THE 11th INTERNATIONAL SYMPOSIUM ON ALPD & CIRRHOSIS
Emerging Therapeutic Targets

ISBRA/ESBRA World Congress, Berlin, Germany, September 2-3, 2016
THE 11TH INTERNATIONAL SYMPOSIUM ON ALPD AND CIRRHOSIS
“Emerging Therapeutic Targets”
ISBRA/ESBRA World Congress, Berlin, Germany, September 2-3, 2016

Organizing Committee: Helmut Karl Seitz (Univ. of Heidelberg, Germany); Samir Zakhari (DISCUS, USA); Bin Gao (NIAAA/NIH, USA); Hidekazu Tsukamoto (Univ. of Southern California, USA)

Friday, September 2, 2016
11:00-11:10 Opening Remarks by Helmut Karl Seitz (Univ. of Heidelberg, Germany)

11:10-12:30 Session-1: Inflammation and Immunology of ALPD
Moderated by Helmut Karl Seitz (Univ. of Heidelberg, Germany) and Craig McClain (Univ. of Louisville, USA)
- Steven Dooley (Univ. of Heidelberg-Mannheim, Germany), Effects of alcohol on TGF-β mediated hepatocyte apoptosis
- Emanuele Albano (Univ. “Amedeo Avogadro” of East Piedmont, Italy), Oxidant stress and adaptive immunity in ASH and NASH
- Hana Algül (Technical Univ. of Munich, Germany), Autophagy and chronic inflammation in the pancreas
- Henrik Thorlacius (Lund Univ., Sweden), NETs trigger trypsin activation, pathological inflammation and tissue damage in severe acute pancreatitis

12:30-14:30 Lunch and Poster Session

14:30-16:00 Session-2: Novel Targets of ALPD
Moderated by Samir Zakhari (DISCUS, USA) and Kazuhiko Koike (Tokyo Univ., Japan)
- Vikas Dudeja (Univ. of Miami, USA), HSP70 as a therapeutic target of pancreatic cancer
- Craig McClain (Univ. of Louisville, USA), Malnutrition and alcoholic liver disease
- Bernd Schnabl (UC San Diego, USA), Targeting intestinal dysbiosis in alcoholic liver disease
- Jonas Rosendahl (Univ. of Leipzig, Germany), Genetics of alcoholic chronic pancreatitis

16:00-16:30 Coffee Break

16:30-18:00 Session-3: Selected Oral Presentation by Travel Awardees
Moderated by Gary Murray (NIAAA) and Yoshiyuki Takei (Mie Univ., Japan)
- 5 selected abstracts (10+5 min each)

Saturday, September 3, 2016
9:00-10:20 Session-4: Liver-Brain Inflammatory Axis in ALPD
Moderated by Hidekazu Tsukamoto (Univ. of Southern California, USA) and Laura Nagy (Cleveland Clinic)
- Yedy Israel (Univ. of Chile), The liver-brain axis: brain acetaldehyde and alcohol addiction
- Tatiana Kisseleva (UC San Diego, USA), IL-17 and alcoholic liver and brain inflammation
- Fulton Crews (UNC-CH, USA), Mechanisms of alcohol-induced brain innate immune gene induction
- Christoph Buettner (Mount Sinai, NY, USA), Hypothalamic dysfunction as a key mechanism for alcoholic liver disease

10:20-10:30 The 12th ALPD Symposium Announcement and Closing Remarks
Kazuhiko Koike (Tokyo Univ., Japan) and Hidekazu Tsukamoto (Univ. of Southern California, USA)
1. EFFECTS OF ALCOHOL ON TGF-β MEDIATED HEPATOCYTE APOPTOSIS

Breitkopf-Heinlein K¹, Meyer C¹, Thomas M², Wahl K³, Bantel H³, Bergheim I⁴, and Dooley S¹.

¹ Universitätsmedizin Mannheim, II. Medical Clinic, Medical Faculty Mannheim at Heidelberg University, Molecular Hepatology – Alcohol Associated Diseases, Mannheim, Germany. ² Dr. Margarete Fischer-Bosch-Institute of Clinical Pharmacology, Stuttgart, Germany. ³ Medizinische Hochschule Hannover OE 6810, Hannover, Germany. ⁴ Institute of Nutritional Sciences, SD Model Systems of Molecular Nutrition, Friedrich-Schiller University Jena, Jena, Germany

Background and aims: Alcohol abuse is a major health concern worldwide and accompanied with elevated levels of the pro-fibrogenic cytokine TGF-β. Aim of this study was to characterize crosstalk of TGF-β and alcohol on hepatocytes.

Methods: Primary isolated mouse hepatocytes were treated with ethanol and TGF-β and cellular fate was determined. Expression patterns of apoptosis-related genes were analyzed on RNA level using Fluidigm technology. To gain mechanistic insight into the crosstalk, we interfered with prominent pathways in vitro. To substantiate in vitro findings, human liver slice cultures and an acute alcohol intoxication animal model were investigated.

Results: TGF-β and ethanol each had a moderate effect on hepatocyte survival. Combined treatment culminated in a massive apoptotic response in primary hepatocytes which was confirmed in human liver tissue treated ex vivo. Alcohol increased the TGF-β pro-apoptotic gene expression signature. The underlying mechanism involves canonical Smad and non-Smad signaling. We define the AKT/GSK3β as a relevant target of ethanol/TGF-β treatment. Noteworthy, alcohol itself – but not its metabolism – mediated these effects as blocking of Cyp2e1 and ADH did not prevent super-induction of apoptosis. In addition, direct application of acetaldehyde could also not mimic the effect of ethanol.

Conclusion: Our study provides molecular data on how ethanol amplifies TGF-β dependent hepatocyte apoptosis. These data indicate that continued alcohol consumption in a pre-damaged liver with TGF-β abundance will likely accelerate and worsen disease development.

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2. OXIDANT STRESS AND ADAPTIVE IMMUNITY IN ASH AND NASH

Albano E, Bruzzi S, Sutti S.

Dept of Health Sciences, University “A. Avogadro” of East Piedmont, Novara Italy.

