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# Alcohol, Inflammation, and Blood-Brain Barrier Function in Health and Disease Across Development

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## Abstract

Alcohol is the most commonly used drug of abuse in the world and binge drinking is especially harmful to the brain, though the mechanisms by which alcohol compromises overall brain health remain somewhat elusive. A number of brain diseases and pathological states are accompanied by perturbations in Blood-Brain Barrier (BBB) function, ultimately exacerbating disease progression. The BBB is critical for coordinating activity between the peripheral immune system and the brain. Importantly, BBB integrity is responsive to circulating cytokines and other immune-related signaling molecules, which are powerfully modulated by alcohol exposure. This review will highlight key cellular components of the BBB; discuss mechanisms by which permeability is achieved; offer insight into methodological approaches for assessing BBB integrity; and forecast how alcohol-induced changes in the peripheral and central immune systems might influence BBB function in individuals with a history of binge drinking and ultimately Alcohol Use Disorders (AUD).

### Keywords

Alcohol; Blood-Brain Barrier; Adolescence; Inflammation; Cytokines

## Introduction

The BBB functions as one of the most vital barriers to protect the CNS from homeostatic disruption in mammalian species. From birth, the barrier serves to closely regulate active and passive transport of blood-borne substances into and out of the brain. This ensures optimal brain health and facilitates communication between the central nervous system (CNS) and peripheral organ systems that mediate normal bodily function (Saunders, Liddelow & Dziegielewaska, 2012; Saili et al., 2017). In the context of inflammation, insult, stress, or exposure to drugs of abuse, and immune signaling factors as well as the drugs themselves are controlled by gating mechanisms mediated by the BBB (Pimentel et al., 2020). The transit of signaling factors from the periphery into the brain confers a degree of

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"awareness" for the CNS as to what is occurring in the periphery (Reinhold & Rittner, 2016; Fuzzati-Armentero, Cerri & Blandini, 2019). Dysfunction of this gatekeeping role often culminates in extreme pathology due to excessive influx of immune signaling factors into the CNS, which in turn creates a cyclical pattern of disruption. Thus, damage or alterations to BBB permeability have far-reaching implications for subsequent brain health (Daneman & Prat, 2015; Profaci et al., 2020).

Ethanol remains one of the most commonly used drugs of abuse in modern society. Owing in part to its cultural/societal acceptance, a reported 85.6% of individuals aged 18 and older self-report consumption of alcohol at some point in their lifetime (SAMHSA, 2019). While there is some data to suggest a small quantity of ethanol consumption can have beneficial impact, 25.8% of individuals 18 and older self-report having engaged in harmful binge consumption within the past month (SAMHSA, 2019). Binge patterns of ethanol consumption and heavy alcohol use are especially harmful to both the brain and organ systems throughout the body. For instance, higher levels of self-reported drinking have been associated with reduced grey matter volumes across the brain (Kvamme et al., 2016; Howell et al., 2013). Binge drinking also strongly predicts a future diagnosis of an Alcohol Use Disorder (AUD), which can compromise overall health across the lifespan (Grant & Dawson, 1997; Dewit et al., 2000).

Chronic alcohol abuse and dependence are associated with pathological increases in inflammation that often leads to organ dysfunction (Wang, Zakhari & Jung, 2010; Leclerq et al., 2017), whereas more moderate doses may produce lower risk levels in some circumstances (Pai et al., 2006; Wang et al., 2008; Bektas, Sen & Ferruci, 2016). One hallmark of alcohol dependence, alcoholic liver disease, is known to involve inflammatory signaling at many different levels of cellular and organ function (Wang et al., 2012). For instance, alcohol can mobilize gut-derived endotoxins, increase inflammatory signaling factors such as cytokines, and increase oxidative stress (Fujimoto et al., 2000). Increased TLR4-mediated signaling in Kupffer cells, hepatocytes, stellate cells, and other important cellular components of the liver are functionally changed by alcohol (Mandrekar & Szabo, 2009). Beyond these peripheral changes, there is a growing body of literature that examines the impact of ethanol on the gut and how these changes may have more far-reaching impact on behavior and brain function. For example, gut microbiome dysfunction can modulate vagus nerve signaling, ultimately amplifying the neuroimmune response in the amygdala and contributing to heightened anxiety and other behavioral changes observed during alcohol withdrawal (Gorky & Schwaber, 2016). Finally, both rodent and human data indicate that prolonged alcohol use and abuse produces a persistent immunosuppressive phenotype that compromises host defense. While this phenotype often may be subclinical in magnitude, when it is combined with an additional immune challenge or insult pathological immunosuppression emerges (Szabo & Saha, 2015).

In preclinical studies with rodents, acute, binge-like alcohol exposure produced fluctuations in pro-inflammatory cytokine expression, with unique cytokine signatures apparent during acute intoxication and a separate pattern evident during alcohol hangover/withdrawal. Specifically, IL-6 and IκBα were markedly increased within the first few hours after ethanol exposure, whereas IL-1β and TNFα were only increased after ethanol clearance

(Doremus-Fitzwater et al., 2014; Walters et al., 2017; Gano et al., 2019). Although the magnitude of these changes varies somewhat across the CNS, they are particularly prevalent in limbic structures such as the hippocampus, amygdala, and the paraventricular nucleus of the hypothalamus (Doremus-Fitzwater et al., 2014). Such changes in neuroimmune factor gene expression are both reproducible and highly conserved, with prior studies reporting equivalent responses in males and females (Gano et al., 2019), and multiple rat strains (Gano, Doremus-Fitzwater, and Deak, 2018). Surprisingly, age of alcohol exposure has emerged as a critical demographic variable which determines the magnitude of neuroimmune signal gene expression response to an acute alcohol challenge. Adolescent rats showed severely diminished neuroimmune responses to alcohol, perhaps in part due to a functional immature neuroimmune system (Doremus-Fitzwater, 2015; Marsland, 2021). In contrast, late aging (24 month) led to a heightened basal expression of cytokines, which made these aged rats refractory (i.e., less responsive) to ethanol-induced neuroimmune changes (Gano et al., 2017). Thus, age of ethanol exposure appears to be a critical determinant of ethanol-related changes in inflammation.

Given the abundance of data that binge or supra-binge levels of ethanol exposure produce a distinct neuroimmune profile, this review will evaluate the potential influence of ethanol evoked inflammation and cytokine induction might have on BBB function. While few studies have directly assessed ethanol's ability to alter BBB function, there is an established body of work demonstrating that inflammatory signaling is mechanistically linked to changes in BBB function. Indeed, inflammatory agents such as LPS have been used in a number of studies to mimic bacterially derived inflammation. Using LPS as a challenge, alterations in BBB permeability may involve a variety of key BBB regulatory mechanisms, including (i) direct damage to the endothelium; (ii) alterations in tight junction (TJ) expression and/or function that "loosen" adhesion of cells; (iii) increased nitric oxide (NO), a transient gas that promotes vasodilation; and (iv) changes in glial (e.g., astrocytes, pericytes, microglia) function and quantity; and many other BBB regulatory mechanisms (for a more thorough review of this literature see Varatharaj & Galea, 2016). As ethanol impacts many of these same physiological parameters and is known to modulate inflammation, it seems reasonable to hypothesize that binge-like alcohol use and abuse compromises BBB function through inflammation-related mechanisms (Figure 1). The present review provides a more detailed framework for pursuing this hypothesis with empirical studies.

## Form and Function of the BBB: Gatekeeper of Neurovascular Unit Function

The BBB plays a vital role in regulating passage of cells and biomolecules into the CNS and is conserved in some form across vertebrate species (O'Brown, Pfau & Gu, 2018). The complex nature of the brain and the processes it governs means that it must gate the passage of infiltrating monocytes and potential toxins and toxicants present in general circulation. At the same time, bidirectional communication is needed to orchestrate effective responses to environmental stimuli. To accomplish this, the BBB functions as a critical gatekeeper of what reaches the parenchyma of the CNS. The BBB is comprised of a variety of cells that work synchronously to provide differing levels of neural protection across the CNS-peripheral nervous system (PNS) and in response to changing physiological

circumstances (Zhao, Nelson, Betsholtz, & Zlokovic, 2015). Brain endothelial cells form the tight physical barrier restricting molecules from entering the brain. Cell-to-cell tight junctions and adherens junctions function as physical barriers to large and/or hydrophilic molecules (Zhao et al., 2015). Tight junctions ensure that only small, lipophilic molecules, estimated to be under 400 Daltons (Da; or 0.4 kDa) can freely pass (Pardridge, 2015). While this aspect of the BBB helps the CNS maintain persistent exclusivity to larger and potentially harmful entities, the brain requires mechanisms that govern passage of a multitude of macromolecular proteins, amino acids, and other building blocks that can't freely pass through the BBB. Movement of these molecules is accomplished via an array of active and passive transport mechanisms. Polar, hydrophilic, small molecules such as amino acids utilize solute carrier membrane transport proteins set within the BBB to gain access to the CNS through facilitated diffusion down the concentration gradient, whereas macromolecule and peptide transit is more frequently handled by more traditional, energy-driven, receptor-mediated transport mechanisms (Banks, 2015; Zhao et al., 2015). All of these systems provide important mechanisms for both paracellular and transcellular crossing into and out of the CNS and serve to highlight the gating dynamics as well as the importance of BBB function to overall brain health.

