ETHANOL, ITS ACTIVE METABOLITES, AND THEIR MECHANISMS OF ACTION: NEUROPHYSIOLOGICAL AND BEHAVIORAL EFFECTS

EDITED BY: Elio Acquas, John D. Salamone and Mercè Correa

PUBLISHED IN: Frontiers in Behavioral Neuroscience

frontiers Research Topics

frontiers

ISSN 1664-8714
ISBN 978-2-88945-516-4
DOI 10.3389/978-2-88945-516-4

About Frontiers

Frontiers is more than just an open-access publisher of scholarly articles: it is a pioneering approach to the world of academia, radically improving the way scholarly research is managed. The grand vision of Frontiers is a world where all people have an equal opportunity to seek, share and generate knowledge. Frontiers provides immediate and permanent online open access to all its publications, but this alone is not enough to realize our grand goals.

Frontiers Journal Series

The Frontiers Journal Series is a multi-tier and interdisciplinary set of open-access, online journals, promising a paradigm shift from the current review, selection and dissemination processes in academic publishing. All Frontiers journals are driven by researchers for researchers; therefore, they constitute a service to the scholarly community. At the same time, the Frontiers Journal Series operates on a revolutionary invention, the tiered publishing system, initially addressing specific communities of scholars, and gradually climbing up to broader public understanding, thus serving the interests of the lay society, too.

Dedication to Quality

Each Frontiers article is a landmark of the highest quality, thanks to genuinely collaborative interactions between authors and review editors, who include some of the world's best academicians. Research must be certified by peers before entering a stream of knowledge that may eventually reach the public - and shape society; therefore, Frontiers only applies the most rigorous and unbiased reviews.
Frontiers revolutionizes research publishing by freely delivering the most outstanding research, evaluated with no bias from both the academic and social point of view. By applying the most advanced information technologies, Frontiers is catapulting scholarly publishing into a new generation.

What are Frontiers Research Topics?

Frontiers Research Topics are very popular trademarks of the Frontiers Journals Series: they are collections of at least ten articles, all centered on a particular subject. With their unique mix of varied contributions from Original Research to Review Articles, Frontiers Research Topics unify the most influential researchers, the latest key findings and historical advances in a hot research area! Find out more on how to host your own Frontiers Research Topic or contribute to one as an author by contacting the Frontiers Editorial Office: researchtopics@frontiersin.org

ETHANOL, ITS ACTIVE METABOLITES, AND THEIR MECHANISMS OF ACTION: NEUROPHYSIOLOGICAL AND BEHAVIORAL EFFECTS

Topic Editors:
Elio Acquas, University of Cagliari, Italy
John D. Salamone, University of Connecticut, United States
Mercè Correa, Universitat Jaume I, Spain

Ethanol, the main psychopharmacologically active ingredient of alcoholic drinks, represents a paradigmatic example of a research subject intrinsically able to perpetually self-generate interdisciplinary cutting-edge investigations.

This eBook was inspired by the aim of providing an up-to-date characterization of the diverse effects of ethanol, of the possible mechanisms of action on different intracellular systems as well as of the hypothesized actions of ethanol and/or its metabolites on various neurotransmitters and neuromodulators.

Indeed, the eBook provides a factual example of an excellent synthesis on the complex relationship between ethanol and its main biologically active metabolites (Chapter 1), on the behavioral and molecular consequences of early exposure to them (Chapter 2), on the recent proposals, advanced by the preclinical research, for new therapeutic approaches to distinct aspects of alcoholism (Chapter 3) and on the most recent and original preclinical evidence of the interactions between ethanol and/or its metabolites and the dopaminergic, adenosinergic and endocannabinoidergic systems (Chapter 4).

Overall we believe that this eBook accomplishes its main goals of widening the perspective on this research subject and offering the readership a newer and, simultaneously, up-to-date and comprehensive scenery on ethanol's and ethanol's active metabolites neurophysiological and behavioral effects.

Citation: Acquas, E., Salamone, J. D., Correa, M., eds. (2018). Ethanol, Its Active Metabolites, and Their Mechanisms of Action: Neurophysiological and Behavioral Effects. Lausanne: Frontiers Media. doi: 10.3389/978-2-88945-516-4

Table of Contents

CHAPTER 3

CHAPTER 4

frontiers in Behavioral Neuroscience

EDITORIAL
published: 17 May 2018
doi: 10.3389/fnbeh.2018.00095

Editorial: Ethanol, Its Active Metabolites, and Their Mechanisms of Action: Neurophysiological and Behavioral Effects

Elio Acquas[1]*, *John D. Salamone*[2]* and *Mercè Correa*[3]*

[1] Department of Life and Environmental Sciences, University of Cagliari, Cagliari, Italy, [2] Department of Psychology, University of Connecticut, Storrs, CT, United States, [3] Department of Psychobiology, Universitat Jaume I, Castelló, Spain

Keywords: acetaldehyde, adenosine, caffeine, D-penicillamine, ethanol metabolism, fenofibrate, nicotine, salsolinol

OPEN ACCESS

Edited and reviewed by:
Nuno Sousa,
Instituto de Pesquisa em Ciências da Vida e da Saúde (ICVS), Portugal

***Correspondence:**
Elio Acquas
acquas@unica.it
John D. Salamone
john.salamone@uconn.edu
Mercè Correa
correa@uji.es

Received: *09 March 2018*
Accepted: *23 April 2018*
Published: *17 May 2018*

Citation:
Acquas E, Salamone JD and Correa M (2018) Editorial: Ethanol, Its Active Metabolites, and Their Mechanisms of Action: Neurophysiological and Behavioral Effects. Front. Behav. Neurosci. 12:95. doi: 10.3389/fnbeh.2018.00095

Editorial on the Research Topic

Ethanol, Its Active Metabolites, and Their Mechanisms of Action: Neurophysiological and Behavioral Effects

Over the last century the neurobiology of ethanol has come a long way since the original proposal that the two main pharmacological effects, intoxication and sedation, could be explained by the ability of ethanol to cause perturbation of neuronal membrane lipids (Meyer, 1901). This view, although questioned on the basis of the advancement of knowledge on the effects of ethanol on membrane proteins and intracellular kinases (Tabakoff and Hoffman, 2013), continued to be highly considered up to the late'80s, whereby disruption of the order or increase of the fluidity of biological membranes was still identified as a critical determinant for its biological and behavioral effects (Kalant, 1975).

Current views on the neurobiology of ethanol have been based on preclinical studies conducted in the last few decades. New approaches are based on processes ranging from identifying ethanol-sensitive molecules to determining the role of such molecules in ethanol-mediated physiological and behavioral changes (bottom-up), but also by establishing the correlation between ethanol-dependent physiological and behavioral effects and the involvement of specific molecular mechanisms (top-down). These views presently allow an in-depth distinction between direct (ion channels, protein kinases) and indirect (intracellular signaling proteins, growth and transcription factors) molecular targets (Abrahao et al., 2017). In addition, by virtue of its peripheral and central metabolism, ethanol generates a number of biologically active molecules. This raises the need for characterization of the relationship between ethanol, its metabolites (mainly acetaldehyde but also acetate and salsolinol), and some of their central and peripheral effects. In this regard, acetaldehyde

in particular has received a great deal of attention. After the serendipitous observation made by Chevens (1953) that patients under treatment with the aldehyde dehydrogenase (ALDH) inhibitor antabuse experienced pleasurable effects upon taking small amounts of ethanol, and the discovery of catalase-mediated central metabolism of ethanol (Aragon and Amit, 1985), it was recognized that acetaldehyde could be significantly mediating ethanol's stimulant effects on behavior. These discoveries led to the recognition of acetaldehyde as a pharmacologically neuroactive molecule (Correa et al., 2012). Along the way there have been long discussions of controversial issues such as the uncertainties surrounding central acetaldehyde determination (discussed in the present topic by the review of Enrico and Diana), and the ability of acetaldehyde to cross the blood brain barrier. More recently, much attention has focused on the differential role of acetaldehyde in acquiring and maintaining voluntary ethanol intake (thoroughly discussed in the contributions by Israel et al. and Peana et al.).

In view of the continued progress being made in research on ethanol and its metabolites, we decided to host this research topic 4 years after another successful research topic "Neuroactive metabolites of ethanol: a behavioral and neurochemical synopsis" (Correa et al., 2014). We reasoned that widening the perspective beyond ethanol's active metabolites to ethanol's neurophysiological and behavioral effects, as well as proposed mechanisms of action, would result in an up-to-date and integrated view of current research on the neurophysiological and behavioral effects of ethanol and its active metabolites.

The four review papers of this topic make a picture of the *state of the art* of preclinical research on the role of ethanol metabolites, ethanol intake, and ethanol-elicited motivated behavior. Israel et al. discuss the recent evidence gained using high-ethanol drinker rats of the UChB line, pointing to the differential role played by acetaldehyde in acquisition, maintenance, and relapse. In particular, whereas acetaldehyde availability seems critical for the initial boost (first hit) of ethanol self-administration (acquisition), and also for relapse after long term withdrawal, this does not apply to the maintenance phase since that is not prevented by the interference with catalase-mediated central metabolism of ethanol. A similar view is presented in the paper by Peana et al. which also discusses the recent hypothesis that 4-methylpyrazole may indirectly affect central as well as peripheral metabolism of ethanol. In fact, mainly known for its ability to inhibit alcohol dehydrogenase (ADH), 4-methylpyrazole may also affect the availability of hydrogen peroxide to act as a factor critical for catalase-mediated oxidation of ethanol. In addition, the reviews by Israel et al., and Peana et al. place a great emphasis on salsolinol as a bioactive molecule that shares locomotor stimulant and motivational properties with ethanol and acetaldehyde. In this regard, the paper by Berrios-Carcamo et al. adding to the recent demonstration that systemic administration of salsolinol exerts central effects such as behavioral sensitization and conditioned place preference, provides fresh and elegant biochemical (classical G protein-adenylate cyclase pathway assessments), and molecular (docking simulations using the crystal structure of the mouse μ opioid receptor) evidence in support of the suggestion that the central actions of salsolinol are mediated by μ opioid receptors.

A distinct and original point of view in regards to the ethanol-acetaldehyde relationship is provided by the contribution of Brancato et al. Besides focusing on the acetaldehyde's mirroring effects of ethanol in the brain, these authors center the discussion of their paper on the relevance of three critical players (a "ménage à trois" on their words) worth of further investigations: (1) the mesolimbic dopamine system, (2) the stress response system, due to the acetaldehyde-mediated activation of the hypothalamic-pituitary-adrenal (HPA) axis and increased CRH and NPY expression, and (3) the endocannabinoid system, due to the ability of CB1 genetic deletion and receptor antagonists to prevent behavioral and neuroendocrine effects of acetaldehyde.

In another review Virgolini et al. discuss the available literature on the ability of the environmental contaminant lead (Pb) to affect the motivational properties of ethanol. This analysis, based on the observation that Pb may affect catalase-mediated central ethanol metabolism as well as ethanol intake, highlights that early exposure to Pb may increase susceptibility to engage in abnormal ethanol taking behaviors through an interference with its central metabolizing enzymes.

Two contributions to this research topic focus on the role that pre-natal exposure to acetaldehyde may have on post-natal acceptance to ethanol as well as on respiratory plasticity in newborns. Gaztañaga et al. report that acetaldehyde acts as a reinforcer in the appetitive learning that occurs upon ethanol exposure during the late gestational days. In particular, this paper shows that pre-natal brain acetaldehyde formation via catalase may be responsible for post-natal acceptance of ethanol, evidence gained by studies involving administration of ethanol and the acetaldehyde-sequestering agent, D-penicillamine, to dams. Moreover, an evaluation of the consequences of exposure of the immature brain to ethanol and acetaldehyde in terms of subsequent self-administration procedures is offered by the study from Acevedo et al. demonstrating that early exposure to both compounds exerts similar effects on respiratory plasticity and thermoregulatory alterations of the neonates as well as on seeking behavior of ethanol as a reinforcer in an operant task in neonate rats.

The ability of previous exposure to ethanol or to environmental enrichment in modulating ethanol consumption in adulthood has been taken into account in the studies by Carrara-Nascimento et al. and Berardo et al. In particular, using a three-bottle choice paradigm to evaluate the escalation into ethanol consumption in adulthood, Carrara-Nascimento et al. showed that rats that received ethanol during adolescence had greater intake of ethanol. Berardo et al. on the other hand, analyzed, in male and female rats, the consequences of early-life exposure to maternal separation (post-natal days 1–21) and environmental enrichment (post-natal days 21–42) on ethanol consumption and found that male

but not female rats exposed to environmental enrichment consume more ethanol during late adolescence in a two-bottle intake procedure than controls, a result not affected by previous experience of maternal separation. Moreover, since heightened exploration of novel stimuli and greater risk-taking behaviors were more evident in male rats exposed to enriched environments, these authors postulate that such increases in ethanol consumption could be due to the effects of exposure to enriched environment upon exploratory and risk-taking behaviors.

The suggestion of potential therapeutic approaches for preventing relapse in alcoholism and abnormal ethanol taking behaviors, which originated based on preclinical evidence, has been dealt with in the contributions by Orrico et al. for the suggestion of D-penicillamine, and by Rivera-Meza et al. for the suggestion of the peroxisome proliferator-activated receptor alpha (PPARα) agonist, fenofibrate. In particular, Orrico et al. discuss recent evidence on the effectiveness of the acetaldehyde sequestering agents in the alcohol deprivation effect, a reliable operant rodent model of relapse-like drinking behavior, which allowed a comparison of the effectiveness of D-Penicillamine with other FDA approved medications such as Acamprosate, Nalmefene and Naltexone. However, based on the conflicting evidence that D-penicillamine may or may not represent a valid pharmacological approach against voluntary ethanol intake in long-term experienced patients, the authors conclude that their suggestion of D-penicillamine as a therapeutic agent against relapse necessitates full clinical testing either alone and in association with other FDA approved medications such as nalmefene.

Using high drinkers UChB rats Rivera-Meza et al. extend their own previous work on the ability of fenofibrate to affect voluntary ethanol intake by a peripheral action linked to increased liver catalase expression and, hence, to increased peripheral acetaldehyde, to the possibility that fenofibrate may also act by a centrally-mediated mechanism. To address this point, the authors evaluated the ability of fenofibrate to affect ethanol-elicited conditioned place preference and voluntary ethanol or saccharine intake. The results of the study show that fenofibrate prevents ethanol-elicited conditioned place preference but also decreases ethanol and saccharin intake, thus supporting the suggestion that its actions might be ascribed to both peripherally- and centrally-mediated mechanisms, perhaps linked to catalase overexpression in the liver but not in the brain.

The contribution by Bassareo et al. provides original evidence of the involvement of nucleus accumbens shell and core dopamine transmission in response-contingent 10% ethanol self-administration under a FR1 schedule of nose-poking, and compares this involvement with that of 20% sucrose and of 10% ethanol + 20% sucrose. The results of this study reveal that active ethanol self-administration similarly increases dopamine transmission in the shell and core subdivisions, whereas under extinction trial this is preferentially increased in the shell of the accumbens. In contrast, under sucrose operant taking and extinction, dopamine transmission increases selectively in the shell overall demonstrating that the 10% ethanol self-administration procedure, without the interference of moving the animals from the home cage to the operant box, increases dopamine in both accumbens subdivisions and that these play different roles in sucrose as compared to ethanol reinforcement stimuli.

