy them. This section will review the types (anti-Aβ and anti-Tau immunotherapy) and some results of immunotherapeutic studies with AD patients.

ANTI-Aβ IMMUNOTHERAPY

Animal studies in which the anti-Aβ immunotherapy strategy has been tested have been promising. Buttini observed that either active (using four different Aβ sequence fragments) or passive immunotherapy (using 3D6 anti-Aβ and 12B4 antibodies) prevent the loss of synapses in PDAPP transgenic mice (Buttini et al., 2005).

The application of a single 3D6 anti-Aβ dose in Tg2576 mice demonstrated that there was no reduction in the number of NPs; nevertheless, there was a reduction in the number of dystrophic neurites (Rozkalne et al., 2009). On the other hand, the application of this antibody in PDAPP mice showed an increase in the number of dendritic spines, which suggests that this anti-Aβ

immunotherapy approach, can increase neuronal plasticity and could contribute to the recovery of neural circuits. Subsequently, the effect of passive immunotherapy was studied with 7B6 anti-Aβ antibodies in Aβ APPswe/PS1dE9 mice (Spires-Jones et al., 2009; Liu et al., 2011). The authors observed that mice had a higher density of mono-aminergic axons in both the cortex and hippocampus. Immunotherapy using anti-Aβ CAD106 antibodies in different transgenic mouse models for APP (APP23 and APP24) showed a reduction in the presence of Aβ in both models (Wiessner et al., 2011).

In humans, some results suggest that anti-Aβ active immunotherapy could be beneficial; however, the frequency of adverse effects has been too high. In a study of active immunotherapy using $A\beta_{1-42}$ pre-aggregating (AN1792) in combination with immunogenic adjuvant QS-21 (a saponin of vegetable origin) observed a slower cognitive decline in subjects responding to the treatment (forming specific antibodies) (Hock et al., 2003; Orgogozo et al., 2003). However, 10% of the participants developed cases of aseptic meningoencephalitis. A similar result was obtained in an additional group of patients who were evaluated after stopping the treatment due to the adverse effects observed. The monitoring of these patients included cognitive testing and MRI scans to determine brain volume. Patients who were classified as antibody producers before the suspension of the study were tested for the presence of antibodies using ELISA; 89% of patients had antibodies at the time of investigation. These patients positive to antibodies production, performed better on cognitive tests than those treated with placebo, which suggests a possible beneficial effect of the treatment. However, brain volume loss was identical for both groups (Vellas et al., 2009).

The high incidence of aseptic meningoencephalitis among the subjects receiving active anti-Aβ immunotherapy could be attributed to an excessive pro-inflammatory response by T lymphocytes. Unfortunately, passive immunization with bapineuzumab, (a humanized murine monoclonal antibody), showed no differences in cognitive performance in subjects treated with bapineuzumab or placebo (Salloway et al., 2009).

ANTI-Tau IMMUNOTHERAPY

Most of the research efforts into AD immunotherapy have focused on anti-Aβ immunotherapy. However, some studies in animal models have also used anti-Tau immunization.

Boutajangout et al. showed that immunotherapy with the complete 441 amino acids peptides of the human Tau protein (hTau) prevented cognitive decline in an hTau transgenic mouse model, which expressed the hTau transgene (Boutajangout et al., 2010). Boimel et al. immunized the Tau transgenic mice presenting NFTs with phosphorylated Tau peptides. A 40% reduction in the number of NFTs was observed (Boimel et al., 2010). Chai et al. studied the effect of passive immunotherapy for Tau in JNPL3 and P301S transgenic mice. Using PHF1 and MC1 antibodies, the authors observed that the peripheral administration of both antibodies significantly reduced Tau pathology compared to controls (Chai et al., 2011). Although these results suggest that anti-Tau immunotherapy could provide favorable results, no clinical trial has been reported to date that evaluated the real effects on humans.

These results indicate that immunotherapy, particularly anti-Aβ active immunotherapy, could be advantageous when a strategy that minimizes its adverse effects is achieved. Further studies are also required with anti-Tau immunotherapy to evaluate whether this therapy could be functional in the future.

CONCLUSION

Currently, several genetic and epidemiological studies have provided an overview of the inflammatory mechanisms involved in AD. Although the molecular basis of the disease remains unknown, the inflammation induced by Aβ has an important role in the neurodegenerative process. The inflammatory process itself is driven by microglial and astrocytic activation through the induction of pro-inflammatory molecules and related signaling pathways, thus leading to synaptic damage, neuronal loss, and the activation of other inflammatory participants.

