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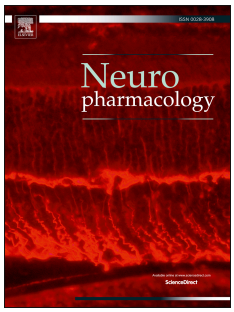

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Title page:

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Keywords : Alcohol use disorders, microbiota, leaky-gut, lipopolysaccharides, peptidoglycans, cytokines, gut-barrier function, probiotics, prebiotics

Abstract:

Preclinical studies have largely supported that alcohol-consumption induces the development of an important neuro-inflammation and this neuro-inflammation contributes to alcohol-drinking behaviors, notably through TLR4 and LPS related mechanisms. The neuro-inflammation originates from a direct interaction of ethanol with the neuronal and immune brain cells, but also from the generation of an inflammation at the periphery. Ethanol in particular interacts with the intestine to develop a gut dysbiosis and an increase in gut permeability, that allows the liberation of bacterial fragments to the systemic circulation and induces a pro-inflammatory response in the systemic circulation and peripheral organs, and in particular the liver. Peripheral cytokines or activated peripheral cells may cross the blood-brain barrier and activate neuro-inflammation. In humans, peripheral inflammation and intestinal dysbiosis are related to symptoms of alcohol use disorders (AUD), such as depression, anxiety and alcohol-craving, However, the dysbiosis, could also participate in a different manner to the symptomatology of the addiction, possibly by interacting with the stress system, by interfering with the sleep processes and altering the abilities for social interactions. The role of the gut suggests that interventions with probiotics or prebiotics might in the future be of interest for the treatment of the addiction.

1- Introduction:

Excessive alcohol consumption is known for several decades to strongly stimulate the innate immune system and to play a major role in the development of alcoholic liver disease (Szabo et al., 2011). Recent observations of neurons using immune signaling as a way to communicate (Boulanger et al., 2001), of the role of immune signaling for cognition (Williamson and Bilbo, 2013) but also that LPS elicited immune activation of the microglia leads to neuro-degeneration (Lehnardt et al., 2003) support a role for the immunity in brain physiology and physiopathology. In the case of AUD, a large literature arising from animal and human studies have suggested a role for neuro-inflammation in the pathophysiology of the disease (Robinson et al., 2014). However, important questions remain as to the origins of the neuro-inflammation. In this article we will discuss the possibility that at least a part of the inflammation arises from peripheral origins and in particular from the gut, giving arguments for potent interventions targeting peripheral sources to decrease the neuro-inflammation and possibly the addiction. Several points will be evoked successively in the manuscript. We will start by describing the importance of neuro-inflammation for psychopathology and the development of AUD, where part of the neuro-inflammation is likely related to a direct effect of ethanol at the brain level. We will then question the role of peripheral sources of inflammation in the development of AUD, and insist on the role of the gut, of the disruption of the gut barrier and the development of a gut dysbiosis in AUD. Other potent sources of inflammation like the gut wall, the liver and the adipose tissue will also be suggested. We will then describe how systemic inflammation and gut barrier are related to the expression of AUD in humans and animals. The mechanisms whereby changes in the gut microbiota may be related to the development of AUD will be evoked and in particular the role of stress, social impairments and sleep disturbances. Finally, we will suggest that the intestine might serve as

a new target for pharmacological or nutritional interventions for the treatment of AUD.

2- Importance of neuro-inflammation in psychopathology and for the development of AUD

a. The sickness behavior and the role of low grade, peripheral inflammation.

The sickness behavior theory (Dantzer and Kelley, 2007) supports that during infections, bacteria or viruses activate the immune system leading to the production of pro-inflammatory cytokines, which reach the brain and induce a sickness reaction characterized by behavioral symptoms such as fatigue, lassitude, inability to concentrate, irritability, loss of appetite and withdrawal from social interactions (Dantzer et al., 2008; Konsman et al., 2002). These symptoms are close to the symptoms of depression, but also to other psychopathological conditions including AUD (Cordovil De Sousa Uva et al., 2010)(Schuckit, 1994), and when the inflammation persists, the symptoms may become more pronounced and chronic, and the condition developed by the individual, fulfill the criteria of a mental disorders, like for instance a major depression disorder (Dantzer et al., 2008; Smith, 1991; Yirmiya, 1996; Yirmiya et al., 1999). A score of experimental and clinical arguments supports the role of inflammation in the development psychiatric disorders. Increasing pro-inflammatory cytokines in healthy volunteers by the intravenous injection of LPS (Krabbe et al., 2005;

Reichenberg et al., 2001) or treating cancer or hepatitis C patients with IFN-α or IL-2 leads to the development of various psychiatric symptoms (Capuron et al., 2001, 2000; Musselman et al., 2001). In major depression, a subgroup of individuals that present with a high baseline plasma level of pro-inflammatory cytokines are improved by medications targeting the immune system (Raison et al., 2013). Finally, altering the immune system of rodents during gestation may induce the development of autism spectrum or schizophrenia-related behavior in the off-springs (Bauman et al., 2014; Hsiao and Patterson, 2011).

b. Existence of a neuro-inflammation in AUDs

Several studies over the past few years have supported both the existence of a neuro-inflammation in AUD, but also that it plays a causal role for its development. Binge intoxication induces an inflammatory response in the brain of rats (Crews et al., 2006)) or mice ((Crews et al., 2013)(Kane et al., 2014), as does a chronic exposure to ethanol of mice (Whitman et al., 2013)(Lippai et al., 2013). This observation is not limited to rodents, as neuro-inflammation is also observed port-mortem in AUD individuals (Crews et al., 2013)(Vetreno et al., 2013)(He and Crews, 2008). Data from studies using microarray analyses of brain genes also support the role of neuro-inflammation in AUD. Several immune genes are overexpressed in the brain of strains of mice selected to present an alcohol-preference (Saba et al., 2006)(Mulligan et al., 2006), and this effect was not related to ethanol administration, consistent with a role of neuro-inflammation in the predisposition to drinking. In addition, exposure of mice to ethanol vapors induce important changes in the expression of immune related genes in astrocytes, microglial, and neuronal cells of the amygdala, the nucleus accumbens and the prefrontal cortex (Osterndorff-Kahanek et al., 2015), suggesting also that ethanol works as an immunomodulator. However, multiple processes could be involved in the development of a neuro-inflammation after exposure to ethanol.