Finally, two research papers address the point of how other highly consumed drugs (nicotine and caffeine) can interfere in ethanol's actions. Coming from the perspective that tobacco use presents a strong positive correlation with alcohol use, the interesting research by Lárraga et al. investigates the relationship between exposure to 10 days of nicotine, or ethanol, or nicotine + ethanol intravenous self-administration, age (adolescents or adults) and sex on ethanol intake in adulthood determined using the two-bottle choice procedure. The results of this longitudinal study indicate major age- and sex-dependent differences whereby adolescent males that appear more sensitive to the reinforcing effects of nicotine + ethanol also result to have greater ethanol intake, suggesting that early exposure to nicotine may determine greater vulnerability to alcohol abuse. Authors conclude, in a translational perspective, that this evidence provides strong support for the suggestion to limit adolescent access to nicotine and tobacco products (including e-cigarettes). The research by López-Cruz et al. focuses on the possible impact that ethanol, caffeine and their interaction may exert on motivation for social contact (recognition and memory) as assessed in CD-1 mice in a three-chambered box. Based on the observations that ethanol affects social interaction in a biphasic manner without affecting social preference, while caffeine reduces social contact and blocks social preference, and that ethanol and caffeine have opposite effects on adenosine system, this study tests the hypothesis that a common mechanism of action, via the adenosine system, may regulate these opposite actions. Results showed that ethanol, at appropriate doses, could reverse the caffeine-mediated reduction of social exploration. However, given that selective antagonists of the adenosine A_1 and A_{2A} receptor subtypes do not mimic the effects of caffeine, the authors conclude, from a translational perspective, that the usefulness of highly caffeinated drinks in counteracting high doses of ethanol-induced impairments in social processes is questionable.

AUTHOR CONTRIBUTIONS

All authors listed have made a substantial, direct and intellectual contribution to the work, and approved it for publication.

ACKNOWLEDGMENTS

EA was supported by Fondazione di Sardegna and Regione Autonoma della Sardegna (F71J090006200002 and B71J11001480002_1), Italy; JDS was supported by NIH/NIMH R03MH112984, USA; MC was supported by a project from U.J.I. P1.1 A 2013-01, Spain.

REFERENCES

Abrahao, K. P., Salinas, A. G., and Lovinger, D. M. (2017). Alcohol and the brain: neuronal molecular targets, synapses, and circuits. *Neuron* 96, 1223–1238. doi: 10.1016/j.neuron.2017.10.032

Aragon, C. M., and Amit, Z. (1985). A two dimensional model of alcohol consumption: possible interaction of brain catalase and aldehyde dehydrogenase. *Alcohol* 2, 357–360.

Chevens, L. C. (1953). Antabuse addiction. *Br. J. Med*. 1, 1450–1451. doi: 10.1136/bmj.1.4825.1450-c

Correa, M., Acquas, E., and Salamone, J. D. (2014). The renaissance of acetaldehyde as a psychoactive compound: decades in the making. *Front. Behav. Neurosci*. 22, 249. doi: 10.3389/fnbeh.2014.00249

Correa, M., Salamone, J. D., Segovia, K. N., Pardo, M., Longoni, R., Spina, L., et al. (2012). Piecing together the puzzle of acetaldehyde as a neuroactive agent. *Neurosci. Biobehav. Rev*. 36, 404–430. doi: 10.1016/j.neubiorev.2011.07.009

Kalant, H. (1975). Direct effects of ethanol on the nervous system. *Fed. Proc*. 34, 1930–1941.

Meyer, H. (1901). Zur Theorie der Alkoholnarkose. Naunyn Schmiedebergs Arch. *Pharmacol*. 46, 338–346.

Tabakoff, B., and Hoffman, P. L. (2013). The neurobiology of alcohol consumption and alcoholism: an integrative history. *Pharmacol. Biochem. Behav*. 113, 20–37. doi: 10.1016/j.pbb.2013.10.009

Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

frontiers in Behavioral Neuroscience

REVIEW
published: 04 April 2017
doi: 10.3389/fnbeh.2017.00057

Acquisition, Maintenance and Relapse-Like Alcohol Drinking: Lessons from the UChB Rat Line

Yedy Israel[1], Eduardo Karahanian[2], Fernando Ezquer[3], Paola Morales[1], Marcelo Ezquer[3], Mario Rivera-Meza[4], Mario Herrera-Marschitz[1] and María E. Quintanilla[1]*

[1]Molecular and Clinical Pharmacology Program, Institute of Biomedical Sciences, University of Chile, Santiago, Chile, [2]Center for Biomedical Research, Universidad Autónoma de Chile, Santiago, Chile, [3]Centro de Medicina Regenerativa, Facultad de Medicina Clínica Alemana-Universidad del Desarrollo, Santiago, Chile, [4]Department of Pharmacological and Toxicological Chemistry, Faculty of Chemical and Pharmaceutical Sciences, University of Chile, Santiago, Chile

This review article addresses the biological factors that influence: (i) the *acquisition* of alcohol intake; (ii) the *maintenance* of chronic alcohol intake; and (iii) *alcohol relapse-like* drinking behavior in animals bred for their high-ethanol intake. Data from several rat strains/lines strongly suggest that catalase-mediated brain oxidation of ethanol into acetaldehyde is an absolute requirement (up 80%–95%) for rats to display ethanol's reinforcing effects and to initiate chronic ethanol intake. Acetaldehyde binds non-enzymatically to dopamine forming salsolinol, a compound that is self-administered. In UChB rats, salsolinol: (a) generates marked sensitization to the motivational effects of ethanol; and (b) strongly promotes binge-like drinking. The specificity of salsolinol actions is shown by the finding that only the R-salsolinol enantiomer but not S-salsolinol accounted for the latter effects. Inhibition of brain acetaldehyde synthesis does not influence the *maintenance* of chronic ethanol intake. However, a prolonged ethanol withdrawal partly returns the requirement for acetaldehyde synthesis/levels both on chronic ethanol intake and on alcohol relapse-like drinking. Chronic ethanol intake, involving the action of lipopolysaccharide diffusing from the gut, and likely oxygen radical generated upon catechol/salsolinol oxidation, leads to oxidative stress and neuro-inflammation, known to potentiate each other. Data show that the administration of N-acetyl cysteine (NAC) a strong antioxidant inhibits chronic ethanol *maintenance* by 60%–70%, without inhibiting its initial intake. Intra-cerebroventricular administration of mesenchymal stem cells (MSCs), known to release anti-inflammatory cytokines, to elevate superoxide dismutase levels and to reverse ethanol-induced hippocampal injury and cognitive deficits, also inhibited chronic ethanol maintenance; further, *relapse-like ethanol drinking* was inhibited up to 85% for 40 days following intracerebral stem cell administration. Thus: (i) ethanol must be metabolized intracerebrally into acetaldehyde, and further into salsolinol, which appear responsible for promoting the *acquisition* of the early reinforcing effects of ethanol; (ii) acetaldehyde is not responsible for the *maintenance* of chronic ethanol intake, while other mechanisms are indicated; (iii) the systemic administration of NAC, a strong antioxidant markedly inhibits the *maintenance* of chronic ethanol intake; and (iv) the intra-cerebroventricular administration of anti-inflammatory and antioxidant MSCs inhibit both the *maintenance* of chronic ethanol intake and *relapse-like drinking*.

Keywords: ethanol, acetaldehyde, catalase, relapse, reinforcement (psychology), inflammation, stem cells, reactive oxygen species

OPEN ACCESS

Edited by:
Merce Correa,
Jaume I University, Spain

Reviewed by:
Carla Cannizzaro,
University of Palermo, Italy
M. Gabriela Chotro,
University of the Basque Country UPV/EHU, Spain

****Correspondence:***
Yedy Israel
yisrael@uchile.cl

Received: *22 November 2016*
Accepted: *20 March 2017*
Published: *04 April 2017*

Citation:
Israel Y, Karahanian E, Ezquer F, Morales P, Ezquer M, Rivera-Meza M, Herrera-Marschitz M and Quintanilla ME (2017) Acquisition, Maintenance and Relapse-Like Alcohol Drinking: Lessons from the UChB Rat Line. Front. Behav. Neurosci. 11:57. doi: 10.3389/fnbeh.2017.00057

INTRODUCTION

This review addresses the biological factors that influence: (i) the *acquisition* of alcohol intake; (ii) the *maintenance* of chronic alcohol intake; and (iii) *alcohol relapse-like* drinking behavior in animals bred for their high-ethanol intake.

Two animal lines derived from the Wistar strain generated 60 years ago (see Mardones and Segovia-Riquelme, 1983; Quintanilla et al., 2006) have been kept for over 90 generations by selective and genetic breeding. These are: an Abstainer rat (UChA) line and a high-ethanol drinker line referred to as Bibulous rat (UChB). The mechanisms leading to alcohol rejection in the virtually abstainer UChA line are mainly related to polymorphisms in nuclear and mitochondrial genes that lead to a slow metabolism of acetaldehyde and to high blood acetaldehyde levels. These UChA studies have been previously described (Sapag et al., 2003; Quintanilla et al., 2005, 2006; Israel et al., 2013) and thus not covered in the present review. Studies conducted in UChB rats are indicated in the text.

Alcohol-Use Disorders: Genetic Aspects

Since alcoholism is 40%–60% genetically determined (Heath et al., 1997; Li, 2000), animals bred to consume high amounts of alcohol while on a constant environment might provide an answer for an elusive single "alcoholism gene" or the lack thereof. It took years for science to conclude that there is no single gene that could promote a high ethanol intake. Such a view could already be derived from crosses between inbred mice with markedly different ethanol intakes: the second generation (F_2) of crosses between high-intake (C57BL) and low intake animals (DBA) results in animals presenting the complete alcohol intake phenotype spectrum spanning their original strains (Phillips et al., 1994).

While in human and animals, analyses of hundreds of genes and genome-wide studies indicate that several polymorphisms or chromosomal markers correlate with alcohol intake and/or alcohol use disorders, these polymorphisms/markers have only minor effects in predicting alcohol-use disorders, compared to the marked effect of the polymorphisms of genes coding for alcohol and acetaldehyde metabolizing enzymes. The reader is referred to a recent review in this area (Tawa et al., 2016).

ACQUISITION OF ETHANOL INTAKE

The Reinforcing Effect of Ethanol-Derived Brain Acetaldehyde

A number of studies in laboratories in Spain, Chile and Italy, using Sprague-Dawley, UChB or Wistar rats, respectively (Tampier and Mardones, 1979; Aragon and Amit, 1992; Peana et al., 2008) have indicated that acetaldehyde generated in the brain by the action of catalase mediates the ethanol reinforcing mechanism. Acetaldehyde generated by the hepatic metabolism of ethanol and present in blood at levels within 10–50 μM, does not cross the blood brain barrier (Eriksson et al., 1977; Lindros and Hillbom, 1979; Petersen and Tabakoff, 1979; Stowell et al., 1980). However, large doses of exogenous acetaldehyde are able to overcome the blood brain barrier limitation. It has been shown that a single intraperitoneal injection of 50 mg acetaldehyde/kg, resulting in blood levels of 350–400 μM acetaldehyde, doubles voluntary ethanol intake in UChB rats (Quintanilla and Tampier, 2003). Thus, a large dose of acetaldehyde sensitizes ethanol reinforcement in UChB rats. This sensitizing effect may be mediated by brain-generated salsolinol formed by the condensation of acetaldehyde and dopamine (*vide infra*).

Operant self-administration studies have shown that rats bred as alcohol high-drinkers (Indiana University, P and HAD rats) will bar-press to self-administer both ethanol and acetaldehyde into the ventral tegmental area (VTA). Noteworthy, the levels of acetaldehyde required for self-administration into the VTA are three orders of magnitude lower for acetaldehyde, in the range of 10 μM than those for ethanol, which are in the range of 10–20 mM (Rodd et al., 2005). These studies indicate that as a reinforcing agent acetaldehyde is more potent than ethanol.

Studies on the mechanisms that generate brain acetaldehyde in Wistar rats show that catalase is responsible for 70% of the brain oxidation of ethanol into acetaldehyde (Zimatkin et al., 2006). Acetaldehyde is rapidly converted into acetate, likely via a low Km aldehyde dehydrogenase (ALDH). In the presence of ethanol, acetate levels in brain homogenates are 7-fold greater than those of acetaldehyde (Zimatkin et al., 2006). Studies by Zimatkin et al. (2006) also suggest that 15% of brain acetaldehyde is generated from CYP2E1 (**Figure 1**).

Two types of studies conducted in UChB rats strongly suggest that the generation of acetaldehyde in the VTA is *an absolute requirement for the acquisition* of alcohol reinforcement:

(a) *Genetic inhibition of catalase synthesis.* **Figure 2A** shows that the intra VTA administration of a lentiviral vector coding for an anticatalase shRNA blocked ethanol intake by 95%. It is noted that ethanol administration to Sprague-Dawley and UChB rats significantly increases dopamine release in nucleus accumbens (Imperato and Di Chiara, 1986; Di Chiara and Imperato, 1988; Quintanilla et al., 2007; Bustamante et al., 2008), while blocking the synthesis of catalase by the injection of the lentiviral vector coding for an shRNA anticatalase abolished the increases in

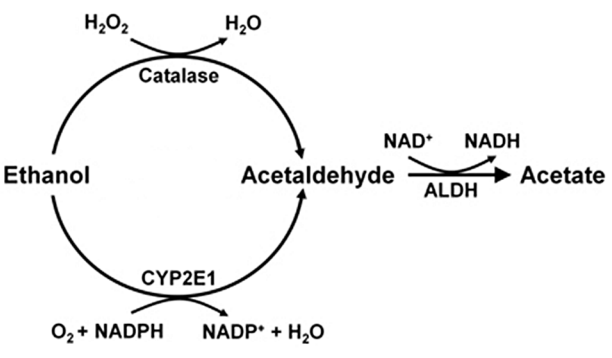

FIGURE 1 | Ethanol metabolism into acetaldehyde and acetate in the brain (Scheme from data of Zimatkin et al., 2006).

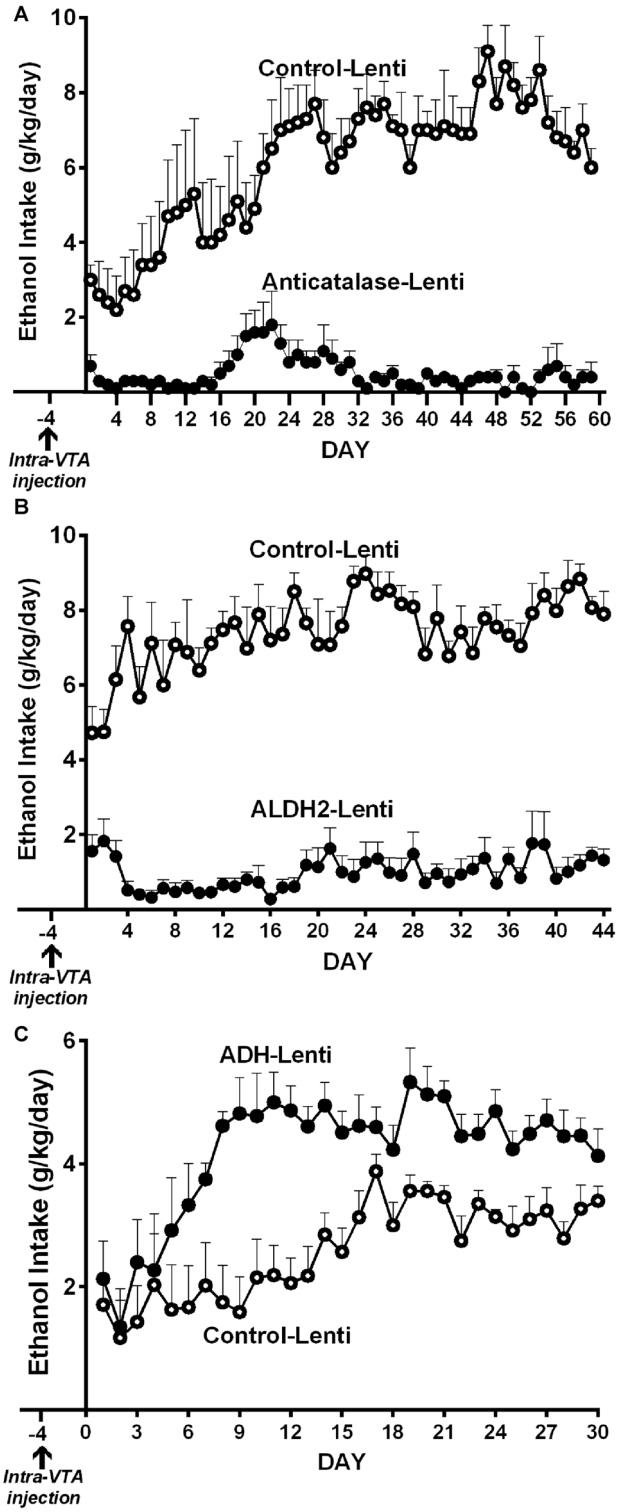

FIGURE 2 | Anticatalase-Lentiviral vector (A) or aldehyde dehydrogenase-2 (ALDH2)-coding Lentiviral vector **(B)** injection into the ventral tegmental area (VTA) of naïve UChB rats markedly reduces voluntary ethanol intake. A liver alcohol dehydrogenase (ADH)-coding Lenti vector injection **(C)** significantly increases ethanol intake in ethanol naïve rats. Arrow (*Continued*)

FIGURE 2 | Continued
indicates the time of administration of either control-lentiviral vector, anticatalase- **(A)**, ALDH2-lentiviral vector **(B)** or ADH-lentiviral vector ($n = 5$ rats per group). UChB rats significantly ($p < 0.001$) reduced their alcohol intake (10% v/v) when injected into the VTA with a lentiviral vector coding for: (i) a shRNA against catalase (anticatalase-Lenti) **(A)**; or (ii) ALDH2-coding Lentiviral vector **(B)**, compared to animals injected with an empty lentiviral vector (control-Lenti). Rats significantly ($p < 0.01$) increased their alcohol l (5% v/v) intake when injected with a lentiviral vector coding liver ADH **(C)** (Panel **A,C** were adapted from Karahanian et al., 2011 and Panel **B** was adapted from Karahanian et al., 2015).

dopamine release induced by ethanol administration. Such an effect, studied in the UChB rat, was specific for ethanol, since dopamine release induced by amphetamine or KCl depolarization was not changed by the intra VTA administration of the shRNA anticatalase coding lentiviral vector (Karahanian et al., 2011)

(b) *Transducing a gene encoding an enzyme that degrades acetaldehyde*. The administration to naïve UChB rats of a lentiviral vector coding for the wildtype high affinity Aldh2, aimed at increasing the VTA ability to degrade acetaldehyde resulted in an 85% inhibition of ethanol (10% v/v) intake (Karahanian et al., 2015; **Figure 2B**).

