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Effects of Ethanol on Brain Extracellular Matrix: Implications for Alcohol Use Disorder

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Abstract

The brain extracellular matrix (ECM) occupies the space between cells and is involved in cell-matrix and cell-cell adhesion. However, in addition to providing structural support to brain tissue, the ECM activates cell signaling and controls synaptic transmission. The expression and activity of brain ECM components are regulated by alcohol exposure. This review will discuss what is currently known about the effects of alcohol on the activity and expression of brain ECM components. An interpretation of how these changes might promote alcohol use disorder (AUD) will be also provided.

Ethanol exposure decreases levels of structural proteins involved in the interstitial matrix and basement membrane, with a concomitant increase in proteolytic enzymes that degrade these components. In contrast, ethanol exposure generally increases perineuronal net (PN) components.

Because the ECM has been shown to regulate both synaptic plasticity and behavioral responses to drugs of abuse, regulation of the brain ECM by alcohol may be relevant to the development of alcoholism. Although investigation of the function of brain ECM in alcohol abuse is still in early stages, a greater understanding of the interplay between ECM and alcohol might lead to novel therapeutic strategies for treating AUD.

Keywords

Extracellular matrix; matrix metalloproteinases; tissue plasminogen activator; perineuronal nets; synaptic plasticity

Alcohol exerts profound and diverse effects on the brain. Prolonged alcohol consumption causes long-lasting neuroadaptations by altering neural transmission and synaptic plasticity. These effects of chronic alcohol exposure are hypothesized to contribute to the development of alcohol use disorder (AUD) and alcohol dependence (Koob and Volkow, 2010).

Molecular and cellular studies have focused for many years on the effects of alcohol exposure on neurotransmitter signaling at the synapse (Zorumski et al., 2014). More recently investigators have begun to appreciate that alcohol also affects the physiology of glial cells, with important ramifications for the interactions between neurons and glia and concomitant

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regulation of synaptic plasticity (Ayers-Ringler et al., 2016; Chastain and Sarkar, 2014; Wilhelm and Guizzetti, 2015). However, another component of the brain, the extracellular matrix (ECM), has emerged as a critical regulator of synaptic plasticity that is altered by several drugs of abuse (Senkov et al., 2014; Smith et al., 2015; Smith et al., 2014; Van den Oever et al., 2010; Xue et al., 2014). Experiments manipulating the ECM in animals have demonstrated that the ECM plays an important role in regulating behavioral responses to drugs of abuse and alcohol. Although the ECM in the brain is structurally different from most connective tissue (Mouw et al., 2014), it plays an important role in normal brain functioning and disease.

This review will briefly introduce the structures and molecules present in the brain ECM and discuss the effects of alcohol on the remodeling of the ECM. The roles of ECM proteins in behavioral responses to ethanol will be described and compared with the function of the ECM in behavioral responses to other drugs of abuse. I will focus on ECM molecules and a subset of the enzymes that remodel the ECM. However, alcohol is also known to dramatically affect the function of cell adhesion molecules during development with implications for fetal alcohol spectrum disorders. This topic is outside the scope of this review, but those interested are directed to the following references (Barker et al., 2012; Fitzgerald et al., 2011; Hong and Krauss, 2012; Ishiguro et al., 2014; Littner et al., 2013).

ECM IN THE BRAIN

Basement Membrane, Neural Interstitial Matrix, and Perineuronal Nets (PNs)

The ECM occupies the space between cells of the brain and is estimated to constitute 10-20% of brain volume. It is organized into three main compartments: the basement membrane, the neural interstitial matrix, and perineuronal nets (PNs) (**Fig. 1**) (Lau et al., 2013). ECM components are produced by both neurons and glial cells (Oohashi et al., 2015; Giamanco and Matthews, 2012) and are secreted into the extracellular space, where they assemble into an insoluble network surrounding cells. Many brain ECM proteins are glycoproteins or proteoglycans that are heavily modified by polysaccharide (sugar) chains composed of heparin sulfate or chondroitin sulfate glycosaminoglycans (GAGs) (Senkov et al., 2014).

The basement membrane (also known as the basal lamina) surrounds blood vessels and functions as part of the blood brain barrier (BBB). The basement membrane is composed primarily of collagen, laminin, fibronectin, and proteoglycans (Baeten and Akassoglou, 2011). The neural interstitial matrix, which occupies the extracellular space of the brain parenchyma and resembles cartilage, is made up of proteoglycans, tenascins, and link proteins (Lau et al., 2013). In addition, a key component of the interstitial ECM is hyaluronan, which is a long linear polysaccharide that is not attached to a protein core but which interacts with and crosslinks ECM proteins (Kwok et al., 2011). Hyaluronan is synthesized by hyaluronan synthases present on the surface of neurons (Kwok et al., 2011; Kwok et al., 2010). Smaller amounts of laminin, fibronectin, and collagen are also present in the interstitial matrix (Lau et al., 2013). Finally, perineuronal nets (PNs) are condensed mesh-like ECM structures that form a network around the soma and proximal dendrites of primarily GABAergic interneurons that express parvalbumin (Hartig et al., 1994; Wegner et

al., 2003). Similar to the interstitial ECM, PNs are composed of hyaluronan, link proteins, chondroitin sulfate proteoglycans (CSPGs), and tenascin-R (Kwok et al., 2011). The main components of ECM in the brain are shown in **Fig. 1**.