c. *Ethanol induced neuro-inflammation is certainly partially related to a direct effect at the brain level*

In AUD, as for other immune related psychiatric disorders like depression (Haroon et al., 2012), the inflammation, could theoretically arise from various sources. Up to now, the studies that allowed demonstrating convincingly of the effects of ethanol on the development of a neuro-inflammation did not question the actual sources of the inflammation within the body. Most studies involved the exposure of the entire body to ethanol that might generate inflammation both at the brain level or at the periphery. They are therefore not conclusive regarding the sources of inflammation. Before examining possible peripheral sources, we will first examine the few studies that support a direct stimulation of inflammation at the brain level by testing the interaction of ethanol with the brain slices, fragments or cells. An inflammatory response has indeed been observed at the mRNA and/or protein level in different cellular or tissue models, such as cultures of rat brain slices (Zou and Crews, 2010)(Zou and Crews, 2014), murine macrophages (Fernandez-Lizarbe et al., 2009), primary microglial (Fernandez-Lizarbe et al., 2008) or astrocyte cells (Alfonso-Loeches et al., 2010). More theoretically, several biochemical changes induced at the brain level by alcohol drinking are expected to generate a neuro-inflammation. The GABA/Glutamate imbalance in neurons leads to excitoxicity and a massive calcium influx within neurons, possibly inducing cell-damage or apoptosis and a subsequent local inflammatory response (Mon et al., 2012). The induction by ethanol of an oxidative stress and the production of acetaldehyde may also cause injury to the brain and an inflammatory response (Almansa et al., 2009) (Sun and Sun, 2001). These observations suggest therefore that direct interaction of ethanol with the brain likely plays an important role in the development of the inflammatory response. How this interacts with inflammation arising from peripheral sources is yet currently unknown.

3- Can peripheral sources of inflammation cause neuro-inflammation and can it generate alcohol addiction?

The potent role of a peripheral inflammation in the development of the neuro-inflammation has largely been demonstrated by studies using peripheral administration of lipopolysaccharides (LPS), to challenge the immune response, as well as other peripheral agents such as polyinosine-polycytidylic acid (poly I: C) in conjunction with endogenous DAMPS co-agonists, such as high-mobility group box (HMGB) proteins (Qin and Crews, 2012). There is an important overlap in genes activated by ethanol and LPS in the brain of mice (Osterndorff-Kahanek et al., 2015). The importance of the LPS for brain stimulation of inflammation is supported by the observation of a large stimulation of TLR4 at the brain level, for which the major ligand is the LPS. Mice lacking TLR 4 have also been shown not to develop a brain inflammation when exposed to ethanol *in vivo* (Kelley and Dantzer, 2011).

Animal models also support that the immune response elicited by LPS may induce alcohol consumption. LPS injections in mice produce a prolonged increase in ethanol self-administration (Blednov et al., 2011), and this effect is mediated by TLR4 receptors or myeloid differentiation primary response gene 88 (MyD88) signaling cascade (Wu et al., 2012) (Liu et al., 2011). Other potent peripheral sources of inflammation as, for instance peptidoglycan, that may generate inflammation by binding to TLR2 receptors have not been studied in detail at the brain level, even though these specific immune pathways could also play a role in the pathophysiology of AUD (Leclercq et al., 2014a), as it does for the pathophysiology of alcoholic liver disease (Gustot et al., 2006).

4- The intestine: a source of inflammation and addictive behaviors in AUD.

a- Description of the gut microbiota and the enteric immune system

The gastro-intestinal tract is inhabited by a vast and dynamic community of micro-organisms (bacteria, viruses, fungi) whose number approximates the number of human cells (Sender et al., 2016). The intestine that contains approximately around 800 different species and 7000 different strains (Bäckhed et al., 2005) of bacteria hence constitutes a extremely diverse potent source of immune reaction, that is located within the body. The microbial colonization begins immediately at birth and influence many aspects of host physiology, including the development of the immune system. The intestinal mucosa is continuously exposed to the microorganisms and constitutes the primary interface between the gut microbiota and internal host tissues. Immune regulation of host-microbes interactions is of critical importance to recognize potential pathogens as well as to induce immune tolerance towards symbiotic microbes. Recent findings indicate that both the innate and adaptive immune responses are involved in the minimization of bacterial invasion into deeper host tissues (Gallo and Hooper, 2012)(Duerkop et al., 2009). Different types of intestinal cells constitute the physical and immunological barrier. Enterocytes, together with the tight junction proteins, are essential to prevent bacterial penetration by limiting the paracellular space between neighboring cells. They also produce and release, with Paneth cells, a variety of antimicrobial substances (defensins, cathelicidins, C-type lectins such as regenerating islet-derived protein (Reg)-3γ) that kill or inactivate micro-organisms and maintain homeostasis (Mukherjee et al., 2008). Goblet cells secrete large quantities of mucin that form a protective layer of mucus to limit bacterial penetration. Finally, the intestinal *lamina propria* contains adaptive immune cells, such as macrophages, dendritic cells, T and B cells which differentiate into plasma cells to

produce bacteria-specific immunoglobulin A (IgA), that also play an essential role in defending the intestinal mucosa against invading bacteria.