Although, the role of amyloid as a potential initiator of inflammation is not obvious, its accumulation exerts an indirect effect by activating caspases and transcription factors, such as NF-κB and AP-1, which produce numerous inflammation amplifiers (IL-1β, TNF-α, and IL-6). Pro-inflammatory cytokines, such as TNF-α and IL-1β and IL-6, could act directly on the neuron and induce apoptosis. Similarly, TNF-α and IL-1β can activate astrocytes, which could release factors that have the capacity to activate microglia.

Furthermore, APP, BACE1, and PSEN expression is governed by factors such as NF-κB. The genes encoding these proteins have sites in their promoter regions, which are recognized by NF-κB; in turn, the expression of these factors is upregulated by the presence of pro-inflammatory cytokines.

Inflammatory mediators acting on neurons contribute to an increase in amyloid production and activate microglia-mediated inflammation. The microglia-neuron communication amplifies the production of factors that contribute to AD-type pathology. However, the neural response is specific for the receptor type expressed in the different neuronal populations. For example, TNF-α binds TNFR1, which activates the cell survival pathway through NF-κB and the apoptotic pathway through the activation of caspases. Conversely, TNFRII signaling only activates NF-κB.

This cascade is primarily mediated by the pro-inflammatory cytokine IL-1β, which is expressed by microglia cells. IL-1β may cause neuronal death via various pathways, which activate microglia and consequently increase the release of IL-1β, thus generating a self-sustaining mechanism that is amplified by itself. This slow but steady inflammation state, generated for long periods in the brain eventually can destroy neurons and contribute to the clinical symptoms observed in the disease (**Figure 1**).

Finally, in accordance with all the above data, particularly because the results of the treatments used have been contradictory so far and there are no clinical trials that shown that anti-inflammatory treatments and the use of immunotherapy are completely safe or beneficial, it is necessary to develop and implement new strategies for AD immunological treatments.

ACKNOWLEDGMENTS

This work was supported by a grant from SEP/CONACYT (No. 157548).

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Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Received: 04 May 2013; paper pending published: 09 June 2013; accepted: 25 July 2013; published online: 13 August 2013.

Citation: Meraz-Ríos MA, Toral-Rios D, Franco-Bocanegra D, Villeda-Hernández J and Campos-Peña V (2013) Inflammatory process in Alzheimer's Disease. Front. Integr. Neurosci. 7:59. doi: 10.3389/fnint.2013.00059

frontiers in
INTEGRATIVE NEUROSCIENCE

ORIGINAL RESEARCH ARTICLE
published: 23 August 2013
doi: 10.3389/fnint.2013.00062

Hyperleptinemia is associated with parameters of low-grade systemic inflammation and metabolic dysfunction in obese human beings

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Leptin is an adipose tissue-derived hormone that has been involved in hypothalamic and systemic inflammation, altered food-intake patterns, and metabolic dysfunction in obese mice. However, it remains unclear whether leptin has a relationship with parameters of systemic inflammation and metabolic dysfunction in humans. We thus evaluated in a cross-sectional study the circulating levels of leptin in 40 non-obese and 41 obese Mexican individuals, examining their relationship with tumor necrosis factor alpha (TNF-α), interleukin (IL) 12, IL-10, central obesity, serum glucose and insulin levels, and serum triglyceride and cholesterol concentrations. Circulating levels of leptin, TNF-α, IL-12, IL-10, and insulin were measured by ELISA, while concentrations of glucose, triglyceride, and cholesterol were determined by enzymatic assays. As expected, serum levels of leptin exhibited a significant elevation in obese individuals as compared to non-obese subjects, showing a clear association with increased body mass index ($r = 0.4173$), central obesity ($r = 0.4678$), and body fat percentage ($r = 0.3583$). Furthermore, leptin also showed a strong relationship with serum TNF-α ($r = 0.6989$), IL-12 ($r = 0.3093$), and IL-10 ($r = -0.5691$). Interestingly, leptin was also significantly related with high concentrations of fasting glucose ($r = 0.5227$) and insulin ($r = 0.2229$), as well as elevated levels of insulin resistance ($r = 0.3611$) and circulating triglyceride ($r = 0.4135$). These results suggest that hyperleptinemia is strongly associated with the occurrence of low-grade systemic inflammation and metabolic alteration in obese subjects. Further clinical research is still needed to determine whether hyperleptinemia may be a potential marker for recognizing the advent of obesity-related metabolic disorders in human beings.