It is well established that chronic hepatic inflammation represents the main driving force in the evolution to fibrosis/cirrhosis of both alcoholic (ASH) and nonalcoholic (NASH) steatohepatitis. So far, innate immune mechanisms have been recognized as the main responsible in supporting inflammatory processes in steatohepatitis. However, growing evidence points on the possible role of adaptive immunity as an additional factor in promoting hepatic inflammation in ASH and NASH. In fact, patient with ASH and NASH, but not those with steatosis only, are characterized by the presence of circulating antibodies and lymphocyte-mediated responses triggered by antigens originating from oxidative stress. Similar immune responses are also detectable in experimental models of ASH and NASH and interference with either oxidative stress or the functions of either helper CD4+ and effector CD8+ T-lymphocytes ameliorates steatohepatitis. In these settings, T-lymphocytes contribute to sustain lobular inflammation and fibrosis by stimulating macrophage activation as well as by promoting natural killer T-cell (NKT) recruitment. Present data also suggest that alterations in regulatory T-cell (Treg) homeostasis and hepatic dendritic cell activation have a role in triggering immune responses during the evolution of steatohepatitis. Clarify the contribution of adaptive immunity in ASH and NASH will open new scenarios on the factors responsible for the inter-individual variability in the disease progression to fibrosis and will provide the rationale for novel treatments based on the application of a wide array of molecules already being used in other conditions characterized by impaired immune regulation or autoimmunity.
3. AUTOPHAGY AND CHRONIC INFLAMMATION IN THE PANCREAS

Little is known about the mechanisms of the progressive tissue destruction, inflammation, and fibrosis that occur during development of chronic pancreatitis. Autophagy is involved in multiple degenerative and inflammatory diseases, including pancreatitis, and requires the protein autophagy related 5 (ATG5). We created mice with pancreas-specific disruption of Atg5 (Ptf1aCreex1;Atg5F/F mice) and compared them to control mice. Mice with pancreas-specific disruption of Atg5 developed atrophic CP, independent of β-cell function; a greater proportion of male mice developed CP than female mice. Pancreata from ATG5-deficient mice had signs of inflammation, necrosis, acinar-to-ductal metaplasia, and acinar-cell hypertrophy; this led to tissue atrophy and degeneration. Based on transcriptome and metabolome analyses, ATG5-deficient mice produced higher levels of reactive oxygen species than control mice, and had insufficient activation of glutamate-dependent metabolism. Pancreata from these mice had reduced autophagy, increased levels of p62, and increases in endoplasmic reticulum stress and mitochondrial damage, compared with tissues from control mice; p62 signaling to Nqo1 and p53 was also activated. Dietary antioxidants, especially in combination with palm oil–derived fatty acids, blocked progression to CP and pancreatic acinar atrophy. Tissues from patients with CP had many histologic similarities to those from ATG5-deficient mice. Mice with pancreas-specific disruption of Atg5 develop a form of CP similar to that of humans. CP development appears to involve defects in autophagy, glutamate-dependent metabolism, and increased production of reactive oxygen species. These mice might be used to identify therapeutic targets for CP.

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Objective: Neutrophils play a pivotal role in local and systemic complications of acute pancreatitis (AP), but the mechanisms regulating neutrophil-induced tissue damage in the inflamed pancreas is not fully understood. Recently, neutrophil extracellular traps (NETs) have been demonstrated to contribute to organ dysfunction in both infective and non-infective diseases. In the present study, we investigated the role of NETs in AP.

Methods: AP was induced in male C57BL/6 mice by infusion of taurocholate into the pancreatic duct. Extracellular DNA was stained by Sytox green and NET formation was quantified by confocal microscopy. To analyze the impact of NET formation in AP, NET depletion was induced by DNase I administration. In separate experiments isolated acinar cells were exposed to NETs.

Results: Taurocholate challenge evoked formation of NET in the pancreas and increased cell-free DNA in plasma. Formation of macrophage inflammatory protein-2 (CXCL2), neutrophil infiltration and tissue damage in the inflamed pancreas and lung were significantly attenuated by DNase I treatment. Moreover, DNase I administration markedly reduced levels of blood amylase, CXCL-2, interleukin-6 and high-mobility groups protein 1 as well as macrophage-1 antigen expression on circulating neutrophils in mice with pancreatitis. NETs triggered trypsin formation and activation of signal transducer and activator of transcription-3 in isolated acinar cells. Histones increased trypsin activation and pre-incubation of NETs with polysialic acid abolished NET-induced activation of trypsin in acinar cells, suggesting that histones are responsible for great part of NET-induced trypsin activation.

Conclusions: Taken together, NET formation regulates local and remote organ inflammation and damage in AP. These novel findings provide new insights in the pathophysiology of pancreatitis and indicate that targeting NETs might be an effective way to ameliorate tissue damage severe AP.

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SESSION 2: Novel Targets of ALPD

1. HSP70 AS A THERAPEUTIC TARGET OF PANCREATIC CANCER

Vikas Dudeja

Dept. of Surgery, University of Miami Miller School of Medicine

Diagnosis of Pancreatic cancer portends poor prognosis due to its aggressive biology and resistance to conventional chemotherapy. Hence there is an urgent need for novel and effective treatments. We have previously shown that heat shock protein 70 (HSP70), a pro-survival protein, is overexpressed in pancreatic cancer cells and its downregulation leads to apoptotic cell death in pancreatic cancer cells. We have also shown that triptolide, a diterpene triepoxide extracted from a Chinese herb, is an effective inhibitor of heat shock response in pancreatic cancer cells. We have evaluated triptolide as a novel therapy for pancreatic cancer and have demonstrated that at nanomolar doses triptolide inhibits HSP70 and induces cell death in various pancreatic cancer cell lines. To facilitate its use in clinics, we have now developed a water soluble pro-drug of triptolide. Minnelide, the pro-drug of triptolide, demonstrates similar efficacy against pancreatic cancer. In an effort to generate pre-clinical data we have now tested Minnelide against pancreatic cancer in various animal models, simulating multiple clinical scenarios. We have shown the Minnelide prevents growth and metastases of pancreatic cancer and also prevents recurrence, even when the treatment is stopped. Currently, Minnelide is in phase I clinical trial against advanced GI malignancies. We are hopeful that Minnelide will emerge as novel and effective therapy against this dreadful disease.