## The Neurovascular Unit

In some ways, the neurovascular unit (NVU) represents a modification and expansion of the classic "tripartite synapse" concept used to summarize synaptic transmission in the CNS (Muoio, Persson, & Sendeski, 2014). At the heart of the NVU, neurons are exceptionally sensitive to changes in blood oxygen, nutrients, and other blood-borne molecules that are carried through the blood supply. Neuronal communication is energy-intensive and, in response to local changes in neuronal activity other components of the NVU, local blood flow to the NVU is altered to match oxygen and nutrient demand. Thus, form and function of the NVU is designed to be responsive to changing neuronal activity and ultimately to optimize neuronal function.

While the "tripartite synapse" incorporates the most direct cellular elements of synaptic communication (neurons and astrocytes), it does not take into account the many other cellular interactions that chemically influence neuronal health and function (Neuwelt et al., 2011). While not every neurovascular interface is identical, it is most frequently comprised of the endothelium, surrounded by the basement membrane, encapsulated by pericytes, and in close contact with both astrocytic end-feet and microglia (see Figure 2). Each of these component cells regulate unique aspects of NVU function as a whole, and are poised to significantly influence BBB permeability (McConnell et al., 2017). Closely aligned astrocytes and microglia play important roles in buffering the cellular microenvironment, often serving as intermediate signaling cells between blood-borne agents and neurons, and vice versa.

Astrocytes function as a direct cellular mediator between neurons and the remainder of the neurovascular unit. Astrocytic end-feet almost completely encapsulate the endothelial cells that comprise the capillaries of the NVU permitting bidirectional communication between both cell types (Figure 2). Through these connections, the astrocytes release a variety of

factors such as prostaglandins and NO that have the capacity to alter blood vessel diameter and therefore blood flow in response to changing neuronal demand (Wolf & Kirkchoff, 2008). Astrocytes also play an active role in the recycling and clearance of neurotransmitters and water from the brain that helps to ensure overall brain function (Sofroniew and Vinters, 2010).

While astrocytic end-feet play an important role in modulating cerebral blood flow, other cells associated with, or encapsulated by, astrocytic end-feet are also critical. The primary contact to the endothelial cells within the NVU is pericytes (Figure 2). Pericytes have emerged as critical modulators of BBB development and functional integrity, since they specifically aid in the attachment of astrocytic end-feet to the NVU (Armulik et al., 2010). Loss of pericytes is known to promote upregulation of several genes (i.e. Angiopoietin 2, Intercellular Adhesion Molecule 1, and Galectin 3) associated with increased vascular permeability, suggesting a role in BBB maintenance and endothelial cell regulation (Daneman et al., 2010). This can help promote angiogenesis and guide vascular branching within the cerebral vasculature (Bergers & Song, 2005; Eilken et al., 2017). More recently, it has also been noted that pericytes also display a direct role in modulating cerebral blood flow. For example, through contraction of the processes that wrap around the vessel, pericytes directly increase or decrease vessel constriction in response to oxygen/nutrient needs of the brain (McConnel et al., 2017).

Microglia, much like astrocytes, express factors that contribute to both the maintenance and degradation of the BBB. Microglia are considered the “first responders” of the CNS, responding to insult, excessive neuronal activity, tissue damage or infection with rapid expression of chemokines and cytokines that forward-propagate neuroinflammation (Liu et al., 2020). Through active monitoring of the NVU, microglia can transition through a number of active states which are accompanied by adaptive changes in the neuroimmune factors they release. One down-stream consequence of microglial activation is impaired BBB function (Haruwaka et al., 2019). A substantial amount of cross-talk occurs between astrocytes and microglia and, through a coordinated response, they orchestrate the NVU’s response to damage (Figure 2). Increases in the total number of active microglia are associated with increased BBB leakiness and multiple diseases associated with BBB dysfunction are correlated with changes in genes unique to microglia such as DAP12 (Sasaki et al., 2015). The final component of the NVU is the basal lamina (Figure 2). The basal lamina perform an important structural role in the NVU by providing anchor points for the astrocytic end-feet. It functions as an additional extracellular matrix comprised primarily of collagen and laminin. Signaling of both endothelial cells and pericytes guide the creation of the basal lamina and ultimately serve as a physical boundary between the two cell types. Thus, the typical NVU is comprised of multiple cell types in close cellular juxtaposition, with extensive cross-talk between each type.

## Molecular Passage Across the BBB

The first line of defense forming the BBB is conferred by brain endothelial cells, which form the vascular wall and constrain passage of blood and its cargo into the interstitial tissue. TJs between adjacent endothelial cells prevent the passage of material between endothelial

cells and are largely comprised of three families of proteins: occludins, claudins, and other adhesion molecules (Luissint et al., 2012). Studies have shown that interference or knockout of any of these three is associated with increased pathological BBB permeability (Nitta et al., 2003). While the semi-permeable nature of tight junctions varies across the CNS, they are generally considered to be relatively impermeable, allowing only very small (<400 Da), lipid-soluble molecules to cross (Pardridge, 2007).

While small, highly lipophilic molecules may cross freely through TJs, the passage of many endogenous and exogenous molecules across the BBB is governed by active transporters. The variety of membrane transport proteins can then be further divided into uptake/influx (into the brain) and efflux (out of the brain). The parenchyma of the brain is carefully guarded and, generally, for any drug or large molecule, access is extremely limited. Essential nutrients such as amino acids and glucose only gain access to the CNS through uptake systems such as organic anion transporters, organic cation transporters, and multidrug and toxin extrusion proteins (Sanchez-Covarrubias et al., 2014). Active transport of these larger polar molecules is facilitated by these membrane proteins. Despite gaining access to the brain, a number of efflux transporters pump out larger, polar molecules that may be present in the CNS. This serves an important regulatory role in preventing over-accumulation of larger molecules, and carefully maintains homeostasis of brain nutrients. Efflux transporters include the Para-glycoprotein (PGP) complex and multidrug resistance-associated proteins (de Boer, van der Sandt, & Gaillar, et al., 2002). The PGP complex specifically plays a vital role in the removal of drugs from the CNS and has remained a focal point in designing new generations of pharmaceuticals that can gain selective access to the brain (for more thorough review see Bendayan, Lee, & Bendayan, 2002; Loscher & Gericke, 2020). The function and quantity of these transporters can be altered in response to a number endogenous and exogenous factors such as neurotransmitters, cytokines, and pathological insult (Avemary et al., 2013; Keaney & Campbell, 2015). Thus, a variety of active transport mechanisms contribute to passage of small molecule and macromolecular complexes across the BBB.

Cytokines interact with the BBB in a number of different ways and many different cytokines transit across the BBB through shared and unique transport mechanisms (see Banks, 2005; Banks, 2008 for excellent reviews). Importantly, cytokine transit is dynamic and can show significant differences in transport rate depending on the animal species, as a result of circadian changes, in region-specific manners, and in response to bodily demands (when challenged). As specific examples, IL-1β is known to cross the BBB into the brain using unidirectional, saturable transport mechanisms (Banks et al., 1991; Davson & Segal, 1996). Similarly, both IL-6 and TNF-α utilize unidirectional saturable transport mechanisms to transit into the brain; however, it is believed that they use explicitly different transporters (Banks et al., 1991; Banks, Kastin & Gutierrez, 1994; Gutierrez, Banks & Kastin, 1993). In contrast, some cytokines, such as neutrophil chemoattracant-1, are able to cross the BBB using diffusion and in some circumstances, such as IL-2, transit is not believed to occur (Pan & Kastin, 2001; Waguespack, Banks & Kastin, 1994). While saturable transport is a primary mechanism by which many cytokines enter the brain, IL-1β, TNF-α, and IL-6 have all been shown to enter the blood from the brain through cerebrospinal fluid absorption (Banks et al., 1991; Chen et al., 1997; Chen & Reichlin, 1998). These differences highlight the ability of both the peripheral and central immune system to influence one another.

## The Role of the BBB in Peripheral to Central Immune Signaling

For an optimal immune response to infection or injury, effective communication must occur between the periphery (and its constituent inflammatory mediators) and the brain (Maier & Watkins, 1998). As such, BBB function is poised to be a key intermediary governing immune-to-brain communication, particularly for immune signaling molecules such as cytokines and chemokines (Banks, 2005; Erickson, Wilson, & Banks, 2020). Studies have shown that TJ permeability is subject to change due to local gene regulation (Balda & Matter, 2009). Inflammatory mediators such as histamines increase permeability in brain endothelial cells in a similar fashion as seen in peripheral capillaries through opening of inter-endothelial cell tight junctions (N. J. Abbott, Ronnback, & Hansson, 2006). In response to hypoxia, increased TJ expression and upregulation of PGP, responsible for rapid transit of compounds out of the brain, result in increased barrier stringency (N. J. Abbott et al., 2006). These findings suggest that BBB permeability is not a static aspect of physiology, but rather constantly changes in response to physiological conditions.