Keywords: leptin, hyperleptinemia, obesity, low-grade inflammation, metabolic disease, type 2 diabetes, human

INTRODUCTION

Obesity is now considered a major health problem worldwide, with a growing prevalence in the Mexican population (Olaiz-Fernández et al., 2006; Popkin, 2011). Obese people show a higher risk to develop type 2 diabetes (T2D), coronary heart disease, stroke, arterial hypertension, non-alcoholic steatohepatitis (NASH), and other obesity-related metabolic disorders (Ritchie and Connell, 2007). These pathologies have been recently associated with a low-grade systemic inflammatory state (Odegaard and Chawla, 2008), characterized by altered circulating levels of inflammatory mediators in the obese subject, including tumor necrosis factor alpha (TNF-α), interleukin (IL-) 12, C-reactive protein (CRP), and IL-10 (Rush et al., 2007; Bremer et al., 2011). TNF-α has been shown to increase with adiposity in mice and human beings (Steinberg et al., 2006). Circulating concentrations of IL-12 and CRP are elevated in overweight and obese individuals, exhibiting a significant association with increased body mass index (BMI) and waist circumference, as well high glucose and triglyceride levels (Visser et al., 1999; Suarez-Alvarez et al., 2013). On the contrary, serum IL-10 levels have been shown to decrease in high-fat diet fed-mice (Gotoh et al., 2012). As it can be seen, circulating proinflammatory factors have received growing attention since they could play a major role in mediating the low-grade inflammatory state, which seems to decisively contribute to the advent of obesity-related metabolic disorders (Ritchie and Connell, 2007; Bertola et al., 2010).

Leptin is an adipose tissue-derived hormone belonging to the class-I helical cytokine family (Trinchieri, 2003). Leptin has been shown to regulate food-intake and energy expenditure in both rodents and humans (Houseknecht et al., 1998). Interestingly, circulating levels of leptin have been shown to increase in high-fat diet fed-mice and obese subjects, leading to a state of hyperleptinemia (Maffei et al., 1995; Lin et al., 2000). However,

although hyperleptinemia strongly correlates with parameters of low-grade systemic inflammation and metabolic dysfunction in animal models of obesity (Munzberg, 2008; Arruda et al., 2011; Stienstra et al., 2011), they show controversial results in human beings. For instance, it has been reported that serum leptin is augmented in obese individuals with metabolic syndrome (MetS) that also show an elevation in the plasma levels of CRP (Kim et al., 2006). In contrast, obese non-diabetic women subjected to a caloric restriction diet show decreased values in plasma leptin without exhibiting a significant diminution in the circulating levels of TNF-α (Agueda et al., 2012). In the same sense, in obese adolescents leptin has been shown to rise independently of the levels of insulin resistance and TNF-α (Aguilar et al., 2012; Cohen et al., 2012). As it can be seen, it is still unclear whether hyperleptinemia has an association with the occurrence of low-grade systemic inflammation and metabolic dysfunction in humans.

We thus studied the serum levels of leptin in non-obese and obese Mexican individuals, examining their possible relationship with parameters of low-grade systemic inflammation (TNF-α, IL-12, and IL-10) and metabolic alteration (elevated serum glucose and insulin, increased level of insulin resistance, high triglyceride and cholesterol concentrations, as well as increasing waist circumference and body fat percentage).

MATERIALS AND METHODS

SUBJECTS

A total of 81 apparently healthy Mexican adult volunteers from the south-central region of Mexico were included in the study. All of the participants provided written informed consent, previously approved by an institutional review board of the General Hospital of Mexico "Dr. Eduardo Liceaga," which guaranteed that the study was conducted in accordance with the principles described at the Helsinki Declaration. Subjects were excluded from the study if they had previous or recent diagnosis of diabetes mellitus, cardiovascular diseases, chronic hepatic or renal disease, blood pressure higher than 140/90 mm Hg, inflammatory or autoimmune disorders, acute or chronic infectious diseases, cancer, and endocrine disorders. We additionally excluded pregnant or lactating women, subjects under any kind of cardiometabolic medication including anti-inflammatory, anti-aggregant, and anti-hypertensive drugs, and subjects without having an 8–12 h overnight fasting. All of the individuals enrolled into the study received full medical evaluation, including the achievement of clinic history and physical examination by a physician.