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2. MALNUTRITION AND ALCOHOLIC LIVER DISEASE

Kirpich IA¹, Feng W¹, Barve SS¹, McClain CJ¹,²

¹ Department of Medicine and Alcohol Research Center, University of Louisville, Louisville, Kentucky, USA. ² Robley Rex VAMC, Louisville, Kentucky, USA

Malnutrition, both protein energy malnutrition (PEM) and deficiencies in individual nutrients, is a frequent complication of alcoholic liver disease (ALD). Severity of malnutrition correlates with severity of ALD. Malnutrition also occurs in patients with cirrhosis due to etiologies other than alcohol, and thus is of global importance to liver disease. The mechanisms for this malnutrition are multifactorial. Alcoholics are frequently consuming 15 or more standard drinks per day and 2,000 or more calories per day which has limited nutritional value. Malnutrition frequently worsens in the hospital due to fasting for procedures and metabolic complications of liver disease, such as hepatic encephalopathy. Aggressive nutritional support is indicated in inpatients with ALD, especially those with severe alcoholic hepatitis (AH). Patients may need to be fed through an enteral feeding tube to achieve protein and calorie goals. Enteral nutritional support clearly improves nutrition status and may improve clinical outcome. Indeed, one multicenter study suggested that aggressive nutritional support in AH was as effective as steroids in reducing short term mortality, and better at reducing long term mortality. Moreover, late-night snacks for outpatient cirrhotics improve nutritional status and lean body mass. Micronutrient deficiencies are common. Zinc deficiency causes gut-barrier dysfunction and plays a critical role in oxidative stress and proinflammatory cytokine production in ALD. Zinc is a critical factor in multiple important zinc finger proteins. Magnesium deficiency is common in alcoholic cirrhotics and often leads to muscle cramps. Vitamin D metabolism is impaired in almost all patients with ALD and can lead to alterations in immune function and gut-barrier function. Altered intake/metabolism of macronutrients, such as dietary fat, is also frequently observed in ALD. Increased intake of linoleic acid plays a critical role in the development of experimental alcohol-related liver injury. Oxidation products of linoleic acid are highly toxic. Moreover, with an increase in omega-6 fatty acids, such as linoleic acid, there is usually an associated decrease in omega-3 fatty acids which are anti-inflammatory and which give rise to specialized pro-resolving mediators. Thus, with no FDA-approved therapy for ALD, careful nutritional intervention should be considered as frontline therapy.

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3. TARGETING INTESTINAL DYSBIOSIS IN ALCOHOLIC LIVER DISEASE

Bernd Schnabl

Department of Medicine, University of California San Diego, La Jolla, CA 92093, USA

The intestinal microbiota and the human body have a symbiotic relationship. A dysbalance of this delicate homeostasis between host and microbes can lead to disease. Alcoholic liver disease is associated with changes in the gut microbiota. Alcohol-associated intestinal dysbiosis is characterized by bacterial overgrowth and changes in the microbial composition. In addition, alcohol abuse results in an intestinal barrier dysfunction. The integrity of the gut barrier is of specific importance to limit bacteria and bacterial products from translocating and reaching extraintestinal sites. The intestinal epithelium has the capacity to segregate microorganisms from the host. A dysfunctional intestinal barrier allows bacterial products to translocate to the portal circulation and reach the liver. This translocation process induces an inflammatory response in the liver and aggravates liver disease. Dysbiosis can induce a failure of the intestinal barrier leading to pathological bacterial translocation and the initiation of an inflammatory response in the liver. The mucosa-associated microbiota has been linked to translocation of viable bacteria through intestinal epithelial cells (transcytosis) to extraintestinal spaces and organs. Recent preclinical studies emphasized the importance of intestinal inflammation for the onset of gut barrier disruption and microbial translocation. Dietary approaches to restore levels of saturated fatty acids in the intestine, maintained eubiosis, stabilized the gut barrier and reduced alcoholic liver disease. In conclusion, the gut microbiota represents an excellent target to prevent the onset and progression of alcoholic liver disease.

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4. GENETICS OF ALCOHOLIC CHRONIC PANCREATITIS

Jonas Rosendahl

University of Leipzig, Germany

Chronic pancreatitis (CP) is a recurring inflammatory disorder of the pancreas with alcohol misuse being the most predominant risk factor. Whereas in patients without alcohol misuse (non-alcoholic CP, NACP) several genetic variants have been captured as risk factors, genetic associations in alcoholic CP (ACP) are rare. This is an interesting finding since only a small percentage of alcohol misuser develops ACP indicating further etiological factors. Recently, a genome-wide association study (GWAs) identified common variants in the PRSS1- and the CLDN2-MORC4 locus to be associated with CP. This finding was replicated in a large European cohort in that association was strongest in the ACP group and in Japanese and Indian CP patients. Functional studies demonstrated that the PRSS1 promoter variant rs4726576 (c.-204C>A) reduces transcription which results in lower intra-pancreatic trypsinogen levels. The mechanism of action of the CLDN2-MORC4 risk variants still remains to some extent unclear although it changes the localization of claudin-2 in carriers. We have conducted a large genome-wide association study in European patients with alcoholic CP and identified new risk loci that have been characterized on a functional level.

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1. P2X7 RECEPTOR ANTAGONISM INHIBITS INFLAMMATORY RESPONSE IN BRAIN INDUCED BY EXPOSURE TO COMBINED ETHANOL AND HIGH-FAT DIET

Asatryan L1, Freire D1, Lazaro RG2, Tsukamoto H2 and Davies DL1

1 School of Pharmacy, University of Southern California, Los Angeles, USA. 2 Research Center for Alcoholic Liver Disease and Pancreatitis and Cirrhosis, School of Medicine, University of Southern California, Los Angeles, USA.