The important role of the gut microbiota in the development of the immune system has been highlighted by recent studies performed in germ free (GF) mice. GF mice are more susceptible to infections and exhibit developmental immune defects at the tissue, cellular and molecular levels suggesting that the absence of gut microbiota impaired the normal development of the immune system (Round and Mazmanian, 2009). More importantly, the microbial colonization early in life seems to play a role in the development of immunity (Kelly et al., 2007) and early life alterations of gut microbiota induced by antibiotics are associated with immune-mediated diseases such as allergic asthma (Russell et al., 2012) and inflammatory bowel diseases (Hviid et al., 2011). Besides its important role for the immune system, most members of the microbiome also play a role in digestion and metabolism, establishing a symbiotic relation to the host (McLoughlin and Mills, 2011). Besides this, the gut microbiota may serve as an endocrine organ, as it secretes more than 100 different metabolites (Clarke et al., 2014) and it also interacts with the vagus-nerve (Forsythe et al., 2014), and with the stress system (Moloney et al., 2014). Altogether, this vast and dynamic microbial community is normally separated from the inner environment by the intestinal barrier that physically limits the inner circulation from the penetration of an excessive quantity of microorganisms or fragments, but allows the absorption of nutrients (Macpherson et al., 2005; Vaishnava et al., 2008; Wells et al., 2011). However, this gut barrier may be altered in specific cases, leading to the leakage of immune products from the intestine to the systemic circulation, and to low grade inflammation, that has been incriminated in the development of various forms of diseases such as for instance obesity and diabetes (Turnbaugh et al., 2006)(Karlsson et al., 2013), but also in psychopathological states such as autistic spectrum disorders (Hsiao et al., 2013).

b- The disruption of the gut barrier function in AUD and the development of a leaky gut

Chronic alcohol exposure have also been shown to induce an increase in gut permeability both in animal models of alcohol addiction and in humans (Bjarnason et al., 1984; Keshavarzian et al., 1999; S. Leclercq et al., 2012; Leclercq et al., 2014c; Parlesak et al., 2000). Several mechanisms have been suggested to explain the ethanol induced disruption of the intestinal barrier and the development of a leaky gut in this population. An important role has been ascribed to acetaldehyde, but also to reactive oxygen species production (Rao, 2008) to local pro-inflammatory cytokine production (IL6, IL-1β, TFNα, IFNγ) (Al-Sadi et al., 2014, 2009) and to NFκB activation (Banan et al., 2007). These oxidative stress or immune local reactions will alter the gut barrier by activating myosin-light chain kinase (Ma et al., 1999), inducing the expression of miRNAs that inhibit the translation of tight junctions (Tang et al., 2008), or up-regulating the expression of intestinal circadian clock gene (Swanson et al., 2011). Recent accumulating evidences also support the role of modifications in the composition of the gut microbiota, in the induction of the leaky gut by ethanol.

c- Intestinal dysbiosis in AUD

In the twentieth century, the identification of microbes necessitated their culture, which largely limited the possibility of analysis of gut microbes since 2/3 of microbes cannot be cultured. New-generation of sequencing techniques allows a much easier determination of gut microbiome (Fraher et al., 2012). These progresses recently permitted to observe important changes of the microbiota after chronic alcohol exposure and the development of a dysbiosis in the gut, defined as a disequilibrium of the different microbial entities which leads to a disruption of symbiosis between the microbiota and the host (McLoughlin and Mills, 2011). Important alterations of the gut microbiota have been observed in rodents chronically exposed

to ethanol (Bull-Otterson et al., 2013; Yan et al., 2011b) and in colonic or jejunal mucosa and in fecal samples of AUD individuals (Chen et al., 2015).

In humans, the dysbiosis measured in fecal samples was only present in a sub fraction of the population and was associated with an increase in gut leakiness (Leclercq et al., 2014d). It was characterized by a major decrease in the global abundance of bacteria within feces but also by large specific decreases in several bacterial taxa such as anti-inflammatory bacteria *Faecalibacterium prausnitzii* and *Bifidobacterium*.. There was also a tendency for a decrease in the diversity of the microbiota, although, this was not statistically significant (Leclercq et al, unpublished data). Conversely the abundance of Proteobacteria (Bull-Otterson et al., 2013; E. A. Mutlu et al., 2012), a major group of gram-negative bacteria, was also largely increased. Protobacteria may constitute an important source of LPS that in case of leaky-gut, can cross the gut mucosa and the gut barrier to reach the systemic circulation. Several studies have shown an increase in blood LPS levels in AUD individuals (Bode et al., 1987; Fujimoto et al., 2000; S. Leclercq et al., 2012; Parlesak et al., 2000) or after episodes of binge drinking (Bala et al., 2014). Diffusion of LPS in the systemic circulation may induce inflammation through activation TLR-4 receptors in various organs, and in particular the liver or the brain as described above in animal models. Chronic ethanol consumption is also associated with alteration of the gut microbiota functionality (Leclercq et al., 2014c; Xie et al., 2013), in particular in subjects that present with a dysbiosis and a leaky gut (Leclercq et al., 2014d). Important changes in various metabolites were observed, but especially an increase in phenol, which is known to be deleterious to the gut barrier and a decrease in indole compounds, which on the contrary present with protective properties. Hence, the changes in functionality associated with the dysbiosis likely also play a role in the alteration of the gut barrier function. The presence of an alteration of the microbiota in only a sub-fraction of actively drinking AUD subjects, was reported by two independent studies (Leclercq et al., 2014c; E.