MEASUREMENT OF ANTHROPOMETRIC PARAMETERS

According to the World Health Organization criteria for BMI, all of the participants were divided into two groups: control non-obese subjects (BMI 18.5–24.9 kg/m^2) and obese subjects (BMI $\geq$ 30 kg/m^2), where BMI resulted of dividing weight by height squared (kg/m^2). Waist circumference was obtained from each study subject, considering the midpoint between the lower rib margin and the iliac crest, using a conventional tape in centimeters (cm). For women, abdominal obesity was considered when their waist circumference were 80 cm or higher, whereas for men it was considered when their waist circumference were 94 cm or higher. Percentage of body fat was individually recorded by using a body composition analyzer (TANITA®, Body Composition Analyzer, Model TBF-300A, Tokyo, Japan).

MEASUREMENT OF METABOLIC PARAMETERS

Blood samples were individually taken after overnight fasting, and collected into pyrogen-free tubes (VacutainerTM, BD Diagnostics, NJ, USA) at room temperature. Collection tubes were then centrifuged at 1000 g/4°C for 30 min, and serum samples obtained and stored at −80°C in numerous aliquots until use. Total cholesterol and triglyceride were individually measured in triplicate by an enzymatic assay according to manufacturer's instructions (Roche Diagnostics, Mannhein, Germany). Serum insulin levels were individually determined in triplicate by means of the enzyme-linked immunosorbent assay (ELISA), following the manufacturer's instructions (Abnova Corporation, Taiwan). Serum glucose levels were individually determined in triplicate by the glucose oxidase assay, following the manufacturer's instructions (Megazyme International, Ireland). The estimate of insulin resistance was individually determined by means of the HOMA-IR, as follows: fasting insulin concentration (mU/L) × fasting glucose concentration (mmol/L) divided by 22.5.

MEASUREMENT OF LEPTIN AND LOW-GRADE SYSTEMIC INFLAMMATION PARAMETERS

Blood samples were individually taken after overnight fasting, and collected into pyrogen-free tubes (VacutainerTM, BD Diagnostics, NJ, USA) at room temperature. Collection tubes were then centrifuged at 1000 g/4°C for 30 min, and serum samples obtained and stored at −80°C in numerous aliquots until use. Serum levels of leptin, TNF-α, IL-10, and IL-12 were determined in triplicate by ELISA, following the manufacturer's instructions (Peprotech, Mexico).

STATISTICAL ANALYSIS

Data from anthropometric and metabolic parameters were analyzed by using the Student's *t*-test for determining significant differences. Data from leptin, TNF-α, IL-10, and IL-12 were analyzed by means of using the Mann-Whitney *U*-test for determining significant differences. The Spearman's correlation coefficient was performed for examining the relationship of leptin with anthropometric, metabolic, and inflammatory parameters. All of the studied groups were matched by gender and age. Statistical analysis was performed using the GraphPad Prism 5 software. Differences were considered significant when $p < 0.05$.

RESULTS

A total of 40 non-obese controls and 41 obese subjects were included in the study. No significant differences were observed in age (for non-obese controls mean age 29.9 ± 10.35 years, whereas for obese subjects mean age 34.9 ± 10.24 years), and women/men proportion (22 women and 18 men in the non-obese control group, and 20 women and 21 men in the obesity group) in the studied groups (**Table 1**). In contrast, BMI, waist circumference, body fat percentage, fasting blood glucose and insulin, circulating levels of triglyceride and cholesterol, and

Table 1 | Anthropometric, metabolic, and inflammatory characteristics of the study subjects.

	Non-obese	Obese	*P*-value
Gender (W/M)	22/18	20/21	N.S.
Age (years)	29.9 ± 10.3	34.9 ± 10.2	N.S.
BMI (kg/m^2)	22.6 ± 1.8	33.7 ± 3.4	$p < 0.0001$
Waist circumference (cm)	79.6 ± 6.5	107.4 ± 9.9	$p < 0.0001$
Body fat percentage	24.6 ± 8.2	37.6 ± 6.9	$p < 0.05$
Fasting blood glucose (mmol/L)	4.3 ± 0.1	5.89 ± 0.3	$p < 0.0001$
Fasting blood insulin (mU/L)	12.6 ± 1.4	15.5 ± 6.7	$p = 0.0088$
HOMA-IR	2.4 ± 0.3	4.08 ± 1.8	$p < 0.0001$
Total cholesterol (mg/dL)	192.8 ± 10.1	198.1 ± 10.3	$p = 0.0219$
Total triglyceride (mg/dL)	137.5 ± 9.1	251.9 ± 14.4	$p < 0.0001$
TNF-α (pg/mL)	271.8 ± 28.05	322.9 ± 58.5	$p < 0.05$
IL-12 (pg/mL)	272.9 ± 13.6	381.5 ± 59.8	$p < 0.0001$
IL-10 (pg/mL)	1145.2 ± 214.6	840.8 ± 96.5	$p < 0.0001$