In addition to the above, transducing into the VTA a gene coding for liver alcohol dehydrogenase (ADH), an enzyme that generates acetaldehyde, *increased* 2–4 fold the reinforcing effect of 5% ethanol (Karahanian et al., 2011; **Figure 2C**).

The above studies would preclude other brain systems in the *acquisition* (development) of ethanol reinforcement in rats.

Systemic Acetaldehyde Can be Both Aversive and Reinforcing

Systemic acetaldehyde generated endogenously at levels that do not cross the endothelial cell layer of the blood brain barrier is aversive (e.g the acetaldehyde protection against alcoholism of East Asians carrying the *ALDH2*2* genotype). However high systemic concentrations of acetaldehyde prior reached when acetaldehyde itself is orally consumed or it is administered intraperitoneally which can cross the blood brain barrier and are reinforcing, as shown by Peana et al. (2010, 2011) in a nose-poking for oral acetaldehyde in an operant model in Wistar rats. Although blood acetaldehyde levels were not reported, these studies further support the reinforcing-motivational role of brain acetaldehyde and are in line with studies of Diana and associates (Foddai et al., 2004) who postulated a preferential reinforcing effect of systemic acetaldehyde over its aversive effects. In UChB rats, Quintanilla and Tampier (2003) showed that the injection of a large dose of acetaldehyde results in conditioned place preference (CPP). Early studies by Brown et al. (1980) demonstrated that infusion of acetaldehyde directly into the left lateral ventricle of the brain of Wistar rats leads to increases in ethanol intake. In this case the peripheral aversive effects are expected to be minimal.

These findings, together with microdialysis studies (Melis et al., 2007; Deehan et al., 2013a,b) show that local administration of acetaldehyde into the posterior VTA leads to increases in

dopamine release in nucleus accumbens further suggesting that the reinforcing effect of acetaldehyde is mediated by activation of dopaminergic neurons. Thus, the effect of acetaldehyde on dopamine release mimics the effects of many drugs of abuse (Di Chiara and Imperato, 1988).

Overall, literature studies support the view that brain acetaldehyde is reinforcing. The possibility that acetaldehyde may be converted into another reinforcing substance is subsequently discussed.

The Reinforcing Effect of Salsolinol: An Acetaldehyde-Derived Product

Ethanol-derived acetaldehyde condenses non-enzymatically with brain dopamine to generate racemic (*R/S*)-salsolinol (*R/S*-SAL; **Figure 3**). Rodd et al. (2008) and Deehan et al. (2013b) have shown that (*R/S*)-SAL at concentrations of 0.03–0.3 μM is self-administered intra VTA by Wistar rats. These concentrations of (*R/S*)-SAL are one to two orders of magnitude lower than the concentrations required for acetaldehyde self-administration in the same brain area.

Microinjections of (*R/S*)-SAL into the VTA of Wistar rats also result in an increased release of dopamine in the nucleus accumbens (Deehan et al., 2013a,b). Rommelspacher et al. (1995) showed that SAL was increased in the blood of alcoholics. Animal studies have shown that chronic ethanol administration to Sprague-Dawley and to high alcohol drinker (HAD) rats results in a significant increase of SAL levels in dopamine-rich areas of the brain (Sjöquist et al., 1982; Matsubara et al., 1987; Rojkovicova et al., 2008).

Several questions arise in relation to the action of SAL: (i) is endogenous dopamine required to generate SAL? (ii) does the chronic administration of (R/S)-SAL generate a sensitized state similar to that generated by chronic ethanol intake, which augments ethanol reinforcement? (iii) does (R/S)-SAL administration to naïve rats induce ethanol motivational effects? (iv) is there an enantiomer specificity distinguishing the effects of R-salsolinol vs. S-salsolinol; and (v) does (R/S)-SAL administration result in locomotor sensitization, as it happens after chronic ethanol administration?

As will be discussed below; the answer to all these questions is "yes":

(i) *In vitro* studies by Melis et al. (2015) showed that inhibition of dopamine synthesis by α-methyl-p-tyrosine, a tyrosine hydroxylase inhibitor, fully abolishes the ability of ethanol and acetaldehyde to activate VTA dopaminergic neurons, an effect that was specific for ethanol and acetaldehyde (as SAL precursors) but was not seen for pre-formed SAL.

(ii) Intra-cerebroventricular (**Figure 4A**) or systemic administration of (*R/S*)-SAL (**Figure 4B**) to ethanol

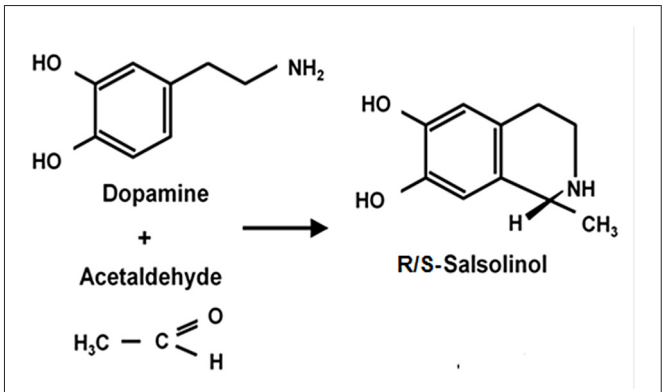

FIGURE 3 | Condensation of dopamine and acetaldehyde yielding salsolinol. Schematic representation of the spontaneous condensation of acetaldehyde with dopamine, yielding salsolinol (adapted from King et al., 1974; Bates et al., 1986).

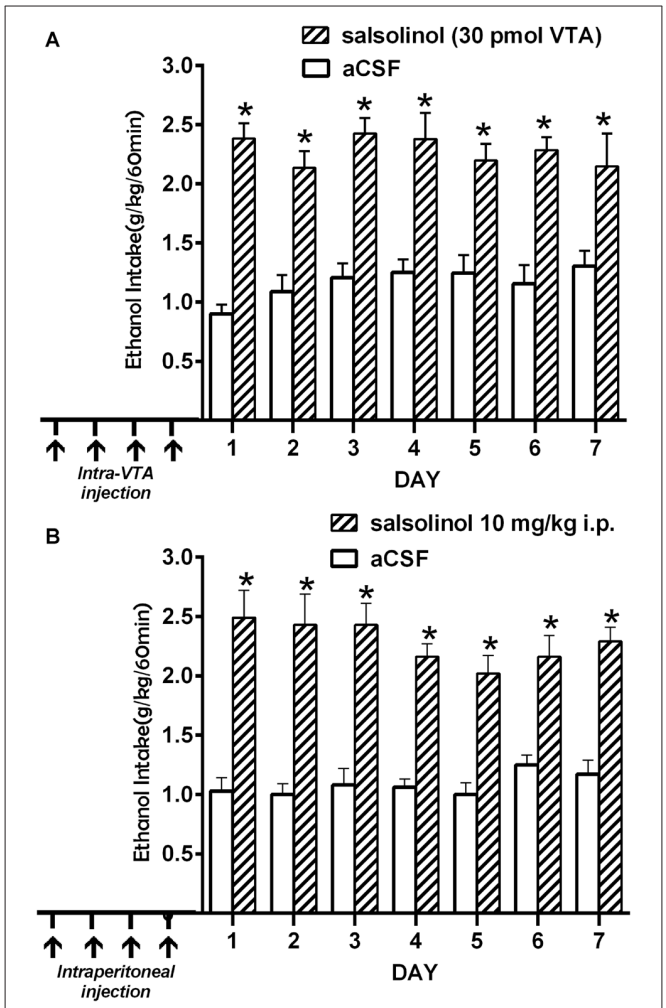

FIGURE 4 | Prior salsolinol administration increases voluntary ethanol intake in UChB rats. (A) UChB rats (n = 5) pretreated with salsolinol into the VTA (30.0 pmol/0.2 μl; days 1, 3, 8, 12; *arrows*) increased ethanol intake (60 min/day) during seven consecutive days (*stippled bars*), vs. rats pretreated with artificial cerebrospinal fluid (aCSF; n = 5) into the VTA (0.2 μl; days 1, 3, 8, 12; arrows; *white bars*). **(B)** UChB rats (n = 5) pretreated with systemic salsolinol (10 mg/kg, i.p.; days 1, 3, 8, 12; *arrows*) increased ethanol intake (60 min/day) during seven consecutive days (*striped bars*), vs. UChB rats (n = 5) pretreated with saline (7 ml/kg, i.p.; days 1, 3, 8, 12; arrows; *white bars*) (Data from Quintanilla et al., 2014). Symbol * means significant difference from aCSF control rats $p < 0.001$ (Two way ANOVA).

naïve UChB rats induces major increases in voluntary ethanol intake (Quintanilla et al., 2014). This effect was also observed by Myers and Melchior (1977) in Sprague-Dawley rats. Noteworthy, the high ethanol intakes are of the same order as those ingested by rats that had consumed ethanol for several weeks and were exposed to the ethanol deprivation condition followed by ethanol re-access (*vide infra*).

(iii) Studies by Quintanilla et al. (2016a), in UChB rats showed that the intra-cerebroventricular or systemic administration of (*R/S*)-SAL increased the motivational effects of ethanol as shown by the place preference technique (**Figure 5**), in line with studies by Matsuzawa et al. (2000) in Sprague-Dawley rats and by Hipólito et al. (2011) in Wistar rats.

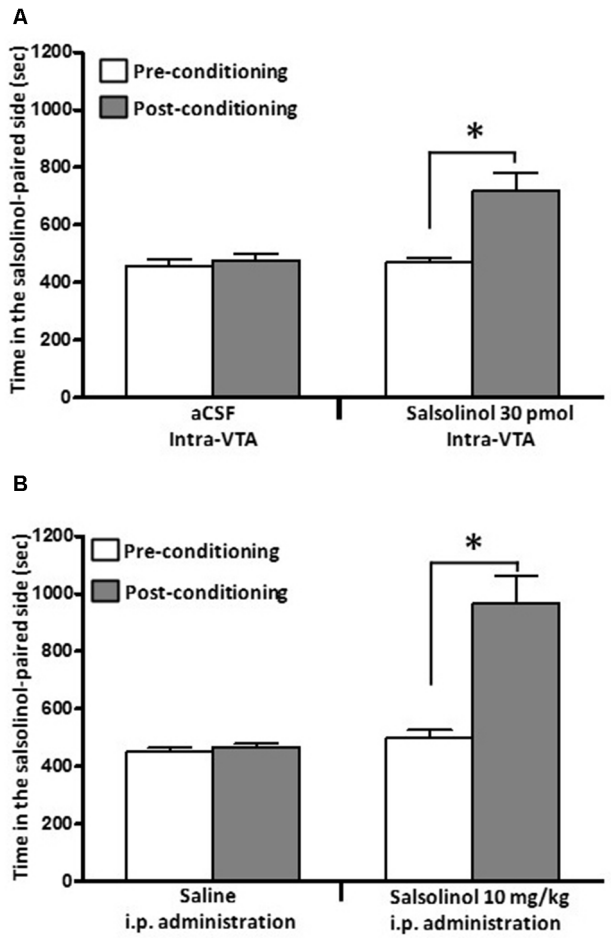

FIGURE 5 | Salsolinol, injected intracerebrally or systemically, produced conditioned place preference (CPP) in UChB rats. Salsolinol or vehicle was administered either **(A)** intra-VTA (30 pmol/left VTA) or **(B)** intraperitoneally (10 mg/kg, i.p.) in UChB rats (n = 28). Data are means ± SEM and represent the time spent in the salsolinol-paired compartment (seconds; means ± SEM, n = 7 for each group; ordinate) during the pre- (white columns) and postconditioning (gray columns) phases. Asterisk represents significant difference in time spent by the salsolinol group on the salsolinol-paired side in the postconditioning phase vs. the preconditioning phase and vs. the vehicle group: *$p < 0.001$ (two-way ANOVA) (Data from Quintanilla et al., 2014).

(iv) Intracerebral administration studies in UChB rats showed that the ethanol motivational and intake sensitization effects of (*R/S*)-SAL is also seen with R-SAL while the S-SAL enantiomer is inactive (**Figure 6**; Quintanilla et al., 2016a). Although the pharmacological mechanisms responsible for the action of *(R/S)*-SAL remain unclear, the specific effect of the R-enantiomer in inducing the motivational effects of ethanol, suggests that *in vivo* the chirality of the C-1 center of *(R/S)*-SAL plays an important role in changing its affinity for transporters or receptors associated with ethanol intake.

(v) The administration of (R/S)-SAL to Wistar or UChB rats induced a sensitization to its locomotor effects (Hipólito et al., 2010; Quintanilla et al., 2014).

In addition to the above, the findings that microinjections of salsolinol into the posterior VTA increase dopamine release in nucleus accumbens (Hipólito et al., 2011; Deehan et al., 2013a) suggest that the reinforcing effect of salsolinol is mediated by the activation of dopaminergic neurons.

Overall, the above studies suggest that brain SAL mediates the effect of ethanol-derived acetaldehyde to motivate the acquisition of ethanol consumption. Further, these studies are also in line with the work of Rodd and associates (Rodd et al., 2008; Deehan et al., 2013b) who showed that rats will self-administer (R/S)-SAL into the posterior VTA at concentrations that are below those required for acetaldehyde self-administration.

MAINTENANCE OF CHRONIC ETHANOL INTAKE

Ethanol-Derived Acetaldehyde Is no Longer Required to Maintain Chronic Alcohol Intake

Studies by Quintanilla et al. (2012) and Karahanian et al. (2015) have shown that *after* the UChB rats have reached a steady state of chronic ethanol intake, the administration into the VTA of either a lentiviral vector coding for an anti-catalase shRNA (**Figure 7A**) or coding for the high affinity Aldh2 do not influence voluntary ethanol intake (**Figure 7B**). It is noted that the unabated ethanol intake seen in these studies after the transduction of genes aimed at lowering acetaldehyde levels is not due to negative reinforcement since addition of quinine (bitter taste) to the ethanol solutions fully inhibits ethanol intake (Quintanilla et al., 2012).

