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Repeated Binge Drinking Increases Perineuronal Nets in the Insular Cortex

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Abstract

Background—Alcohol exposure leads to changes in the extracellular matrix (ECM) in the brain, which profoundly impacts neuronal plasticity. Perineuronal nets (PNs) are specialized ECM structures that enclose subpopulations of neurons in the cortex. Adolescent exposure to alcohol induces long-lasting increases in the expression of PN components in the cortex in adult mice. However, it has not been determined whether binge alcohol exposure in young adults alters PNs. Here, we examined PNs and their core components in the insula and primary motor cortex after repeated binge-like ethanol consumption in adult mice.

Methods—The 4 day drinking in the dark (DID) procedure was performed in mice for 1 or 6 weeks to model binge alcohol consumption. The impact of ethanol drinking on PNs was examined by fluorescent staining of brain sections using a marker for PNs, *Wisteria floribunda* agglutinin (WFA). In another set of experiments, cortex was dissected and Western blots and quantitative real-time PCR (qPCR) were performed to evaluate the expression of the PN proteins aggrecan, brevican, and phosphacan.

Results—Binge-like ethanol drinking for 6 weeks caused a significant increase in PNs in the insula, as measured by WFA binding. Aggrecan, brevican and phosphacan protein expression, and aggrecan mRNA expression, were also elevated in the insula after 6 weeks of ethanol drinking. In contrast, expression of PN components did not change after 1 week of DID. The increase in PNs appears to be specific to the insula, since alterations were not observed in the primary motor cortex.

Conclusions—Our results provide the first evidence that insular PNs increase after long-term binge drinking. The insula mediates compulsive alcohol use. Since PNs influence neuronal firing and plasticity, increased PNs in the insula after multiple binge cycles may contribute to restricted neuronal plasticity and lead to the development of compulsive alcohol use.

Keywords

Binge drinking; perineuronal nets; insular cortex; chondroitin sulfate proteoglycans

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INTRODUCTION

Perineuronal nets (PNs) are extracellular matrix (ECM) structures that form around the soma and proximal dendrites of neurons. PNs play an important role in synaptic plasticity and increase during brain development, a process that restricts plasticity (Pizzorusso et al., 2002, Wang and Fawcett, 2012). PNs are altered in neurological and psychiatric disorders. For example, PNs are decreased in the cortex and amygdala of human subjects with schizophrenia (Berretta et al., 2015). PNs also appear to play an important role in alcohol and drug addiction (Lubbers et al., 2014). Adolescent exposure to alcohol increases the expression of PN components in the orbital frontal cortex in adulthood (Coleman et al., 2014). This increase in PNs correlates with decreased behavioral flexibility. In contrast, PN components are reduced in the prefrontal cortex of rats after long-term abstinence from heroin, and increased after cue-induced reinstatement of heroin self-administration. In the amygdala, digestion of PNs using the enzyme chondroitinase ABC (ChABC) during extinction of cocaine and morphine conditioned place preference (CPP) behavior prevents drug-induced reinstatement of place preference (Xue et al., 2014). Similarly, extinction of heroin and cocaine self-administration is facilitated and reinstatement of drug-seeking behavior is attenuated after ChABC infusion into rat amygdala (Xue et al., 2014). ChABC treatment in the prefrontal cortex also decreases the acquisition and reconsolidation of cocaine CPP (Slaker et al., 2015). Together, these studies implicate PNs as a contributing factor in the formation and persistence of detrimental drug memories.

Although the majority of PNs condense around GABAergic interneurons that produce the calcium-binding protein parvalbumin (PV), a low percentage of PNs also surround glutamatergic pyramidal cells in the cortex (Alpar et al., 2006, Wegner et al., 2003, Hartig et al., 1992). PNs are lattice-like structures that are composed of several components, including chondroitin sulfate proteoglycans (CSPGs), hyaluronan, link proteins, and tenascin proteins (Wang and Fawcett, 2012). The CSPGs are comprised of a core protein known as a lectican (aggrecan, brevican, neurocan, versican, or phosphacan) that is modified by the attachment of chondroitin sulfate glycosaminoglycan chains (Wang and Fawcett, 2012). *Wisteria floribunda* agglutinin (WFA) is a plant lectin that has high affinity for chondroitin sulfate N-acetylgalactosamine and is used as a tool to visualize PNs in the nervous system (Hartig et al., 1992).