Abbreviations: W, women; M, men; BMI, body mass index; HOMA-IR, homeostatic model assessment-insulin resistance; TNF-α, tumor necrosis factor alpha; IL, interleukin; N.S., non-significant differences.
Data are presented as mean ± standard deviation.
Differences were considered significant when p < 0.05.

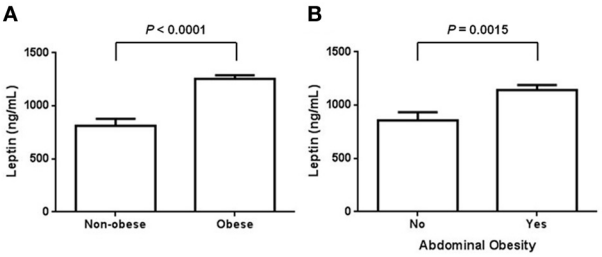

FIGURE 1 | Serum levels of leptin in obese and non-obese individuals. **(A)** Circulating leptin levels were assessed in normal weight and obese subjects, defining obesity according to the World Health Organization criteria for body mass index. In our study population, serum leptin was also evaluated in terms of abdominal obesity **(B)**. For women, abdominal obesity was considered when the waist circumference was 80 cm or higher, whereas for men, it was considered when the waist circumference was 94 cm or higher. Data are expressed as mean ± S. E. Differences were considered significant when $p < 0.05$.

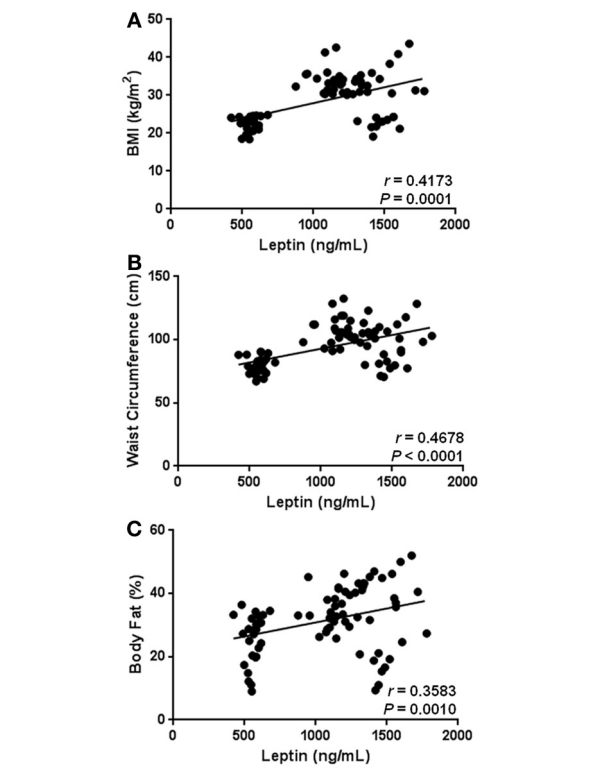

FIGURE 2 | Statistical correlation between serum levels of leptin and anthropometric parameters of obesity. Serum levels of leptin were positively associated with body mass index (BMI) **(A)**, waist circumference **(B)**, and body fat percentage **(C)**. Coefficients (*r*) and *P*-values were calculated by using the Spearman's correlation model. The correlation level was considered significant when $p < 0.05$.

serum concentrations of TNF-α and IL-12 were clearly increased in obese individuals as comparing with non-obese control subjects (**Table 1**). Concomitantly, serum levels of IL-10 were significantly lower in obese individuals than in non-obese subjects (**Table 1**). It merits to mention that BMI was clearly correlated with waist circumference ($r = 0.9297$, $p < 0.0001$) and body fat percentage ($r = 0.7655$, $p < 0.0001$) in our study population. In a similar way, there was also a significant association between waist circumference and body fat percentage ($r = 0.7655$, $p < 0.0001$).