The failure of the anticatalase or ALDH2 coding lentiviral vectors to reduce ethanol intake in rats that had consumed alcohol for 2 months suggests that following chronic alcohol consumption other signaling pathways might be recruited. Chronic consumption of drugs of abuse induces changes at the molecular, cellular and neurocircuitry levels that mediate the transition from occasional, controlled substance use to loss of control in drug intake and chronic addiction (Koob and Le Moal, 2008). Although chronic drug consumption induces changes in several neurotransmitter systems, including dopamine, GABA, cannabinoids and opioid systems (see Fattore and Diana, 2016),

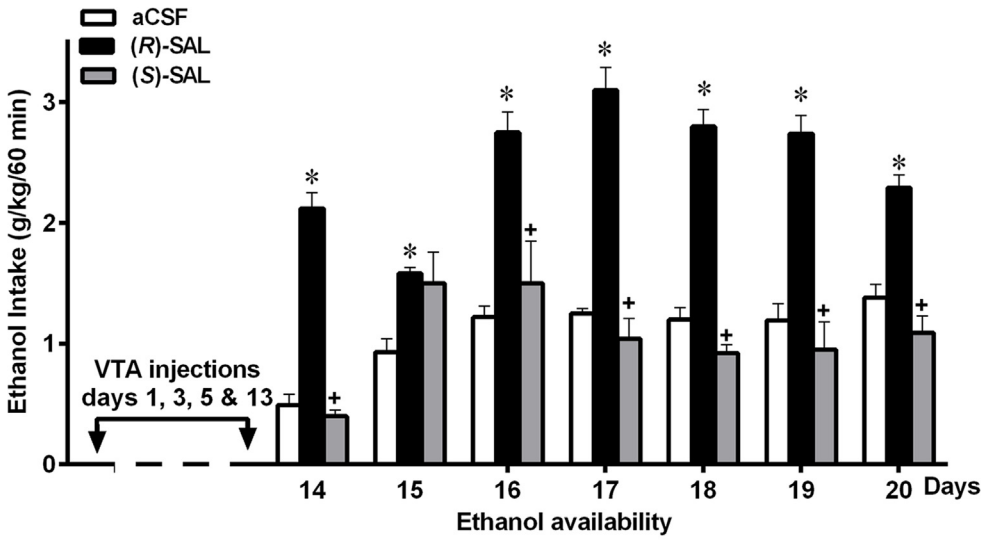

FIGURE 6 | Prior repeated administration of (*R*)-SAL, but not of (*S*)-SAL, increases voluntary ethanol intake in ethanol-naïve UChB rats. UChB rats (n = 5 rats per group) were pretreated intra-VTA with (*R*)-SAL (black columns), (*S*)-SAL (gray columns) or control aCSF (white columns) on days 1, 3, 5 and 13 (*arrows*); then (from day 14) animals were exposed to ethanol for 1 h/day. Asterisk symbol (*$P < 0.05$) indicates that the ethanol intake is significantly higher than that of the control (aCSF) group of the same day. Single plus (+ cross-like) sign indicates that the ethanol intake of (S)-SAL treated animals is significantly different lower ($p < 0.001$) than that of the (R)-SAL group on the same day (Data from Quintanilla et al., 2016a).

without implying a lesser role for other neurotransmitter systems, we mainly focus our review on glutamate transmission since: (i) many addictive drugs lead to an increased glutamatergic signaling (Wolf et al., 2003; Koob and Volkow, 2010); which (ii) drive the enhanced motivation to obtain several drugs, including cocaine (Kalivas and McFarland, 2003; Pickens et al., 2011) and, now also ethanol (Sari et al., 2013; Das et al., 2015); and (iii) can be modified by the administration of drugs that normalize the glutamate homeostasis (*vide infra*).

The Hyperglutamatergic Hypothesis

Mechanisms that lead subjects to maintain drug intake involve: (a) learned cues (see Volkow et al., 2002; Hyman et al., 2006; Berridge et al., 2009); for ethanol likely its odor (see Bragulat et al., 2008); and (b) an increased glutamatergic tone (see Reissner and Kalivas, 2010). An increased glutamatergic tone has been shown in Sprague-Dawley rats to be associated with the *maintenance* of chronic ethanol intake, as well as with the relapse of several drugs of abuse (Weiland et al., 2015). Alcohol preferring rats (P strain) on a chronic ethanol intake schedule show marked increases in extracellular glutamate in nucleus accumbens (Ding et al., 2012), resulting from a reduction in the levels of the Na^+-glutamate (exchange) transporter (GLT1) in astrocytes of tripartite glutamatergic synapses. Sari et al. (2013) studied the *maintenance* of chronic ethanol intake in P rats. In these studies, the administration of ceftriaxone, a drug that increases the levels of GLT1, resulted in a 60%–70% reduction in chronic ethanol intake. Das et al. (2015) confirmed an elevation of extracellular glutamate in nucleus accumbens of P rats exposed to ethanol chronically, and showed that ceftriaxone markedly inhibited chronic ethanol intake.

Studies in Sprague-Dawley rats show that extracellular glutamate levels are regulated not only by the astrocyte Na+ gradient-dependent GLT1 transporter but also by the astrocyte exchange of cystine for glutamate via the cystine/glutamate exchanger (Herrera-Marschitz et al., 1996; Baker et al., 2003). Scofield and Kalivas (2014) demonstrated that a number of drugs of abuse reduce the levels of the GLT1 transporter and increase the extracellular levels of glutamate. In rodents, operant cocaine self-administration reduces both the nucleus accumbens cystine-glutamate exchange and glutamate transport by the GLT-1 transporter. Most importantly, administration of the antioxidant drug N-acetyl cysteine (NAC) normalizes these two processes. Reissner et al. (2015) concluded that restoring GLT-1, not the cystine-glutamate exchange, is the key mechanism whereby daily NAC reduces the hyperglutamatergic state.

The Administration of N-Acetyl Cysteine in Drug Dependence and Motivation

A number of studies have shown that NAC reduces relapse (or reduces CPP) of many addictive drugs including: (i) cocaine (Madayag et al., 2007; Moussawi et al., 2009; Reichel et al., 2011; Kupchik et al., 2012; Reissner et al., 2015); (ii) nicotine (Ramirez-Niño et al., 2013; Bowers et al., 2016; Moro et al., 2016); (iii) heroin (Zhou and Kalivas, 2008); and now (iv) ethanol (Quintanilla et al., 2016b).

Most of the above studies were conducted in rodents, while only one clinical study has reported the effect of NAC on a drug use disorder (LaRowe et al., 2013). These investigators indicated that their studies failed to demonstrate that NAC

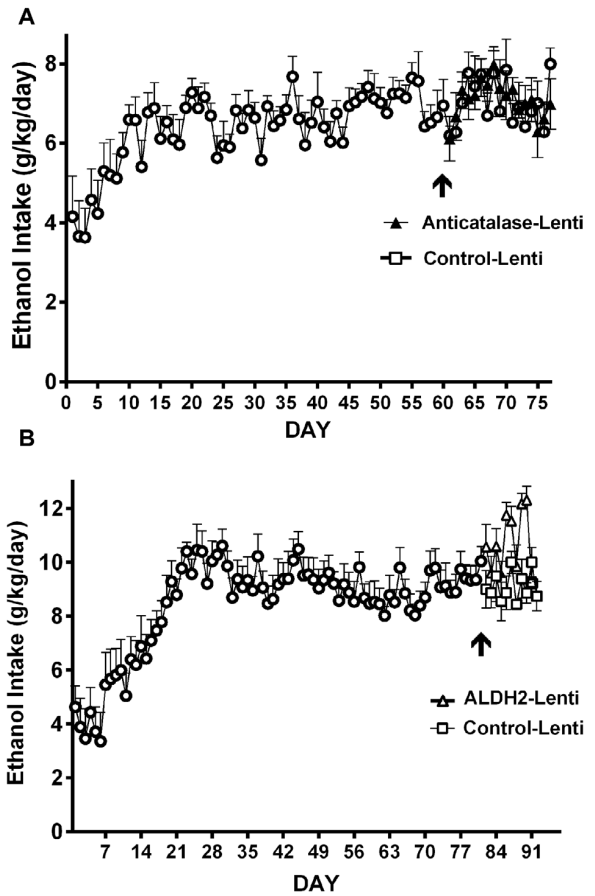

FIGURE 7 | Injection of an shRNA anti-catalase (A) or ALDH2 coding vector **(B)** into the VTA did not reduce alcohol (10%) intake in UChB rats that had chronically ingested ethanol. Arrow indicates the time of administration in **(A)** control-lentiviral vector (-□-) or anticatalase-lentiviral vector (-▲-) and in **(B)** the control-lentiviral vector (-□-) or the ALDH2-coding vector (-△-). The number of animals was 8 or 10 rats/group. Neither the anticatalase, the ALDH2-coding vector nor the control vector modified the voluntary ethanol intake of the animals that had already been under free choice ethanol consumption for 60 days (Panel **A** was adapted from Quintanilla et al., 2012 and Panel **B** was adapted from Karahanian et al., 2015).

reduced cocaine use in cocaine-dependent individual actively self-administering the drug. However, they also report that NAC prevented the return to cocaine use (relapse) in individuals who had already achieved abstinence from cocaine. In the latter, NAC administration reduced cocaine relapse by 90% (LaRowe et al., 2013; **Figure 2**). Despite the promising studies showing an inhibition of cocaine relapse in animals, these studies in abstinent cocaine users will require confirmation before their clinical application.

A possible explanation for the effect of NAC in preventing cocaine relapse only in detoxified patients may relate to the dual effect of cocaine in generating oxygen radicals (ROS) in the brain: (i) cocaine inhibits both the dopamine transporter and the norepinephrine transporter (Dohi et al., 2002), thus increasing the exposure of both extracellular dopamine and norepinephrine to a physiological pH, which leads to the autooxidation of catecholamines, generating one-electron oxidant semi-quinones; and (ii) both neurotransmitters are deaminated by monoamine oxidases generating hydrogen peroxide (Kopin, 1994). Thus, NAC is expected to be considerably less active when cocaine continues to be self-administered, while most active in a condition where only the *remaining* cocaine-induced ROS self-potentiating effects promote drug relapse. Amphetamine-like drugs have similar properties as cocaine in generation of ROS as amphetamines release not only dopamine but also norepinephrine (Rothman et al., 2001). McClure et al. (2014) suggest that NAC may prove to be an ideal relapse prevention aid when given after periods of abstinence or when combined with other forms of pharmacological and/or behavioral treatments to promote abstinence.

Despite the above, NAC markedly inhibited the chronic intake of ethanol of rats (*vide infra*). Animals were chronically self-administering alcohol; thus not in an abstinent condition (Quintanilla et al., 2016b), suggesting that the ROS/neuroinflammation generated by chronic ethanol intake is less intense than that generated by drugs that increase the extracellular levels of both dopamine and norepinephrine. We are not aware of clinical studies aimed at testing the effect of NAC as a treatment of alcoholism, whether prior or after abstinence. However, a recent clinical study showed that when successful, NAC treatment of marihuana users also reduced their alcohol use (Squeglia et al., 2016).

As shown by Quintanilla et al. (2016b) the daily administration of NAC, although not inhibiting the *acquisition* of chronic ethanol intake (**Figure 8A**), was a strong inhibitor (70%–75% reduction) of ethanol intake *maintenance* (**Figure 8B**). These results are in line with the findings of Doyle et al. (2014) indicating that glutamatergic signaling in the nucleus accumbens of Sprague-Dawley rats, although not essential for modifying initial cocaine use in non-addicted stages, becomes critical for post withdrawal relapse after the addiction has developed.

Oxidative Stress and Neuroinflammation: A General Role in the Addiction Process

Several studies indicate that oxidative stress is a relevant mechanism contributing to neural cytotoxicity and behavioral changes associated with drug addiction (see Cunha-Oliveira et al., 2013). Oxidative stress in the nervous system has been found upon *in vivo* exposure to amphetamine or amphetamine derivatives (Frey et al., 2006; Jung et al., 2010) and heroin (Qiusheng et al., 2005; Xu et al., 2006). Withdrawal from cocaine or heroin also induces oxidative stress in rodent's brain (Cemek et al., 2011; Pomierny-Chamiolo et al., 2013). The effect NAC in both normalizing glutamate levels and reducing drug relapse (Reissner et al., 2015) likely results from its high antioxidant activity. N-acetylcysteine is also used to treat an acetaminophen overdose due to its high antioxidant activity, being a precursor of cysteine and glutathione (Lucyk et al., 2016).

A number of studies in Wistar and Sprague-Dawley rats have shown that chronic ethanol administration leads *to both oxidative stress* and *neuroinflammation* (reviewed by Crews et al., 2015; Crews and Vetreno, 2016). Noteworthy, oxidative

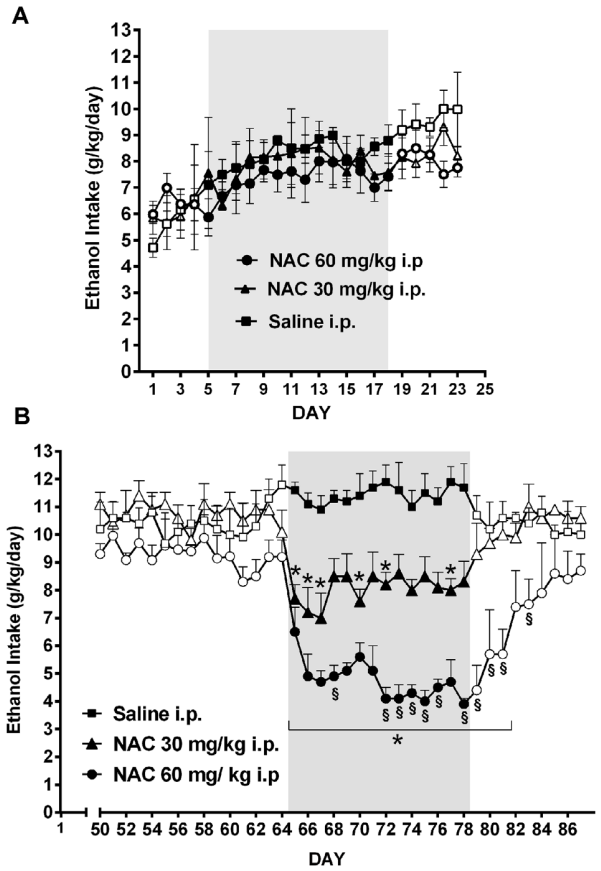

FIGURE 8 | Chronic N-acetyl cysteine (NAC) administration (30 or 60 mg/kg, i.p.) administered during the acquisition of alcohol consumption did not reduce ethanol intake (A), but given during the chronic ethanol maintenance phase markedly reduced ethanol intake **(B)** in UChB rats. Three groups of rats (n = 5 rats per group) under continuous access to 10% ethanol and water were treated with either saline (black square), 30 mg/kg NAC i.p. (black triangles), or 60 mg/kg N AC i.p (black circles) from day 5–18 (acquisition phase) of ethanol intake **(A)**. Different groups of rats under continuous access to 10% ethanol and water rats (n = 5 rats per group) were treated with either saline (black square), 30 mg/kg NAC (black triangles), or 60 mg/kg NAC (black circles) from day 65 to day 78 (maintenance phase) of ethanol intake **(B)**. Data are means ± SEM of daily ethanol intake. Asterisk symbol *$p < 0.05$, indicates that the ethanol intake is significantly lower than that of the saline control group of the same day. (§) Symbol $p < 0.001$, indicates that the ethanol intake of NAC 60 mg/kg group is lower than that of NAC 30 mg/kg group (Data from Quintanilla et al., 2016b).

stress and neuroinflammation potentiate each other via the oxidation of IκB with activation of NFκB and the generation of inflammatory cytokines; the latter in turn generate oxygen radicals via mitochondrial uncoupling (Kastl et al., 2014). Montesinos et al. (2016) reviewed the direct relationship between neuroinflammation and brain injury. In alcoholics, a marked hippocampal cell loss and injury has been shown (Sullivan et al., 1995). Long Evans rats that consume alcohol for several months in nutritionally adequate liquid diets also display marked hippocampal damage (Walker et al., 1980). Studies in C57/BL mice have shown that chronic alcohol intake increases brain TLR4 and NF-κB, both involved in the generation of inflammatory cytokines (Alfonso-Loeches et al., 2010).