A. Mutlu et al., 2012) and it correlated strongly with the increase in intestinal permeability (Leclercq et al., 2014c).

These modifications may also be of clinical significance, as the subjects that presented with the highest intestinal permeability at the beginning of withdrawal were also those that presented with the largest scores of craving, depression and anxiety at the end of the detoxification, suggesting that dysbiosis and increased permeability are not necessarily associated with the disorder, but rather with a severe form of AUD. The exact reasons for the existence of two distinct subpopulations of AUD individuals, that present, or do not present with a dysbiosis are unknown. A first hypothesis would be that the dysbiotic subpopulation would present with a preceding gut disorder, like an Intestinal Bowel Syndrome (IBS). However, there were no specific signs of gut alterations in that subpopulation (de Timary et al., 2015), and for instance, no signs of diarrhea at the end of detoxification. A second hypothesis would be the existence of a primary dysbiosis, which would precede the development the AUD. In IBS for instance, the existence of a primary dysbiosis has been shown. It is not impossible that such a primary dysbiosis exist in that subpopulation of AUD individuals and would hence make them vulnerable for the development of the disorder, like genetic factors have been shown to be predisposition factors (Schuckit, 2014). If it was the case, various origins might be suggested for a primary dysbiosis, such as the microbial colonization early in life from the mother's microbes, the host genetic characteristics, that may influence the microbiome by a host-microbiota interactions (Goodrich et al., 2014), or finally the exposure to specific earlier stresses that might also influence the selection of microbial species, as will be developed later in the manuscript. Conversely the dysbiosis might also be secondary to the development of specific eating or drinking habits. Leclercq et al (2014c, supplemental material) for instance observed a trend towards a higher proportion of spirits compared to wine or beer in the average regimen of the subpopulation of AUD patients

with an increased permeability, suggesting that the alcohol beverages might influence the permeability.

Consistent with the role of the alcoholic beverages, the intestinal permeability decreased to normal within 3 weeks after alcohol cessation, as well as the LPS levels. However, the gut microbiota composition and the systemic inflammation remained largely altered (S. Leclercq et al., 2012; Leclercq et al., 2014c; E. A. Mutlu et al., 2012) at the end of detoxification. Hence the increase in gut leakiness does not depend solely on the presence of an alteration of the gut microbiota composition but also on the exposure to alcohol drinking. Although ethanol needs to be consumed for the development of the gut leakiness, it is not yet clear whether alcohol consumption is also at the origin of the development of the dysbiosis and how to explain why a sub-fraction of AUD subjects did not present a gut dysbiosis, while the two subpopulations consumed the same quantity of ethanol (Leclercq et al., 2014d). The existence of a reciprocal interaction between the gut barrier and the microbiota suggest several possible explanations for the effect of ethanol: ethanol could directly lead to alterations of the gut microbiota composition and hence to a gut leakiness, or conversely, ethanol could directly affect the gut barrier with potent modifications of the expression of antimicrobial peptides involved in a regulation by the gut of the composition of the microbiota (Cash et al., 2006). A recent study where feces from human AUD individuals that have developed a leaky gut and liver alterations were transplanted to germ free mice clearly suggested the role of the gut microbiota in the alteration of the gut barrier function, but this effect was again also dependent on alcohol exposure, as the transplanted mice were also exposed to a Lieber De Carli diet supplemented with ethanol (Llopis et al., 2015).

Although intestinal bacteria seem to play crucial functions for health and diseases, viruses (including bacteriophages) and eukaryotes like fungi likely interact with the bacteria as well as with the host. Like the microbiome, once established, the healthy adult human virome is

relatively stable with >95% of viral genotypes showing minimal variation in their relative abundance (Reyes et al., 2010). Also, alterations of the intestinal mycobiome, which is usually studied by targeting the internal transcribed spacer (ITS) (Cui et al., 2013), have recently been associated with inflammatory bowel diseases (Ott et al., 2008), hepatitis B cirrhosis (Chen et al., 2011) and obesity probably by impairing the immune system. PAMPs of fungi, such as α-mannans, β-mannans, and β-glucans are indeed recognized by TLRs and dectins (Netea et al., 2006)(Iliev et al., 2012) and can therefore shape the immune response. The effect of chronic alcohol abuse on virome and mycobiome have not been investigated yet.

d- The gut, the peripheral blood mononuclear cells (PBMCs) and the development of the systemic inflammatory response

AUD in humans is characterized by the existence of a chronic low-grade systemic inflammation as evidenced by elevated plasma levels of TNFα, IL-1β, IL-6, IL-8, IL-10 and hsCRP (Achur et al., 2009; S. Leclercq et al., 2012; Leclercq et al., 2014b) and this inflammation could not be related to the existence of a systemic viral or bacterial inflammation or liver damages, as in these studies, the population samples were collected in AUD inpatients that were selected not to present with liver cirrhosis or steatohepatitis (Leclercq et al., 2012). The gut is potentially a large source of immune products and PBMCs (lymphocytes and monocytes) are essential to defend the organism from gut-derived bacterial immune products. Hence, they likely contribute to the observed low-grade inflammation. A mechanistic study has analyzed in details the inflammatory pathways of PBMCs of AUD subjects, both at the beginning and the end of alcohol-withdrawal (Leclercq et al., 2014b). The observation of a stimulation of the expression and activation of the LPS-receptors TLR4 and CD14, supported the role of LPS which derive from gram-negative bacteria. The same study also revealed the importance of peptidoglycans (PGN) originating essentially from

gram-positive bacteria, as revealed by elevated plasma PGN levels, an increased expression and activation of PGN-receptor TLR2 but also of NOD2, an intracellular receptor which binds muramyl diphosphate, the bioactive structure of PGN (Leclercq et al., 2014b). PGN had previously been shown to be increased in peripheral blood of rats after acute ethanol administration (Tabata et al., 2002).

The activation of LPS and PGN dependent pathways led to the production of IL-1β and IL-8, through the activation of the transcription factor AP-1 and NLRP3 inflammasome. This contrasts with a down-regulation of the expression of TNFα and IL-6 in PBMCs, as well as a down regulation of the NFκB pathway in these immune cells. Altogether, these observations suggest that IL-1β and IL-8 are produced by PBMCs, while peripheral TNFα and IL-6 likely come from other sources.