One of the key components responsible for communication between peripheral and central immune responses are cytokines. Cytokines are produced by microglial cells, endothelial cells, astrocytes, macrophages, and many other types of cells both within the CNS and in the periphery. They are released rapidly during the acute inflammatory process and are able to cross the BBB via passive diffusion in CNS regions, only where the BBB is weak or absent, such as the circumventricular organs and the OVLT (Buller, 2001; Buller, 2002; Roth et al., 2004). Many cytokines are also subject to active transport mechanisms, which specifically transport cytokine proteins across the BBB into the CNS; although, the precise mechanism of cytokine transit across the BBB is largely species and context-dependent (Banks, 2005). Importantly, many cytokines also disrupt the BBB. Pro-inflammatory cytokines and other inflammatory signaling factors cause TJ degradation, thereby increasing permeability of the BBB (Coisne & Engelhardt, 2011; Wan, Chen, & Li. 2013; Al-Obaidi & Desa. 2018). This is, at least partially, caused through disruption of adhesion molecules on endothelial cells. When combined with chemokine signaling, leukocyte recruitment permits extravasation, the movement of white blood cells from the blood capillaries into the CNS often towards the site of insult. Cytokines can also damage the basal lamina by inducing matrix metalloproteinase (MMP) expression by endothelial cells and microglia, which further impairs the structural integrity of the BBB (Klein & Bishcoff, 2011; Gurney, Estrada, & Rosenberg, 2006).

In addition to active and passive transport across the BBB, many inflammatory signaling molecules use neural pathways, humoral pathways, and direct interaction with endothelial cells to propagate *de novo* inflammatory signals on the brain side of the BBB (Goehler et al., 1999; Laflamme & Rivest, 2001; Disabato, Quan, & Godbout. 2016). In this way, inflammatory signals originating in the periphery communicate with the CNS through transcellular communication. Many peripheral cytokines and other inflammatory mediators also bind to peripheral nerves or nerve associated lymphoid tissue (NALT), causing the peripheral inflammatory signal to be transcribed into neural impulses, which in turn may orchestrate central inflammation signaling (Kennedy & Silver, 2016). Thus, most mammalian species have evolved multiple physiological mechanisms for PNS-CNS immune communication that bypasses direct passage of peripherally-derived cytokines into the CNS.

Alterations in BBB function are frequently noted in response to challenges that evoke inflammation or a profound immune response. High levels of proinflammatory cytokines such as IL-1β & IL-6, often released by microglia, lead to increased permeability of the BBB that in times of more extreme pathology allow the infiltration of peripheral macrophages and other immune cells into the CNS (Jin, Silverman, & Vannucci, 2009). Microglia, the prominent and resident immune cell of the CNS, migrate to the damaged area in response to focal injury or conventional immune challenges and, through a variety of different modalities, forward-propagate the expression of chemokines, cytokines, and other signaling molecules crucial for tissue repair (in the case of damage) or host defense (in the case of infection). This increase in inflammatory tone can evoke significant changes in the BBB including increasing local permeability. Proinflammatory cytokines are integral for leukocyte trafficking across the BBB (Blond, Campbell, Butchart, Perry, & Anthony, 2002), one mechanism by which the peripheral immune response may modify central immune function. Minocycline administration, a tetracycline antibiotic that reduces microglial activity, has also been shown to reduce BBB permeability induced by LPS exposure, suggesting a potential role for microglia in inflammation-mediated BBB change (Moretti et al., 2015). LPS exposure in neonatal rats (PND0-PND8) has also been shown to increase BBB permeability, but only once animals reach adulthood and not at P20 (Stolp et al., 2011). These changes suggest a potential developmental vulnerability to some of the inflammation-induced BBB permeability changes. Correlations have also been observed between multiple pathophysiological conditions such as Alzheimer's disease, cancer, and multiple sclerosis, heightened cytokine levels, and the BBB (Moretti et al., 2015; Schenk & de Vries, 2016; Wardill et al., 2016).

Sex differences have been well documented regarding changes in BBB function and data have shown that sex differences exist in many cellular components of the BBB (Torres & Bynoe, 2018). Some of the differences noted in BBB permeability are believed to stem from endogenous differences in estrogen and the potential role estrogen may have in protecting against BBB degradation. Female-typical hormones such as estrogens and progestins also have anti-inflammatory properties (Nadkarni & McArthur, 2013), which may short-circuit inflammation-dependent alterations in BBB permeability. In addition, estrogen has been shown to enhance tight junctions, limit lymphocyte extravasation, and protect against BBB degradation resulting from LPS administration (Maggioli et al., 2016). In contrast, some data have shown that certain strains of mice may show heightened female vulnerability to changes in BBB permeability after LPS challenge (Erickson et al., 2018). Given the number of factors capable of modifying the components of the BBB and NVU, additional studies directly assessing sex differences in BBB regulation are needed.

## Approaches for Assessment of the BBB

Several simple and straight-forward approaches for assessment of BBB integrity are used routinely, each with their strengths and limitations. One of the most common methods for quantifying BBB function is through the assessment of individual genes, proteins, or cells that are known to be involved in formation of the BBB. There is no shortage of work examining factors such as claudins, occludins, integrins, and other adhesion molecules (Liebner et al., 2000; Dias et al., 2019). Similarly, direct visualization of endothelial cells,

astrocytes, perivascular microglia or pericytes offer additional opportunities to investigate the NVU in relation to BBB integrity (Kovacs et al., 2017; Brown et al., 2020). While these approaches address cellular and structural mechanisms contributing to the BBB, they do not provide a direct *functional* assessment of BBB permeability. In addition, a number of *in vitro* model systems have been used to assess BBB permeability, offering unique opportunities to assess specific BBB regulatory mechanisms (Sivandzade & Cucullo, 2018). Comprehensive *in vitro* modeling of the BBB requires functional incorporation of the various cellular components of the NVU, an incredibly challenging task. However, to truly assess the functional integrity of the BBB, permeability characteristics of the BBB must be probed through the use of dye-based or radioligand-based, *in vivo* assessments.

One of the oldest and most common approaches for measuring BBB permeability is through the use of dyes or labeled compounds to directly quantify brain access. Dating back to Rossner and Temple (1966), Evans Blue (a vital dye that binds to serum albumin and other macromolecules) has been used as a metric for how much albumin is able to cross into the brain. Albumin is a relatively large molecule (~70 kDa) that, under normal conditions, should demonstrate little access to the brain. Due to its function as a vital dye, this allows for a quick and relatively simple quantification of dye extravasation in a variety of tissue compartments following a single *in vivo* exposure. These studies often use i.v. administration of Evans Blue dye followed by quantification or visualization of the amount of dye that accumulates in brain tissue as a metric for BBB permeability, either through tissue slicing and direct visualization; or through gross dissection of brain regions, homogenization of tissue, and quantification of dye using a spectrophotometer. More recently, however, a number of questions have been raised about the validity of the Evans Blue dye assessment. For instance, we now know that Evans Blue does not to bind exclusively to albumin, suggesting the scientific premise underlying this approach may be flawed (see Saunders et al., 2015 for review). Further, 70 kDa is a comparatively large compound relative to many nutrients and humoral signaling molecules commonly distributed in the blood. While it certainly addresses extreme instances of BBB pathology, it does not address smaller molecule permeability that could still have important biological significance. For instance, many pro-inflammatory cytokines range from 14–20 kDa in size, suggesting significant passage of these molecules at levels of BBB disruption that would not be effectively quantified with an Evans Blue dye extravasation test.

Another common method to study BBB permeability is the use of horseradish peroxidase after i.v. administration. Horseradish peroxidase is a roughly 45 kDa glycoprotein that, when combined with the proper substrate, can produce a colorimetric or fluorometric derivative allowing it to be easily quantified. As with Evans Blue dye, this allows for simple *in vivo* administration to be quantified in a variety of tissue compartments. The reaction product can be visualized using electron microscopy, allowing even more detailed assessment of BBB permeability. While this technique was shown to work effectively in mice, the method has been questioned because in rats, horseradish peroxidase alone can cause histamine and serotonin release, both of which increase BBB permeability and may potentially confound results of such an analysis (Majno et al., 1961). Moreover, many of the toxic consequences of HRP seem to be dose- and species-specific, making interpretation across experiments and laboratories complicated (Cotran et al., 1968; Clementi. 1970; Ross et al., 1977).

To more specifically probe BBB integrity across a spectrum of molecular sizes, more recent studies have used fluorophore-tagged dextran molecules (Natarajan, Northrop, & Yamamoto, 2017). Dextrans are complex polysaccharides that can be purchased across a range of sizes (ranging from as small as 4 kDa to as large as 2000 kDa). In this model, transcardial perfusion of Dextran of the target molecular weight is followed by tissue slicing and visualization of fluorescent dye in the interstitial tissue. By probing a range of molecular weights, a more refined and sensitive assessment of subtle, yet biologically important fluctuations in BBB integrity is possible.