In the search for novel targets for chronic alcohol-induced inflammatory responses, we recently started exploring the therapeutic potential of ATP-gated purinergic P2X7 receptors (P2X7Rs). Despite the recognized important role in several neurodegenerative pathologies and inflammatory conditions, the role of P2X7Rs in ethanol-induced inflammation and organ damage is still unknown. Using a chronic ethanol exposure model of alcohol liver disease that combines intragastric (iG) ethanol feeding and high fat diet (Hybrid) in C57BL/6J mice, our recent work demonstrated an increased expression of P2X7Rs that paralleled neuroinflammatory changes in several ethanol-sensitive brain regions. These findings served the basis for the hypothesis that there is a functional link between P2X7Rs and chronic ethanol-induced inflammatory response leading to organ damage. To further test this hypothesis, in the present study we tested the effects of a P2X7R pharmacological inhibitor on inflammatory responses in brain and liver using a 4 week Hybrid treatment schedule. A specific P2X7R antagonist, A804598 (5 mg/kg), was applied 3 times a week through an iG catheter. After 4 weeks of vehicle or antagonist treatment, brain (hippocampus, amygdale) and liver tissues were isolated and tested for 1) changes in histological features and 2) gene expression of critical mediators linked to inflammation, apoptosis and signaling. The antagonist treatment abolished Hybrid-induced astrocyte proliferation observed as reduction in GFAP-positive cell number in hippocampal slices. Using custom Taqman gene expression plates and qRT-PCR, we found that the antagonist reversed the increases in inflammatory cytokines, chemokines and signaling molecules including IL1β, TNFa, CCL2, CXCR2, TLR3, Nlrp3, IL1R in both hippocampus and amygdala. In parallel, initial results demonstrated that there was a small decrease in the extent of liver steatosis with the antagonist. Ongoing work is testing changes in gene expression for inflammatory markers in liver tissues. Collectively, the findings support a role for P2X7Rs in chronic ethanol-induced inflammatory response and suggest that P2X7R antagonism may represent a novel therapy against ethanol-induced brain damage. [Support: NIAAA/NIH AA017243, INIA West Pilot Project, Zumberge Individual Research Award (LA), A022448 (DD) and the USC School of Pharmacy]
2. STEAROYL-COA DESATURASE IN ALCOHOLIC LIVER FIBROSIS AND CANCER

Lai KKY1, Kweon SM1, Chi F1, Hwang E1, Kabe Y2, French S1,3, Murali R1,4, Ntambi JM5, Tsukamoto H1,6

1 Southern California Research Center for ALPD and Cirrhosis and Department of Pathology, University of Southern California, Los Angeles, CA, USA. 2 Department of Biochemistry, Keio University School of Medicine, Tokyo, Japan. 3 Harbor-UCLA Medical Center, Torrance, CA, USA. 4 Department of Biomedical Sciences, Cedars-Sinai Medical Center, Los Angeles, CA, USA. 5 Departments of Biochemistry and Nutritional Sciences, University of Wisconsin-Madison, Madison, WI, USA. 6 Department of Veterans Affairs Greater Los Angeles Healthcare System, Los Angeles, CA, USA.

Background: Evidence suggests that alcohol promotes liver fibrosis and hepatocellular carcinoma (HCC) by activation of Wnt/β-catenin pathway. Yet, Wnt targets essential for activation of hepatic stellate cell (HSC) and liver tumor-initiating stem cell-like cells (TICs), the primary cell types responsible for liver fibrosis and cancer, respectively, are unknown. Stearoyl-CoA desaturase (SCD) catalyzes the biosynthesis of the monounsaturated fatty acids (MUFA), oleic acid and palmitoleic acid, and is implicated in metabolic syndrome, tumorigenesis, and stemness, but SCD’s role in liver fibrosis and cancer is elusive.

Aim: Our study aimed to determine whether and how SCD promotes liver fibrosis and cancer.

Methods: To identify SCD as a Wnt-dependent gene, rodent primary HSCs, mouse liver TICs, and human HCC lines were assayed for Wnt pathway inhibition. Functional significance of SCD was tested by pharmacologic and genetic inhibition in mouse models of liver fibrosis, chronic alcoholic steatohepatitis (ASH), alcoholic neutrophilic hepatitis (AH), and alcohol-promoted TIC-dependent liver tumorigenesis. Nano-bead pull-down assay, LC-mass spectrometry, computational modeling, and ribonucleoprotein immunoprecipitation disclosed a novel mechanism of Wnt-SCD crosstalk.

Results: A shift from Scd1 to Scd2 expression is evident in chronic ASH and AH livers and in mouse TICs from alcohol-promoted HCC. Scd2 (rodent) and SCD (human) are novel Wnt target genes induced in TICs from alcohol-promoted HCC, HSCs from hepatotoxic and cholestatic liver fibrosis, and human HCC cell lines. Scd2 inhibition/knockdown ameliorates liver tumorigenesis and fibrosis in the respective models. Wnt co-receptor LRP5/6 is also induced in the models when SCD/Scd2 is induced. Mechanistically, the Wnt effector β-catenin enhances SREBP-1-dependent Scd transcription and is in turn stabilized by MUFA generated by SCD/Scd2. This positive loop is caused by HuR-induced mRNA stabilization of LRP5/6. As such, SCD/Scd2 inhibition reduces cytosolic HuR, LRP5/6 stability, β-catenin stabilization, HSC activation and TIC self-renewal, and attenuates liver fibrosis and tumorigenesis in vivo.

Conclusion: The newly disclosed Wnt-SCD/Scd2-LRP5/6 loop may serve as a novel therapeutic target for alcohol-promoted liver fibrosis and tumorigenesis.

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3. THE MECHANISM OF ALCOHOL-INDUCED GOLGI FRAGMENTATION IN HEPATOCYTES

Pi-Wan Cheng\textsuperscript{1,3}, Carol A. Casey\textsuperscript{2,3} and Armen Petrosyan\textsuperscript{1}

\textbf{1} Department of Biochemistry and Molecular Biology, College of Medicine, and \textbf{2} Department of Internal Medicine, University of Nebraska Medical Center, Omaha, NE, USA. \textbf{3} Omaha Western Iowa Health Care System, VA Service, Department of Research Service, Omaha, NE, USA.