A general function of the ECM is to provide support to cells by allowing for cell-ECM adhesion and cell-cell adhesion. The ECM also initiates intracellular signal transduction cascades because of direct interactions with cell surface receptors such as the integrins. In the brain the interstitial ECM and PNs have specialized functions including the formation and stabilization of synapses, sequestration of growth factors, regulation of extracellular ion concentrations, and control of neurotransmitter receptor diffusion in the plasma membrane (Frischknecht and Gundelfinger, 2012). All of these functions are important for proper neuronal functioning and synaptic plasticity, which has implications for learning and memory (Senkov et al., 2014). The structure and function of some of the main components of ECM proteins are introduced below. Since there are approximately 300 categorized ECM proteins in mammals (Naba et al., 2016), I will focus on only those that are currently known to be altered by exposure to alcohol and/or other drugs of abuse.

Fibronectin

Fibronectin is a glycoprotein and a component of the basement membrane and interstitial ECM in the brain. It is secreted from cells as soluble dimers and assembles into fibrils at the cell surface, forming into a detergent-insoluble multimeric matrix (Potts and Campbell, 1994). Fibronectin is able to bind to multiple constituents of the ECM and link the ECM to cell surface receptors, thus acting as a scaffolding molecule for many proteins in addition to heparin sulfate proteoglycans and CSPGs. Fibronectin is composed of three types of repeating modular domains, known as Fn1-3 (Pankov and Yamada, 2002). Fibronectin directly binds to integrins at the cell surface through its Fn3 domain. Integrins are receptors that are important for the assembly of fibrils, yet they also have multiple cellular functions through their ability to connect the ECM with the actin cytoskeleton and activate intracellular signaling cascades. Fibronectin regulates synaptic plasticity through its interactions with integrins (Bernard-Trifilo et al., 2005; Chun et al., 2001).

Tenascins

The tenascin family of proteins consists of four glycoproteins in vertebrates, three of which (tenascin-C, tenascin-R, and tenascin-X) are expressed in the brain (Chiquet-Ehrismann and Tucker, 2011). Tenascin-C is expressed during brain development and is induced by tissue injury and inflammation, tenascin-R is expressed throughout the adult brain, and tenascin-X is expressed in the choroid plexus and leptomeningeal trabecula (Imura and Sato, 2009; Chiquet-Ehrismann and Tucker, 2011). Tenascin proteins contain several fibronectin (Fn3) domains, epidermal growth factor (EGF)-like repeats and fibrinogen-related domains. Tenascins form a unique six-armed or “hexabrachion” structure and function as trimers. Tenascins and fibronectin bind to each other, and tenascins also interact with integrin receptors at the cell surface through their Fn3 domains (Chiquet-Ehrismann et al., 1988; Chiquet-Ehrismann et al., 1991; Katoh et al., 2013; Saito et al., 2007; Tucker and Chiquet-Ehrismann, 2015; Yokosaki et al., 1998). In addition to fibronectin, tenascins bind to CSPGs. Notably, tenascin-C, -R, and -X knockout mice exhibit altered behavior,

demonstrating the importance of these proteins in normal brain functioning (Senkov et al., 2014; Freitag et al., 2003; Kawakami and Matsumoto, 2011; Morellini and Schachner, 2006). In addition to motor and cognitive changes, tenascin-R and tenascin-X knockout mice show increased anxiety-like behavior (Freitag et al., 2003; Fukamauchi et al., 1998), whereas tenascin-C knockout mice demonstrate decreased anxiety-like behavior (Morellini and Schachner, 2006). In accord with deficits in learning and memory, tenascin knockout mice also show altered hippocampal long-term potentiation (LTP), which is a cellular measure of synaptic plasticity (Senkov et al., 2014; Bukalo et al., 2007; Evers et al., 2002; Saghatelian et al., 2001; Saghatelian et al., 2000).

Laminins and Collagen

Laminins are glycoproteins that are primarily located in the basement membrane of the BBB and are also found in the choroid plexus and pial basement membrane (Lau et al., 2013). Each laminin isoform is a trimer consisting of three chains, α , β and γ , which are encoded by several different genes (Halper and Kjaer, 2014). Laminin chains assemble into a cross-, Y- or rod-like shape (Mouw et al., 2014). Similar to fibronectin, laminins interact with integrins at the cell surface and collagen to assemble into the fibrous mesh structure of the basement membrane. Laminins also contain EGF-like repeats. Like tenascins and fibronectin, laminins can regulate neurite outgrowth, LTP and synaptic plasticity (Cowen et al., 1997; Nakagami et al., 2000; Smalheiser, 1989). Collagen is also an important protein component of the basement membrane. Collagen molecules assemble into a triple helical structure, either as homotrimers or heterotrimers of collagen α -chains (Mouw et al., 2014).

PNs and CSPGs

A key component of PNs that surround neuronal cell bodies and dendrites are the CSPGs. CSPGs bind to hyaluronan, tenascin-R, and the cartilage link protein 1 to form PNs. The protein core of CSPGs is a lectican. Aggrecan, brevican, neurocan, versican, and phosphacan are all lecticans that are covalently modified by chondroitin sulfate GAG chains on serine residues (Wang and Fawcett, 2012). PNs play an important role in restricting neuronal plasticity. During development, PNs condense around neurons in an activity-dependent manner at the end of synaptogenesis. A well-studied example is in the visual cortex, where PNs form around neurons during the closure of the critical window of visual system development, when ocular dominance plasticity due to monocular deprivation ends (Pizzorusso et al., 2002; Vorobyov et al., 2013). Aggrecan is essential to the formation of PNs, and its expression is induced by neuronal activity (Giamanco and Matthews, 2012; Giamanco et al., 2010). In addition to playing an important role in brain development, CSPGs act as inhibitory signals to restrict axon regeneration after injury (Kwok et al., 2011).