In a most relevant study *causally linking neuroinflammation to* an increased ethanol intake, Blednov et al. (2011) showed long-lasting increases in ethanol intake in C57/BL mice following the administration of a single dose of bacterial lipopolysaccharide, a well know neuroinflammatory agent, thus increasing the reinforcing effect of ethanol. Ethanol intake, via gut-generated acetaldehyde, induces the entrance of intestinal lipopolysaccharide into the blood (Ferrier et al., 2006), which via TNF-α generates neuroinflammation (see Crews et al., 2015). Additionally, in the brain itself a pro-oxidant and pro-inflammatory agent generated from ethanol is salsolinol; an oxygen radical-generating agent when oxidized into semi-quinones by metals ions present in biological systems (Jung and Surh, 2001). Thus, oxygen radicals and lipopolysaccharide potentiate each other in generating a neuroinflammation.

An additional link between neuroinflammation and an increased glutamatergic signal has been recently reported (David et al., 2016). The authors demonstrated a significant reduction in the primary astrocytic glutamate transporter, GLT-1 and increases in extracellular glutamate levels induced by neuroinflammation following an infection due to toxoplasma administration to mice. Thus, these studies further support the sequence oxidative stress/neuroinflammation—low glutamate transporter—hyperglutamatergic state.

Overall, the studies reviewed indicate that different mechanisms are responsible for the *acquisition of ethanol intake* and for its chronic *maintenance*. A number of studies strongly support the view that chronic ethanol intake is maintained by mechanisms known to increase the extracellular levels of brain glutamate, likely in nucleus accumbens. The inhibition of chronic ethanol intake by NAC, a strong antioxidant, further suggests that the reactive oxygen species/neuroinflammation system plays a role in chronic ethanol *maintenance*. The reactive oxygen radical species and inflammatory cytokines (e.g., TNF-alpha) are known to potentiate each other.

RELAPSE-LIKE ALCOHOL INTAKE

Following Ethanol Deprivation, Ethanol Intake Upon Re-Access Is Again Dependent on Brain Acetaldehyde

In UChB rats ingesting ethanol chronically for 2-months, a 4-week ethanol deprivation leads to a partial recovery of the inhibitory effect of the anti-catalase vector on ethanol intake (**Figure 9**; Quintanilla et al., 2012). The fact that the effect on 24-h ethanol intake is not seen immediately after ethanol re-access may relate to the marked intake in the first few hours following the alcohol deprivation and re-access, in line with studies of Hölter and Spanagel (1999); Rodd et al. (2009) and Vengeliene et al. (2014) for Wistar and HAD rats.

The relapse-like drinking also known as the "alcohol deprivation effect" (ADE) is a condition in which animals subjected to chronic ethanol intake followed by a long

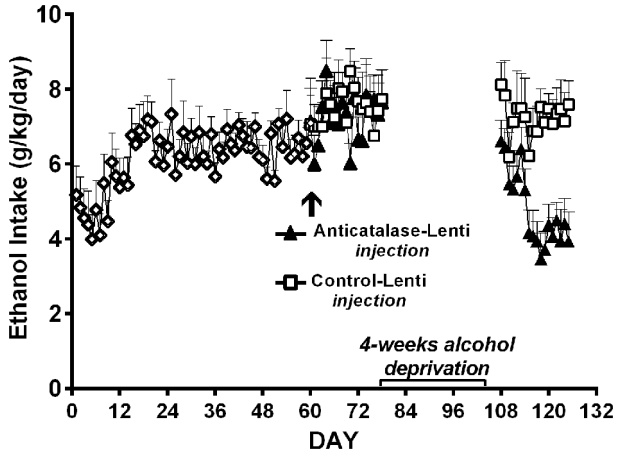

FIGURE 9 | A period of alcohol deprivation is required to reduce voluntary alcohol intake following the anticatalase-lentiviral vector administration into the VTA in UChB rats. UChB rats (n = 10 rats per group) allowed to access 10% ethanol and water on a 24-h basis for 2 months did not change their voluntary alcohol intake when these were subsequently injected into the VTA with a single dose of a lentiviral vector coding for an shRNA against catalase (anticatalase-Lenti), but significantly (ANOVA; $F_{(1,33)} = 111.54$, $p < 0.001$) reduced (50%) their alcohol intake following 4 weeks of alcohol deprivation when compared to the ethanol intake of animals (n = 10) that received a control lentiviral vector (control-Lenti). After the alcohol deprivation period, the animals were returned to a free access of 10% ethanol and water on a 24-h basis (Data from Quintanilla et al., 2012).

deprivation, consume intoxicating amounts of ethanol in as little as 60-min upon ethanol re-access. The ADE paradigm in animals has good predictive value in representing relapse-like drinking in humans as it is inhibited by three medications used clinically to reduce ethanol intake, namely: naltrexone, nalmefene and acamprosate-Ca (Spanagel and Zieglgänsberger, 1997; Orrico et al., 2014; Spanagel et al., 2014), indicating that several neurotransmitter systems- including a hyperglutamatergic tone and importantly the opiate system also mediate ADE. As will be discussed below, acetaldehyde also plays a role in relapse-like ADE drinking.

In the ADE model in UChB rats, chronic ethanol intake for 1–3 months is interrupted by an alcohol deprivation of 7–15 days before animals are allowed ethanol re-access. Upon ethanol re-access, animals consume intoxicating amounts of ethanol of the order of 2–2.5 g ethanol/kg/in the first 60 min of re-access (Tampier et al., 2013; Karahanian et al., 2015). In the UChB model, ethanol intake studies in the ADE condition were aimed at dissociating ADE ethanol intake from the ethanol "drinking-in-the-dark" condition, where high ethanol intakes are observed primarily if water is not offered (Thiele et al., 2014). Since, as shown in Wistar and P rats, the endogenous opiate tone is increased upon food intake (Jalowiec et al., 1981) which is *per se* involved in ethanol intake (Froehlich et al., 1990), the studies in UChB rats were conducted at 1–2 PM (on a 7 AM to 7 PM normal light cycle). Animals rapidly and almost exclusively approach the alcohol solution bottles and not the water bottles, consuming minimal amounts of water upon re-access. A large ADE-induced intake is observed mainly during the initial hour of ethanol re-access (Tampier et al., 2013; Karahanian et al., 2015), in line with studies of Hölter and Spanagel (1999) and Rodd et al. (2009) for HAD and Wistar rats, respectively. The latter authors have demonstrated that on ethanol re-access in the ADE condition, animals are willing to work for alcohol to a greater extent (e.g., to a higher bar-pressing breakpoint), suggesting a more rewarding effect of ethanol in such condition (Hölter et al., 1998). An additional characteristic of the ADE mechanism is "kindling-like" effect that increases the post ADE ethanol intake following several periods of ethanol deprivation and ethanol reinstatement (Hölter and Spanagel, 1999; Rodd et al., 2009), a characteristic also observed in UChB rats (Tampier et al., 2013; Karahanian et al., 2015; **Figures 10A,B**). **Figures 10A,B** further show that the deprivation period partly allows the recovery of the inhibitory effect on ethanol intake exerted by the intra-VTA administration of a lentiviral vector coding for an anticatalase shRNA or an ALDH enzyme (Tampier et al., 2013; Karahanian et al., 2015). These findings are in line with the observations of Muggironi et al. (2013) and Orrico et al. (2013) who showed that administration of penicillamine, an acetaldehyde trapping agent, partly inhibited the relapse-like alcohol intake in Wistar rats. Vengeliene et al. (2005) showed in Wistar rats that the i.p. administration of ethanol prior to oral re-access (which *per se* generates acetaldehyde before oral ethanol intake occurs) partly reduced ADE ethanol intake upon re-access.

Overall, studies show that in animals that have consumed ethanol chronically and are subjected to a protracted abstinence followed by ethanol re-access, brain-derived acetaldehyde plays a significant role in the relapse-like drinking. Noteworthy, relapse drinking is a characteristic of alcoholism in humans.

Maintenance and Relapse-Like Alcohol Drinking: Stem Cells Administration

As indicated above, neuroinflammation leads to cognitive dysfunction and increases chronic alcohol intake (Blednov et al., 2011; Crews and Vetreno, 2016; Montesinos et al., 2016). These studies suggest that reducing neuro-inflammation could reduce both chronic ethanol intake (*maintenance*) and possibly relapse-like drinking. Developments in the stem cell field have shown that most tissues contain mesenchymal stem cells (MSCs; Prockop et al., 2010), known to be activated by inflammatory mediators (e.g., TNFα) in damaged areas, leading to the generation of anti-inflammatory cytokines including IL-10 (Lee et al., 2016) and a soluble TNFα receptor, which neutralizes TNFα (Yagi et al., 2010). MSCs can be isolated and expanded from a number of tissues, such as bone marrow and adipose tissue (Contador et al., 2015; Ezquer et al., 2016).

Yang et al. (2015) showed that hippocampal apoptosis and neurocognitive impairments generated by chronic ethanol administration in Sprague-Dawley rats could be reversed by the infusion of mesenchymal bone marrow stem cells. The study indicated that increases in hippocampal superoxide dismutase (which lowers oxidative stress and likely neuroinflammation) as well as increases in neural growth factor were associated with the reversal of apoptosis and cognitive deficits.

Recent work in UChB rats (Israel et al., 2017) tested whether MSCs from bone marrow or adipose tissue of ethanol-naïve rats

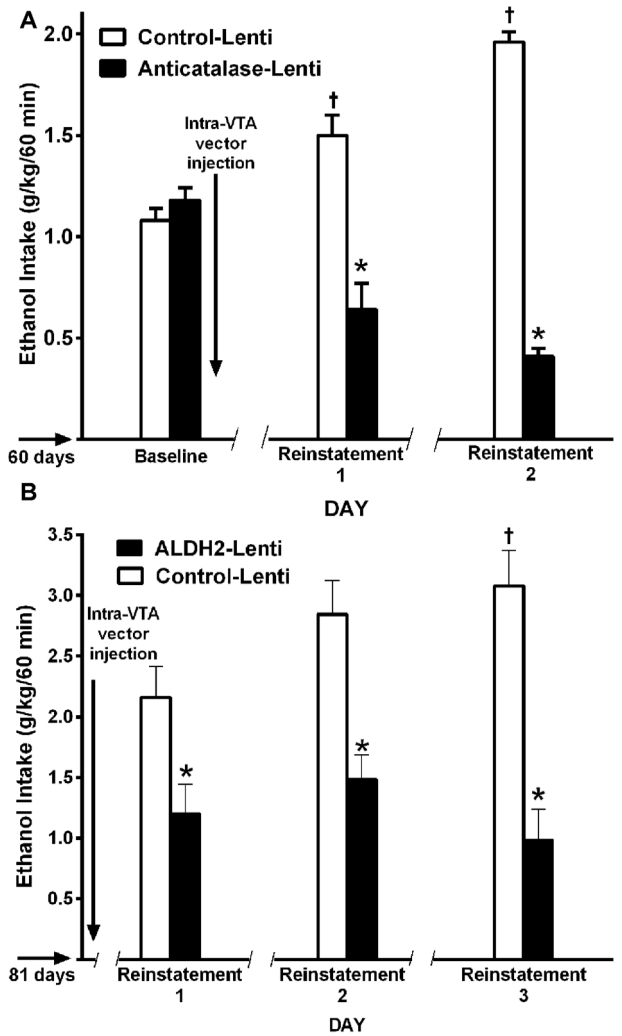

FIGURE 10 | A single VTA area injection of a shRNA anticatalase-coding lentiviral vector (A) or an ALDH2-coding lentiviral vector **(B)** inhibit first-hour ethanol (EtOH) relapse-like ethanol intake after the first and second and third deprivation periods. Baseline data correspond to the average of EtOH intake restricted to only 1 h a day, for 7 days immediately prior to alcohol deprivation before intra-VTA injections **(A,B)** by UChB rats. Control-Lenti or Anticatalase-Lentiviral vector (n = 5 rats per group) were injected during the first day of deprivation **(A)**. Control-Lenti or ALDH2-lentiviral vector (n = 5 rats per group) were injected during the first day of deprivation **(B)**. The -/ /- symbol in the x-axis represents the 15-day deprivation period. The first, second and third re-exposure consumptions were *symbol, means significant different from control-Lenti $p < 0.001$; and †symbol, means significant different from its own baseline value $p < 0.01$ (Panel **A** was adapted from Tampier et al., 2013 and Panel **B**, was adapted from Karahanian et al., 2015).

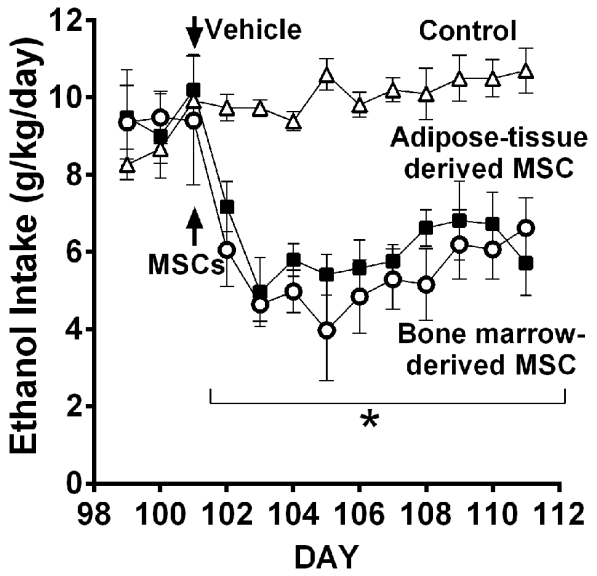

FIGURE 11 | Intra cerebroventricular injection of bone marrow-derived mesenchymal stem cell (MSC) or adipose tissue—MSC reduces chronic 24-h ethanol consumption in UChB rats. Rats under chronic ethanol access were injected with bone marrow-derived or adipose tissue—derived MSCs or vehicle into the left cerebral ventricle (n = 5 rats per group). Ethanol consumption is shown as g ethanol/kg/day. Asterisk *$p < 0.001$ indicates a reduction of ethanol intake compared to vehicle during all MSCs post-treatment days (Data from Israel et al., 2017).

injected intra-cerebroventricularly could inhibit chronic ethanol intake both in the *maintenance* condition and in the *relapse-like* condition induced by the ethanol deprivation effect (ADE). **Figure 11** shows the inhibitory effect of MSCs on intake of ethanol of rats that had freely ingested 10% ethanol for 3 months. Data show that a significant inhibition of ethanol intake exerted by the MSCs; a single dose of MSCs (5×10^5 cells in 5 μL) injected into the brain lateral ventricle reduced by 40%–60% the *maintenance* of chronic alcohol intake for the 10 days studied.

Relapse-like alcohol intake as affected by MSCs under the ADE condition, was studied in a separate group of UChB rats. Animals that had freely consumed ethanol solutions for 87 days were deprived of ethanol for 14 days. On the fourth day of deprivation animals were administered the MSCs and on deprivation day 15 animals had re-access to ethanol solutions. Prior to the alcohol deprivation, animals displayed a basal alcohol intake of 1.1 g ethanol/kg/60 min (**Figure 12**), intake which was doubled after repeated alcohol deprivation (ADE) and re-access cycles, reaching 2.2 g alcohol/kg/60 min (equivalent to the consumption of over 10 standard drinks/70 kg in a 1-h sitting). Data in **Figure 12** show that animals treated with MSCs reduced up to 80%–85% their relapse-like alcohol intake compared to sham control rats. There were no significant differences between the effects of bone marrow-derived MSCs and adipose tissue-derived MSCs. It is noted that a single intra-cerebroventricular injection of both MSC types inhibited relapse-like drinking for the 40 days investigated, suggesting a marked inhibition of the ADE-activated brain reward systems.