Previous work from our laboratory has probed BBB permeability in response to acute ethanol challenge using several approaches. Using the Evans Blue procedure described above, we assessed the sensitivity of the method using a high dose LPS injection (500 μg/kg) as a presumptive positive control previously documented as producing increased BBB permeability (Ghosh et al., 2014; Banks et al., 2015). We saw no substantial differences in brain dye content relative to no dye or vehicle injected controls (Fig. 3A). These data mirror small pilot studies that were executed to determine whether any observable changes in Evan's Blue dye could be quantified following ethanol exposure. Given our concern over assay sensitivity, we piloted adolescent intermittent ethanol (AIE) exposure evoked changes in BBB permeability using a more sensitive protocol, the FITC-dextran procedure, and observed substantial increases in permeability in adult rats with a history of AIE ethanol exposure at the 20 kDa size (Fig. 3B). Such an assessment would not have been possible without the flexibility of size provided by dextran, and it is highly likely that such a difference would have been missed entirely at the 70 kDa assessment size relevant to Evans Blue.

It is critical to note that 70 kDa passage across the BBB represents a degree of permeability that would imply a highly pathological level of infiltration into the brain. In contrast, the 20 kDa weight range used with dextran is mechanistically relevant as the pro-inflammatory cytokines (IL-6, IL-1β, TNFα) evoked by ethanol are similar in weight. Since these dextrans are available tagged to a variety of fluorophores, they offer an easy method for both visualization of brain tissue as well as quantification through fluorometry. Despite the advantages of this method, as with Evans Blue and HRP, repeated dextran administration has also been shown to be toxic (Edlund, 1952) and depending on the structure and size of the polysaccharide branching this may increase toxicity producing a confound. In addition, as dextran is a sugar it is possible that the inherent mechanisms responsible for detecting and responding to blood glucose levels could potentially respond to and facilitate the transfer of smaller dextrans across the BBB. Nevertheless, the FITC-tagged dextran approach offers significant advantages over earlier approaches.

Whereas all methods of assessing BBB integrity have strengths and limitations, all of the methods discussed above are readily available and easy to implement for *in vivo* studies. In selection of a model, one must consider a number of critical issues, including (i) the size of molecule and how it relates to sensitivity of the intended test of BBB integrity; (ii) potential toxicity risks associated with the target compound/dye that will be used; and (iii) potential off-target binding activity of the target compound/dye that may obfuscate conclusions. From our perspective, the ability to procure multiple dextran sizes and probe

BBB integrity at different macromolecular weights represents a substantial advantage in study design. This method ensures a high degree of sensitivity in probing BBB permeability to an array of different compounds and minimizes potential type 2 errors that may occur if an experiment using Evans Blue was employed. Overall, careful scrutiny should be employed when evaluating or designing studies to probe BBB integrity to avoid potential type I error (stemming from toxicity of the administered probes) as well as potential type II error (dismissing a potential change in BBB permeability because only one size was tested).

## Regulation of BBB in Development

In rodent models, the BBB forms across embryonic development and is largely considered to be stably formed by birth (Engelhardt, 2003; Blanchette & Daneman, 2015). Prenatal BBB development is largely guided by canonical Wnt/beta-catenin signaling and VEGF release, which promotes angiogenesis and normal BBB development (Anderson et al., 2011). Genetic disruption of this signaling in early embryonic development leads to both abnormal vessel guidance and morphology (Daneman et al., 2009) and also leads to the vessels displaying abnormal phenotypes that at times miss critical BBB junction regulating proteins such as claudin (Liebner et al., 2008). A number of good reviews outlining specific mechanisms that govern BBB permeability in prenatal development and how exposure to teratogens can alter BBB permeability across the lifespan are available (see Goasdue et al., 2017). In contrast, much less work has attempted to directly assess changes in the BBB across postnatal development.

Though few studies have examined BBB permeability across the postnatal and adolescent periods, evidence suggests that the BBB continues to mature. Saunders, Liddelow, and Dziegielewska (2012) hypothesized that the prenatal and newborn BBB was “immature” relative to the adult BBB. While this claim has been challenged by others (Mallard et al., 2018), it is possible that maturation of the BBB is non-linear, and instead fluctuates to meet demands uniquely at different developmental epochs. For example, 2-hour old rat pups displayed markedly lower BBB permeability in response to pro-inflammatory cytokine injection than postnatal day (PND) 21 rats (Anthony et al., 1997). Similarly, adult rats displayed increased BBB permeability after acute stroke, an effect that was not observed in neonatal rats (Fernandez-Lopez et al., 2012). In addition, functional imaging studies have revealed that increased neural activity almost always corresponded with simultaneous increases in blood flow in adults, and that this coupling was not consistent in neonatal and developing brains, suggesting again that the dynamics that regulate the NVU in the adult brain are not operating in an identical fashion throughout development (Kozberg & Hillman, 2016). Much of the remainder of the evidence supporting potential BBB immaturity during early development comes from changes observed in the components of the NVU across time. While as mentioned previously, endothelial cells, neurons, and even pericytes are in place relatively early in embryonic development, astrocytic end-feet placement tends to occur postnatally in rodents (Rowitch & Kriegstein, 2010). Changes in important regulators of BBB transport, such as endothelial transporters, are present in the newborn rat; these undergo intense periods of growth that ended in a near quadrupling of receptor quantity by the time rats hit early adolescence (Vanucci, 1994). While the role that development may play in “normal” BBB function has yet to be untangled, pathological challenges that occur

during these unique stages of brain development can have profound, long-lasting effects on BBB integrity. These parallel developmental differences in CNS and PNS function relative to age-specific physiological demands.

Extending BBB function into a lifespan framework, late aging is associated with natural breakdown of the transport mechanisms governing glucose, amino acid, and hormone transit, as well as lower PGP activity relative to younger individuals (Mooradian, 1994; Toornvliet et al., 2006). These changes functionally mimic an overall decline in BBB permeability, an effect that seems to parallel a marked shift in inflammatory tone later in life. Aging is associated with both an increase in inflammatory pathology such as atherosclerosis as well as an overall increase in systemic and CNS inflammation (Walker et al., 2018; Perkins et al., 2021). This is accompanied by an array of changes in the NVU including alterations in microglial activity (Ronaldson & Davis, 2012), impaired LTP and neurogenesis (Blau et al., 2012), TJ degradation (Elahy et al., 2015), and loss of pericyte coverage (Berthiaume et al., 2018). All of these changes represent different mechanistic ways in which BBB function could change and ultimately, seem to interact negatively with many of the pathologies that often worsen substantially in late aging (Alzheimer's Disease (AD), vascular dementia, ischemic stroke).

## Alcohol Effects on BBB Permeability

### Adolescence Is A Time of Unique Vulnerability to The Neurotoxic Consequences of Ethanol

Alcohol use has been estimated to contribute to 3.3 million deaths per year globally and nearly $223.5 billion in monetary expenses to the United States alone (Control, 2014; Organization, 2014). Nearly 75% of this is attributed to binge drinking, defined by NIAAA as ethanol consumption resulting in Blood Ethanol Concentrations (BECs) of 0.08 mg/dL or higher (Control, 2014). This type of alcohol consumption is particularly common among adolescents (White, Kraus, & Swartzwelder, 2006). Although substantial progress has been made in curtailing adolescent binge drinking, the highest prevalence of binge drinking across age groups continues to occur among adolescents (Chung et al., 2018). These patterns of intake tend to peak in late adolescence and emerging adulthood (21–25 years of age), with consumption then tapering off throughout adulthood and into late aging. Even more concerning is the emergence of excessive drinking, termed "High Intensity" drinking, where BECs often approach 2–3 times the NIAAA definition of binge (Patrick & Azar, 2018). Such patterns of drinking are particularly hazardous and high frequency binge drinking is strongly correlated with a later AUD diagnosis (Crews, Vetreno, Broadwater, & Robinson, 2016). Adolescents display altered sensitivity to many of the positive and negative effects of ethanol. This often manifests as a heightened susceptibility to the positive effects of ethanol such as subjective perception of reward (Pautassi et al., 2008) and social facilitation (Willey et al., 2009) and increased resilience to the negative consequences of ethanol such as social inhibition (Varlinskaya & Spear, 2002), sedation (Silveri & Spear, 1998) and aversion (Vetter-O'Hagen et al., 2009). This pattern of consequences is believed to contribute to the increased levels of ethanol consumption observed in adolescents.

Beyond the concerns of early adolescent ethanol use on later alcohol use, the adolescent period displays heightened vulnerability to many long-lasting effects of ethanol use. At least some of the adolescent-like phenotypes discussed above display a “locking-in” like effect that persists into adulthood (Spear & Swartzwelder, 2014). In addition, the neurotoxic effects of ethanol are particularly profound during adolescence. Several studies have highlighted that adolescent ethanol exposure promotes inflammatory brain damage (Pascual et al., 2007), unique patterns of cortical brain damage (Crews et al., 2000), inhibited neurogenesis (Crews et al., 2006) and much more (Crews, Braun, Hoplight, Switzer, & Knapp, 2000; Guerri & Pascual, 2010). It is worth noting that most studies do not include comparisons of equivalent alcohol exposures in adults, making it difficult to attribute these findings to a vulnerability that is specific to adolescence.