These effects on plasticity can be reversed by treatment with chondroitinase ABC (ChABC), an enzyme that cleaves the chondroitin sulfate GAG chains. Interestingly, synapses are located in open “holes” in PNs and it has been hypothesized that PNs stabilize synapses and are thus the sites for storage of long-term memories (Tsien, 2013). For example, ChABC digestion of PNs in the amygdala facilitates the erasure of a fear memory (Gogolla et al., 2009). In addition, ChABC digestion of PNs in the hippocampus reduces LTP and long-term

depression (Bukalo et al., 2001). These studies provide evidence for PNs in the stabilization of synapses and formation of long-term memories.

ECM Proteases: Matrix Metalloproteinases (MMPs) and Tissue Plasminogen Activator (tPA)/Plasminogen

The ECM is not a static structure, but is rather remodeled in a dynamic fashion through the activity of extracellular proteases. Fibronectin, laminin, collagen, tenascins, and proteoglycans are all processed by a number of different proteases, including plasmin, tPA, MMPs, a disintegrin and metalloproteinases (ADAMs), and ADAMTs (ADAMs with a thrombospondin motif) (Bonnans et al., 2014). These proteases are directly implicated in the regulation of synapse formation, synaptic plasticity, and learning and memory (Ethell and Ethell, 2007; Nagy et al., 2006; Pang et al., 2004; Baranes et al., 1998; Salazar et al., 2016).

The tPA/plasminogen system has been well characterized for its role in dissolving blood clots (thrombolysis). This system is also active in the brain and plays a key role in synaptic plasticity, learning and memory, and anxiety-like behavior (Tsilibary et al., 2014; Baranes et al., 1998; Pang et al., 2004). tPA is a serine protease that converts plasminogen to plasmin, which is also a serine protease that cleaves ECM proteins. In addition to cleaving ECM components such as fibronectin and laminin, tPA interacts with the GluN2B subunit of the N-methyl-D-aspartate (NMDA) receptor and the lipoprotein receptor-related proteins (LRP), but appears to regulate synaptic plasticity through these receptors independent of its proteolytic activity (Norris and Strickland, 2007; Pawlak et al., 2005; Zhuo et al., 2000). In addition, tPA/plasmin cleaves growth factors such as brain derived neurotrophic factor (BDNF) to their active mature form (Pang et al., 2004). The activity of tPA is tightly regulated by serine protease inhibitors (serpins) such as plasminogen activator inhibitor-1 (PAI-1).

Several MMPs are expressed in the brain and induced by tissue injury or neuronal activity (Huntley, 2012; Ethell and Ethell, 2007). Substrates for MMP-2 and MMP-9 include collagens and fibronectin. MMP-3 cleaves aggrecan, laminin, plasminogen, and MMP-9 in addition to collagens and fibronectin (Bonnans et al., 2014). MMP-9 has been studied extensively for its role in synaptic plasticity and learning and memory (Huntley, 2012; Tsilibary et al., 2014). MMP-9 protein levels and activity are rapidly increased in the hippocampus by stimulation that induces long-term potentiation (LTP) and MMP-9 knockout mice show deficits in LTP and hippocampal-dependent associative learning (Nagy et al., 2006). The activity of MMPs activity is tightly controlled by tissue inhibitors of matrix metalloproteinases (TIMPs) (Khokha et al., 2013).

REGULATION OF THE ECM BY ALCOHOL

Fibronectin, Laminin, and Collagen

Several protein components of the interstitial matrix and basement membrane in the brain are altered by exposure to ethanol. Changes induced by ethanol are listed in **Table 1**. In primary cortical astrocytes and organotypic hippocampal cultures, the cholinergic agonist carbachol increases fibronectin and laminin protein (Giordano et al., 2011; Guizzetti et al.,

2010). Treatment of astrocytes or hippocampal slices with ethanol for 24 hours blocks the carbachol-stimulated increase in fibronectin and laminin (Giordano et al., 2011; Guizzetti et al., 2010). The effects of carbachol and ethanol on fibronectin and laminin protein levels correlate with the extent of neurite outgrowth in hippocampal slices and in primary cultured hippocampal neurons, suggesting that secretion of these ECM components by astrocytes might directly impair neuronal development. Consistent with this finding, blocking fibronectin and laminin function with antibodies reduces neurite-outgrowth stimulated by carbachol (Giordano et al., 2011; Guizzetti et al., 2010). Similarly, astrocytic C6 glioma cells stimulated with the endotoxin lipopolysaccharide (LPS) and a protein kinase C activator (phorbol 12-myristate 13-acetate, PMA) show elevated fibronectin mRNA and protein. This stimulated increase in fibronectin is blocked by 5-9 days of continuous ethanol exposure (Ren et al., 2000). A recent study in ferrets also demonstrated decreases in laminin proteins. Ferrets were injected with IP ethanol every other day during postnatal days 10-30, which mimics the third trimester of human gestation; astrocytes from the ferrets were cultured for 4 weeks after *in vivo* ethanol exposure (Trindade et al., 2016). Conditioned media from astrocytes from animals treated with ethanol contained lower levels of several laminin subunits compared with media from astrocytes from animals treated with saline. Together, these studies suggest that ethanol reduces the expression of fibronectin and laminin by astrocytes, which consequently adversely affects neuronal morphology. However, Vangipuram and colleagues observed an increase in *LAMA1* mRNA, encoding the laminin α -1 subunit, after exposure of fetal human neurosphere cultures to ethanol for 72 hours (Vangipuram et al., 2008). This inconsistency may be due to the timing and dose of ethanol treatment or differences in cell types examined.