In accordance with previous reports, circulating levels of leptin were significantly increased in obese subjects when comparing to non-obese control individuals. In terms of BMI, leptin exhibited a significant 1.5-fold increase in obese subjects as comparing with normal weight controls (**Figure 1A**). In obese individuals, the mean value of leptin was 1256.1 ± 207.7 ng/mL, whereas in the non-obese group it was around 812.1 ± 417.8 ng/mL (**Figure 1A**). Interestingly, serum values of leptin still showed a significant elevation when examining in subjects with abdominal obesity (**Figure 1B**). For this case, the mean value of leptin in subjects with abdominal obesity was 1141.8 ± 343.6 ng/mL, while it decreased to 858.3 ± 419.6 ng/mL in individuals exhibiting a normal waist perimeter (**Figure 1B**).

As expected, our results show that circulating levels of leptin increase with obesity-related anthropometric parameters. Indeed, leptin was significantly correlated with increased BMI ($r = 0.4173$, $p = 0.0001$), central obesity ($r = 0.4678$, $p < 0.0001$), and body fat percentage ($r = 0.3583$, $p = 0.0010$) (**Figures 2A–C**, respectively). Furthermore, leptin also exhibited a clear association with parameters of metabolic alteration. In this sense, serum leptin was significantly related with increased levels of blood glucose ($r = 0.5227$, $p < 0.0001$) and insulin ($r = 0.2229$, $p = 0.0455$) (**Figures 3A,B**, respectively). There was also a significant relationship between leptin and the level of insulin resistance,

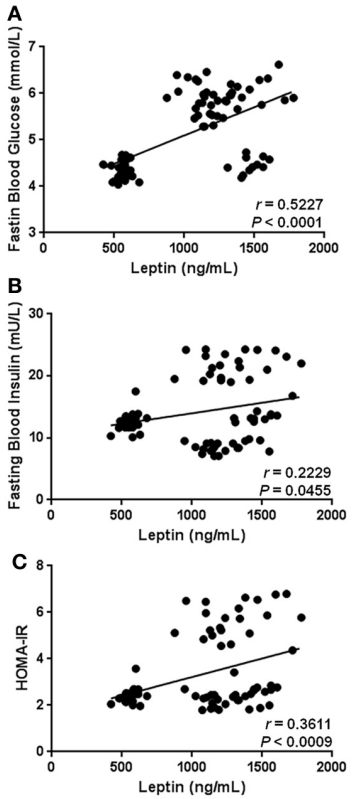

FIGURE 3 | Statistical correlation of the serum levels of leptin with fasting blood glucose, fasting blood insulin, and insulin resistance. Serum concentrations of leptin exhibited a positive significant relationship with high levels of glucose **(A)** and insulin **(B)**, as well increased insulin resistance **(C)**. The level of insulin resistance was estimated using the HOMA-IR index, which results of multiplying fasting insulin concentration (mU/L) by fasting glucose concentration (mmol/L), then divided by the constant 22.5. Coefficients (*r*) and *P*-values were calculated by the Spearman's correlation model. The correlation level was considered significant when $p < 0.05$.

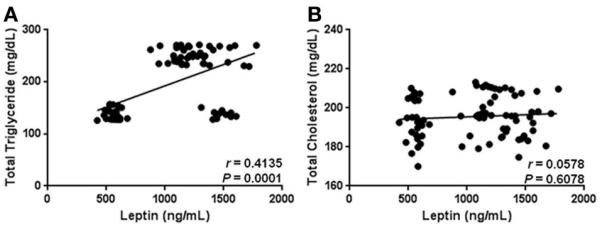

FIGURE 4 | Statistical correlation of the serum levels of leptin with total triglyceride and cholesterol levels. Serum concentrations of leptin showed a positive significant relationship with high levels of triglycerides **(A)**, but not with total cholesterol **(B)**. Coefficients (*r*) and *P*-values were calculated by the Spearman's correlation model. The correlation level was considered significant when $p < 0.05$.