Overall, the inhibition of ethanol intake by MSCs, both under chronic and relapse-like conditions, further supports the view that chronic ethanol intake is maintained by brain oxidative stress/neuroinflammatory conditions, also indicating a role of inflammatory mechanisms on relapse-like ethanol intake. Noteworthy is the long-lasting inhibition afforded by a

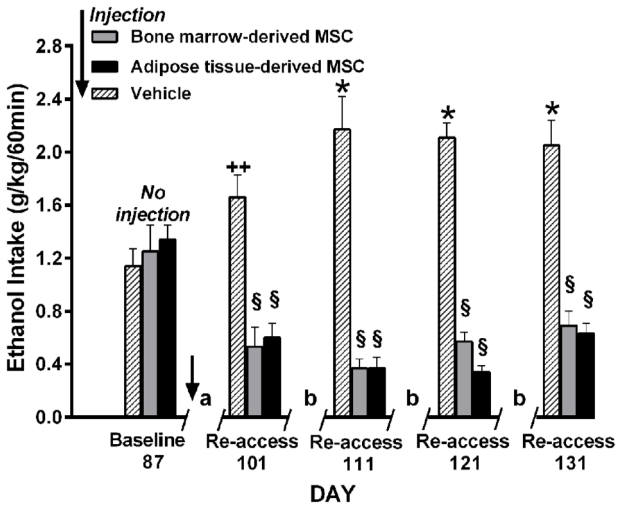

FIGURE 12 | Intra cerebroventricular injection of bone marrow-derived or adipose-tissue derived MSCs block relapse-like (60-min) ethanol intake in UChB rats. Rats allowed 87 days of free-choice ethanol access were injected on the fourth day of deprivation with bone marrow-derived MSC (gray columns), adipose tissue-derived MSC (black columns) or vehicle (dashed columns) in the left cerebral ventricle (n = 5 rats per group) and deprived of ethanol for 14-days, after which ethanol re-access was allowed. Animals were further subjected to three additional cycles of 3 days of *ad libitum* ethanol drinking and further 7-days of deprivation prior to the next ethanol re-access. The symbol / / represents the deprivation period prior to ethanol re-access: **(a)** 14-day deprivation, **(b)** 7-day deprivation. $^{*}p < 0.001$ and $^{++}p < 0.05$ indicate ethanol intake increases vs. baseline. $^{\S}p < 0.001$ indicates a reduction of ethanol intake compared to baseline value for each of the MSC types (Data from Israel et al., 2017).

single administration of MSCs on the relapse-like ethanol intake condition.

CONCLUSIONS

The studies presented:

(a) Confirm, by the use of genetic modifications, studies by several groups in several rat strains that had indicated that catalase-mediated brain oxidation of ethanol into acetaldehyde is required for animals to initiate (*acquisition*) chronic ethanol intake. In the UChB rat, brain-derived acetaldehyde was shown to be an absolute requirement (80%–95%) for the initiation of chronic ethanol intake.

(b) Demonstrate that after a steady chronic ethanol intake (*maintenance*) has been attained, brain acetaldehyde generation is no longer required to perpetuate its intake. This effect seen in UChB and Wistar rats has also been observed in operant ethanol self-administration studies in mice. Following a protracted ethanol deprivation, acetaldehyde is again required to induce a relapse-like condition.

(c) Demonstrate that the daily administration of the antioxidant drug N-acetyl cysteine to UChB rats that have consumed ethanol chronically markedly inhibits (70%–75%) voluntary ethanol intake. Noteworthy, N-acetyl cysteine did not inhibit the initial acquisition of ethanol intake of naïve animals.

(d) Demonstrate in UChB rats that the intracerebral administration of salsolinol—the condensation product of dopamine and acetaldehyde—results in enhanced ethanol reinforcement, leading to binge-like ethanol intakes (up to 3 g ethanol/kg in 60 min, equivalent to 15 drinks/70 kg) and in an enhancement of ethanol motivational effects as shown by the place preference technique.

(e) Demonstrate that a single intra-cerebroventricular administration to UChB rats of mesenchymal stem cells, known to have marked anti-inflammatory and antioxidant properties, inhibited *relapse-like ethanol drinking* by 60%–85% for 40 days.

(f) Overall, studies indicate that ethanol-derived metabolites are by themselves involved in the *acquisition* of ethanol intake; while these metabolites are indirectly involved in *maintenance* of chronic ethanol intake *and in relapse-like ethanol drinking*. A new element playing a role in *maintenance and relapse-like* drinking is neuroinflammation, partly mediated by acetaldehyde increasing the diffusion of gut lipopolysaccharide into the systemic circulation and possibly by oxygen radicals generated in the oxidation of salsolinol.

A final note, while many of the studies discussed have been conducted in rodents of different species and strains or in cells, extrapolation of these findings to humans requires caution.

AUTHOR CONTRIBUTIONS

All the authors contributed to the writing and approved the final manuscript.

ACKNOWLEDGMENTS

Supported by Fondo Nacional de Desarrollo Científico y Tecnológico (Fondecyt) #1130012, #1150589, #1150850, 11130241, Comisión Nacional de Investigación Científica y Tecnológica (Conicyt) ACT1411 and Millennium Institute #P09-015-F. We appreciate the expert support of Jimena López and Juan Santibañez.

REFERENCES

Alfonso-Loeches, S., Pascual-Lucas, M., Blanco, A. M., Sanchez-Vera, I., and Guerri, C. (2010). Pivotal role of TLR4 receptors in alcohol-induced neuroinflammation and brain damage. *J. Neurosci.* 30, 8285–8295. doi: 10.1523/JNEUROSCI.0976-10.2010

Aragon, C. M., and Amit, Z. (1992). The effect of 3-amino-1,2,4-triazole on the voluntary ethanol consumption: evidence for brain catalase involvement in the mechanism of action. *Neuropharmacology* 31, 709–712. doi: 10.1016/0028-3908(92)90150-n

Baker, D. A., McFarland, K., Lake, R. W., Shen, H., Tang, X. C., Toda, S., et al. (2003). Neuroadaptations in cystine-glutamate exchange

underlie cocaine relapse. *Nat. Neurosci.* 6, 743–749. doi: 10.1038/nn1069

Bates, H. A., Bagheri, K., and Vertino, P. M. (1986). Effect of pH on the regioselectivity of Pictet-Spengler reactions of 3-hydroxyphenethylamines with formaldehyde and acetaldehyde. *J. Org. Chem.* 51, 3061–3063. doi: 10.1021/jo00365a041

Berridge, K. C., Robinson, T. E., and Aldridge, J. W. (2009). Dissecting components of reward: 'liking', 'wanting', and learning. *Curr. Opin. Pharmacol.* 9, 65–73. doi: 10.1016/j.coph.2008.12.014

Blednov, Y. A., Benavidez, J. M., Geil, C., Perra, S., Morikawa, H., and Harris, R. A. (2011). Activation of inflammatory signaling by lipopolysaccharide produces a prolonged increase of voluntary alcohol intake in mice. *Brain Behav. Immun.* 25, S92–S105. doi: 10.1016/j.bbi.2011.01.008

Bowers, M. S., Jackson, A., Maldoon, P. P., and Damaj, M. I. (2016). N-acetylcysteine decreased nicotine reward-like properties and withdrawal in mice. *Psychopharmacology* 233, 995–1003. doi: 10.1007/s00213-015-4179-4

Bragulat, V., Dzemidzic, M., Talavage, T., Davidson, D., O'Connor, S. J., and Kareken, D. A. (2008). Alcohol sensitizes cerebral responses to odors of alcoholic drinks: an fMRI study. *Alcohol. Clin. Exp. Res.* 32, 1124–1134. doi: 10.1111/j.1530-0277.2008.00693.x

Brown, Z. W., Amit, Z., and Smith, B. (1980). Intraventricular self-administration of acetaldehyde and voluntary consumption of ethanol in rats. *Behav. Neural Biol.* 28, 150–155. doi: 10.1016/s0163-1047(80)91487-9

Bustamante, D., Quintanilla, M. E., Tampier, L., Gonzalez-Lira, V., Israel, Y., and Herrera-Marschitz, M. (2008). Ethanol induces stronger dopamine release in nucleus accumbens (shell) of alcohol-preferring (bibulous) than in alcohol-avoiding (abstainer) rats. *Eur. J. Pharmacol.* 591, 153–158. doi: 10.1016/j.ejphar.2008.06.069

Cemek, M., Büyükokuroğlu, M. E., Hazman, O., Konuk, M., Bulut, S., and Birdane, Y. O. (2011). The roles of melatonin and vitamin E plus selenium in prevention of oxidative stress induced by naloxone-precipitated withdrawal in heroin-addicted rats. *Biol. Trace Elem. Res.* 142, 55–66. doi: 10.1007/s12011-010-8744-8

Contador, D., Ezquer, F., Espinosa, M., Arango-Rodriguez, M., Puebla, C., Sobrevia, L., et al. (2015). Dexamethasone and rosiglitazone are sufficient and necessary for producing functional adipocytes from mesenchymal stem cells. *Exp. Biol. Med. (Maywood)* 9, 1235–1246. doi: 10.1177/1535370214566565

Crews, F. T., Sarkar, D. K., Qin, L., Zou, J., Boyadjeiva, N., and Vetreno, R. P. (2015). Neuroimmune function and consequences of alcohol exposure. *Alcohol Res.* 37, 331–351.

Crews, F. T., and Vetreno, R. P. (2016). Mechanisms of neuroimmune gene induction in alcoholism. *Psychopharmacology* 233, 1543–1557. doi: 10.1007/s00213-015-3906-1

Cunha-Oliveira, T., Rego, A. C., and Oliveira, C. R. (2013). Oxidative stress and drugs of abuse: an update. *Org. Chem.* 10, 321–324. doi: 10.2174/1570193x113106660026

Das, S. C., Yamamoto, B. K., Hristov, A. M., and Sari, Y. (2015). Ceftriaxone attenuates ethanol drinking and restores extracellular glutamate concentration through normalization of GLT-1 in nucleus accumbens of male alcohol-preferring rats. *Neuropharmacology* 97, 67–74. doi: 10.1016/j.neuropharm.2015.05.009

David, C. N., Frias, E. S., Szu, J. I., Vieira, P. A., Hubbard, J. A., Lovelace, J., et al. (2016). GLT-1-dependent disruption of CNS glutamate homeostasis and neuronal function by the protozoan parasite *Toxoplasma gondii*. *PLoS Pathog.* 12:e1005643. doi: 10.1371/journal.ppat.1005643

Deehan, G. A. Jr., Engleman, E. A., Ding, Z. M., McBride, W. J., and Rodd, Z. A. (2013a). Microinjections of acetaldehyde or salsolinol into the posterior ventral tegmental area increase dopamine release in the nucleus accumbens shell. *Alcohol. Clin. Exp. Res.* 37, 722–729. doi: 10.1111/acer.12034

Deehan, G. A. Jr., Hauser, S. R., Wilden, J. A., Truitt, W. A., and Rodd, Z. A. (2013b). Elucidating the biological basis for the reinforcing actions of alcohol in the mesolimbic dopamine system: the role of active metabolites of alcohol. *Front. Behav. Neurosci.* 7:104. doi: 10.3389/fnbeh.2013.00104

Di Chiara, G., and Imperato, A. (1988). Drugs abused by humans preferentially increase synaptic dopamine concentrations in the mesolimbic system of freely moving rats. *Proc. Nat. Acad. Sci. U S A* 85, 5274–5278. doi: 10.1073/pnas.85.14.5274

Ding, Z. M., Engleman, E. A., Rodd, Z. A., and McBride, W. J. (2012). Ethanol increases glutamate neurotransmission in the posterior ventral tegmental area of female wistar rats. *Alcohol. Clin. Exp. Res.* 36, 633–640. doi: 10.1111/j.1530-0277.2011.01665.x

Dohi, T., Kitayama, S., Kumagai, K., Hashimoto, W., and Morita, K. (2002). Pharmacology of monoamine neurotransmitter transporters. *Nihon Yakurigaku Zasshi* 120, 315–326. doi: 10.1254/fpj.120.315

Doyle, S. E., Ramôa, C., Garber, G., Newman, J., Toor, Z., and Lynch, W. J. (2014). A shift in the role of glutamatergic signaling in the nucleus accumbens core with the development of an addicted phenotype. *Biol. Psychiatry* 76, 810–815. doi: 10.1016/j.biopsych.2014.02.005

Eriksson, C. J. P., Sippel, H. W., and Forsander, O. A. (1977). The determination of acetaldehyde in biological samples by head-space gas chromatography. *Anal. Biochem.* 80, 116–124. doi: 10.1016/0003-2697(77)90631-5

Ezquer, M., Urzúa, C. A., Montecino, S., Leal, K., Conget, P., and Ezquer, F. (2016). Intravitreal administration of multipotent mesenchymal stromal cells triggers a cytoprotective microenvironment in the retina of diabetic mice. *Stem Cell Res. Ther.* 7:42. doi: 10.1186/s13287-016-0299-y

Fattore, L., and Diana, M. (2016). Drug addiction: an affective-cognitive disorder in need of a cure. *Neurosci. Biobehav. Rev.* 65, 341–361. doi: 10.1016/j.neubiorev.2016.04.006

Ferrier, L., Bérard, F., Debrauwer, L., Chabo, C., Langella, P., Buéno, L., et al. (2006). Impairment of the intestinal barrier by ethanol involves enteric microflora and mast cell activation in rodents. *Am. J. Pathol.* 168, 1148–1154. doi: 10.2353/ajpath.2006.050617

Foddai, M., Dosia, G., Spiga, S., and Diana, M. (2004). Acetaldehyde increases dopaminergic neuronal activity in the VTA. *Neuropsychopharmacology* 29, 530–536. doi: 10.1038/sj.npp.1300326

Frey, B. N., Valvassori, S. S., Gomes, K. M., Martins, M. R., Dal-Pizzol, F., Kapczinski, F., et al. (2006). Increased oxidative stress in submitochondrial particles after chronic amphetamine exposure. *Brain Res.* 1097, 224–229. doi: 10.1016/j.brainres.2006.04.076

Froehlich, J. C., Harts, J., Lumeng, L., and Li, T. K. (1990). Naloxone attenuates voluntary ethanol intake in rats selectively bred for high ethanol preference. *Pharmacol. Biochem. Behav.* 35, 385–390. doi: 10.1016/0091-3057(90)90174-g

Heath, A. C., Bucholz, K. K., Madden, P. A., Dinwiddie, S. H., Slutske, W. S., Bierut, D. J., et al. (1997). Genetic and environmental contributions to alcohol dependence risk in a national twin sample: consistency of findings in women and men. *Psychol. Med.* 27, 1381–1384. doi: 10.1017/s0033291797005643

Herrera-Marschitz, M., You, Z. B., Goiny, M., Meana, J. J., Silveira, R., Godukhin, O. V., et al. (1996). On the origin of extracellular glutamate levels monitored in the basal ganglia of the rat by *in vivo* microdialysis. *J. Neurochem.* 66, 1726–1735. doi: 10.1046/j.1471-4159.1996.66041726.x

Hipólito, L., Martí-Prats, L., Sánchez-Catalán, M. J., Polache, A., and Granero, L. (2011). Induction of conditioned place preference and dopam ine release by salsolinol in posterior VTA of rats: involvement of μ-opioid receptors. *Neurochem. Int.* 59, 559–562. doi: 10.1016/j.neuint.2011.04.014

Hipólito, L., Sánchez-Catalán, M.-J., Zornoza, T., Polache, A., and Granero, L. (2010). Locomotor stimulant effects of acute and repeated intrategmental injections of salsolinol in rats: role of μ-opioid receptors. *Psychopharmacology* 209, 1–11. doi: 10.1007/s00213-009-1751-9

Hölter, S. M., Engelmann, M., Kirschke, C., Liebsch, G., Landgraf, R., and Spanagel, R. (1998). Long-term ethanol self-administration with repeated ethanol deprivation episodes changes ethanol drinking pattern and increases anxiety-related behaviour during ethanol deprivation in rats. *Behav. Pharmacol.* 9, 41–48.

Hölter, S. M., and Spanagel, R. (1999). Effects of opiate antagonist treatment on the alcohol deprivation effect in long-term ethanol-experienced rats. *Psychopharmacology* 145, 360–369. doi: 10.1007/s002130051069

Hyman, S. E., Malenka, R. C., and Nestler, E. J. (2006). Neural mechanisms of addiction: the role of reward-related learning and memory. *Ann. Rev. Neurosci.* 29, 565–598. doi: 10.1146/annurev.neuro.29.051605.113009

Imperato, A., and Di Chiara, G. (1986). Preferential stimulation of dopamine-release in the accumbens of freely moving rats by ethanol. *J. Pharmacol. Exp. Ther.* 239, 219–228.