Laminin is also affected by ethanol exposure in the adult mouse. Mice chronically fed an ethanol diet for 2 weeks, or mice that have intermittently consumed ethanol in a binge-drinking paradigm for 4 weeks demonstrate decreased laminin protein in the cornu ammonis one (CA1) region of the hippocampus (Rubio-Araiz et al., 2016; Skrzypiec et al., 2009). In both of these ethanol exposure paradigms, laminin levels were measured at 6 or 24 hours of ethanol withdrawal, respectively. Notably, deleting the laminin γ -1 gene protects neurons in the hippocampus from neurodegeneration induced by ethanol withdrawal, suggesting an important role for laminin in ethanol-stimulated neurodegeneration in the hippocampus (Skrzypiec et al., 2009).

Similar to laminin and fibronectin, collagen-IV levels are reduced by chronic ethanol exposure. Rubio-Araiz and colleagues found that collagen-IV is decreased in the dorsolateral prefrontal cortex of human alcoholics (Rubio-Araiz et al., 2016). The tight-junction protein claudin-5, a component of the BBB, was also decreased, along with an increase in the number of leukocytes infiltrating the brain (Rubio-Araiz et al., 2016). These data add to the literature that the BBB is compromised with chronic alcohol use (Pratt et al., 1990; Thomsen et al., 1994; Alikunju et al., 2011; Haorah et al., 2007; Haorah et al., 2005). Interestingly, 72 hours of ethanol treatment of brain microvascular endothelial cells (BMVEC) cultured on a collagen substrate results in degradation of collagen-IV (Haorah et al., 2008). Since BMVEC are the major vascular cell type of the BBB, this links the endothelial cells of the BBB to regulation of the basement membrane in response to ethanol exposure. Collagen-IV is also decreased in the CA1 region of the hippocampus in mice that have undergone 4

weeks of repeated binge-like drinking episodes in a drinking in the dark (DID) paradigm and 24 hours of withdrawal (Rubio-Araiz et al., 2016). In general, it appears that structural components of the interstitial matrix and basement membrane in the brain are decreased by ethanol exposure.

MMPs

As mentioned above, the ECM is tightly regulated by protease systems and ethanol affects the activation and production of proteases involved in ECM degradation. Two groups found that MMP-9 activity is increased with ethanol exposure (Haorah et al., 2008; Rubio-Araiz et al., 2016). Rubio-Araiz and colleagues observed an increase in MMP-9 activity and protein levels in the dorsolateral prefrontal cortex of post-mortem brains from human alcoholics compared to controls (Rubio-Araiz et al., 2016). MMP-2 activity was not elevated in human alcoholics in this brain region, demonstrating some specificity in the activation of MMPs in response to alcohol. However, secreted MMP-1, -2, and -9 activity and protein levels were all increased in BMVEC media after 48 hours of ethanol treatment (Haorah et al., 2008). This discrepancy may be due to the tissue examined (BMVEC vs. brain) or the duration of ethanol treatment (48 hours vs. lifetime chronic alcohol consumption). In contrast with these findings, Wright and colleagues found that MMP-9, but not MMP-2, activity was decreased in the hippocampus and prefrontal cortex of rats treated intraperitoneally with ethanol for 2, 4 or 6 days in a dose-dependent manner (Wright et al., 2003). In these experiments, MMP-9 activity was measured immediately after a battery of motor tests. The discrepancy between increased vs. decreased MMP-9 activity after ethanol treatment could be due to a number of factors, such as the ethanol exposure paradigm (acute injections vs. chronic ethanol drinking), species (rat vs. human), or the timing of measurement after the last ethanol exposure. However, it appears from these three studies that ethanol can alter MMP activity, which is important for ECM remodeling and synaptic plasticity (Huntley, 2012). Increased MMP-9 activity is expected to decrease levels of extracellular fibronectin and collagen due to proteolysis, since these proteins are substrates for MMP-9 (Bonnans et al., 2014). This is consistent with studies finding decreased fibronectin and collagen seen after alcohol exposure. Finally, TIMP-1 and TIMP-2 were reduced after 48 hours of ethanol exposure in BMVEC (Haorah et al, 2008) and TIMP-1 was decreased in the media of astrocytes from ferrets that had been exposed to ethanol during postnatal days 10-30 (Trindade et al., 2016). Since TIMPs inhibit MMPs, a decrease in TIMPs would increase MMP activity because the ratio of MMP to TIMP determines overall proteolytic activity of the MMPs (Bonnans et al., 2014; Khokha et al., 2013).

tPA/Plasminogen

Another protease system that controls the structure of the ECM is tPA/plasminogen. The first report of ethanol regulating activity of this system in the brain came from the laboratory of Sidney Strickland (Pawlak et al., 2005). In this report, mice were given a chronic ethanol liquid diet to induce physical dependence. tPA activity was examined immediately after two weeks of ethanol treatment and 6 hours after the last ethanol exposure. tPA activity was increased in the hippocampus and central nucleus of the amygdala after ethanol treatment and during ethanol withdrawal. The increase in tPA activity in the hippocampus was confirmed and extended by demonstrating a specific increase in the CA1 region of the