## Acute Ethanol Alters BBB Function

Several studies have examined the impact of acute and chronic ethanol on BBB function alone or in conjunction with other forms of challenge. Going back to 1990, postmortem human analysis revealed that individuals with a history of alcohol use disorders were documented as having abnormal or dysfunctional BBBs (Pratt et al., 1990; Thomsen, Kaatsch, & Asmus, 1994). Rodent studies from the 1970s have also hinted at a possible role that ethanol may have in modulating BBB permeability in response to stab wounds (Rosengren, Persson et al., 1979), and after chronic vapor exposure when challenged with starvation as a stressor (Phillips & Cragg, 1982). More recent *in vitro* studies have also shown that acute ethanol produced BBB dysfunction through TJ degradation in brain microvascular endothelial cells (Haorah et al., 2007) (Figure 4). Several other studies have shown that chronic ethanol exposure degraded many of the proteins associated with normal TJ function (Yu et al., 2017), and enhanced BBB permeability produced by LPS exposure (Singh et al., 2007). While no consistent mechanism has been identified as being responsible for these changes, ethanol interferes with normal TJ function (Haorah et al., 2007), angiogenesis (Muneer et al., 2012), and endothelial transport receptors (Chang et al., 2018).

Another potential mechanism by which ethanol produces substantial changes in BBB permeability is cytokine release. The TLR4 signaling pathway has been associated with BBB dysfunction (Szabo & Lippai, 2014; Wardill et al., 2016). At binge- or supra-binge levels of ethanol exposure, ethanol increased the functional release of high mobility group box-1 (HMGB1), a danger-associated molecular pattern (DAMP) that serves as an endogenous ligand at TLR4 (Ge et al., 2014; Zou & Crews, 2014; Wang et al., 2015; Crews & Vetreno, 2018). For this reason, high dose ethanol seems to recapitulate at least some of the effects observed with LPS, which also binds to TLR4 (Pandey, 2012; Alfonso-Loeches, 2010). Both human and animal studies have supported a role of ethanol-induced TLR4 signaling in BBB dysfunction. For instance, TLR4 KO mice displayed nearly none of the conventional neuroinflammatory consequences of ethanol and showed decreased BBB permeability (Alfonso-Loeches, Urena-Peralta, Morillo-Bargues, Gomez-Pinedo, & Guerri, 2016). TLR4 KO mice exposed to binge drinking paradigms showed reduced levels of several proteins that are used as biomarkers of BBB permeability and hippocampal BBB impairment (Rubio-Araiz et al., 2016). The same studies have also found increased TJ

degradation in the brains of human alcohol abusers (Rubio-Araiz et al., 2016). Alcohol preferring P-rats displayed increased LPS-induced BBB abnormalities in comparison to normal rats (Singh, Jiang, Gupta, & Benlhabib, 2007). Thus, accumulating evidence posits TLR4 signaling as a potential mechanism for ethanol-induced disruption of the BBB, yet few studies have linked ethanol evoked TLR4 signaling with quantifiable differences in BBB permeability.

A final mechanism by which ethanol is known to affect the BBB is through altered blood flow. Even relatively small doses of ethanol substantially increase systolic, diastolic, and mean blood flow velocities (Stendel et al., 2016). This is believed to occur through dilation of the cerebral arteries, and significantly increased blood volume and access to the brain (Gazzieri et al., 2006). In contrast, reduced cerebral blood flow was observed during ethanol withdrawal in ethanol dependent individuals (Matthew et al., 1986). These changes can significantly modify stroke vulnerability and outcome. Consumption of small quantities of ethanol protected against ischemic stroke, whereas heavier alcohol consumption patterns exacerbated injury, presumably by increasing vulnerability to hemorrhage (Li et al., 2020). In contrast, ethanol substantially increased vulnerability to hemorrhagic stroke, and not only aggravated hemorrhagic volume, but also increased microglial activation and overall inflammation (Liew et al., 2016). These changes result in the hemorrhagic stroke being more severe, and significantly increased mortality and degree of impairment following stroke resolution (Daniel & Bereczki, 2004).

## Adolescent Ethanol Exposure Produces Sex-Specific Changes in Ethanol-Induced Inflammation

Acute ethanol exposure during adolescence is known to evoke a significant neuroimmune signaling response and these neuroimmune signaling changes persist into adulthood. Human data supports that individuals with a history of AUD show increases in many immune signaling molecules such as HMGB1 that correlate with lifetime alcohol exposure (Crews et al., 2013). In addition, both human data as well as rodent studies have indicated a persistent increase in TLR expression, including TLR4, following AIE (Crews et al., 2017). Long-term changes in histone acetylation as well as brain BDNF levels have been observed following AIE and this correlates strongly with changes in neuroimmune gene expression and ultimately decreased neurogenesis (Broadwater et al., 2014; Sakharkar et al., 2016). While these data highlight the potential for AIE to produce lasting changes in immune gene expression, fewer studies have assessed whether the sex of the animal would modify these results and if these changes also reflect lasting differences in peripheral immune activation.

In previous work, male rats with a history of AIE showed a unique (not observed in their female counterparts), robust suppression of adult peripheral cytokine gene expression in response to either an LPS or a restraint stress challenge in adulthood (Vore et al., 2017). Follow-up work reported that male rats with a history of AIE also displayed a suppressed neuroimmune response to adult ethanol challenge when brain cytokine protein was measured using large molecule microdialysis (Gano et al., 2019). When male and female rats with a history of AIE were challenged with an identical dose of ethanol in adulthood, both sexes displayed elevated hippocampal IL-6 and IκBα, however, only male

rats showed this increase in the amygdala (Vore et al., 2021 [Under Review]). Collectively, this work highlights that male rats may be uniquely susceptible to long-lasting changes in inflammation after AIE, with females being resistant to such changes.

In Vore et al., 2021, male rats with a history of AIE also displayed decreased VEGF-A (a known BBB permeability inducing factor) gene expression in response to an adult ethanol challenge that was not observed in female rats. Using the identical experimental manipulation, male rats also showed altered hippocampal ethanol kinetics, achieving significantly higher brain ethanol concentrations more rapidly than adolescent vehicle-exposed counterparts (Gano et al., 2019). The shift in ethanol transit into the CNS among AIE-exposed males indicates the potential for a change in the neurobehavioral response to ethanol. When rats exposed to the same AIE and adult ethanol challenge were assessed for Loss of Righting Reflex (LORR) sensitivity, male rats with a history of AIE showed an almost 50% reduction in sleep time that was not observed in female comparators. Male rats showed no lingering signs of tolerance or altered ethanol metabolism when BEC curves were assessed after a challenge with 0.75, 1.5, or 3.0 g/kg ethanol. The data discussed above highlight critical sex differences in adolescent ethanol sensitivity that require further exploration and could accompany BBB disruption and portend significant changes in later-life pathology.

## Alterations in BBB Permeability Exacerbate Brain Pathology

An abundance of data supports the notion that alterations in BBB permeability are associated with more severe instances of brain pathology. Small differences in access to and from the brain can have widespread impact on brain health in the long-term. The link between vascular dysfunction and Alzheimer's disease (AD) has been studied both as a mechanistic component of the disorder as well as how altered BBB function may worsen disease pathology. Vascular dementia is the second most common form of dementia following AD and perhaps best represents the substantial increase in pathological changes in cerebrovasculature can create (Gorelick et al., 2011). Cerebrovascular disease can result in cognitive impairment ranging from mild to full dementia, similar to what is observed in AD patients. Due to the important role the cerebral vasculature plays in supplying neurons as well as other components of the NVU with oxygen and nutrients, even small vascular alterations can contribute to cognitive impairment. These include factors such as microbleeds, microinfarcts, arteriosclerosis, and vascular stiffening and all of these are connected to reductions in global cerebral perfusion as well as different severities of dementia and cognitive impairment (see Iadecola 2013 for relevant review). The summated impact of these pathogenic factors may ultimately lead to white matter damage, and increased BBB permeability, leading to increased brain cytokine levels, microglial activation, and oxidative stress. This highlights how even small perturbations in the homeostasis governed by the BBB can lead to substantial physiological and behavioral dysfunction.