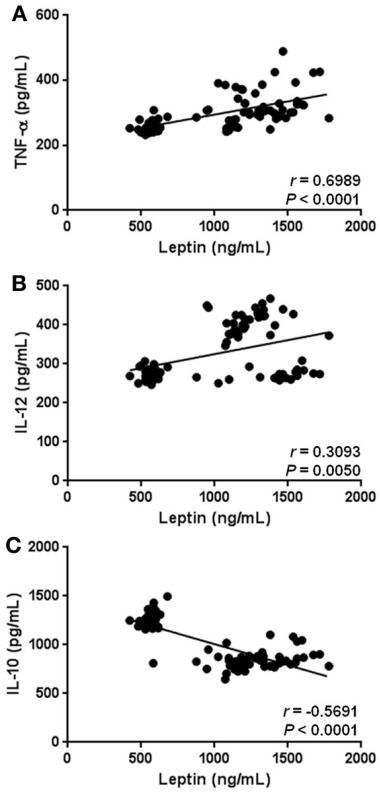

FIGURE 5 | Statistical correlation of the serum levels of leptin with parameters of low-grade systemic inflammation. Serum concentrations of leptin showed a positive significant relationship with increased serum levels of TNF-α **(A)**, and IL-12 **(B)**. At the same time, hyperleptinemia exhibited a significant inverse association with circulating concentrations of IL-10 **(C)**. Coefficients (*r*) and *P*-values were calculated by the Spearman's correlation model. The correlation level was considered significant when $p < 0.05$.

estimated by means of the HOMA-IR ($r = 0.3611$, $p < 0.0009$) (**Figure 3C**). Interestingly, leptin had a positive association with increased triglyceride levels ($r = 0.4135$, $p = 0.0001$), but not with total cholesterol (**Figures 4A,B**, respectively).

Besides having significant relationships with anthropometric and biochemical parameters associated with obesity-related metabolic alterations, hyperleptinemia also showed a strong relation with markers of low-grade systemic inflammation in the study subjects. In fact, circulating leptin was positively correlated with serum levels of TNF-α ($r = 0.6989$, $p < 0.0001$) and IL-12 ($r = 0.3093$, $p = 0.0050$) (**Figures 5A,B**), whereas a significant negative relationship between leptin and IL-10 was observed in the study population ($r = -0.5691$, $p < 0.0001$) (**Figure 5C**).

DISCUSSION

As mentioned, it has been consistently shown that serum concentrations of leptin increase in high-fat diet fed-mice and obese humans. In mice, hyperleptinemia is related to hyperphagia and fat depot augmentation, while it is associated with increased white adipose tissue (WAT) mass and body weight gain in human beings (Stanley et al., 2005). However, recent experimental evidence from animal models of obesity suggests that leptin is not only a marker of weight gain but also seems to have a

relationship with developing a systemic state of low-grade inflammation and metabolic disturbance. In fact, obese mice exhibiting hyperleptinemia show increased plasma levels of inflammatory cytokines (Dube et al., 2008; Yang et al., 2010; Enos et al., 2013), accompanied by numerous metabolic disorders including hyperglycemia (Yang et al., 2010), hyperinsulinemia (Stienstra et al., 2011), hyperlipidemia (Kang et al., 2011), liver steatosis (Shih et al., 2010), and insulin resistance (Yang et al., 2010). Taking this experimental evidence into account, it is important to evaluate whether hyperleptinemia could also be associated to the establishment of systemic inflammation and metabolic dysfunction in human beings with high metabolic risk, such as obese individuals.

In humans, the relationship of leptin with a state of metabolic dysfunction has been barely studied, showing inconclusive results to date. Indeed, a study conducted in a group of obese adolescents revealed that high serum levels of leptin correlate with increased BMI and waist circumference, without having a significant relation with the insulin resistance level (Aguilar et al., 2012). In contrast, recent data from a cross-sectional survey conducted in obese adults demonstrated that hyperleptinemia is clearly associated with BMI, hyperinsulinemia, and insulin resistance (Martins Mdo et al., 2012). Our results are consistent with the last study, since high levels of leptin were significantly correlated with hyperglycemia, hyperinsulinemia, increased HOMA-IR, and hypertriglyceridemia in our study population. A possible explanation to understand this apparently controversial finding may involve the age of the subjects included in each study. As it is widely known, the level of sex-steroid hormones reaches a plateau during maturity, in comparison to both childhood and adolescence where numerous hormonal variations are normally observed (Stanhope and Brook, 1988; Rogol, 2004). Interestingly, it has been recently reported that sex-steroid hormones are able to upregulate the leptin expression in human and rat cells (Feng et al., 2011; Gambino et al., 2012). Therefore, it is feasible to expect that synthesis of leptin may be enhanced during adulthood, which may contribute to decrease the leptin levels in children/adolescents in comparison with adults. However, additional clinical studies considering the influence of sex-steroid hormones upon the systemic levels of leptin are necessary in order to address major conclusions.