Israel, Y., Ezquer, F., Quintanilla, M. E., Morales, P., Ezquer, M., and Herrera-Marschitz, M. (2017). Intracerebral stem cell administration inhibits relapse-like alcohol drinking in rats. *Alcohol Alcohol.* 52, 1–4. doi: 10.1093/alcalc/agw068

Israel, Y., Rivera-Meza, M., Karahanian, E., Quintanilla, M. E., Tampier, L., Morales, P., et al. (2013). Gene specific modifications unravel ethanol and acetaldehyde actions. *Front. Behav. Neurosci.* 7:80. doi: 10.3389/fnbeh.2013.00080

Jalowiec, J. E., Panksepp, J., Zolovick, A. J., Najam, N., and Herman, B. H. (1981). Opioid modulation of ingestive behavior. *Pharmacol. Biochem. Behav.* 3, 477–484. doi: 10.1016/0091-3057(81)90280-x

Jung, B. D., Shin, E. J., Nguyen, X. K., Jin, C. H., Bach, J. H., Park, S. J., et al. (2010). Potentiation of methamphetamine neurotoxicity by intrastriatal lipopolysaccharide administration. *Neurochem. Int.* 56, 229–244. doi: 10.1016/j.neuint.2009.10.005

Jung, Y., and Surh, Y. (2001). Oxidative DNA damage and cytotoxicity induced by copper-stimulated redox cycling of salsolinol, a neurotoxic tetrahydroisoquinoline alkaloid. *Free Radic. Biol. Med.* 30, 1407–1417. doi: 10.1016/s0891-5849(01)00548-2

Kalivas, P. W., and McFarland, K. (2003). Brain circuitry and the reinstatement of cocaine-seeking behavior. *Psychopharmacology* 168, 44–56. doi: 10.1007/s00213-003-1393-2

Karahanian, E., Quintanilla, M. E., Tampier, L., Rivera-Meza, M., Bustamante, D., Gonzalez-Lira, V., et al. (2011). Ethanol as a prodrug: brain metabolism of ethanol mediates its reinforcing effects. *Alcohol. Clin. Exp. Res.* 35, 606–612. doi: 10.1111/j.1530-0277.2011.01439.x

Karahanian, E., Rivera-Meza, M., Tampier, L., Quintanilla, M. E., Herrera-Marschitz, M., and Israel, Y. (2015). Long-term inhibition of ethanol intake by the administration of an aldehyde dehydrogenase-2 (ALDH2)-coding lentiviral vector into the ventral tegmental area of rats. *Addict. Biol.* 20, 336–344. doi: 10.1111/adb.12130

Kastl, L., Sauer, S. W., Ruppert, T., Beissbarth, T., Becker, M. S., Süss, D., et al. (2014). TNF-α mediates mitochondrial uncoupling and enhances ROS-dependent cell migration via NF-κB activation in liver cell. *FEBS Lett.* 588, 175–183. doi: 10.1016/j.febslet.2013.11.033

King, G. S., Goodwin, B. L., and Sandler, M. (1974). Isosalsolinol formation: a secondary reaction in the Pictet-Spengler condensation. *J. Pharm. Pharmacol.* 26, 476–478. doi: 10.1111/j.2042-7158.1974.tb09323.x

Koob, G. F., and Le Moal, M. (2008). Review. Neurobiological mechanisms for opponent motivational processes in addiction. *Philos. Trans. R. Soc. Lond. B Biol. Sci.* 363, 3113–3123. doi: 10.1098/rstb.2008.0094

Koob, G. F., and Volkow, N. D. (2010). Neurocircuitry of addiction. *Neuropsychopharmacology* 35, 217–238. doi: 10.1038/npp.2009.110

Kopin, I. J. (1994). Monoamine oxidase and catecholamine metabolism. *J. Neural Transm. Suppl.* 41, 57–67. doi: 10.1007/978-3-7091-9324-2_7

Kupchik, Y. M., Moussawi, K., Tang, X. C., Wang, X., Kalivas, B. C., Kolokithas, R., et al. (2012). The effect of N-acetylcysteine in the nucleus accumbens on neurotransmission and relapse to cocaine. *Biol. Psychiatry* 71, 978–986. doi: 10.1016/j.biopsych.2011.10.024

LaRowe, S. D., Kalivas, P. W., Nicholas, J. S., Randall, P. K., Mardikian, P. N., and Malcolm, R. J. (2013). A double-blind placebo-controlled trial of N-acetylcysteine in the treatment of cocaine dependence. *Am. J. Addict.* 22, 443–452. doi: 10.1111/j.1521-0391.2013.12034.x

Lee, H. J., Oh, S.-H., Jang, H. W., Kwon, J.-H., Lee, K. J., Kim, C. H., et al. (2016). Long-term effects of bone marrow-derived mesenchymal Stem Cells in dextran sulfate sodium-induced murine chronic colitis. *Gut Liver* 10, 412–419. doi: 10.5009/gnl15229

Li, T. K. (2000). Pharmacogenetics of responses to alcohol and genes that influence alcohol drinking. *J. Stud. Alcohol.* 61, 5–12. doi: 10.15288/jsa.2000.61.5

Lindros, K. O., and Hillbom, M. E. (1979). Acetaldehyde in cerebrospinal fluid: its near-absence in ethanol-intoxicated alcoholics. *Med. Biol.* 57, 246–247.

Lucyk, S. N., Yarema, M. C., Sivilotti, M. L., Johnson, D. W., Nettel-Aguirre, A., Victorino, C., et al. (2016). Outcomes of patients with premature discontinuation of the 21-h intravenous N-acetylcysteine protocol after acute acetaminophen overdose. *J. Emerg. Med.* 50, 629–637. doi: 10.1016/j.jemermed.2015.12.004

Madayag, A., Lobner, D., Kau, K. S., Mantsch, J. R., Abdulhameed, O., Hearing, M., et al. (2007). Repeated *N*-Acetylcysteine administration alters plasticity-dependent effects of cocaine. *J. Neurosci.* 27, 13968–13976. doi: 10.1523/JNEUROSCI.2808-07.2007

Mardones, J., and Segovia-Riquelme, N. (1983). Thrity two years of selection of rats by ethanol preference: UChA and UChB strains. *Neurobehav. Toxicol. Teratol.* 5, 171–178.

Matsubara, K., Fukushima, S., and Fukui, Y. (1987). A systematic regional study of brain salsolinol levels during and immediately following chronic ethanol ingestion in rats. *Brain Res.* 413, 336–343. doi: 10.1016/0006-8993(87)91025-0

Matsuzawa, S., Suzuki, T., and Misawa, M. (2000). Involvement of μ-opioid receptor in the salsolinol-associated place preference in rats exposed to conditioned fear stress. *Alcohol. Clin. Exp. Res.* 24, 366–372. doi: 10.1097/00000374-200003000-00020

McClure, E. A., Gipson, C. D., Malcolm, R. J., Kalivas, P. W., and Gray, K. M. (2014). Potential role of N-acetylcysteine in the management of substance use disorders. *CNS Drugs* 28, 95–106. doi: 10.1007/s40263-014-0142-x

Melis, M., Carboni, E., Caboni, P., and Acquas, E. (2015). Key role of salsolinol in ethanol actions on dopamine neuronal activity of the posterior ventral tegmental area. *Addict. Biol.* 20, 182–193. doi: 10.1111/adb.12097

Melis, M., Enrico, P., Peana, A. T., and Diana, M. (2007). Acetaldehyde mediates alcohol activation of the mesolimbic dopamine system. *Eur. J. Neurosci.* 26, 2824–2833. doi: 10.1111/j.1460-9568.2007.05887.x

Montesinos, J., Alfonso-Loeches, S., and Guerri, C. (2016). Impact of the innate immune response in the actions of ethanol on the central nervous system. *Alcohol. Clin. Exp. Res.* 40, 2260–2270. doi: 10.1111/acer.13208

Moro, F., Orrù, A., Marzo, C. M., Di Clemente, A., and Cervo, L. (2016). mGluR2/3 mediates short-term control of nicotine-seeking by acute systemic N-acetylcysteine. *Addict. Biol.* doi: 10.1111/adb.12443 [Epub ahead of print].

Moussawi, K., Pacchioni, A., Moran, M., Olive, M. F., Gass, J. T., Lavin, A., et al. (2009). *N*-Acetylcysteine reverses cocaine-induced metaplasticity. *Nat. Neurosci.* 12, 182–189. doi: 10.1038/nn.2250

Muggironi, G., Fois, G. R., and Diana, M. (2013). Ethanol-derived acetaldehyde: pleasure and pain of alcohol mechanism of action. *Front. Behav. Neurosci.* 7:87. doi: 10.3389/fnbeh.2013.00087

Myers, R. D., and Melchior, C. L. (1977). Differential actions on voluntary alcohol intake by tetrahydroisoquinolines or a β-carboline infused chronically in the ventricle of the rat. *Pharmacol. Biochem. Behav.* 7, 381–392. doi: 10.1016/0091-3057(77)90235-0

Orrico, A., Hipólito, L., Sanchez-Catalán, J. M., Martí-Prats, L., Zornoza, T., Granero, L., et al. (2013). Efficacy of D-penicillamine, a sequestering acetaldehyde agent, in the prevention of alcohol relapse-like drinking in rats. *Psychopharmacology* 228, 563–575. doi: 10.1007/s00213-013-3065-1

Orrico, A., Martí-Prats, L., Cano-Cebrián, M. J., Granero, L., Polache, A., and Zornoza, T. (2014). Improved effect of the combination naltrexone/D-penicillamine in the prevention of alcohol relapse-like drinking in rats. *J. Psychopharmacol.* 28, 76–81. doi: 10.1177/0269881113515063

Peana, A. T., Enrico, P., Assaretti, A. R., Pulighe, E., Muggironi, G., Nieddu, M., et al. (2008). Key role of ethanol-derived acetaldehyde in the motivational properties induced by intragastric ethanol: a conditioned place preference study in the rat. *Alcohol. Clin. Exp. Res.* 32, 249–258. doi: 10.1111/j.1530-0277.2007.00574.x

Peana, A. T., Muggironi, G., and Diana, M. (2010). Acetaldehyde-reinforcing effects: a study on oral self-administration behavior. *Front. Psychiatry* 1:23. doi: 10.3389/fpsyt.2010.00023

Peana, A. T., Muggironi, G., Fois, G. R., Zinellu, M., Vinci, S., and Acquas, E. (2011). Effect of opioid receptor blockade on acetaldehyde self-administration and ERK phosphorylation in the rat nucleus accumbens. *Alcohol* 45, 773–783. doi: 10.1016/j.alcohol.2011.06.003

Petersen, D. R., and Tabakoff, B. (1979). Characterization of brain acetaldehyde oxidizing systems in the mouse. *Drug Alcohol Depend.* 4, 137–144. doi: 10.1016/0376-8716(79)90054-1

Phillips, T. J., Crabbe, J. C., Metten, P., and Belknap, J. K. (1994). Localization of genes affecting alcohol drinking in mice. *Alcohol. Clin. Exp. Res.* 18, 931–941. doi: 10.1111/j.1530-0277.1994.tb00062.x

Pickens, C. L., Airavaara, M., Theberge, F., Fanous, S., Hope, B. T., and Shaham, Y. (2011). Neurobiology of the incubation of drug craving. *Trends Neurosci.* 34, 411–420. doi: 10.1016/j.tins.2011.06.001

Pomierny-Chamiolo, L., Moniczewski, A., Wydra, K., Suder, A., and Filip, M. (2013). Oxidative stress biomarkers in some rat brain structures and peripheral

organs underwent cocaine. *Neurotox. Res.* 23, 92–102. doi: 10.1007/s12640-012-9335-6

Prockop, D. J., Kota, D. J., Bazhanov, N., and Reger, R. L. (2010). Evolving paradigms for repair of tissues by adult stem/progenitor cells (MSCs). *J. Cell. Mol. Med.* 14, 2190–2199. doi: 10.1111/j.1582-4934.2010.01151.x

Qiusheng, Z., Yuntao, Z., Rongliang, Z., Dean, G., and Changling, L. (2005). Effects of verbascoside and luteolin on oxidative damage in brain of heroin treated mice. *Pharmazie* 60, 539–543.

Quintanilla, M. E., Bustamante, D., Tampier, L., Israel, Y., and Herrera-Marschitz, M. (2007). Dopamine release in the nucleus accumbens (shell) of two lines of rats selectively bred to prefer or avoid ethanol. *Eur. J. Pharmacol.* 573, 84–92. doi: 10.1016/j.ejphar.2007.06.038

Quintanilla, M. E., Israel, Y., Sapag, A., and Tampier, L. (2006). The UChA and UChB rat lines: metabolic and genetic differences influencing ethanol intake. *Addict. Biol.* 11, 310–323. doi: 10.1111/j.1369-1600.2006.00030.x

Quintanilla, M. E., Rivera-Meza, M., Berríos-Cárcamo, P., Cassels, B. K., Herrera-Marschitz, M., and Israel, Y. (2016a). (*R*)-Salsolinol, a product of ethanol metabolism, stereospecifically induces behavioral sensitization and leads to excessive alcohol intake. *Addict. Biol.* 21, 1063–1071. doi: 10.1111/adb.12268

Quintanilla, M. E., Rivera-Meza, M., Berríos-Cárcamo, P., Salinas-Luypaert, C., Herrera-Marschitz, M., and Israel, Y. (2016b). Beyond the "First Hit": marked inhibition by N-acetyl cysteine of chronic ethanol intake but not of early ethanol intake. parallel effects on ethanol-induced saccharin motivation. *Alcohol. Clin. Exp. Res.* 40, 1044–1051. doi: 10.1111/acer.13031

Quintanilla, M. E., Rivera-Meza, M., Berríos-Cárcamo, P. A., Bustamante, D., Buscaglia, M., Morales, P., et al. (2014). Salsolinol, free of isosalsolinol, exerts ethanol-like motivational/sensitization effects leading to increases in ethanol intake. *Alcohol* 48, 551–559. doi: 10.1016/j.alcohol.2014.07.003

Quintanilla, M. E., and Tampier, L. (2003). Acetaldehyde-reinforcing effects: differences in low-alcohol-drinking (UChA) and high-alcohol-drinking (UChB) rats. *Alcohol* 31, 63–69. doi: 10.1016/j.alcohol.2003.07.001

Quintanilla, M., Tampier, L., Karahanian, E., Rivera-Meza, M., Herrera-Marschitz, M., and Israel, Y. (2012). Reward and relapse: complete gene-induced dissociation in an animal model of alcohol dependence. *Alcohol. Clin. Exp. Res.* 36, 517–522. doi: 10.1111/j.1530-0277.2011.01606.x

Quintanilla, M. E., Tampier, L., Valle-Prieto, A., Sapag, A., and Israel, Y. (2005). Complex I regulates mutant mitochondrial aldehyde dehydrogenase activity and voluntary ethanol consumption in rats. *FASEB J.* 19, 36–42. doi: 10.1096/fj.04-2172com

Ramirez-Niño, A. M., D'Souza, M. S., and Markou, A. (2013). N-acetylcysteine decreased nicotine self-administration and cue-induced reinstatement of nicotine seeking in rats: comparison with the effects of N-acetylcysteine on food responding and food seeking. *Psychopharmacology* 225, 473–482. doi: 10.1007/s00213-012-2837-3

Reichel, C. M., Moussawi, K., Do, P. H., Kalivas, P. W., and See, R. E. (2011). Chronic N-acetylcysteine during abstinence or extinction after cocaine self-administration produces enduring reductions in drug seeking. *J. Pharmacol. Exp. Ther.* 337, 487–493. doi: 10.1124/jpet.111.179317

Reissner, K. J., Gipson, C. D., Tran, P. K., Knackstedt, L. A., Scofield, M. D., and Kalivas, P. W. (2015). Glutamate transporter GLT-1 mediates N-acetylcysteine inhibition of cocaine reinstatement. *Addict. Biol.* 20, 316–323. doi: 10.1111/adb.12127

Reissner, K. J., and Kalivas, P. W. (2010). Using glutamate homeostasis as a target for treating addictive disorders. *Behav. Pharmacol.* 21, 514–522. doi: 10.1097/FBP.0b013e32833d41b2