One of the hallmark characteristics of Alzheimer's Disease is the steady accumulation of harmful amyloid beta plaques as the individual ages. These Aβ plaques are then believed to ultimately create the cognitive disturbance and many other neurological symptoms observed

in AD (Cleary et al., 2005; Lesne et al., 2006; Butterfield et al., 2007). The BBB plays a fundamental role in both amyloid beta production as well as clearance out of the brain. Recent work in both rodent and human models has shown that peripheral amyloid beta contributes to the ultimate accumulation of brain Aβ (Eisele et al., 2010; Sagare et al., 2011). In addition, peripheral expression of a variety of Aβ sequestering agents such as anti-amyloid beta antibodies have been shown to help slow brain Aβ accumulation by reducing peripheral Aβ contribution (DeMattos et al., 2002). Much of the shuttling of Aβ into the brain and the continued propagation of Aβ is mediated through the receptor for advanced glycation end products (RAGE) (Deanne et al., 2003). RAGE expression directly embedded within the brain endothelium permits direct transit of Aβ as well as peripheral monocytes into the brain (another marker of heightened peripheral inflammation) and ultimately helps to propagate Aβ toxicity (Giri et al., 2000). Conversely, low density lipoprotein receptor-related protein 1 (LRP1) also located in the brain endothelium plays a vital role in the shuttling of brain Aβ back into the blood (Deanne et al., 2004; Jaeger et al., 2009). Altered LRP1 expression in brain microvessels was associated with the accumulation of Aβ during both natural ageing as well as pathological AD (Shibata et al., 2000; Donahue et al., 2006). Clearly, the BBB and brain vasculature as a whole is inextricably linked with both the genesis of AD as well as the rate of its progression and ultimate severity. This has led to more recent studies that highlight a vascular hypothesis of AD wherein vascular injury ultimately leads to BBB dysfunction that produces the aberrant accumulation of Aβ plaques, which ultimately contributes to tau pathology (Zlokovic et al., 2011).

Similar dysfunctions in BBB integrity have been reportedly associated with Parkinson's pathology and potentially its prognosis (Kortekaas et al., 2005; Hirano et al., 2008; Lee & Pienar, 2014). Parkinson's disease (PD) is the most common movement disorder in the elderly and is generally considered the second most common degenerative neurological disorder in senescence (Desai et al., 2007). It is characterized by the pathological loss of dopamine neurons and the presence of Lewy bodies that ultimately produce significant motor dysfunction such as tremor, bradykinesia, and instability. While the exact mechanisms underlying this dysfunction have yet to be confirmed, animal models have consistently demonstrated elevated inflammatory tone and increased inflammatory cytokine presence in the brain of PD animal models (Barcia et al., 2003). While it was originally believed that PD patients did not show altered BBB function, more recent work using sensitive, modern methods of probing BBB integrity such as FITC-labeled albumin have shown significantly increased BBB permeability in the striatum and other components of the nigrostriatal pathway (Carvey et al., 2006). These differences were observed both in animals following 6-hydroxydopamine lesions (Carvey et al., 2006) as well as MPTP exposure (Zhao et al., 2007). The latter authors also noted that minocycline treatment (known to inhibit microglial activation) has been shown to prevent the changes in leakage associated with MPTP-induced Parkinsonian symptoms (Zhao et al., 2007). These changes, coupled with an overall increase in microglia numbers in areas where BBB leakage and neuronal degeneration was observed, further suggests a role for inflammation and subsequent BBB damage in PD pathology (Teismann et al., 2004). This was further supported by data showing that TNF-α KO mice showed neither microglial dysfunction nor the increased BBB permeability following MPTP exposure (Zhao et al., 2007). Finally, substantial increases in vascular density/angiogenesis

were observed in the substantia nigra of PD patients (Faucheux et al., 1999). Increased angiogenesis occurred in close proximity to areas of high neuronal death and correlated well with heightened levels of VEGF (Barcia et al., 2005). The authors speculated that in response to neuronal degeneration, increased VEGF may be the body's attempt to supplement blood and nutrient flow as well as removal of cellular debris. This activity may help in the short-term but ultimately allows further peripheral macrophage infiltration and exacerbates neuronal degeneration and disease progression (Desai et al., 2007).

## Natural Aging and Inflammation

Both PD and AD are common neurodegenerative disorders that frequently occur in mid-to-late aging. As discussed above, both disorders are characterized by pathological inflammation that co-occurs with, or perhaps ultimately produces, changes in BBB permeability. This allows the unrestricted transit of toxins or harmful molecules into the brain. This potentially triggers the onset of pathology, which ultimately can initiate a cycle of increased inflammation, disrupted BBB activity, and decreased efflux of toxins producing a more profound pathology. While these disorders represent extreme instances of pathology, natural aging is also characterized by well documented changes in inflammatory tone as well as increased BBB leakage (Perkins et al., 2021). As "normal" individuals age a chronic, low-grade inflammation dubbed "inflammaging" gradually develops (Franceschi et al., 2018). This persistent activation of the innate immune system is believed to reflect natural changes that occur during senescence. Ultimately, the inflammaging phenotype develops which is believed to contribute to increased vulnerability to bacterial, viral, and other forms of infection seen in aging (Dall'Olio et al., 2013).

While the exact cause of inflammaging has yet to be identified, it is likely due to the summation of several different CNS changes. The first is the gradual accumulation of cellular debris and damaged molecules that can occur during aging, owing in part to aberrant production and slowing clearance of the same (Franceschi et al., 2014). A variety of different compounds are recognized by different sensors as "danger signals" that trigger an innate immune reaction to prompt resolution of this dysfunction. However, as the body ages and these signals begin to accumulate, it can overwhelm CNS clearance mechanisms and become a chronic condition (Franceschi et al., 2000). Another potential contributor to this chronic immune activation could be increased leakiness of the gut microbiome as an individual ages. While the gut normally traps microbes and their products, over time heightened leakiness may trigger the release of these products into surrounding tissue producing cytokine release and subsequent innate immune activation (Biagi et al., 2011). It is also likely that natural senescence in cells could contribute to increased inflammatory tone. Normally, the development of senescence in cells serves an important biological function in preventing the rapid proliferation of aged cells that can contribute to cancer; however, the accumulation of senescent cells that release pro-inflammatory cytokines could serve to drive age-related pathology. In mice, the removal of senescent cells has been shown to ameliorate age-related pathology (Coppe et al., 2010). It is also plausible that inflammaging represents a natural decline in immune function. As individuals age, adaptive immunity naturally declines and a possible compensatory mechanism is that innate immunity undergoes a mild increase, again triggering a mild, persistent increase

in inflammatory tone (Shaw et al., 2010). Complementing the presence of inflammaging and the gradual increase of inflammatory signaling molecules across aging, “normal” aging is associated with increased BBB leakiness (Zhang et al., 2018), an effect that could be worsened by a lifetime history of alcohol consumption. The combination of increased BBB permeability and heightened inflammation is a natural progression and likely contributes to the significant increase in overall disease vulnerability noted in older individuals.

## Implications for Drug/Alcohol Use and Abuse

Several post-mortem studies have documented BBB pathology in individuals with a history of AUD (Pratt et al., 1990; Thomsen, Kaatsch & Asmus, 1999). *In vitro* models of the BBB have shown that ethanol and acetaldehyde augmented BBB permeability (Haorah et al., 2008). In addition, neuroimaging studies revealed significantly increased BBB permeability in the hippocampus of social drinkers (Ivanidze, Mackay & Hoang, 2019). Other studies examining water distribution using diffusion tensor imaging showed abnormal brain diffusivity, reflective of BBB leakage, in the fronto-temporal regions of the brains of individuals with current AUD in contrast to healthy comparators (Monnig et al., 2013). The same study documented persistent damage in the parietal regions of individuals with at least one year of abstinence (Monnig et al., 2013). These changes may enhance transit of cytokines and other inflammatory signaling molecules into the brain of individuals with a history of AUD. Subsequently, it could substantially alter the ability of ethanol and other drugs of abuse to cross the BBB. Much as heroin is considered to have a higher degree of subjective reinforcement than morphine due to its ability to more rapidly transit into the brain (Schaefer et al., 2017), even small changes in brain ethanol kinetics (such as those observed in Gano et al., 2019) could substantially alter ethanol’s reinforcing property. Similarly, changes in BBB permeability may significantly alter acetaldehyde levels. Normally, blood acetaldehyde levels do not reflect what is observed in the brain as it is screened out by the BBB (Tabakoff, Anderson & Ritzmann, 1976). While it is believed that *de novo* acetaldehyde is produced through the metabolism of CNS ethanol by regional ADH (Martinez et al., 2001), increased access of peripheral acetaldehyde into the brain may produce biologically active levels capable of producing reinforcement as observed when acetaldehyde was administered directly into the brain (Myers et al., 1984; Amit & Smith, 1985; Brown, Amit & Rockman, 1979; Rodd et al., 2002; Quertemont, 2004). In addition, when chronic or high levels of ethanol are consumed, the microsomal ethanol oxidation system (MEOS) and induction of CYP2E1 is also utilized to oxidize ethanol into acetaldehyde (Crabb & Liangpunsakul, 2007). Unlike with ADH, with chronic exposure MEOS enzymes increase in number and are not inhibited by the presence of acetaldehyde (Crabb & Liangpunsakul, 2007). AIE could readily induce CYP2E1 activity in a regionally specific manner given the presence of MEOS systems across the brain (Zimatkin & Dietrich, 1997) that may render specific brain areas vulnerable to acetaldehyde accumulation and subsequent inflammation and behavioral changes.