5- Other potent peripheral sources of inflammation in AUD

As evoked above in this article, in humans, the activation of LPS and PGN dependent pathways in PBMCs led to the production of IL-1β and IL-8, through the activation of the transcription factor AP-1 and NLRP3 inflammasome. This contrasted with a down-regulation of the expression of TNFα and IL-6 in PBMCs. However, TNFα and IL-6 were very much increased in the plasma circulation in humans and were correlated with markers of severity of AUD (Leclercq et al., 2014a). Altogether, these observations suggest that part of the inflammation measured in AUD patients do not come from the PBMCs but rather from other peripheral sources, which are as yet not completely known. Several sources may be evoked for the peripheral inflammation, including the gut wall, the liver or the adipose tissue.

a. The gut wall

The gut wall is the first organ between the gut lumen and the systemic circulation and may also constitute a source of inflammation. Pro-inflammatory cytokines IL-1-β or TNF-α are increased in the intestine of ethanol fed mice (Chen et al., 2015). Recently an important inflammatory infiltrate was observed in the jejunum of a model of alcohol fed mice, characterized by an increase in TNF-α producing macrophages and monocytes in the *lamina propria*. This increase was not observed in the ileum and the colon. Similar observations could be made in the duodenum of human AUD individuals. This proximal inflammation was directly related to the development of the leaky gut and the liver disease in mice. *Lamina propria* monocytes and macrophages appear to be the source for increased cytokine production. The jejunal inflammation may also play a role in the development of the systemic inflammation and in particular in the elevated level of circulating TNF-α measured in the systemic circulation.

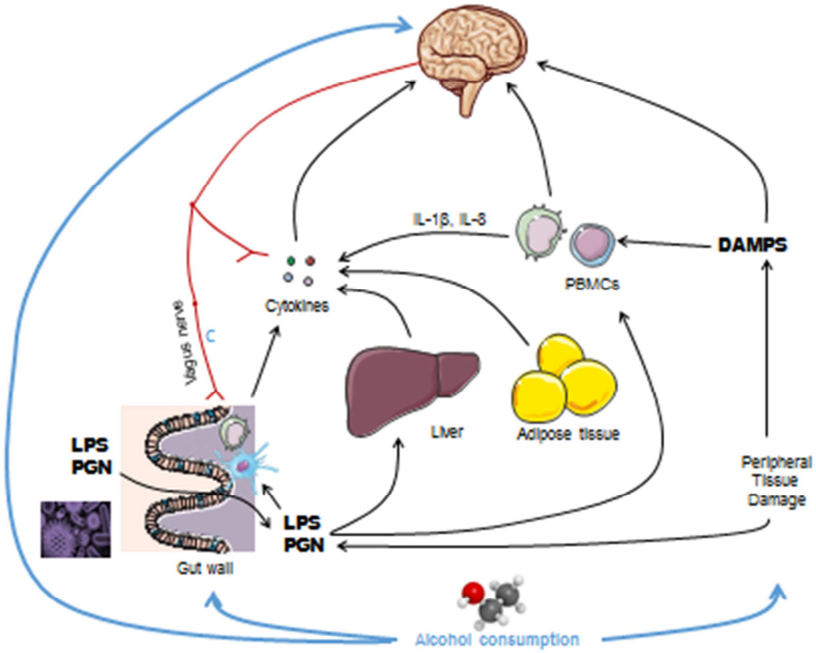

Figure-1 legend : The various sources of inflammation in AUD.

Alcohol-consumption may directly impact inflammation at the brain level or at the level of peripheral organs, but also lead to tissue damage, influence the composition of the gut microbiota and gut permeability. The important alterations in the gut microbiota compositon and in the gut barrier function will lead, while the AUD individuals are in a drinking period, to a leakage of lipopolysaccharides and peptidoglycans from the gut lumen. Ethanol induced tissue damage may also liberate PGN in the peripheral circulation. LPS and PGN will induce specific inflammatory reactions at the level of the gut wall itself, of peripheral blood mononuclear cells (PBMCs) and of peripheral organs, and in particular the liver. Arguments also support the existence of an inflammatory response at the level of the adipose tissue. These are different sources of inflammation that may influence brain functioning either through circulating cytokines, by the permeation of PBMCs through the blood brain barrier, or the activation of the Vagus Nerve. Finally, peripheral tissue damage will also induce damage-associated molecular patterns (DAMPS) that may also induce inflammation either in periphery or in the CNS.

b The liver and a possible gut/liver/brain axis.

Both quantitative changes in the composition of the gut microbiota manifested by an intestinal bacterial overgrowth and qualitative changes have been related to alcoholic-liver disease in rodent exposed to ethanol and in human AUD (Ece A. Mutlu et al., 2012) (Mutlu et al., 2009)(Kirpich et al., 2012)(Yan et al., 2011a)(Chen et al., 2015). The severity of the bacterial overgrowth correlated with that of cirrhosis (Casafont Morencos et al., 1996) and induction of a bacterial overgrowth in the small intestine results in the translocation of bacterial products and subsequent liver injury. On the other hand intestinal decontamination with antibiotics

decreases bacterial translocation, plasma LPS, and reduces the level of liver injury in rodents (Adachi et al., 1995). These observations support the role of intestinal bacteria for the development of alcoholic liver disease.