An interesting finding in this cross-sectional study involves the relationship of hyperleptinemia with a systemic state of low-grade inflammation in obese human beings. A recent study demonstrated that leptin is overexpressed in the subcutaneous adipose tissue (SAT) of obese individuals with MetS, as comparing with SAT from healthy obese subjects and non-obese individuals (Farb et al., 2011). Furthermore, increasing in the leptin expression is accompanied by macrophage infiltration and overexpression of proinflammatory cytokines such as IL-1β, IL-6, and IL-8 in the SAT of those same patients (Bremer et al., 2011; Farb et al., 2011). Consistent with this previous study, our results show that hyperleptinemia is significantly associated with high serum levels of TNF-α and IL-12, as well as reduced concentrations of IL-10 in subjects with central obesity, hyperglycemia, increased insulin resistance, and hypertriglyceridemia. IL-12 is a cytokine with the ability to induce synthesis of interferon-gamma (IFN-γ) in T cells and natural killer cells (Trinchieri, 2003). IFN-γ is a key mediator in releasing of TNF-α by classically activated macrophages (Odegaard and Chawla, 2008). Taking into consideration that serum IFN-γ has been shown to increase during obesity (Azar Sharabiani et al., 2011), it is conceivable to expect a positive relationship among TNF-α, IL-12, and leptin in our study population. Another intriguing issue concerning the positive association among leptin, TNF-α, and IL-12, involves the ability of leptin to regulate the expression of inflammatory cytokines. It has been recently reported that leptin is able to stimulate the *in vitro* production of TNF-α and IL-1β in human mononuclear cells (Tsiotra et al., 2013). Thus, it is now proposed that high levels of leptin may induce the production of proinflammatory cytokines in obese people, contributing in this way to the systemic inflammation observed in these subjects. Nevertheless, before establishing a possible cause-and-effect relation among leptin, TNF-α, and IL-12 in the scenario of obesity, additional prospective clinical research is required. Moreover, consistent with the installation of a systemic state of low-grade inflammation, we observed a significant reduction in the circulating levels of IL-10 in obese individuals as comparing with non-obese subjects. IL-10 is a cytokine with potent anti-inflammatory properties in mice and humans (Saraiva and O'garra, 2010). However, the role of IL-10 during systemic inflammation and metabolic dysfunction is still poorly understood (Formoso et al., 2012; Tajik et al., 2012). For this reason, it is important to mention that the present work is one of the first contributions showing a significant inverse correlation between serum IL-10 and hyperleptinemia in obese individuals. Collectively, these findings suggest that obesity-related hyperleptinemia is accompanied by a low-grade inflammatory profile, characterized by increased circulating levels of TNF-α and IL-12, and reduced concentrations of IL-10. Whether hyperleptinemia is cause or consequence of the systemic inflammatory milieu in humans, is a matter worthy of being considered in further basic and clinical studies.

Present results demonstrate that high circulating levels of leptin are significantly associated with a systemic state of low-grade inflammation and metabolic dysfunction in obese subjects. Additional prospective clinical studies are still required to evaluate whether hyperleptinemia may be used as a marker for recognizing the advent of obesity-related metabolic disorders in human beings.

ACKNOWLEDGMENTS

We thank Jose Alejandro Perez Gonzalez, Guadalupe Gonzalez Galicia, Maria de Jesus Mata Rivera, Alejandra Lopez, Maria Esther Ruiz Escalera, Yazmin Saldaña Arenas, Patricia Enriquez Lemus, and Yolanda Camarillo Ornelas for their technical assistance in the enrollment of the study subjects. This work was supported by grant no. CB-2009-01-129316 from Consejo Nacional de Ciencia y Tecnologia (CONACYT) to Galileo Escobedo. S. Leon-Cabrera thanks the support from the Programa del Mejoramiento del Profesorado (PROMEP)-Mexico. Dr. L. Graue corrected the English version of this manuscript.

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Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Received: 02 May 2013; paper pending published: 27 May 2013; accepted: 01 August 2013; published online: 23 August 2013.

Citation: Leon-Cabrera S, Solís-Lozano L, Suárez-Álvarez K, González-Chávez A, Béjar YL, Robles-Díaz G and Escobedo G (2013) Hyperleptinemia is associated with parameters of low-grade systemic inflammation and metabolic dysfunction in obese human beings. Front. Integr. Neurosci. 7:62. doi: 10.3389/fnint.2013.00062

This article was submitted to the journal Frontiers in Integrative Neuroscience.