While this review has focused predominantly upon alcohol, a number of other common drugs of abuse also produce alterations in BBB permeability. Continuous administration of cocaine produces an almost 50% increase in BBB permeability (Sharma et al., 2009) believed to be modulated through shared mechanistic factors such as increased

TNF-α (Sharma et al., 2009); TJ degradation (Dietrich, 2009); and altered astrocyte (Wang et al., 2021) and microglia function (Buch et al., 2012). Similar changes have been shown to occur following methamphetamine administration (Northrop & Yamamoto, 2015). Methamphetamine evoked changes in oxidative stress (Ramirez et al., 2009), changes in astrocytic end-feet contacts (Northrop & Yamamoto, 2012), and potentially methamphetamine-associated excitotoxicity in pericytes (Montiel-Eulefi et al., 2012) have all been identified as potential mechanisms through which methamphetamine may alter BBB integrity. Morphine and other opiates show similar alterations in BBB homeostasis (Kousik et al., 2012) and also inhibit PGP expression, reducing morphine removal from the brain and increasing the analgesic and potentially reinforcing consequences of such drugs (Seleman et al., 2014). Finally, chronic nicotine exposure also disrupts TJ proteins (Kousik et al., 2012) and direct binding to nicotinic receptors localized on brain endothelial cells induces NO release and can further compromise BBB function (Mazzone et al., 2010). The present review has highlighted many shared mechanisms by which drugs of abuse with completely different pharmacodynamic properties can result in the same changes of BBB integrity. Polysubstance abuse is common in adolescents and heavy cannabis use is associated with a higher risk for future illicit drug use (Patton et al., 2007). It is possible that adolescent consumption of one drug may alter BBB permeability, subsequently modifying the subjective response to future drug exposures (both to the same drug and to others). Beyond changes in drug access to the brain, alterations in BBB permeability may render drug abusing individuals more vulnerable to comorbid disease states such as HIV infection (Fitting et al., 2010) and hepatitis that are often observed in higher percentages of substance abusing individuals in contrast to individuals not currently abusing drugs (Nath, 2010).

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## Abbreviations

| | |
|---|---|
| **AIE** | Adolescent Intermittent Ethanol |
| **ADH** | Alcohol Dehydrogenase |
| **AUD** | Alcohol Use Disorder |
| **AD** | Alzheimer’s Disease |
| **Aβ** | Amyloid Beta |
| **BBB** | Blood Brain Barrier |
| **BECs** | Blood Ethanol Concentrations |
| **BDNF** | Brain Derived Neurotrophic Factor |
| **CNS** | Central Nervous System |

| | |
|---|---|
| **CYP2E1** | Cytochrome P4502E1 |
| **Da** | Daltons |
| **DAMP** | Danger-Associated Molecular Pattern |
| **FITC** | Fluorescein Isothiocyanate |
| **HMGB1** | High Mobility Group Box-1 |
| **HRP** | Horseradish Peroxidase |
| **IL** | Interleukin |
| **I.P.** | Intraperitoneal |
| **I.P.** | Intravenous |
| **KO** | Knockout |
| **LPS** | Lipopolysaccharide |
| **LTP** | Long-term Potentiation |
| **LORR** | Loss of Righting Reflex |
| **LRP1** | Low Density Lipoprotein Receptor-Related Protein 1 |
| **MEOS** | Microsomal Ethanol Oxidation System |
| **NIAAA** | National Institute of Alcohol Abuse and Alcoholism |
| **NALT** | Nerve Associated Lymphoid Tissue |
| **NVU** | Neurovascular Unit |
| **NO** | Nitric Oxide |
| **IκBα** | Nuclear Factor of Kappa Light Polypeptide Gene Enhancer in B-Cells Inhibitor, Alpha |
| **PGP** | Para-glycoprotein |
| **PD** | Parkinson’s Disease |
| **PNS** | Peripheral Nervous System |
| **PND** | Postnatal Day |
| **RAGE** | Receptor for Advanced Glycation End Products |
| **SAMHSA** | Substance Abuse and Mental Health Services Administration |
| **TJ** | Tight Junction |
| **TNF** | Tumor Necrosis Factor |

| | |
|---|---|
| **TLR** | Toll-Like Receptor |
| **VEGF** | Vascular Endothelial Growth Factor |

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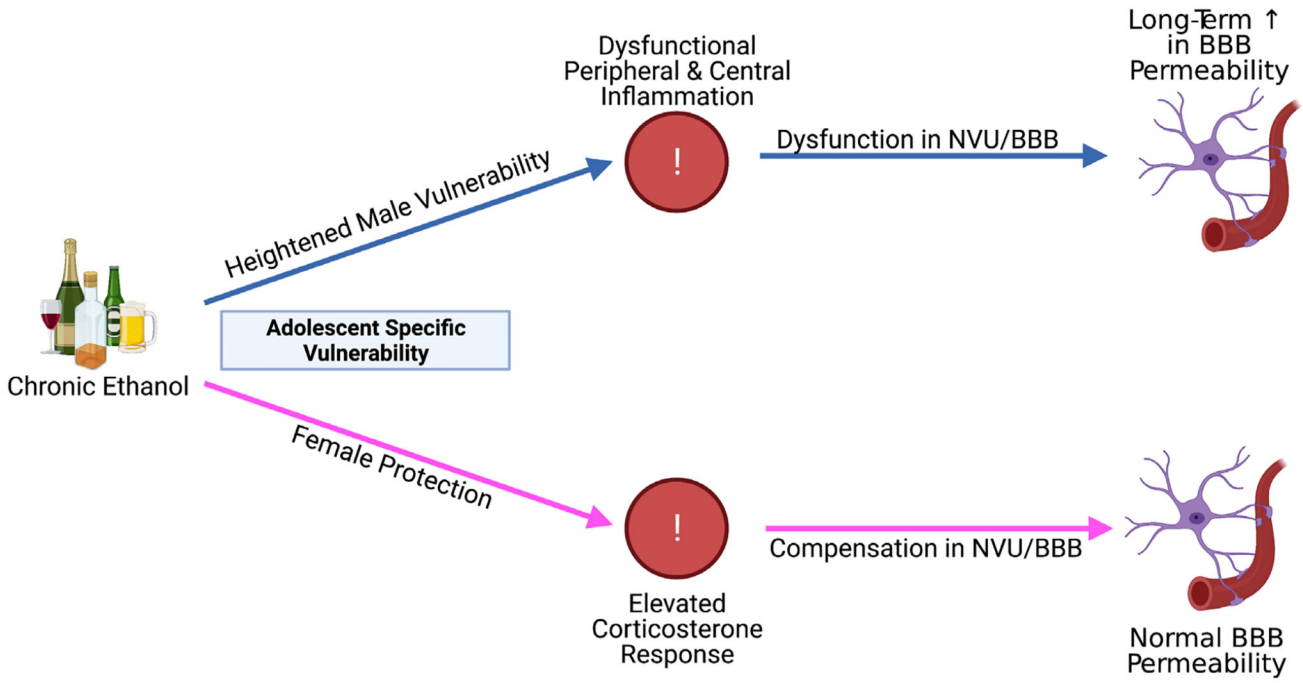


**Figure 1: Hypothetical Mechanistic Outline by which AIE Alters BBB Permeability.**

This figure illustrates the hypothesized mechanism through which chronic ethanol exposure during the important developmental period of adolescence may produce sex-specific changes in inflammation, ultimately resulting in elevated male BBB permeability.