Here, we examined WFA binding to PNs and the expression of several CSPGs in the cortex after binge-like ethanol consumption in C57BL/6J mice. To model binge drinking, we performed the drinking in the dark (DID) procedure, in which mice drink enough ethanol to achieve pharmacologically relevant blood ethanol concentrations (BECs) (Rhodes et al., 2005). Binge drinking is the most common pattern of excessive alcohol use in the United States, with a prevalence of approximately 30% among adults 18-34 years old (Kanny et al., 2013). Binge drinking is associated with negative health outcomes, impaired cognition, and the development of alcohol dependence (Bonomo et al., 2004, Courtney and Polich, 2009). The goal of this study was to determine if there are alterations in PNs in the cortex that occur after binge drinking in young adult mice. We examined changes in PNs after 1 and 6 weeks of the 4 day DID procedure (Rhodes et al., 2005) to compare short-term and long-term binge drinking behavior. PNs were analyzed in the cortex since they are prominently

observed surrounding cortical neurons, and several cortical areas are dysregulated by alcohol abuse (Koob and Volkow, 2010).

MATERIALS AND METHODS

Animals

Male C57BL/6J mice were purchased from The Jackson Laboratory at the age of 8 weeks (Bar Harbor, ME, USA). Mice were individually housed in a temperature- and humidity-controlled room with a 12 hour reversed light/dark cycle (lights off at 10 am) for 2 weeks prior to beginning experiments. Food and water were available *ad libitum*. All procedures with mice were conducted according to the National Institutes of Health Guide for the Care and Use of Laboratory Animals and approved by the UIC Animal Care and Use committee.

Chemicals and Antibodies

Biotinylated WFA, glycoprotein-free blocking solution, and Dylight 488-conjugated streptavidin were purchased from Vector Laboratories (Burlingame, CA, USA). Mouse anti-calcium/calmodulin-dependent protein kinase II alpha (CaMKII- α) antibody was ordered from Millipore (Billerica, MA, USA). Mouse anti-glutamic acid decarboxylase (GAD67) antibody was purchased from Abcam (Cambridge, MA, USA). Mouse anti-parvalbumin (PV) and mouse anti- β -actin antibodies were ordered from Sigma (St. Louis, MO, USA). Alexa Fluor 594-conjugated anti-mouse IgG was from Jackson ImmunoResearch (West Grove, PA, USA). Mouse anti-aggreCAN antibody was purchased from Millipore. Mouse anti-brevican and mouse anti-phosphacan antibodies were purchased from BD Transduction Labs (San Jose, CA, USA). HRP-conjugated anti-mouse IgG was from Santa Cruz (Dallas, TX, USA).

Drinking in the Dark and Measurement of Blood Ethanol Concentrations (BECs)

Mice were given access to a single sipper tube of 20% ethanol (v/v in water) or water (as a control) for 4 h per day, 4 days per week (Monday through Thursday), starting 3 h into the dark cycle (Rhodes et al., 2005). The 4 day DID procedure was conducted for 1 or 6 weeks. All mice were given their first access to alcohol at the age of 10 weeks, such that mice in the 1-week group were matched with the 6-week group for the age of first ethanol exposure, rather than the age at euthanasia. Volumes of fluid consumed after each 4 h DID session were recorded and amounts consumed were calculated as g ethanol or ml water consumed per kg body weight. To obtain BECs, 20 μ L of blood was collected into heparinized capillary tubes via tail vein puncture immediately after the 4th DID session in weeks 1 and 6. BECs were determined using an NAD-ADH enzymatic assay as described by Carnicella *et al* (Carnicella et al., 2009).