Specific pathogen-associated molecular patterns (PAMPs) such as LPS, peptidoglycan, or bacterial DNA (Akira et al., 2006), are recognized by the liver innate immune system through Toll-like receptors (TLRs). In rodents, most of the studies support a role for LPS and TLR4 receptors in the development of hepatic injury. TLR4 mutant C3H/Hej mice and TLR4-knockout mice – that present with a gut permeability similar to that of wild-type mice after alcohol feeding – show less hepatic inflammation (Hritz et al., 2008)(Uesugi et al., 2001). In humans, TNF alpha and Il1-beta are elevated in the liver and likely contribute to the systemic levels of inflammation. In contrary to what has been established in rodents, it remains unclear whether this response is principally mediated by the response of the TLR4 receptor system in human liver. Strikingly, IL6 levels are suppressed in human liver exposed to alcohol, suggesting that the liver in not the source of the elevated systemic IL6 levels found in AUD patients, the origin of which is still not known (Stärkel, own unpublished data(Leclercq et al., 2014a))

c- The adipose tissue

The role of the adipose tissue as a peripheral source of inflammation with possible consequences on the brain has been suggested in particular in the case of obese individuals where a bidirectional relation to exist between obesity and depression (Haroon et al., 2012). However in the case of obesity, de development of the fat mass may lead to the compression of the local vasculature and to hypoxia, oxidative stress and adipocyte necrosis and hence to

inflammation (Nathan, 2008). The production by the adipocytes of obese subjects of MCP-1 and MCP-2 will lead to the migration of macrophages to the adipose tissue, where and inflammatory reaction develops, as well as the production of cytokines such as TNFα, IL-6 and IL-1β (Nathan, 2008). However, in humans, contrarily to what is observed in obesity, the subpopulation of AUD subjects that present with the largest consumption of ethanol, that also present with the most elevated level of inflammation (Leclercq et al., 2014a), the fat mass is decreased (de Timary et al., 2012)(Santolaria et al., 2003)(Addolorato et al., 2000)(Addolorato et al., 1998). However, in rats at least, chronic ethanol exposure is associated with the infiltration of macrophages into the adipose tissue and an increase in the mRNA expression of MCP1, IL6 and TNF α (Kang et al., 2007). Moreover, adiponectin levels are elevated in heavy drinkers, but rapidly decreases after they stop drinking, demonstrating a reactivity of the adipose tissue to alcohol exposure in vivo (Buechler et al., 2009)(Hillemacher et al., 2009). Hence, the adipose tissue may also constitute a source of inflammation in AUD.

6- Systemic inflammation and gut barrier function correlate with psychological symptoms of AUD in humans and animal models

In the beginning of alcohol withdrawal, plasma pro-inflammatory cytokines of AUD patients, positively correlated with scores of depression, anxiety and alcohol craving (S. Leclercq et al., 2012). These symptoms of AUD, reflect the tendency for drinking in situations of negative affects (Cordovil De Sousa Uva et al., 2010) and likely reflect the expression of a negative reinforcement process (Koob and Le Moal, 2005),. The association between inflammation and alcohol craving was confirmed in another study (Leclercq et al., 2014b) where the improvement of OCDS craving scores during short-term alcohol withdrawal and more

particularly of the obsessional sub score, strongly correlated with the decrease the expression of IL-1β and IL-8 in PBMCs. IL-8 was the best predictor and is to our knowledge essentially secreted by PBMCs in humans. Although systemic inflammation and psychological symptoms decreased during short-term detoxification, their levels were still elevated compared to controls and remained correlated (S. Leclercq et al., 2012). By contrast, the anti-inflammatory cytokine IL-10 was negatively correlated with depression, anxiety and craving at the end of a 3-week detoxification (S. Leclercq et al., 2012). These data in humans are consistent with the numerous observations made in rodents supporting a relation between inflammation and the alcohol addiction.

Data in animal models demonstrate a relation between brain inflammation and alcohol preference and that genetic deletions of the inflammatory genes were associated with a decrease in alcohol preference and consumption (Blednov et al., 2012), offer stronger arguments than that obtained in humans where the observation is only correlational. However, the existence of a relation between gut permeability or gut microbiota and symptoms of addiction have also been observed in humans, were AUD dysbiotic subjects presented with stronger signs of severity of AUD at the end of a 3 week detoxification period than non dysbiotic ones (Leclercq et al., 2014d).

However, altogether, both human and animal models support the existence of a kind of vicious circle between drinking and inflammation, and data in humans (Sophie Leclercq et al., 2012), (Leclercq et al., 2014a)(Leclercq et al., 2014d) support that this relations could at least partially be related to negative reinforcement processes and to changes in gut permeability.

7- Potent mechanisms that may link the changes in the gut microbiota and the development of AUD

The vast literature on the role of the gut microbiota and the gut barrier function, has suggested their potent importance for the development of diseases, including psychiatric diseases, by diverse mechanisms (Dantzer, 2009). In this section of the article, we will describe several potent gut related processes, that may play a role in the development of AUD, besides the involvement of inflammation and sickness behavior, although their relation to AUD has not been described yet. Changes in the composition of the gut microbiota have other physiological consequences than the development of an inflammation. Furthermore, negative reinforcement processes only represent an aspect of AUD and are far from explaining the whole complexity of the behaviors. Subjects presenting with alcohol use disorders also present with deficits in executive functions, with impulsivity, with profound alterations in the their social interactions and with profound sleep disturbances. Three of these aspects, that have been described to have some relation with the gut, will be developed in this section.

a - Stress and changes in the gut barrier and gut microbiota.

Early life trauma and stresses induced by maternal separation induces large and prolonged changes in intestinal physiology, as manifested by a leaky gut, an increased gut secretion, an increase motility and an hyperalgesia (Kuhn and Schanberg, 1998). However, gut physiology and postnatal microbial colonization is also related to the physiology of the hypothalamus-pituitary-adrenal axis (Sánchez et al., 2001). These data support that perinatal adversity may durably alter both the gut physiology and the regulation of the stress response, which are important for the expression psychopathological symptoms, throughout the entire life. These effects on colonic function and HPA regulation are reversible upon administration of probiotic during the separation period (Gareau et al., 2007), supporting the importance of the microbiota. At the adult age, exposure to prolonged stresses or to social defeat later in life, will also induce modifications of the gut microbiota, of the gut barrier function of inflammation, that is related to a decrease in social interactions (Bharwani et al., 2016).