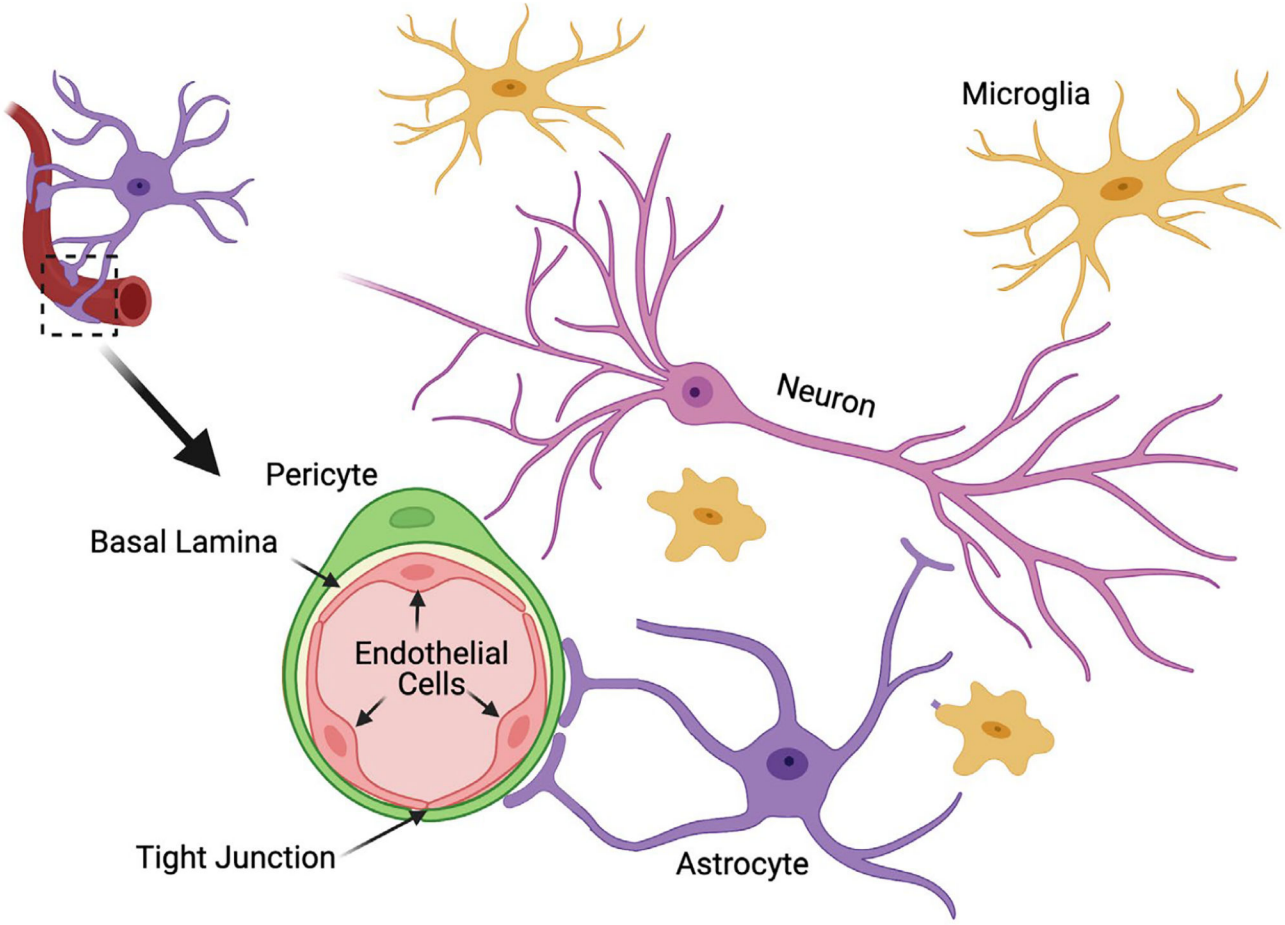


**Figure 2: Schematic Representation of the Neurovascular Unit.**

This schematic highlights the complex array of cell-to-cell interactions that influence the BBB and molecular access into the CNS. The endothelial cells that form tight junctions are surrounded by the basal lamina as well as pericytes that directly communicate with the endothelial cells. Bidirectional communication occurs at each level between astrocytes, neurons, and microglia as well as astrocytic end-feet connections directly modifying pericyte signaling.

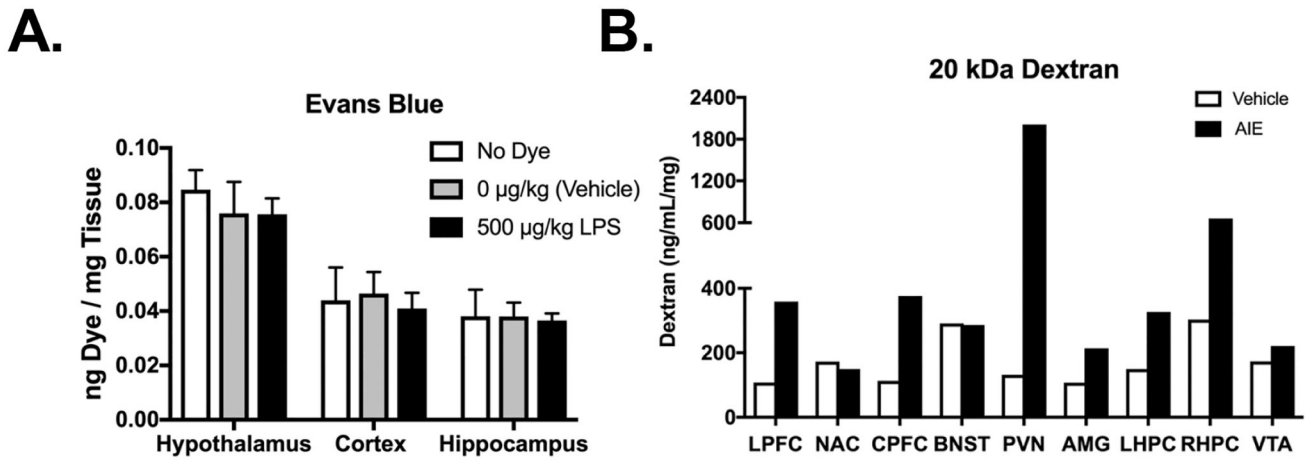


**Figure 3: Comparison of Different Methods of Probing BBB Permeability.**

Adult male, Sprague-Dawley rats received 500 µg/kg i.p. LPS injection and 15 hours later BBB permeability was assessed with i.v. 1.0 mL/kg, 2.0% Evans Blue Dye. No differences in dye concentration were observed in gross-dissected brain tissue, evidenced by dye levels being comparable to no dye controls, despite evidence that high dose LPS increased BBB permeability (Ghosh et al., 2014; Banks et al., 2015) (A). In a subsequent pilot, rats (n=2–3) with a history of adolescent intermittent ethanol (AIE) under **basal** conditions, revealed significant differences in 20 kDa dextran permeability in a range of brain regions (B).

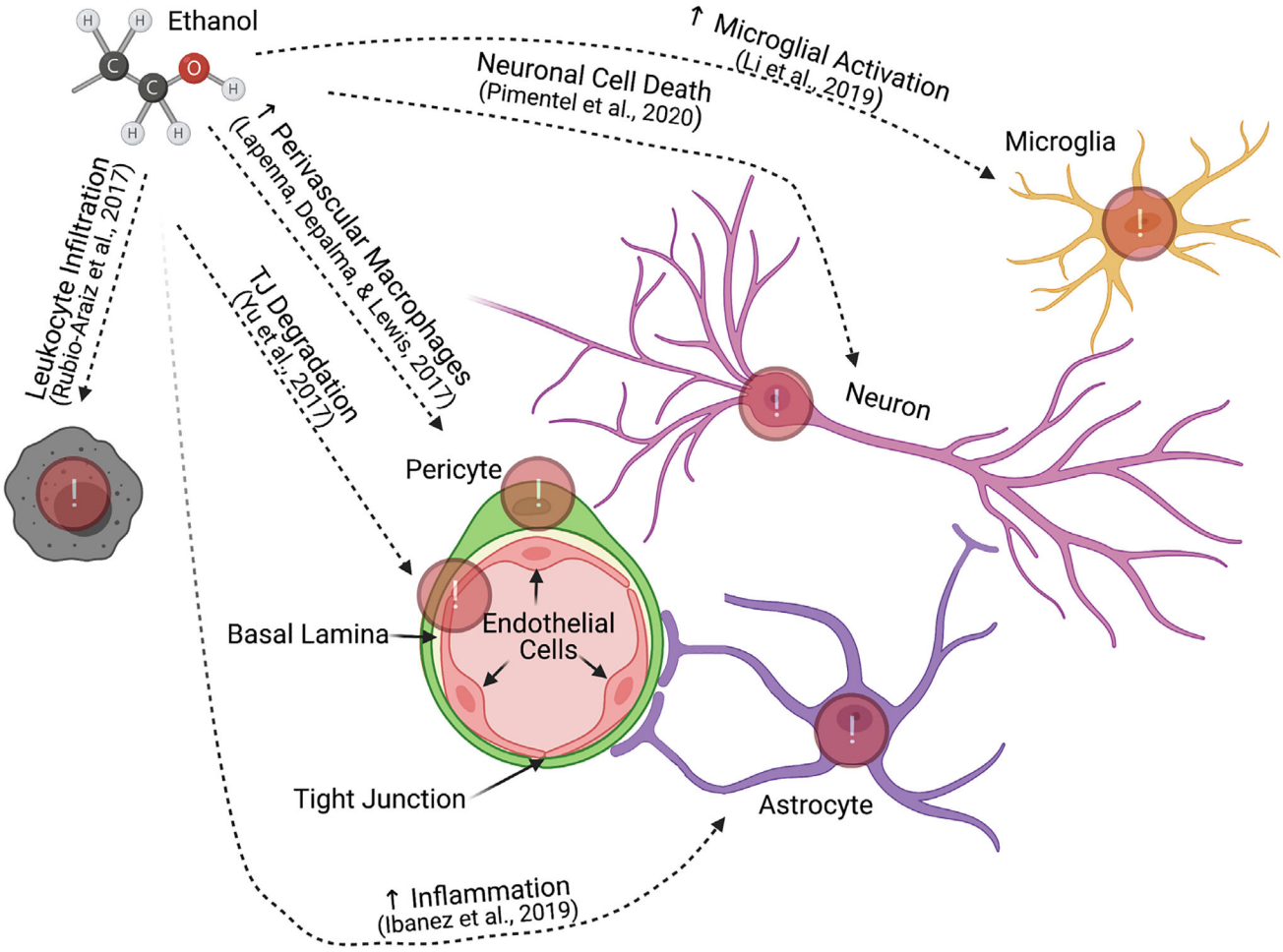


**Figure 4: Evidence of Ethanol's Ability to Disrupt the NVU.**

Studies indicate potential mechanisms by which ethanol-evoked changes may disrupt BBB permeability are indicated by exclamation points on relevant NVU components. While many of the papers referenced in this schematic highlight the specific role that inflammation plays, there exists substantial correlational evidence that long-term ethanol exposure could alter BBB permeability directly.