The abnormalities in the Golgi apparatus function are important for the development of alcoholic liver injury, but mechanism and consequences have not been defined. Previously, we found that formation of compact Golgi requires dimerization of the largest Golgi matrix protein, giantin, which is catalyzed by protein disulfide isomerase A3 (PDIA3). Here, in both HepG2 cells expressing alcohol dehydrogenase and hepatocytes isolated from alcohol-fed rats, we show that ethanol administration induces crucial Golgi disorganization, as reflected by conversion of its main body to the several mini-Golgi structures, exhibiting swollen and distended cisternae. This Golgi fragmentation was accompanied by reduced level of giantin and its dimer form, and surprisingly, by decreased content of Sar1a, the small GTPase which initiates formation of COPII vesicles. Further analysis revealed that ethanol blocks activation of Sar1a, thus preventing formation of COPII. We found that PDIA3 employs a COPII-dependent mechanism for Golgi targeting and that after ethanol treatment, this enzyme is arrested in the endoplasmic reticulum (ER), thus blocking formation of giantin dimer. Notably, Sar1a gene silencing in hepatocytes mimics the effect of ethanol: dedimerization of giantin, trapping PDIA3 in the ER, and large-scale alterations in Golgi morphology. EtOH induced Golgi disorganization appears to have no effect on ER-to-Golgi transportation of the hepatic asialoglycoprotein receptor (ASGP-R), but it does result in its deposition in cis-medial-, but not trans-Golgi, thereby preventing its delivery to the plasma membrane. Further, we found that EtOH administration results in S1943 phosphorylation of non-muscle Myosin IIA (NMIIA) heavy chain, thus facilitating its connection with Golgi enzymes, as detected by biochemical approaches and 3D Structured Illumination Microscopy. We revealed that NMIIA-P-S1943 competes with giantin for the Rab6a GTPase dimer, which was converted to monomer after Golgi fragmentation. Therefore, Rab6a plays a dual role in the Golgi, serving as master regulator of Golgi organization/disorganization, and NMIIA and Giantin have a ‘tag-of-war’ to modulate Golgi organization. Downregulation of NMIIA or overexpression of NMHC-IIAtailpiece, as well overexpression of dominant negative Rab6a(T27N), a GDP-bound mutant, preserved compact Golgi phenotype. Thus, targeting of NMIIA-P-S1943 may be important for prevention of alcohol metabolism’s damaging effects on the cell.

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4. DIFFERENTIAL ROLE FOR THE TRANSCRIPTIONAL AND NON-TRANSCRIPTIONAL FUNCTIONS OF IRF-3 IN CHRONIC ETHANOL-INDUCED LIVER INJURY

Sanz-Garcia C¹, Roychowdhury S¹, Chattopadhyay S², McMullen MR¹, Sen GC², Nagy LE¹

Departments of ¹Pathobiology and ²Molecular Genetics, Center for Liver Disease Research, Lerner Research Institute, Cleveland Clinic, Cleveland, Ohio, USA.

Background and Aims: Increased exposure of hepatic macrophages to lipopolysaccharide (LPS) and activation of TLR4 are important contributors to alcoholic liver disease (ALD). Interferon regulatory factor 3 (IRF3) is a master regulator of host responses to viral infection, but is also activated via the TLR4-MyD88-independent pathway. Upon activation, IRF3 is phosphorylated and translocated to the nucleus where it initiates a specific transcriptional program. Recently, two non-transcriptional functions for IRF3 have been identified: 1) a novel pro-apoptotic function termed RIG-I-induced IRF3-mediated pathway of apoptosis (RIPA) and 2) an interaction with the p65 subunit of NFκB that prevents p65 translocation to the nucleus. While IRF3 has been implicated in the progression of ethanol-induced liver injury, the transcriptional versus non-transcriptional functions of IRF3 have not been studied yet. Here we made use of IRF3-deficient mice (IRF3 KO), as well as a novel knock-in of a mutated IRF3 that cannot translocate to the nucleus and only retains the non-transcriptional functions of IRF3 (nt-IRF3 KI).

Methods: Wild-type C57BL/6, IRF3-KO and nt-IRF3 KI mice were allowed free access to an ethanol containing Lieber-deCarli diet (up to 32% ethanol, 25d) or pair-fed control diets. Measures of liver injury were assessed.

Results: Chronic ethanol feeding increased hepatic steatosis, plasma ALT and AST activities, expression of inflammatory cytokines and hepatocyte apoptosis in wild-type mice. IRF3 KO mice had reduced hepatic steatosis and hepatocyte apoptosis, but expression of TNFα protein, as well as ALT and AST concentrations, were still increased in response to ethanol feeding. In contrast, nt-IRF3 KI mice had reduced TNFα protein, ALT/AST and hepatic steatosis. Interestingly, while hepatocyte apoptosis was reduced in nt-IRF3 KI mice, apoptosis of cells with the morphologic appearance of hepatic macrophages was increased.

Conclusion: Taken together, these data suggest that there are distinct transcriptional and non-transcriptional functions of IRF3 in chronic ethanol-induced liver injury. Interestingly, increased macrophage apoptosis in the nt-IRF3 KI mice was associated with a protection from ethanol-induced inflammatory responses, suggesting a potential protective role for the non-transcriptional activities of IRF3 in the progression of chronic ethanol-induced liver injury.