Immunofluorescence Staining

Mice were euthanized approximately 20 h after the final drinking session using a lethal dose of pentobarbital followed by transcardial perfusion with 4% paraformaldehyde. Brains were post-fixed overnight and incubated in 30% sucrose. Every 4th section from 40 μ m serial coronal sections were chosen from the area of the brain approximately 1.5 to 0.8 mm anterior to bregma (Franklin and Paxinos, 2007) for analysis, giving a total of 4 sections per

mouse. Sections were blocked in glycoprotein-free blocking solution and incubated with biotinylated WFA (1:2000). Sections were then incubated with Dylight 488-conjugated streptavidin (1:200). Images of primary motor cortex (medial/lateral: 1 to 1.7 mm; dorsal/ventral: -1 to -1.5 mm) and granular insula (medial/lateral: 2.7 to 3.4 mm; dorsal/ventral: -3 to -3.5 mm) from layers 2-5 were captured using an Olympus BX51 fluorescence microscope (Center Valley, PA, USA) equipped with a 10X objective lens. Quantification of fluorescence intensity was measured using NIH Image J software from the left hemisphere of 4 sections per mouse and the average integrated density measure from ImageJ obtained. A threshold was applied during image analysis to normalize background staining. Images were acquired with the same magnification and camera settings. For co-localization of WFA and PV, sections were blocked again in 5% donkey serum after WFA staining and incubated with PV antibody (1:2000), followed by incubation with Alexa Fluor 594-conjugated mouse IgG (1:200) for the visualization of PV positive neurons. An extra antigen retrieval step was included in double staining of WFA and CaMKII- α , or WFA and GAD67. Sections were heated in 10 mM citrate buffer (pH 8.5) at 80°C for 30 min, and then subjected to the staining protocol above. CaMKII- α and GAD67 antibodies were diluted 1:500 in blocking solution. A Zeiss LSM710 confocal microscope (Carl Zeiss, Thornwood, NY, USA) was utilized to capture images of the double fluorescent staining.

Western Blotting

Mice were euthanized approximately 20 h after the final drinking session by CO₂ inhalation and rapidly decapitated. The brain was removed, rinsed in cold PBS, and sectioned on ice into 1 mm-thick coronal sections using an adult mouse brain matrix (Zivic Instruments, Pittsburgh, PA USA). The insula and adjacent primary motor cortex (1 mm³ of tissue) were dissected from one section approximately 1.2 mm anterior of bregma. For the insula, dorsal edge of the piriform cortex was used as a boundary. The cingulum was used to mark the medial edge of the primary motor cortex (Franklin and Paxinos, 2007). Tissue collected from the left hemisphere was used for Western blotting, whereas tissue from the right hemisphere was used for gene expression analysis by qPCR (described below). Tissue was snap frozen on dry ice in 1.5 ml centrifuge tubes. Samples were homogenized in 100 μ l of lysis buffer (20 mM Tris-HCl, 150 mM NaCl, 1 mM EDTA, 1 mM EGTA, 1% Triton X-100, 2.5 mM sodium pyrophosphate, 1 mM β -glycerophosphate, 1 mM Na₃VO₄, 1 μ g/ml leupeptin, and 1 μ g/ml aprotinin) containing the Complete protease inhibitor cocktail (Roche, Indianapolis, IN, USA). Protein concentrations were determined using the BCA Protein Assay Kit (Fisher Scientific, Pittsburgh, PA, USA). Equal amounts of protein (20 μ g) were subjected to SDS-PAGE and transferred to PVDF membranes. Membranes were blocked with 5% milk in TBST (25 mM Tris-HCl, 137 mM NaCl and 0.1% Tween 20) and incubated with primary antibodies overnight at 4°C (anti-aggrecan, 1:1000; anti-brevican, 1:1000; anti-phosphacan, 1:1000; anti- β -actin, 1:5000). The membranes were then incubated with HRP-conjugated secondary antibody (1:2000) at room temperature and developed with enhanced chemiluminescence (ECL) detection reagents (GE Healthcare, Pittsburgh, PA, USA). Band intensities were quantified using NIH Image J software and protein levels were normalized to the densities of corresponding β -actin protein in each sample.