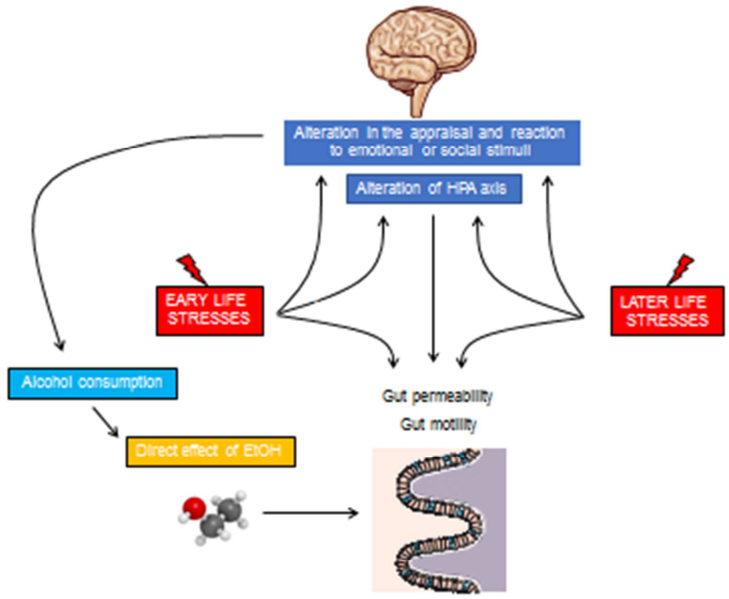

Figure 2 legend : The relation between stress exposure and changes in gut barrier function.

Early life stresses are known to profoundly and durably affect the HPA axis functioning, notably through epigenetic modifications of the expression of central cortisol receptors. Early life stresses also affect gut permeability, gut motility and possibly the gut microbiota composition. Furthermore, the stimulation of the HPA axis, related to early or later life stresses may profoundly influence the gut barrier function through a mechanism that involves the activation mastocytes by CRH. Hence, the stress system and the gut barrier function seem largely intertwined through processes that may possibly keep a memory of early life events.

Exposure to physical or social stress alters the gut barrier function, resulting in the uptake of bacterial derived pro-inflammatory molecules. Evidence support that this process is at least partially mediated by the local activation of mucosal mast cells by neuronal terminations releasing acetylcholine or corticotrophin-releasing factor (Söderholm and Perdue, 2001).

In AUD, the cortisol level is increased at rest in actively drinking or recently abstinent AD subjects (Wand and Dobs, 1991)(Sinha, 2009)(de Timary et al., 2012). The HPA axis regulation in response to stresses and to exposure to ethanol is also altered, due to alteration of the negative feedback between cortisol and the pituitary gland, as evidences by modified cortisol to ACTH ratio and by an altered autonomic response (Sinha et al., 2011). Furthermore, the disruption of the cortisol response to stress or to ethanol exposure is predictive of relapse risk in AUD (Junghanns et al., 2005). These observations suggest an important role of the HPA axis in the development of the addiction. However, the potent role of the gut microbiota or of the gut barrier function in the deregulation of the HPA axis has not been tested in AUD yet. We suspect an interrelation between the gut and the HPA axis regulation that may be both linked at the beginning, to the exposures to early life or adult age stresses and possibly reinforced later on by alcohol consumption.

b – Gut dysbiosis and the impairment of social relations in alcohol-use disorders?

AUD patients interact difficultly with others: they present true deficits in the interpretation of non-verbal language (Kornreich et al., 2016) are hypersensitive to situations of social exclusion (Maurage et al., 2012) and present with important impairments in theory of mind (Maurage et al., 2015)(Maurage et al., 2016)(Uekermann et al., 2007) , which may all alter the quality of social interactions in this population. Difficulties in social interaction are a frequent cause of relapse in recently sober AUD individuals (Zywiak et al., 2003). The relation

between the gut barrier function or gut microbiota and social interactions have never been assessed in AUD, but the role of the gut has been related to difficulties in social interaction in human (Adams et al., 2011)(Finegold et al., 2010)(De Angelis et al., 2013) and animal (Hsiao et al., 2013) models of autistic spectrum disorders, and at least a part of the effect was attributed to metabolites that were produced by the gut microbiota (Raison et al., 2013). Furthermore, the activation of the maternal immune system of rodent or primates models of autistic spectrum disorders is reported to induce important brain molecular changes, where IL-6 generation plays a crucial mediator, and that ultimately results in autism-and schizophrenia-related behavior in offspring (Bauman et al., 2014; Hsiao and Patterson, 2011). Hence the possibility of a relation between gut alterations and difficulties in social interactions deserves being evaluated.

c- Sleep disturbance

AUD individuals also present important sleep difficulties and anomalies in sleep architecture (Colrain et al., 2009), (Ehlers and Slawecki, 2000) and these difficulties are related to higher relapse rate after detoxification (Brower, 2003). The sleep disturbances are related to stress exposure, to the HPA axis regulation and to the expression of clock genes (Perreau-Lenz and Spanagel, 2015). Alterations in the clock gene expression has been shown to induce an increased intestinal permeability in response to ethanol in mice, an effect mediated by differences in the expression of the tight junction proteins occludins (Summa et al., 2013). Arguments therefore exist for a relation between gut alterations, clock genes and sleep disturbances in AUD (Weinstock and Walters, 2011).

8- The intestine: a target for pharmacological or nutritional interventions?