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Inflammatory cell recruitment is a major feature of alcoholic liver injury however; the signals and cellular sources regulating the migration of these cells to the liver are still not well defined. C-C chemokine receptor type 2 (CCR2) is mainly expressed by active hepatic stellate cells (HSC) and is a key recruitment signal. Active HSC are also important sources of hydrogen peroxide resulting from the activation of NADPH oxidase 4 (NOX4). As the role of this NOX in early alcoholic liver injury has not been well studied, we hypothesized that NOX4 via hydrogen peroxide modulates the mRNA stability of CCR2 thereby inducing recruitment of inflammatory cells. Methods: NOX4 was studied in healthy human livers and in patients with alcoholic liver disease. Fl/fl and HSC specific [GFAP-cre NOX4 knockout mice (KO)] were pair-fed with the Lieber deCarli or control isocaloric diets, and CCR2, MCP-1, TNFα, IL-1β, IL-6, and Ly6C expression in the livers were assessed by real time qPCR. In vitro, NOX4 expression was studied in primary HSC exposed to acetaldehyde (Ac). CCR2 mRNA stability was assessed 1) in primary wt or NOX4KO HSC, and 2) in LX2 human HSC line transfected by Ad-NOX4 or control vector. Immunohistochemistry and western blots were done to detect the mRNA binding protein HuR subcellular localization and phosphorylation. Results: NOX4 was co-localized with αSMA-expressing activated HSC in liver biopsies of patients with alcoholic hepatitis. NOX4 mRNA was significantly induced in patients, as well as in mice on the Lieber deCarli diet. In the fl/fl mice on Lieber deCarli diet, TG content, lipid peroxidation and the expression of CCR2, TNFα, IL-6, MCP-1, Ly6C were significantly increased compared to the HSCNOX4Komice (p <0.05). NOX4 was induced in primary HSC by Ac treatment (p<0.05). NOX4 has significantly increased transcript stability of CCR2 (p<0.05) coinciding with cytoplasmic shuttling and phosphorylation of HuR. In conclusion: NOX4 is induced in early alcoholic liver injury in HSC and regulates CCR2 mRNA stability thereby promotes recruitment of inflammatory cells and production of proinflammatory cytokines.

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SESSION 4: Liver-Brain Inflammatory Axis in ALPD

1. THE LIVER-BRAIN AXIS: BRAIN ACETALDEHYDE AND ALCOHOL ADDICTION

Israel Y1, Quintanilla ME1, Karahanian E2, Rivera-Meza M1, Morales P1, Berrios-Carcamo P1, Salinas-Luypaert C1, Herrera-Marschitz M1

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Studies in Canada, Spain, Italy and Chile have shown that in animals the oxidation of ethanol by brain catalase is needed to generate endogenous brain acetaldehyde, which initiates chronic ethanol intake. Studies show that inhibition of brain catalase synthesis or increasing brain acetaldehyde oxidation fully prevent the development of chronic ethanol intake. Brain acetaldehyde spontaneouly condenses with dopamine forming salsolinol, a strong oxidant which may add to the brain inflammation induced by LPS. Salsolinol administration (i.p. or intra-VTA) leads to marked daylight binge-drinking in ethanol-naïve animals (2 to 2.5 g ethanol/kg in 60 minutes).

After a steady chronic ethanol intake is achieved the perpetuation of ethanol intake is no longer dependent on acetaldehyde but on a brain system that recognizes mechanisms which are markedly inhibited (70-75%) by the administration of N-acetyl cysteine a strong anti-oxidant, which does not inhibit the initiation of chronic ethanol intake. Other anti-oxidant mechanisms directed to the brain may to reduce chronic ethanol and virtually abolish binge drinking.

Alcoholic hepatitis is accompanied by increased inflammatory cytokines and oxidative stress (known to potentiate each other). Noteworthy, a meta-analysis of 22 clinical trials of alcoholic hepatitis treatment showed that while corticosteroids – well known for their anti-inflammatory activity- reduced patient mortality by 45%, the addition to corticosteroids of N-acetyl cysteine resulted in an 85% reduction of mortality. Upon discontinuation of N-acetyl cysteine administration, mortality return to expected levels.

Overall, both liver and brain react positively to the administration of a clinically approved drug such as N-Acetyl cysteine, which may become a treatment of choice for alcohol use disorders. [Supported Fondecyt 1130012, 1130241, 1120079, Millennium P09015F]

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2. IL-17 AND ALCOHOLIC LIVER AND BRAIN INFLAMMATION

Tatiana Kisseleva

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Alcoholic liver disease (ALD) progresses from a normal liver, to alcoholic steatohepatitis, fibrosis and hepatocellular carcinoma (HCC). Alcohol produces a systemic effect on other tissues and organs, including liver-brain axis and intestinal permeability. Liver is directly affected by alcohol. Alcohol-induced chronic hepatotoxicity results in systemic release of proinflammatory microbial products, toxic lipids (such as ceramides) and cytokines into the circulation, and exacerbates cytotoxic effect of alcohol on other organs, including development of insulin resistance and oxidative stress. Central nervous system (CNS) is the other major target of alcohol toxicity. Chronic alcohol consumption causes direct neurotoxic effects, such as neuronal apoptosis and astrogliosis, neurocognitive impairment. In addition, alcohol misuse establishes a liver-brain axis of neurodegeneration mediated by toxic lipid trafficking across the blood-brain barrier. Despite of intensive studies, the pathogenesis of alcohol-induced damage of the liver-brain axis is poorly understood. Furthermore, the role of Interleukine 17A (IL-17) cytokine in regulation of alcohol-mediated hepatо- and neurotoxicity has not been evaluated. IL-17-producing T helper (Th17) cells originate from naïve Th0 cells via TGF-β1/IL-6/IL-23-dependent activation of retinoid-related orphan receptor γt (ROR γt), which plays the central role in Th17 proliferation and IL-17 production. IL-17 signaling was implicated in mediation of autoimmunity, psoriasis, rheumatoid arthritis, fibrosis and tumorigenesis. Here we demonstrate that genetic deletion of IL-17 signaling in mice devoid of IL-17 Receptor A (IL-17RA/-/- mice) attenuates development of alcohol-induced progression steatohepatitis to fibrosis and HCC, and blocking IL-17 with anti-IL-17 antibody have a therapeutic effect on alcoholic liver fibrosis. In addition, administration of anti-IL-17 antibodies to mice with alcohol-induced liver fibrosis also prevented neuronal apoptosis and astrogliosis, suggesting that blocking of IL-17 may effectively prevent alcohol-induced hepatо- and neurotoxicity. If proven, IL-17 may become an attractive target for anti fibrotic therapy of patients with alcohol-induced liver fibrosis and HCC.