QPCR

Total RNA was isolated from tissue using the GeneJet RNA Purification Kit (Thermo Scientific, Schaumburg, IL, USA) according to the manufacturer's instructions. RNA was reverse transcribed using the Maxima First Strand cDNA Synthesis Kit (Thermo Scientific) and qPCR was performed using Maxima qPCR Master Mix (Thermo Scientific). Sequences of primers were as follows: aggrecan (*Acan*) forward primer: 5'-CGTTGCAGACCAGGAGCAAT-3', *Acan* reverse primer, 5'-CGGTCATGAAAGTGGCGGTA-3'; brevican (*Bcan*) forward primer: 5'-TTTTGTGAGGCTCCCGGC-3', *Bcan* reverse primer: 5'-GGAAGAACTTGCGAGGTCCC-3; phosphacan (*Ptprz1*) was detected with a pre-designed TaqMan® gene expression assay from Life Technologies (Grand Island, NY, USA). Amplification of β -actin (*Actb*) was used as a normalization control for total RNA input. Relative expression levels of *Acan*, *Bcan* and *Ptprz1* were calculated using the dCq method.

Statistical Analysis

Data were expressed as the mean $\pm$ SEM and analyzed using Prism software (GraphPad, LaJolla, CA). A *p* value of less than 0.05 was accepted as statistically significant. DID data were analyzed using a 1 way repeated measures (RM) ANOVA followed by Tukey's multiple comparisons test (*n* = 22). BEC data were analyzed using a Student's *t*-test (*n* = 6 per group). WFA fluorescence intensity, Western blots, and qPCR were analyzed with a 2 way ANOVA followed by Tukey's multiple comparisons test (*n* = 6 mice per group).

RESULTS

Characterization of PNs in the Insula and Primary Motor Cortex

We first investigated the PN distribution pattern in the insula and primary motor cortex of ethanol-naïve mice in order to determine the type of neurons that are surrounded by these ECM structures. We performed double fluorescent labeling using WFA as a marker for PNs and antibodies to the GABAergic neuronal marker GAD67 or the glutamatergic neuronal protein CaMKII- α (Jones et al., 1994). A schematic of the insula and primary motor cortex regions analyzed is shown in Fig. 1A (Franklin and Paxinos, 2007). Results of PN-positive cell counts from the insula and motor cortex are summarized in Table 1. Approximately 80% of the cells surrounded by PNs were also positive for GAD67 (Fig. 1B), while only 6% of PNs surrounded CaMKII- α -expressing neurons (Fig. 1C). We also examined co-localization of PNs with PV since PNs have been well characterized to surround GABAergic PV-expressing neurons in the rat cortex (Hartig et al., 1994, Hartig et al., 1992, Kosaka and Heizmann, 1989). We found that approximately 70% of PNs in the insula and motor cortex surrounded cells expressing PV (Table 1 and Fig. 1D). Together, these data indicate that the majority of neurons surrounded by PNs are GABAergic PV-expressing neurons.

Ethanol Consumed and BECs During 6 Weeks of DID

We measured the amount of ethanol and water consumed during the DID procedure. In the group of mice that received ethanol for only 4 days of DID (1 cycle), the average 4 h ethanol consumption was 3.85 ± 0.22 g/kg and BECs were 73 ± 19.3 mg/dl. In the group of mice

given ethanol for 6 cycles of DID, the average 4 h consumption over 6 weeks was 5.06 g/kg. As shown in Fig. 2A, average ethanol consumption was lower in week 1 (4.2 g/kg) compared to weeks 2-6 (*e.g.* week 2, 5.0 g/kg and week 6, 5.4 g/kg). A 1 way RM ANOVA indicated a significant effect of time ($F(5,105) = 11.82, p < 0.0001$). Post-hoc multiple comparisons tests demonstrated that ethanol intake during the first week was significantly different from weeks 2-6 (week 2, $p = 0.0003$; weeks 3-6, $p < 0.0001$). To verify that blood ethanol levels achieved during week 6 were higher than those in week 1, we measured BECs on the 4th day of drinking in weeks 1 and 6 in one cohort of mice ($n=6$ per group). BECs increased from 70.9 in the first week to 138 mg/dl in week 6, consistent with increased ethanol intake observed in week 6 (Fig. 2B, $t(10) = 2.25, p = 0.049$). No significant differences were found in ethanol consumption or BECs between mice that drank for only 1 cycle compared to the 1st cycle of the 6-week experiment. Control mice that were given water in sipper tubes (instead of ethanol) during the DID procedure consumed significantly different amounts of water in each week, with peak consumption occurring in week 3 at 52.4 ml/kg (Fig. 2C, 1 way RM ANOVA, $F(5,105) = 7.58, p < 0.0001$). Post hoc multiple comparisons tests demonstrated that water consumption during week 1 was significantly lower than weeks 2-5 (week 2, $p = 0.041$; week 3, $p < 0.0001$; week 4, $p = 0.0083$; week 5, $p = 0.011$). In contrast to the ethanol-drinking pattern, water consumption in week 6 (42.7 ml/kg) dropped to similar levels as observed in week 1 (40.8 ml/kg).