hippocampus (Skrzypiec et al., 2009). Increased tPA activity was also observed in the nucleus accumbens one hour after acute ethanol injections of various doses (Bahi and Dreyer, 2012), and in the cingulate cortex and thalamus of postnatal day 7 mice treated with two IP injections of ethanol in a model of fetal alcohol syndrome (Noel et al., 2011). The increase in tPA activity was observed as soon as 30 minutes after the final injection and persisted for 24 hours. Consistent with these findings, tPA mRNA and protein were increased in rat cortical astrocyte cultures treated for 24 hours with ethanol (Zhang et al., 2014b). This suggests that ethanol increases tPA activity in the brain through its effects on astrocytes, although tPA is also released from neuronal cells in an activity-dependent manner (Gualandris et al., 1996). Secretion of the inhibitor of tPA, PAI-1, from astrocytes is also reduced after one day of ethanol treatment (Guizzetti et al., 2010) and in conditioned media from astrocytes obtained from ferrets exposed to ethanol during postnatal days 10-30 (Trindade et al., 2016). The net result of decreased PAI-1 is an increase in activated tPA. Similar to the MMPs, an increase in tPA activity would lead to proteolysis of ECM components such as laminin, collagen and fibronectin. In fact, the decrease in laminin protein induced by chronic ethanol consumption and withdrawal is likely due to increased tPA activity, since levels of laminin in the CA1 region of the hippocampus are not decreased during ethanol withdrawal in tPA knockout mice (Skrzypiec et al., 2009). Notably, ethanol withdrawal-induced neurodegeneration is also absent in tPA and plasminogen knockout mice, demonstrating an important role for this system in promoting neurodegeneration in the hippocampus (Skrzypiec et al., 2009).

PNs and CSPGs

In contrast to what has been observed for components of the basement membrane and neural interstitial matrix, the structural components of PNs are increased with ethanol exposure (**Table 2**). PNs are visualized in the brain using labeled *Wisteria floribunda* agglutinin (WFA), which is a plant lectin that binds to sulfated GAGs containing N-acetylgalactosamine (Hartig et al., 1992). Alternatively, PNs can be measured with antibodies to specific PN proteins, such as aggrecan, brevican, or neurocan. Increased WFA reactivity was found in the insular cortex of adult mice exposed to 6 weeks of binge-like ethanol consumption and one day of withdrawal (Chen et al., 2015). Consistent with the increase in WFA reactivity after chronic ethanol exposure, aggrecan and phosphacan protein levels were also augmented by alcohol. Interestingly, the increase in PNs was not observed in the motor cortex, indicating some regional specificity in response to ethanol. The insular cortex is thought to play a role in addiction by regulating craving and drug seeking behavior under conditions of high risk (Naqvi et al., 2014) and to contribute to compulsive alcohol consumption (Seif et al., 2013).

Increases in PNs in cortical regions after chronic ethanol exposure might be expected to increase synaptic plasticity (LTP) of pyramidal neurons because of restrictive effects of PNs on inhibitory interneuron firing (Dityatev et al., 2007). Supporting this hypothesis, chronic ethanol exposure decreases the threshold needed to induce LTP in the hippocampus (Fujii et al., 2008), while ChABC digestion of PNs surrounding interneurons in hippocampal slices decreases LTP in CA1 pyramidal neurons (Bukalo et al., 2001). This is consistent with a potential increase in PNs surrounding interneurons in the CA1 after ethanol exposure. Seif

and colleagues have shown that glutamatergic activity from cortical regions to the nucleus accumbens is important for compulsive alcohol drinking (Seif et al., 2013). Thus, increased PNs in cortical regions after chronic ethanol exposure might promote excessive ethanol consumption. However, PNs also surround excitatory neurons. Interestingly, in the CA2 region of the hippocampus, the majority of PNs surround pyramidal neurons and ChABC digestion of PNs in this region leads to enhanced LTP, indicating that PNs can also directly restrict the plasticity of excitatory neurons (Carstens et al., 2016).

Coleman and colleagues observed an increase in WFA reactivity in the orbitofrontal cortex in adult mice after a binge-like ethanol administration protocol given during adolescence (Coleman et al., 2014). Concomitant with the increase in WFA, brevican and neurocan levels were also higher in mice that had been treated with alcohol during adolescence. These changes were associated with learning deficits, specifically behavioral flexibility as measured by reversal learning, after adolescent alcohol exposure (Coleman et al., 2014). The increase in PNs surrounding interneurons in the orbitofrontal cortex after alcohol exposure thus might reduce behavioral flexibility. Notably, chronic ethanol exposure in adult mice increases the intrinsic excitability and glutamatergic signaling of neurons in the orbitofrontal cortex and the prefrontal cortex (Kroener et al., 2012; Nimitvilai et al., 2016). It is possible that increased PNs surrounding GABAergic interneurons after chronic ethanol exposure in these brain regions is partially responsible for these effects.

Astrocytes are likely to be involved in the increase in PNs after ethanol exposure. Treatment of rat cortical astrocytes for 24 hours with ethanol led to an increase in sulfated GAGs and chondroitin-4-sulfate, found in CSPGs (Zhang et al., 2014a). This appears to be due to ethanol inhibiting the activity of the arylsulfatase B enzyme, which removes sulfate groups from chondroitin-4-sulfate (Zhang et al., 2014a). In addition to sulfated GAGs, ethanol treatment of astrocytes increased neurocan mRNA and protein levels (Zhang et al., 2014a). Similar changes were found in the hippocampus of postnatal rats treated with intragastrically with two doses of ethanol from postnatal days 4-9 (Zhang et al., 2014a), indicating that PNs in the hippocampus are affected by ethanol exposure during fetal brain development and may play a key role in neurite outgrowth. Collectively, the data indicate that overall ethanol increases PN components, which may alter neuronal plasticity and neurite outgrowth due to the inhibitory effect of CSPGs (Miller and Hsieh-Wilson, 2015).