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3. MECHANISMS OF ALCOHOL-INDUCED BRAIN INNATE IMMUNE GENE INDUCTION

CrewsFT\textsuperscript{1}, VetrenoR\textsuperscript{1}, MasseyV\textsuperscript{1}, QinL\textsuperscript{1}, ColemanL\textsuperscript{1}, ZouJ\textsuperscript{1}

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Ethanol increases expression of innate immune genes that contribute to the development of alcoholic pathology. Innate immune genes induced by binge drinking include HMGB1, multiple Toll-like receptors, RAGE, multiple cytokines, and chemokines that are increased in blood, liver, and brain. There are multiple mechanisms of ethanol induction of immune signaling proteins. In brain slice cultures, ethanol treatment releases HMGB1 from cells and increases NFkB-DNA binding and transcription of CCL2, TNFalpha, RAGE, other cytokines, and Toll-like receptors. In vivo treatment of mice with ethanol for 10 days sensitizes mice to systemic and brain proinflammatory gene induction by the TLR agonists, LPS (TLR4), Poly I:C (TLR3), and imiquimod (TLR7), in part due to ethanol induction of multiple TLR receptors in multiple tissues. Data on ethanol induction of endogenous TLR agonists and receptors will be presented. Specifically, HMGB1-TLR4, Let7-TLR7, and TNFSF15 (TL1A)-TNFSF25 (Death Receptor 3) signaling induction by ethanol will be presented. Also discussed will be studies finding each of these immune signaling agonist-receptor pairs is increased in mice treated with ethanol (C57BL6-5gm/kg/day-10days) and in post-mortem brains of alcoholics. This is consistent with ethanol consumption inducing innate immune genes in alcoholics and genetic risk being related to induction of innate immune genes. Another mechanism of increased brain innate immune signaling involves systemic TNFalpha and other cytokine activation of brain innate immune responses. Ethanol increases blood endotoxin and systemic proinflammatory cytokines, and stress combined with ethanol further increases blood endotoxin. However, responses vary depending on the amount of ethanol consumed and dynamic changes during acute exposure and withdrawal, with prominent increases once ethanol has cleared. Further, many systemic changes occur in abstinence following intoxication. Interestingly, ethanol induction in the brain shows a long-lasting persistence not found systemically. Neuroimmune signaling and glutamate excitotoxicity are linked to alcoholic neurodegeneration. Further, alcohol-induced, long-lasting increases in brain neuroimmune gene expression promote persistent and long-term increases in alcohol consumption, suggesting alcohol-induced neuroimmune activation contributes to the neurobiology of alcohol use disorders. Funded by NIAAA

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Hepatic steatosis is a key feature in alcoholic liver disease (ALD) and is also thought to represent an important pathophysiological step during ALD development. Insulin is a key regulator of metabolism and exerts some of its effect by signaling in the CNS, where it restrains hepatic glucose production and lipolysis in adipose tissue for example, the latter through dampening of sympathetic outflow to adipose tissue. Our laboratory has previously demonstrated that binge drinking impairs brain insulin action in rats, defined as the ability of brain insulin to suppress hepatic glucose production and white adipose tissue (WAT) lipolysis (Lindtner et al, SciTraMed, 2013). Preventing brain insulin resistance during binge drinking through the pharmacological inhibition of protein-tyrosine phosphatase 1β (PTP1β), a negative regulator of insulin signaling, prevented the glucose intolerance seen after binge drinking. Thus, we propose that alcohol binging impairs brain insulin action, which increases sympathetic outflow to WAT resulting in unrestrained lipolysis and hepatic steatosis. Here we present studies examining the role of adipose tissue lipolysis in alcohol induced adipose tissue inflammation and hepatic steatosis using both conditional knock out mouse models and pharmacological inhibition of lipolysis. These studies provide support for the paradigm that the brain-adipose axis plays an important role in ALD.
1. ADOLESCENT INTERMITTENT ETHANOL REDUCES ADULT RAPHE NUCLEUS SEROTONERGIC NEURONS AND INCREASES INNATE IMMUNE EXPRESSION THAT IS PREVENTED BY EXERCISE

Vetreno RP, Crews FT

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Serotonergic neurons of the raphe nucleus regulate sleep, mood, endocrine function, and other processes that mature during adolescence. Alcohol abuse and binge drinking are common during human adolescence. We hypothesized that adolescent intermittent binge ethanol exposure would persistently alter the developing serotonergic system into adulthood. Using a Wistar rat model of adolescent intermittent ethanol (AIE; 5.0 g/kg, i.g., 2-day on/2-day off from postnatal day [P]25 to P55), we found a loss of dorsal raphe nucleus (DRN) serotonin (5-HT)-immunoreactive (+IR) neurons in late adolescence (P56) that persisted into adulthood (P220). Hypothalamic and amygdalar DRN serotonergic projections were reduced following AIE. Tryptophan hydroxylase 2, the rate-limiting 5-HT synthesizing enzyme, and vesicular monoamine transporter 2, which packages 5-HT into synaptic vesicles, were also reduced in the young adult midbrain following AIE treatment. Adolescent intermittent ethanol treatment increased expression of phosphorylated (activated) NF-κB p65 as well as markers of microglial activation (i.e., Iba-1 and CD11b) in the adult DRN. Administration of lipopolysaccharide to mimic AIE-induced microglial activation reduced 5-HT+IR and increased phosphorylated NF-κB p65+IR similar to AIE treatment. Voluntary exercise during adolescence through young adulthood prevented the AIE-induced loss of 5-HT+IR neurons, and blunted microglial marker and phosphorylated NF-κB p65+IR in the DRN. Together, these data report that AIE reduces serotonergic neurons in the adult brain, possibly through an innate immune mechanism, which might impact adult cognition, arousal, or reward sensitivity. Further, exercise prevents the deleterious effects of AIE on the serotonergic system of the young adult DRN.

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2. AP-001 FOR TREATING PANCREATIC ADENOCARCINOMA

Edderkaoui M, Chheda C, Soufi B, Murali R, Pandol SJ

Cedars-Sinai, UCLA and VA Los Angeles.