Long-term Binge Ethanol Consumption Increases PNs in the Insula

We next evaluated the effect of binge-like ethanol consumption on PNs in the insula and primary motor cortex using fluorescent labeling of WFA. Fig. 3A-D shows that WFA fluorescence intensity in the insula increased after 6 weeks of ethanol DID when compared to 6 weeks of water drinking. Notably, WFA binding did not increase in the insula after 1 week of ethanol DID. Quantification of fluorescence intensity demonstrated that WFA binding in the insula increased by 44% in mice that drank ethanol for 6 weeks, with an ANOVA indicating a significant effect of treatment, time, and a treatment by time interaction (Fig. 3E, 2 way ANOVA, effect of treatment: $F(1,83) = 4.51, p = 0.037$; time: $F(1,83) = 8.09, p = 0.0056$; interaction: $F(1,83) = 8.09, p = 0.0056$). In contrast, WFA fluorescence intensity did not change in the primary motor cortex after 1 or 6 weeks of ethanol DID (Fig. 3F-J).

Enhanced WFA binding in the insula after 6 weeks of ethanol DID could be due to increased deposition of PNs surrounding the same neurons at 1 and 6 weeks, or might be the result of PNs condensing around new neurons. Although we could not image the same neurons at 1 and 6 weeks, we sought to address this question by counting PN-positive cells in the insula after 1 and 6 weeks of ethanol drinking. The number of cells surrounded by PNs did not change between 1 and 6 weeks of ethanol drinking (data not shown). Therefore, it appears that the increase in WFA binding intensity after binge-like ethanol drinking is due to increased deposition of PN components around neurons with existing PNs.

Aggrecan, Brevican, and Phosphacan Levels Increase After 6 Weeks of Ethanol Drinking

To characterize in more detail the increase in PNs in the insula after ethanol drinking, we measured the expression of the CSPGs aggrecan, brevican, and phosphacan in the insula and

primary motor cortex by Western blotting. Fig. 4A shows that aggrecan expression increased by 131% after 6 weeks, but not 1 week, of ethanol DID in comparison to water drinking (2 way ANOVA, effect of treatment: $F(1,20) = 5.30, p = 0.032$; time: $F(1,20) = 5.18, p = 0.034$; interaction: $F(1,20) = 5.18, p = 0.034$). Similar to aggrecan, both brevican and phosphacan levels increased in the insula by 94% and 109%, respectively, after 6 weeks of ethanol DID when compared to the water-drinking controls (Fig. 4C, E). For brevican, a 2 way ANOVA indicated a significant effect of treatment ($F(1,20) = 5.41, p = 0.031$), but the effect of time ($F(1,20) = 2.75, p = 0.11$) and the treatment by time interaction ($F(1,20) = 2.75, p = 0.11$) were not statistically significant. For phosphacan, a 2 way ANOVA indicated a significant effect of treatment, time, and a treatment by time interaction (treatment: $F(1,20) = 5.59, p = 0.028$; time: $F(1,20) = 5.26, p = 0.033$; interaction: $F(1,20) = 5.26, p = 0.033$). We did not observe any significant changes in the levels of aggrecan, brevican, or phosphacan proteins in the primary motor cortex after ethanol drinking (Fig. 4B, D, F).

To determine if the changes in protein expression in the insula also occurred at the level of gene transcription, we examined aggrecan, brevican, and phosphacan gene expression by qPCR. Aggrecan expression was higher in the insula after 6 weeks of ethanol drinking (Fig. 5A, 2 way ANOVA, effect of treatment: $F(1,20) = 5.84, p = 0.025$; time: $F(1,20) = 4.70, p = 0.042$; interaction: $F(1,20) = 5.34, p = 0.032$). In contrast, we did not observe any changes in mRNA levels of brevican and phosphacan (Fig. 5B-F).