EVIDENCE THAT REGULATION OF THE ECM AFFECTS ALCOHOL ABUSE

tPA/Plasminogen

The numerous changes in ECM components induced by ethanol suggest that regulation of the ECM might be causally involved in the development or maintenance of AUD. Although there are only a few studies that have directly examined the role of ECM components in behaviors related to alcohol abuse, they provide evidence that regulation of the ECM may be involved in alcohol addiction. For instance, withdrawal from chronic ethanol withdrawal induces seizures in mice. Deletion of the tPA gene in mice attenuates ethanol withdrawal-induced seizures, demonstrating a role for elevated tPA activity in the brain in ethanol withdrawal (Pawlak et al., 2005). In addition, tPA activity in the amygdala is elevated by corticotropin-releasing factor (CRF) and stress, and tPA knockout mice exhibit lower levels

of CRF- and stress-induced anxiety (Matys et al., 2004; Pawlak et al., 2003). During ethanol withdrawal, CRF levels in the amygdala are increased, which promotes a negative affective state that is thought to drive continued excessive alcohol consumption (Koob and Volkow, 2010). Thus, tPA may be a downstream target of CRF that promotes alcohol consumption during withdrawal through proteolytic processing of the ECM, although this remains to be tested directly.

In addition to affecting brain responses to ethanol withdrawal, tPA appears to be involved in the rewarding effects of ethanol. Viral-mediated overexpression or knockdown of tPA in the nucleus accumbens bi-directionally alters the rewarding properties of ethanol in mice. Increased tPA in the nucleus accumbens enhances ethanol conditioned place preference, while knockdown of tPA reduces ethanol conditioned place preference (Bahi and Dreyer, 2012).

MMPs

Similar to tPA, MMPs may also be involved in excessive alcohol consumption during ethanol withdrawal. Smith and colleagues induced ethanol dependence in rats using chronic intermittent ethanol vapor exposure and then tested them for operant ethanol self-administration. Rats that were dependent on ethanol showed escalated ethanol intake compared to control, non-dependent rats (Smith et al., 2011). Interestingly, treatment with a broad spectrum MMP inhibitor, FN-439, chronically or acutely during ethanol withdrawal, prevented the escalation of ethanol self-administration (Smith et al., 2011). This suggests that preventing ECM degradation by inhibiting MMPs might be a useful strategy for decreasing ethanol consumption in ethanol-dependent individuals.

Genetic studies in humans and mice also implicate genes encoding ECM components in alcohol dependence. A polymorphism in the MMP-9 gene was found to be more prevalent in alcoholics compared to controls (Samochowiec et al., 2010). A genome-wide association study also discovered polymorphisms in a region of chromosome 1, which contain the tenascin-N (*TNN*) and tenascin-R (*TNR*) genes, associated with alcohol dependence (Zuo et al., 2012). In mice, a quantitative trait locus on chromosome 1 that is associated with differential predisposition to alcohol withdrawal severity contains the *Adams4* gene, which encodes a protease in the ADAMTs family (Denmark and Buck, 2008). These limited studies provide evidence that variants in genes encoding ECM components can increase the risk for AUD.

COMPARISON OF ALCOHOL EFFECTS ON ECM WITH OTHER DRUGS OF ABUSE

Many studies have examined the effects of several drugs of abuse (opiates, psychostimulants, and nicotine) on ECM structure and remodeling by extracellular proteases and have provided causal evidence that remodeling of the brain ECM is involved in behaviors related to substance abuse. An excellent summary of these effects can be found in recent reviews (Lubbers et al., 2014; Slaker et al., 2016; Mizoguchi et al., 2011; Mulholland

et al., 2016). I will briefly describe how these other substances compare with alcohol in changing ECM components.

tPA/Plasminogen

Cocaine, methamphetamine, nicotine, and morphine all increase the activity of tPA in the brain, similar to what has been found after ethanol exposure (Bahi and Dreyer, 2008; Bahi and Dreyer, 2012; Hashimoto et al., 1998; Nagai et al., 2005; Nagai et al., 2004; Pawlak et al., 2005; Nagai et al., 2006). In addition, behavioral studies generally show that animals deficient in tPA exhibit reduced rewarding and reinforcing responses to drugs of abuse (Bahi and Dreyer, 2012; Nagai et al., 2005; Nagai et al., 2004). This raises the possibility that targeting the tPA/plasminogen system might be a novel strategy for reducing substance use and AUD.

MMPs

In general, treatment of animals with drugs of abuse increases MMP activity in the brain. Morphine, methamphetamine, cocaine, and nicotine have all been shown to increase MMP activity (Brown et al., 2008; Mizoguchi et al., 2007; Nakamoto et al., 2012; Smith et al., 2014; Natarajan et al., 2013). This is in contrast to the finding of Wright and colleagues, who found a decrease in MMP-9 activity after ethanol injections (Wright et al., 2003). However, as mentioned above, MMP-9 activity is increased in the brains of alcoholics, suggesting that the timing of measuring MMP activity after ethanol exposure may yield different results. Cocaine abusers who died from cocaine intoxication were found to have decreased MMP-9 protein levels in the brain (Mash et al., 2007). Taken together, these data suggest that there is a dynamic regulation of MMP-9 in the brain in response to drugs of abuse and alcohol. Interestingly, though, concordance has been observed with treatment with a broad-spectrum MMP inhibitor, FN-439 in animal models of addiction. MMP inhibition has been shown to decrease ethanol self-administration, nicotine reward, and reinstatement of heroin-seeking behavior (Natarajan et al., 2013; Smith et al., 2011; Van den Oever et al., 2010). In addition, specific MMP-2 and MMP-9 inhibitors reduce the reinstatement of cue- and cocaine-induced drug seeking behavior (Smith et al., 2014). Thus, treatment with MMP inhibitors may also be a novel strategy to reduce illicit substance use and AUD, although the timing of inhibitor treatment may be important because of fluctuations in MMP activity after alcohol and drug exposure and withdrawal.