Pancreatic ductal adenocarcinoma (PDAC) is a deadly disease with no effective treatment. We have developed a new drug AP-001 that targets at the same time the glycogen synthase kinase 3 beta (GSK-3β) and histone deacetylase (HDAC), important mediators of cancer progression. PDAC and normal cells were treated with different doses of AP-001 and cell survival, apoptosis, epithelial to mesenchymal transition (EMT) markers, and invasion were measured. Treatments were performed alone and in combination with gemcitabine or exposure to irradiation. In vivo, KrasLSL-G12D/+; Pdxcre (KC) mice were exposed to cigarette smoke and alcohol to induce cancer progression. KC mice were intraperitoneally injected with GSK-3β and HDAC-I/II inhibitors (4mg/Kg and 50mg/Kg, respectively) trice/week for 6 weeks. KrasLSL-G12D/+;Trp53LSL-R172H/+;Pdxcre (KPC) mice were intraperitoneally injected with AP-001 (5mg/Kg) trice/week until death. Survival and number of metastasis were determined. Pancreatic lesions and tumor grades, fibrosis and inflammation were measured by immunohistochemistry and Western.

AP-001 significantly (at nanomolar concentrations) decreased cancer cell survival and increased apoptosis in several PDAC cells. The same doses did not affect survival of normal hepatocytes and pancreatic ductal cells. AP-001 increased histone acetylation, inhibited GSK-3β activity, decreased expression of markers of EMT/metastasis and cancer stemness, and prevented cancer cell migration. Furthermore, AP-001 sensitized PDAC cells to gemcitabine and radiotherapy. In vivo, Smoking and alcohol exposure increased GSK-3β and HDAC activities and stimulated formation of pancreatic intraepithelial neoplasia (PanIN) lesions in KC mice. Combination of GSK-3β and HDAC inhibitors prevented this effect. Treatment with AP-001 significantly increased KPC mice survival by 42%. Histologic examination showed that AP-001 treatment inhibited formation of both PanIN lesions and late lesions (carcinoma) in the pancreas of these animals. Distal metastasis was decreased from 29% in control KPC mice to 0% in AP-001 treated KPC mice.

We have designed a novel drug that shows a significant anti-cancer effect in a very aggressive mouse model of experimental PDAC. Importantly, AP-001 increased the survival of KPC mice and prevented metastasis in vivo with no significant toxicity to normal cells.

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3. SUB-CHRONIC ETHANOL EXPOSURE SENSITIZES THE LIVER TO IMIQUIMOD-INDUCED INFLAMMATORY RESPONSE IN MICE

Massey VL\textsuperscript{1}, Qin L\textsuperscript{1}, Crews FT\textsuperscript{1}

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Background. Chronic liver inflammation is a driving factor in the development of alcoholic liver disease (ALD). Toll-like receptors (TLRs) are a family of pattern recognition receptors that mediate the innate immune response through the recognition of pathogen-associated molecular patterns. TLR7 is an endosomal TLR that is activated by single stranded RNA, including viral RNA and some endogenous micro-RNAs. Ethanol exposure increases hepatic immune gene expression including induction of TLR. Therefore, the purpose of this study was to test the hypothesis that sub-chronic alcohol exposure alters expression of TLR7 and sensitizes the liver to imiquimod, a TLR7 agonist, in mice.

Methods. Male C57Bl6/J mice were exposed to a binge-like dose of ethanol (5 g/kg, i.g.) once per day for 10 days. On the day following the last dose of ethanol, mice were administered a TLR7 agonist (imiquimod; 2.5 mg/kg, i.p.). Quantitative PCR was used to determine hepatic mRNA expression and paraffin-embedded liver tissue was stained with hematoxylin & eosin.

Results. Although ethanol exposure did not increase proinflammatory cytokine or TLR8 mRNA, it significantly increased hepatic mRNA for TLR7 by more than 200% and TLR4 by 300%. Imiquimod alone did not significantly affect TLR expression. Pretreatment with ethanol followed by imiquimod significantly increased the hepatic mRNA expression of TNF\textsubscript{a}, MIP-2, and MCP-1 by 700%, 600%, and 1000%, respectively, compared to control. Interestingly, MyD88, a key TLR signaling protein, TLR9, as well as IFN\textsubscript{a} and IFN\textsubscript{b} showed an increasing trend with either ethanol or imiquimod exposure, whereas combined exposure increased expression of these genes by at least 600%. Neither ethanol nor imiquimod caused overt morphologic changes in the liver at the time point studied.

Conclusions. These findings support the hypothesis that sub-chronic ethanol exposure can sensitize the liver to TLR7 induced cytokine responses. This sensitization may be mediated, at least in part, via up-regulation of hepatic TLR7 expression.

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4. PROINFLAMMATORY SIGNALING REGULATES VOLUNTARY ALCOHOL INTAKE AND STRESS INDUCED CONSUMPTION AFTER EXPOSURE TO SOCIAL DEFEAT STRESS IN MICE

Karlsson C¹, Schank JR², Rehman F³, Stojakovic A¹, Björk K¹, Barbier E¹, Solomon M³, Tapocik JD³, Engblom D¹, Thorsell A¹, Heilig M¹

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Proinflammatory activity has been postulated to play a role in addictive processes and stress responses, but the underlying mechanisms remain largely unknown. Here, we examined the role of interleukin 1 (IL-1) and tumor necrosis factor-α (TNF-α) in regulation of voluntary alcohol consumption, alcohol reward and stress-induced drinking. Mice with a deletion of the IL-1 receptor I gene (IL-1RI KO) exhibited modestly decreased alcohol consumption. However, IL-1RI deletion affected neither the rewarding properties of alcohol, measured by conditioned place preference (CPP), nor stress-induced drinking induced by social defeat stress. Previous studies show that TNF-α signaling can compensate for phenotypic consequences of IL1-RI deletion. We then hypothesized that double knockout (KO) mice lacking both IL-1RI and TNF-1 receptors (TNF-1R) may be involved in regulation of alcohol intake. Double KOs consumed significantly less alcohol than control mice over a range of alcohol concentrations. The combined deletion of TNF-1R and IL-1RI did not influence alcohol reward, but did prevent increased alcohol consumption resulting from exposure to repeated bouts of social defeat stress. Taken together, these data indicate that IL-1RI and TNF-1R contribute to regulation of stress induced, negatively reinforced drinking perhaps through overlapping signaling events downstream of these receptors, while leaving rewarding properties of alcohol largely unaffected.

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