The observed relation between gut dysbiosis, inflammation and the symptoms of AUD suggest that the intestine might be a target for pharmaceutical interventions, with the ambition of improving the symptomatology of the disease. Several agents that target directly neuro-inflammation at the brain level have shown promising positive effects on ethanol addiction (George, 1989), ethanol induced inflammation (Zou and Crews, 2014) or neurotoxicity (Pascual et al., 2007). Some of the agents tested for their anti-inflammatory properties were antibiotics, such as Minocyclin, that was shown both to reduce the degree of activation of the microglia by ethanol (Qin and Crews, 2012) and to reduce alcohol preference in mice (Agrawal et al., 2011) or Rapamycin that reduced ethanol induced ROS generation, was neuroprotective (Chen et al., 2012), but also decreased alcohol behavioral effects and decreased alcohol intake in mice (Neasta et al., 2010). However, it cannot be excluded that at least part of these anti-inflammatory effects of antibiotics were related to changes in the composition of the gut microbiota, as these dimensions were not controlled for in these studies. A study addressing the role of the gut microflora on the effects of ethanol on the gut permeability had indeed shown that antibiotics (ampicillin and neomycin that do not target the same bacteria as minocyclin and rapamycin) were decreasing the permeability and reducing acetaldehyde generation (Ferrier et al., 2006).

However, alternate methods that target the gut microbiota might also improve the outcome of AUD. In rodents, the gut microbiota may be improved by the use of probiotics or dietary fibers that also reduce gut leakiness, endotoxemia, inflammation and improves liver function (Forsyth et al., 2009; Keshavarzian et al., 2001; Tang et al., 2009). Probiotic interventions in humans, by a supplementation of *Bifidobacterium bifidum* and *Lactobacillus plantarum 8PA3* in the regimen of AUD subjects during detoxification reduced liver enzymes (Kirpich et al., 2008) and the administration of *Lactobacillus casei Shirota* improved neutrophil phagocytic capacity of alcoholic cirrhosis patients (Stadlbauer et al., 2008). However, the benefits of

these interventions were obtained in the field of hepatology and liver disease. Studies are steel needed to test whether these pro- or prebiotics could improve the evolution of AUD in a psychopathological standpoint.

Prebiotics are carbohydrate chains that are non digestible in the upper gastrointestinal tract, that are selectively fermented in the colon and allow specific changes, both in the composition and/or activity in the gastrointestinal microbiota that confers benefits upon host well-being and health (Roberfroid, 2007). They exert their beneficial effects through the production of specific metabolites such as short-chain fatty acids (acetate, propionate, butyrate) that present with antimicrobial activity, the inhibition of pathogen growth by lowering the intestinal pH, the reinforcement the defense barrier of the colon and the development of anti-inflammatory properties (Hamer et al., 2008). Prebiotics seem to be safer than probiotics as they likely do not induce infections in organisms that present with altered gut barrier function. They are also more efficient since they modify more largely the gut microbial ecosystem and modify the abundance of more than 100 bacterial taxa (Dewulf et al., 2013; Everard et al., 2011; Gibson et al., 1995). In the specific case of AUD, *Faecalibacterium prausnitzii* and *Bifidobacteria*, that are both largely decreased in the feces of dysbiotic individuals (Leclercq et al., 2014d) exhibit strong anti-inflammatory properties (Preising et al., 2010; Sokol et al., 2008). The consumption of prebiotics galacto-oligosaccharides or inulin-type fructans by healthy volunteers or obese patients (Dewulf et al., 2013) increase their proportion, but no data is currently available in AUD.

9- Conclusion

Current biological models of AUD essentially focus on the role of ethanol on brain neurotransmitters for the development of the disporder. However, recent researches have

provided strong arguments supporting the role of neuro-inflammation . The neuro-inflammation is generated both from a direct interaction of ethanol with the brain and from the induction of inflammation at the level of peripheral organs. Several arguments support that a major source of inflammation at the periphery is the intestine and the development of a gut leakiness that is related to profound alterations in the composition of the gut microbiota. This suggests that besides the brain, the gut is also an important target for the effects of ethanol. A gut-brain inflammatory axis could be an important player in the pathophysiology of AUD, that would complement the role of a gut-brain endocrine axis that had already been supported by previous observations (Leggio et al., 2011).To ascertain the existence of this gut-brain axis, further experiments would however be needed, that would test the relation between gut barrier function and the brain functioning in AUD, as one study has tested so far, in healthy participants (Tillisch et al., 2013). Moreover, the peripheral inflammation cannot be completely explained by the intestinal source and other peripheral organs also participate to the generation of the inflammation. The liver in particular, when exposed to ethanol and gut-derived bacterial products develops an important inflammation, that will participate to peripheral inflammation, suggesting the role of a gut-liver-brain axis. Given the complexity of the inflammatory pathways, and the fact that they may be variously stimulated depending on the organs but also on the species, important efforts should be made to investigate the exact sources of inflammation in AUD. Several studies in animal models support that the gut derived peripheral inflammation may induce an increase in alcohol consumption. In humans, studies show that gut derived inflammation, but also gut dysbiosis and leaky gut are related to symptoms of AUD, such as craving, depression and anxiety. However, theoretically, leaky gut and dysbiosis could also affect alcohol-consumption though other mechanisms, such as an alteration of the stress system, sleep disturbance and alterations of the social relations. The exact role of the gut microbiota in the regulation of the stress axis is not completely

understood, but recent studies support a tight relation between acute stress and gut permeability or changes in the composition of the gut microbiota (Karl 2017, (Leclercq et al., 2016)). How the relation between the gut dysbiosis and stress are at the origin of episodes of drinking should also be assessed. The development within the brain of a memory of past stressful events is essential in the understanding of the expression of mental health disorders. Whether the complexity of the gut microbiota participate to a memory of past events would also deserve being tested. Finally, the best proof for the role of the gut microbiota in the development of AUD, would be to decrease its severity by a modification of its composition, by various types of interventions, such as fecal transplant or the administration of probiotics or prebiotics and observe whether these interventions help decrease neuro-inflammation and improve the outcome of AUDs.

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Highlights :

A review on peripheral sources of inflammation in AUD is proposed.

The intestine appears as a major source of peripheral inflammation.

Other peripheral sources of inflammation like the gut-wall or the liver are also suggested.

Mechanisms for transduction of peripheral inflammation to the CNS are evoked

Hypotheses regarding the relation between peripheral inflammation and the symptoms of AUD is are described