PNs

The effect of ethanol on PNs seems to differ from other abused substances. Several reports have demonstrated that ethanol treatment and withdrawal increases PNs, whereas abstinence from heroin decreases PNs (Chen et al., 2015; Coleman et al., 2014; Van den Oever et al., 2010). However, PNs in the cerebellum are increased by chronic cocaine treatment and withdrawal, similar to what has been observed with ethanol exposure in cortical regions (Vazquez-Sanroman et al., 2015). To date, no studies have been performed to examine how specifically manipulating PNs might alter behaviors related to alcohol abuse. However, digestion of PNs with ChABC in specific brain regions has been shown to attenuate behaviors related to cocaine, morphine, and heroin reward and drug-seeking behavior (Slaker et al., 2015; Xue et al., 2014). In addition, heterozygous brevicin knockout mice

exhibit enhanced cocaine conditioned place preference, which can be rescued by overexpression of brevican in the dorsal hippocampus (Lubbers et al., 2015). These studies indicate that manipulations of PN components could feasibly decrease behaviors related to alcohol abuse.

FUTURE DIRECTIONS AND GAPS IN KNOWLEDGE

It has become apparent that ethanol affects the regulation of the ECM in the brain in various ways and at all developmental stages (fetal, adolescent and adult). Since the ECM plays an important role in synaptic processes that are altered by ethanol exposure, it is possible that remodeling of the brain ECM is important in the pathophysiology of AUD. Research on the role of brain ECM, although promising, is still in early stages. Future experiments should determine the role of specific ECM proteins in behaviors related to alcohol abuse, either through gene targeting strategies in mice or pharmacological manipulation of ECM proteins in the brains of animals.

For instance, infusion of specific antagonists to MMPs into the brain would be helpful in determining if MMP-9 or another MMP is responsible for promoting ethanol consumption before and after the development of dependence, similar to what has been done with studies on cocaine relapse in rats (Levin et al., 2001; Smith et al., 2014; Agamennone et al., 2016). Conditional knockout or overexpression of PN proteins, such as aggrecan, brevican, neurocan, and tenascins in a site-specific and cell-type specific manner would also be useful, since it is currently not clear how these proteins affect behaviors related to AUD. It is also important to realize that there are regional differences in the expression of ECM proteins and that identifying the specific neuroanatomical location of changes in ECM proteins in response to ethanol and their roles in behavioral responses to ethanol will be necessary (Dauth et al., 2016).

Another outstanding issue that remains is the time in which critical ECM adaptations after ethanol exposure contribute to excessive ethanol consumption and dependence. It is apparent from the current literature that changes in some proteins, such as tPA, occur in brain regions involved in AUD even after a single dose of ethanol (Bahi and Dreyer, 2012) and that tPA activity is also increased after chronic ethanol consumption and withdrawal (Pawlak et al., 2005; Skrzypiec et al., 2009). Perhaps the persistence of ECM changes during ethanol withdrawal is important for the development of AUD. A more comprehensive examination of the endurance of these changes after acute and chronic ethanol treatment, and their coordinate effects on neuronal firing and synaptic plasticity will be helpful in sorting out this issue. In addition, examination of function of these proteins on intracellular signal transduction cascades should provide valuable new mechanistic insights.

The ECM is also involved in sequestering secreted proteins that act as ligands at cell surface receptors that play a role in synaptogenesis. Risher and colleagues found that thrombospondins TSP2 and TSP4 levels increase after ethanol administration in an adolescent binge-like protocol (Risher et al., 2015). Another secreted molecule, midkine, binds to chondroitin sulfate and may also play a role in synaptogenesis (Zhou et al., 1997; Solera et al., 2016). ECM remodeling after ethanol exposure may release these types of

molecules that have direct effects on synaptic plasticity. This is an exciting area for future research.

Finally, ECM proteases are linked to the inflammatory response (Khokha et al., 2013). Since the neuroimmune system is also altered by ethanol exposure and plays a key role in behaviors related to alcohol abuse (Cui et al., 2014), investigations into the interplay between the brain ECM and neuroimmune activation will likely yield important new knowledge regarding cellular mechanisms that contribute to alcohol abuse.

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Fig. 1.

Schematic diagram of extracellular matrix (ECM) compartments in the brain (A). Basement membrane located on the basolateral side of endothelial cells of blood vessels is part of the blood-brain barrier (BBB) and comprises collagen, laminin, fibronectin, and heparin sulfate proteoglycans. (B). Interstitial matrix is found between neuronal and glial cells of the brain parenchyma and contains proteoglycans, hyaluronan, tenascins, and fibronectin. Small amounts of laminin and collagen can also be found in the interstitial matrix. Regulation of ECM is controlled by proteases such as the matrix metalloproteinases (MMPs). Extracellular matrix proteins such as fibronectin bind to integrins located on the cell surface. Integrin signaling leads to remodeling of the actin cytoskeleton. (C). Perineuronal nets (PNs) form lattice-like structures around neurons and contain chondroitin sulfate proteoglycans, tenascins, hyaluronan, link proteins, and tenascin-R.

Table 1

Brain Interstitial Matrix and Basement Membrane Altered by Ethanol Exposure. DID, drinking in the dark; BMVEC, brain microvascular endothelial cells; MMP, matrix metalloproteinase; TIMP, tissue inhibitor of metalloproteinase; tPA, tissue plasminogen activator; PAI-1, plasminogen activator inhibitor-1; IP, intraperitoneal; CA1, cornus ammonis 1 region of the hippocampus.