DISCUSSION

Our primary conclusion is that multiple cycles of binge-like ethanol consumption in mice cause increased accumulation of PNs around insular neurons. This data is supported by higher expression of the CSPGs aggrecan, brevican, and phosphacan after 6 weeks of ethanol drinking. To our knowledge, this is the first demonstration that PN dynamics in the insula change after voluntary alcohol drinking. Alterations in PNs after long-term binge drinking could affect synaptic plasticity in the insula and consequently alter alcohol-drinking behavior. Our first key finding is that the increased intensity of PNs in the insula is observed after 6, but not 1, cycles of ethanol DID. Based on this, it appears that long-term or multiple bouts of binge-like ethanol consumption are required for this adaptation to occur. We believe that the changes in PNs in the insula after 6 weeks of DID are due to long-term exposure to ethanol, rather than an ethanol withdrawal effect. Cox et al (Cox et al., 2013) found that mice that have undergone 6 weeks of DID do not exhibit any traditional signs of withdrawal 1 day after the last drinking session, such as increased anxiety or handling-induced convulsions. However, we cannot formally rule out the possibility that the changes in PNs are due to a subtle ethanol withdrawal effect.

We have not studied intermediate time points, but it would be interesting to know if the change in PNs occurs earlier than 6 weeks. It is also important to point out that in this study, mice in the 1 week and 6 week groups were matched for age at first ethanol exposure (10 weeks old), rather than their age at euthanasia. It is clear that animals and humans respond differently to alcohol at different points in the lifespan (Squeglia et al., 2014). A caveat to the approach taken in this study is that it is possible that slightly older mice (15 weeks old)

might be more sensitive to the effect of ethanol on the expression of PNs and we have not controlled for this in our experimental design.

Our second key finding is that the increase in PNs is not global. We examined the primary motor cortex, which has a similar number and distribution of PNs as the insula, and did not find greater PN intensity in this brain region after alcohol drinking. Similarly, we found that PN intensity did not change in the anterior cingulate after ethanol exposure (Chen and Lasek, data not shown). Together, these data suggest that neurons and glia in the adult insula may be particularly sensitive to the effects of binge-like alcohol drinking and respond by increasing the production of ECM components.

Consistent with our observation that the intensity of WFA binding increases after 6 weeks of alcohol drinking, levels of the CSPGs aggrecan, brevican, and phosphacan were elevated at this time point in the insula but not the primary motor cortex. When we examined mRNA expression, aggrecan transcript was increased after 6 weeks of DID, consistent with the increased protein levels. However, brevican and phosphacan mRNA levels were unchanged. The discrepancy between mRNA and protein expression of brevican and phosphacan may be due to the time point at which we measured expression, or because of increased post-translational stability of brevican and phosphacan in response to alcohol that is independent of transcriptional regulation.

Consistent with our data, Coleman *et al* observed an increase in WFA binding in the orbital frontal cortex in adult mice that had previously been treated intermittently with ethanol injections during adolescence (Coleman et al., 2014). In addition, they found that brevican and neurocan, but not phosphacan, expression increased after adolescent alcohol exposure. We examined neurocan expression by qPCR in adult mice subjected to 6 weeks of binge-like drinking and did not see any changes in expression (Chen and Lasek, data not shown). Neurocan is predominantly a juvenile extracellular matrix protein and increased levels of neurocan may be related to delayed or altered maturation of PNs (Coleman et al., 2014), so it is not surprising that neurocan expression did not change in adult mice after binge-like ethanol consumption. We did not examine PN intensity or CSPG expression after binge drinking in the orbital frontal cortex, but it would be interesting to determine if these alterations occur in this brain region in adult mice.

Aggrecan, brevican, and phosphacan are all components of PNs in the adult brain, but are produced by different cell types (Giamanco and Matthews, 2012). For example, aggrecan is produced by neurons, whereas most other PN proteins are produced by astrocytes (Giamanco and Matthews, 2012, Yamada et al., 1997). Aggrecan is an essential component of PNs, since cortical neurons from aggrecan knockout mice do not develop these structures (Giamanco et al., 2010). Aggrecan expression increases with neuronal activity and corresponds with the formation of PNs during the closure of critical periods of plasticity during brain development (Giamanco and Matthews, 2012, Nabel and Morishita, 2013). Chronic ethanol exposure increases the activity of neurons by creating a hyper-glutamatergic state during withdrawal, which could result in increased transcription of aggrecan (Holmes et al., 2013). Ethanol is also known to profoundly impact the functioning of astrocytes and alters the production of astrocyte-derived PN components (Zhang et al., 2014). Thus,

ethanol might affect the production of PN proteins in both neurons and glia. PNs are protective against several neurotoxic insults, including elevated glutamate and reactive oxygen species (Suttkus et al., 2014). The chondroitin sulfate component of CSPGs is thought to mediate this effect (Sato et al., 2008). We hypothesize that elevated glutamate and/or oxidative stress induced by repeated bouts of binge drinking results in a compensatory increase in PNs surrounding insular neurons, thereby protecting these cells from ethanol-induced neurotoxicity (Ward et al., 2009).

Since PNs in the mouse insula have not been well characterized, we performed co-localization studies to determine the types of neurons surrounded by PNs in this brain region. We found that approximately 80% of the neurons surrounded by PNs were GABAergic. Our data are consistent with other studies showing that approximately 67% of PNs surround GABAergic neurons in mouse somatosensory and rat parietal cortex (Karetko-Sysa et al., 2014, Wegner et al., 2003). In particular, the vast majority (67-90%) of PNs in mouse somatosensory cortex and rat cortex are associated with PV-expressing GABAergic neurons (Hartig et al., 1992, Karetko-Sysa et al., 2014, Kosaka and Heizmann, 1989). We found that approximately 70% of PNs surrounded PV-positive neurons in both the mouse insula and primary motor cortex, also consistent with other reports. In contrast, we observed only about 6% of PNs surrounding CamKII- α positive neurons, whereas Wegner et al (Wegner et al., 2003) found 30% of PNs surrounded glutamate-positive neurons. These PNs were described as diffuse and low intensity. We may have missed some of these PNs in our analysis of CaMKII- α positive cells due to the antigen retrieval method, which reduces WFA binding. Although our results with the CaMKII- α immunostaining are not consistent with Wegner et al, our data is in accord with the percentage of PNs that surround GABA neurons. We conclude that the increase in PNs specifically in the insula (compared to the motor cortex) after repeated binge-like ethanol consumption is likely not due to differences in the types of neurons surrounded by PNs in these brain regions. In addition, since we did not observe an increase in the number of neurons in the insula surrounded by PNs after binge-like ethanol consumption, it appears that the increase in PN intensity results from greater deposition of PN components around neurons surrounded by existing PNs prior to alcohol exposure. This suggests that the increase PNs after repeated binge drinking likely involves PV-positive GABAergic neurons.

It is interesting to speculate about the behavioral consequences of increasing PNs in the insula after repeated binge-like ethanol exposure. The insula integrates interoceptive states into conscious feelings and decision-making and is involved in drug addiction (Naqvi et al., 2014). Recent evidence supports the importance of the insula in alcohol use disorders. Ethanol cues induce greater activity in the insula compared to control stimuli, which is correlated with the severity of alcohol dependence in humans (Claus et al., 2011, Schacht et al., 2013). Glutamatergic efferents from the insula to the nucleus accumbens promote aversion-resistant (*i.e.* compulsive) ethanol consumption in rats (Seif et al., 2013). In addition, an antagonist of the GABA_A receptor inhibits ethanol-induced fMRI changes in the rat insula (Tsurugizawa et al., 2010). Here, we show that PNs increase in the insular cortex after long-term binge-like ethanol drinking in mice, which might have implications for the development of compulsive alcohol use.

PV-expressing, GABAergic interneurons are likely to be affected by changes in PNs after ethanol exposure, since they comprise the vast majority of neurons surrounded by PNs. PV-positive interneurons are fast-spiking, have high firing frequencies, and are important for inhibitory control in the cortex and proper cognitive functioning (Lewis et al., 2012). Altering the structure of PNs surrounding PV positive interneurons would therefore be expected to disrupt inhibitory activity in the insula. In ventral hippocampus slices, digestion of PNs with ChABC results in greater firing of pyramidal neurons, indicating that the loss of PNs surrounding GABA interneurons decreases their firing rate (Shah and Lodge, 2013). In contrast, digestion of PNs in hippocampal cultures increases the firing of interneurons (Dityatev et al., 2007). At this point, it is not known how an increase in PNs in the insula would affect inhibitory control and alcohol drinking behavior, but this is an important question to be answered by future research.