ECM component	Species	Brain region or cell type tested	Ethanol exposure method	Ethanol withdrawal	Effect of ethanol	References
Fibronectin	Rat	Hippocampus from postnatal day 5 (organotypic slices)	24 hours incubation	No	Decreased protein	Giordano et al. (2011)
		Cortical astrocyte cultures from embryonic day 21	24 hours incubation	No	Decreased protein	Guizzetti et al. (2010)
		C6 glial cultures	5-9 days incubation	No	Decreased mRNA and protein	Ren et al. (2000)
Collagen-IV	Human	Dorsolateral prefrontal cortex	Alcoholic	Unknown	Decreased protein	Rubio-Araiz et al. (2016)
	Mouse	Hippocampus (CA1)	Intermittent consumption (DID) for 4 weeks	24 hours	Decreased protein	Rubio-Araiz et al. (2016)
	Human	BMVEC	72 hours incubation	No	Decreased protein	Haorah et al. (2008)
Laminin	Mouse	Hippocampus (CA1)	Intermittent consumption (DID) for 4 weeks	24 hours	Decreased protein	Rubio-Araiz et al. (2016)
	Mouse	Hippocampus (CA1)	Chronic ethanol liquid diet for 2 weeks	6 hours	Decreased protein	Skrzypiec et al. (2009)
	Rat	Hippocampus from postnatal day 5 (organotypic slices)	24 hours incubation	No	Decreased protein	Giordano et al. (2011)
	Rat	Cortical astrocyte cultures from embryonic day 21	24 hours incubation	No	Decreased protein	Guizzetti et al. (2010)
	Human	Neurosphere cultures	72 hours incubation	No	Increased mRNA (<i>LAMA1</i> gene)	Vangipuram et al. (2008)
	Ferret	Cortical astrocyte cultures from	IP injections every	4 weeks	Decreased laminin protein	Trindade et al. (2016)

ECM component	Species	Brain region or cell type tested	Ethanol exposure method	Ethanol withdrawal	Effect of ethanol	References
		postnatal day 31	other day between postnatal day 10-30		(subunits $\alpha 2$, $\alpha 4$, $\beta 1$, $\beta 2$, and $\gamma 1$)	
MMP-9	Human	Dorsolateral prefrontal cortex	Alcoholic	Unknown	Increased activity and protein	Rubio-Araiz et al. (2016)
	Rat	Hippocampus, prefrontal cortex	2, 4, or 6 IP injections	No	Decreased activity	Wright et al. (2003)
MMP-1, MMP-2, & MMP-9	Human	BMVEC	48 hours incubation	No	Increased activity and protein	Haorah et al. (2008)
TIMP-1 & TIMP-2	Human	BMVEC	48 hours incubation	No	Decreased protein	Haorah et al. (2008)
TIMP-1	Ferret	Cortical astrocyte cultures from postnatal day 30	IP injections every other day between postnatal day 10-30	4 weeks	Decreased protein	Trindade et al. (2016)
tPA	Mouse	Hippocampus (CA1), amygdala (medial, central)	Chronic ethanol liquid diet for 2 weeks	6 hours	Increased activity	Pawlak et al. (2005), Skrzypiec et al. (2009)
	Mouse	Nucleus accumbens	Single IP ethanol injection	No	Increased activity	Bahi and Dreyer (2012)
	Mouse (postnatal day 7)	Cingulate cortex, thalamus	Two IP injections	No	Increased activity	Noel et al. (2011)
	Rat	Cortical astrocyte cultures from embryonic day 21	24 and 48 hours	No	Increased mRNA and protein	Zhang et al. (2014b)
PAI-1	Mouse (postnatal day 7)	Forebrain	Two IP injections	No	Initial increase in protein, followed by a decrease at 24 hours withdrawal	Noel et al. (2011)
	Rat	Cortical astrocyte cultures from embryonic day 21	24 hours	No	Decreased protein	Guizzetti et al. (2010)
PAI-1	Ferret	Cortical astrocyte cultures from postnatal day 30	IP injections every other day between postnatal day 10-30	4 weeks	Decreased protein	Trindade et al. (2016)

Table 2

Perineuronal Net (PN) Components Altered by Ethanol Exposure. PNs, perineuronal nets; DID, drinking in the dark; WFA, *Wisteria floribunda* agglutinin; GAGs, glycosaminoglycans; CA1, cornu ammonis 1 region of the hippocampus; IG, intragastric.

PN component	Species	Brain region or cell type tested	Ethanol exposure method	Ethanol withdrawal	Effect of ethanol	References
PNs	Mouse	Insula	Intermittent consumption (DID) for 6 weeks	24 hours	Increased WFA reactivity	Chen et al. (2015)
	Mouse	Orbitofrontal cortex	Six IG treatments administered over 10 days of adolescence	10 weeks	Increased WFA reactivity	Coleman et al. (2014)
Aggrecan	Mouse	Insula	Intermittent consumption (DID) for 6 weeks	24 hours	Increased protein and mRNA	Chen et al. (2015)
Phosphacan	Mouse	Insula	Intermittent consumption (DID) for 6 weeks	24 hours	Increased protein	Chen et al. (2015)
Neurocan	Mouse	Orbitofrontal cortex	Six IG treatments administered over 10 days of adolescence	10 weeks	Increased protein	Coleman et al. (2014)
	Rat	Cortical astrocyte cultures from embryonic day 21	24 hours	No	Increased mRNA and protein	Zhang et al. (2014a)
	Rat	Hippocampus (CA1)	Two IG treatments from postnatal days 4-9	No	Increased protein	Zhang et al. (2014a)
Brevican	Mouse	Orbitofrontal cortex	Six IG treatments administered over 10 days of adolescence	10 weeks	Increased protein	Coleman et al. (2014)
Sulfated GAGs	Rat	Cortical astrocyte cultures from embryonic day 21	24 hours	No	Increased	Zhang et al. (2014a)
	Rat	Hippocampus (CA1)	Two IG treatments from postnatal days 4-9	No	Increased	Zhang et al. (2014a)