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Fig. 1.

Distribution of PNs in the insula of ethanol-naïve mice. (A) A schematic of a coronal brain section (1.2 mm anterior to bregma) showing the insula and primary motor cortex regions analyzed in this study. Yellow shaded region shows the area of primary motor cortex and the blue shaded region indicates the insula. Images were obtained from cortical layers 2-5. (B-D) Representative confocal microscope images demonstrating fluorescent WFA binding to PNs (green) and immunofluorescence with the (A) GAD67 antibody (red), (B) CaMKII-α antibody (red), or (C) PV antibody (red). Note the substantial co-localization of WFA with GAD67-expressing neurons in (B) and the absence of WFA co-localized with CaMKII-α-expressing neurons in (C). In panel (D), examples of cells labeled with WFA and PV antibody are indicated by white arrows and cells labeled with WFA but not expressing PV are marked by yellow arrowheads. Scale bar = 50 μm.

Fig. 2.

Ethanol and water consumption and BECs in the DID procedure. **(A)** Average 4 h ethanol consumption (in g/kg), shown averaged for each week from 1-6. *** $p < 0.001$, **** $p < 0.0001$ when compared to week 1 by Tukey's multiple comparisons test. **(B)** BECs at the end of the 4 h drinking session on the 4th day of weeks 1 and 6. * $p < 0.05$ by t-test. **(C)** Average 4 h water consumption in the control mice (in ml/kg), shown for each week from 1-6. * $p < 0.05$, ** $p < 0.01$, **** $p < 0.0001$ when compared to week 1 by Tukey's multiple comparisons test.

Fig. 3. PNs increase in the insula after 6 weeks of ethanol drinking. Representative images of fluorescent WFA binding in the insula (A-D) and primary motor cortex (F-I). W1, 1 week of water DID; E1, 1 week of ethanol DID; W6, 6 weeks of water DID; E6, 6 weeks of ethanol DID. Note the increase in WFA binding after 6 weeks of ethanol consumption in the insula, but not in the primary motor cortex. (E, J) Quantification of PN intensity in the insula (E) and primary motor cortex (J). Data are expressed as the percentage change from water DID within each time point. Significant main effects by 2 way ANOVA, $*p < 0.05$ and $**p < 0.01$; # significant treatment by time interaction.

Fig. 4.

Increased CSPGs in the insula after 6 weeks of ethanol drinking. Insula and primary motor cortex were obtained after 1 or 6 weeks of DID. Representative Western blots are shown above each graph. Graphs show quantification of protein expression by densitometry using ImageJ. Protein levels were normalized for each sample based on actin blots. (A, B) Aggrecan expression in the insula (A) and primary motor cortex (B). (C, D) Brevican expression in the insula (C) and primary motor cortex (D). (E, F) Phosphacan expression in the insula (E) and primary motor cortex (F). (G, H) Representative actin western blots from the insula (G) and primary motor cortex (H). Significant main effects by 2 way ANOVA, $*p < 0.05$; $\#$ significant treatment by time interaction.

Fig. 5.

Aggrecan mRNA expression is increased in the insula after 6 weeks of ethanol drinking. Insula and primary motor cortex were dissected after 1 or 6 weeks of DID. Quantification of *Acan* (A, D), *Bcan* (B, E) and *Ptprz1* (C, F) mRNA expression in the insula (A, B, C) and primary motor cortex (D, E, F) by qPCR. Significant main effects by 2 way ANOVA, * $p < 0.05$; # significant treatment by time interaction.

Table 1

Perineuronal Net (PN) Distribution in the Insula and Primary Motor Cortex

Brain region	GAD67	CaMKII- α	PV
Insula	77.8 $\pm$ 3.9	5.8 $\pm$ 2.9	67.1 $\pm$ 4.1
Motor Cortex	82.1 $\pm$ 4.9	6.0 $\pm$ 2.1	68.6 $\pm$ 5.3

Shown are the percentages of PN-positive neurons (Mean $\pm$ SEM) expressing GAD67, CaMKII- α , and PV in the insula and primary motor cortex.