Rodd, Z. A., Bell, R. L., Kuc, K. A., Murphy, J. M., Lumeng, L., and McBride, W. J. (2009). Effects of concurrent access to multiple ethanol concentrations and repeated deprivations on alcohol intake of high-alcohol-drinking (HAD) rats. *Addict. Biol.* 14, 152–164. doi: 10.1111/j.1369-1600.2008.00140.x

Rodd, Z. A., Bell, R. L., Zhang, Y., Murphy, J. M., Goldstein, A., Zaffaroni, A., et al. (2005). Regional heterogeneity for the intracranial self-administration of ethanol and acetaldehyde within the ventral tegmental area of alcohol-preferring (P) rats: involvement of dopamine and serotonin. *Neuropsychopharmacology* 30, 330–338. doi: 10.1038/sj.npp.1300561

Rodd, Z. A., Oster, S. M., Ding, Z. M., Toalston, J. E., Deehan, G., Bell, R. L., et al. (2008). The reinforcing properties of salsolinol in the ventral tegmental area: evidence for regional heterogeneity and the involvement of serotonin and dopamine. *Alcohol. Clin. Exp. Res.* 32, 230–239. doi: 10.1111/j.1530-0277.2007.00572.x

Rojkovicova, T., Mechref, Y., Starkey, J. A., Wu, G., Bell, R. L., McBride, W. J., et al. (2008). Quantitative chiral analysis of salsolinol in different brain regions of rats genetically predisposed to alcoholism. *J. Chromatogr. B Analyt. Technol. Biomed. Life Sci.* 863, 206–214. doi: 10.1016/j.jchromb.2008.01.016

Rommelspacher, H., Sllström Baum, S., Dufeu, P., and Schmidt, L. G. (1995). Determination of (*R*)- and (*S*)-salsolinol sulfate and dopamine sulfate levels in plasma of nonalcoholics and alcoholics. *Alcohol* 12, 309–315. doi: 10.1016/0741-8329(95)00004-b

Rothman, R. B., Baumann, M. H., Dersch, C. M., Romero, D. V., Rice, K. C., Carroll, F. I., et al. (2001). Amphetamine-type central nervous system stimulants release norepinephrine more potently than they release dopamine and serotonin. *Synapse* 39, 32–41. doi: 10.1002/1098-2396(20010101)39:1<32::AID-SYN5>3.0.CO;2-3

Sapag, A., Tampier, L., Valle-Prieto, A., Quintanilla, M. E., Moncada, C., and Israel, Y. (2003). Mutations in mitochondrial aldehyde dehydrogenase (ALDH2) change cofactor affinity and segregate with voluntary alcohol consumption in rats. *Pharmacogenetics* 13, 509–515. doi: 10.1097/00008571-200308000-00009

Sari, Y., Franklin, K. M., Alazizi, A., Rao, P. S., and Bell, R. L. (2013). Effects of ceftriaxone on the acquisition and maintenance of ethanol drinking in peri-adolescent and adult female alcohol-preferring (P) rats. *Neuroscience* 241, 229–238. doi: 10.1016/j.neuroscience.2013.03.016

Scofield, M. D., and Kalivas, P. W. (2014). Astrocytic dysfunction and addiction: consequences of impaired glutamate homeostasis. *Neuroscientist* 20, 610–622. doi: 10.1177/1073858413520347

Sjöquist, B., Liljequist, S., and Engel, J. (1982). Increased salsolinol levels in rat striatum and limbic forebrain following chronic ethanol treatment. *J. Neurochem.* 39, 259–262. doi: 10.1111/j.1471-4159.1982.tb04730.x

Spanagel, R., Vengeliene, V., Jandeleit, B., Fischer, W. N., Grindstaff, K., Zhang, X., et al. (2014). Acamprosate produces its anti-relapse effects via calcium. *Neuropsychopharmacology* 39, 783–791. doi: 10.1038/npp.2013.264

Spanagel, R., and Zieglgänsberger, W. (1997). Anti-craving compounds for ethanol: new pharmacological tools to study addictive processes. *Trends Pharmacol. Sci.* 18, 54–59. doi: 10.1016/s0165-6147(97)89800-8

Squeglia, L. M., Baker, N. L., McClure, E. A., Tomko, R. L., Adisetiyo, V., and Gray, K. M. (2016). Alcohol use during a trial of N-acetylcysteine for adolescent marijuana cessation. *Addict. Behav.* 63, 172–177. doi: 10.1016/j.addbeh.2016.08.001

Stowell, A., Hillbom, M., Salaspuro, M., and Lindros, K. O. (1980). Low acetaldehyde levels in blood, breath and cerebrospinal fluid of intoxicated humans as assayed by improved methods. *Adv. Exp. Med. Biol.* 132, 635–645. doi: 10.1007/978-1-4757-1419-7_66

Sullivan, E. V., Marsh, L., Mathalon, D. H., Lim, K. O., and Pfefferbaum, A. (1995). Anterior hippocampal volume deficits in nonamnesic, aging chronic alcoholics. *Alcohol. Clin. Exp. Res.* 19, 110–122. doi: 10.1111/j.1530-0277.1995.tb01478.x

Tampier, L., and Mardones, J. (1979). Catalase mediated oxidation of ethanol by rat brain homogenates. *ICRS Med. Sci.* 7:389.

Tampier, L., Quintanilla, M. E., Karahanian, E., Rivera-Meza, M., Herrera-Marschitz, M., and Israel, Y. (2013). The alcohol deprivation effect: marked inhibition by anticatalase gene administration into the ventral tegmental area in rats. *Alcohol. Clin. Exp. Res.* 37, 1278–1285. doi: 10.1111/acer.12101

Tawa, E. A., Hall, S. D., and Lohoff, F. W. (2016). Overview of the genetics of alcohol use disorder. *Alcohol Alcohol.* 51, 507–514. doi: 10.1093/alcalc/agw046

Thiele, T. E., Crabbe, J. C., and Boehm, S. L. II (2014). "Drinking in the Dark" (DID): a simple mouse model of binge-like alcohol intake. *Curr. Protoc. Neurosci.* 68, 9.49.1–9.49.12. doi: 10.1002/0471142301.ns0949s68

Vengeliene, V., Bachteler, D., Danysz, W., and Spanagel, R. (2005). The role of the NMDA receptor in alcohol relapse: a pharmacological mapping study using the alcohol deprivation effect. *Neuropharmacology* 48, 822–829. doi: 10.1016/j.neuropharm.2005.01.002

Vengeliene, V., Bilbao, A., and Spanagel, R. (2014). The alcohol deprivation effect model for studying relapse behavior: a comparison between rats and mice. *Alcohol* 48, 313–320. doi: 10.1016/j.alcohol.2014.03.002

Volkow, N. D., Fowler, J. S., Wang, J., and Goldstein, R. Z. (2002). Role of dopamine, the frontal cortex and memory circuits in drug addiction: insight from imaging studies. *Neurobiol. Learn. Mem.* 78, 610–624. doi: 10.1006/nlme.2002.4099

Walker, D. W., Barnes, D. E., Zornetzet, S. F., Hunter, B. E., and Kubanis, P. (1980). Neuronal loss in hippocampus induced by prolonged ethanol consumption in rats. *Science* 209, 711–713. doi: 10.1126/science.7394532

Weiland, A., Garcia, S., and Knackstedt, L. A. (2015). Ceftriaxone and cefazolin attenuate the cue-primed reinstatement of alcohol-seeking. *Front. Pharmacol.* 6:44. doi: 10.3389/fphar.2015.00044

Wolf, M. E., Mangiavacchi, S., and Sun, X. (2003). Mechanisms by which dopamine receptors may influence synaptic plasticity. *Ann. N. Y. Acad. Sci.* 1003, 241–249. doi: 10.1196/annals.1300.015

Xu, B., Wang, Z., Li, G., Li, B., Lin, H., Zheng, R., et al. (2006). Heroin-administered mice involved in oxidative stress and exogenous antioxidant-alleviated withdrawal syndrome. *Basic Clin. Pharmacol. Toxicol.* 99, 153–161. doi: 10.1111/j.1742-7843.2006.pto_461.x

Yagi, H., Soto-Gutierrez, A., Navarro-Alvarez, N., Nahmias, Y., Goldwasser, Y., Kitagawa, Y., et al. (2010). Reactive bone marrow stromal cells attenuate systemic inflammation via sTNFR1. *Mol. Ther.* 18, 1857–1864. doi: 10.1038/mt.2010.155

Yang, H.-Y., Wu, X.-M., Liu, Y., and He, D. (2015). Transplantation of bone marrow mesenchymal stem cells promotes learning and memory functional recovery and reduces hippocampal damage in rats with alcohol-associated dementia. *Transplantation* 99, 492–499. doi: 10.1097/TP.0000000000000535

Zhou, W., and Kalivas, P. W. (2008). N-acetylcysteine reduces extinction responding and induces enduring reductions in cue- and heroin-induced drug-seeking. *Biol. Psychiatry* 63, 338–340. doi: 10.1016/j.biopsych.2007.06.008

Zimatkin, S. M., Pronko, S. P., Vasiliou, V., Gonzalez, F. J., and Deitrich, R. A. (2006). Enzymatic mechanisms of ethanol oxidation in the brain. *Alcohol. Clin. Exp. Res.* 30, 1500–1505. doi: 10.1111/j.1530-0277.2006.00181.x

Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

frontiers in Behavioral Neuroscience

REVIEW
published: 11 May 2017
doi: 10.3389/fnbeh.2017.00081

Mystic Acetaldehyde: The Never-Ending Story on Alcoholism

Alessandra T. Peana[1]†, María J. Sánchez-Catalán[2†], Lucia Hipólito[2†], Michela Rosas[3], Simona Porru[3], Federico Bennardini[1], Patrizia Romualdi[4], Francesca F. Caputi[4], Sanzio Candeletti[4], Ana Polache[2], Luis Granero[2] and Elio Acquas[3,5]**

[1]Department of Chemistry and Pharmacy, University of Sassari, Sassari, Italy, [2]Department of Pharmacy, Pharmaceutical Technology and Parasitology, University of Valencia, València, Spain, [3]Department of Life and Environmental Sciences, University of Cagliari, Cagliari, Italy, [4]Department of Pharmacy and Biotechnology, University of Bologna, Bologna, Italy, [5]Centre of Excellence on Neurobiology of Addiction, University of Cagliari, Cagliari, Italy

After decades of uncertainties and drawbacks, the study on the role and significance of acetaldehyde in the effects of ethanol seemed to have found its main paths. Accordingly, the effects of acetaldehyde, after its systemic or central administration and as obtained following ethanol metabolism, looked as they were extensively characterized. However, almost 5 years after this research appeared at its highest momentum, the investigations on this topic have been revitalized on at least three main directions: (1) the role and the behavioral significance of acetaldehyde in different phases of ethanol self-administration and in voluntary ethanol consumption; (2) the distinction, in the central effects of ethanol, between those arising from its non-metabolized fraction and those attributable to ethanol-derived acetaldehyde; and (3) the role of the acetaldehyde-dopamine condensation product, salsolinol. The present review article aims at presenting and discussing prospectively the most recent data accumulated following these three research pathways on this never-ending story in order to offer the most up-to-date synoptic critical view on such still unresolved and exciting topic.

Keywords: ethanol, acetaldehyde, salsolinol, ethanol metabolism, epigenetics, neuroinflammation, mesolimbic system, dopamine

OPEN ACCESS

Edited by:
Rainer Spanagel, Central Institute of Mental Health, Germany

Reviewed by:
Sheketha R. Hauser, Indiana University School of Medicine, USA
CJ Peter Eriksson, University of Helsinki, Finland

****Correspondence:***
Alessandra T. Peana
apeana@uniss.it
Elio Acquas
acquas@unica.it

†These authors have contributed equally to this work.

Received: *26 January 2017*
Accepted: *18 April 2017*
Published: *11 May 2017*

Citation:
Peana AT, Sánchez-Catalán MJ, Hipólito L, Rosas M, Porru S, Bennardini F, Romualdi P, Caputi FF, Candeletti S, Polache A, Granero L and Acquas E (2017) Mystic Acetaldehyde: The Never-Ending Story on Alcoholism. Front. Behav. Neurosci. 11:81. doi: 10.3389/fnbeh.2017.00081

INTRODUCTION

The investigations on the role of acetaldehyde and ethanol metabolism in the central effects of ethanol have been a long-standing issue of interest and controversy (McBride et al., 2002; Quertemont et al., 2005; Correa et al., 2012). Thus, although numerous lines of research have focused on the role of acetaldehyde in different aspects of ethanol effects, the role of its main metabolite in the biological basis of its effects and, in particular, of its reinforcing properties, are still not fully understood. Accordingly, contrasting theories have arisen suggesting, on one hand, that ethanol is a molecule responsible of the reinforcing properties of alcoholic drinks and, on the other hand, that ethanol acts as pro-drug and hence owns most of its central effects to other compounds generated, directly or indirectly, from its metabolism. Followers of the first view suggest that ethanol exerts its properties within the brain by affecting numerous neurotransmitter systems, that there is no significant evidence that its metabolites cross the blood brain barrier and that the metabolites occur for only short periods to mediate the effects of ethanol intoxication. The working hypothesis that envisions ethanol as a pro-drug, on the other hand, suggests that its activating and reinforcing properties are supported by the central actions of its metabolites

(Deng and Deitrich, 2008; Deitrich, 2011; Karahanian et al., 2011; Correa et al., 2012; Hipólito et al., 2012; Peana and Acquas, 2013; Israel et al., 2015), no matter if generated centrally or peripherally. In the latter case, according to the pro-drug theory, acetaldehyde's plasma levels, following ethanol intake, would reach concentrations sufficient to significantly affect its targets within the central nervous system (CNS). Another possibility predicts that the activating and reinforcing properties, mostly mediated through the involvement of the dopaminergic mesolimbic system, could depend on the actions of ethanol itself in combination with those of its metabolites produced within the brain from local metabolism of ethanol (Sánchez-Catalán et al., 2008; Hipólito et al., 2009; Martí-Prats et al., 2013, 2015). Accordingly, after ethanol administration, the net effect on the activity of dopamine (DA) neurons would be the *algebraic* consequence of the activation, due to the action of the ethanol derivatives, and the depression, due to ethanol itself (Martí-Prats et al., 2013, 2015). Indeed, the concentrations and duration of the effects of ethanol and its derivatives determine the final effect on DA neurons, which is ultimately governed by the rate of ethanol metabolism (Martí-Prats et al., 2013, 2015).

Significant behavioral evidence implicates acetaldehyde in the mechanisms underlying the psychopharmacological effects of ethanol (Correa et al., 2012; Peana and Acquas, 2013; Peana et al., 2016). Acetaldehyde has reinforcing properties on its own (Correa et al., 2012; Peana et al., 2016), induces euphoria at low concentrations (Eriksson, 2001) and has been involved in alcohol addiction (Deng and Deitrich, 2008; Deehan et al., 2013). Moreover, support to the critical role of acetaldehyde in the reinforcing properties of ethanol was provided by the observations that a negative interference with the peripheral or central metabolism of ethanol to acetaldehyde, as well as a reduction of its bioavailability, prevents several ethanol actions, including its reinforcing effects (Foddai et al., 2004; Melis et al., 2007; Peana et al., 2008, 2010a,b, 2015; Enrico et al., 2009; Martí-Prats et al., 2013, 2015; Orrico et al., 2013, 2014). This is in agreement with the original observation, made by Chevens (1953). In fact, he reported that his patients did not perceive aversive effects by taking low amounts of ethanol when they were under treatment with disulfiram, an inhibitor of aldehyde dehydrogenase (ALDH), suggesting that ALDH inhibition could increase the euphoric and pleasurable effects of small doses of ethanol by increasing acetaldehyde's availability (Brown et al., 1980).

In addition to the above, acetaldehyde has multiple tissue damage effects and these also should be appreciated as a feature of another never-ending story. In fact, humans are frequently exposed to acetaldehyde from various sources including alcoholic beverages, tobacco smoke and foods and even microbes are responsible for the bulk of acetaldehyde production from ethanol both in saliva and in the Helicobacter pylori-infected and achlorhydric stomach (Salaspuro, 2011). Moreover, acetaldehyde is also usually used as a food additive and aroma agent. Unfortunately, acetaldehyde is mutagenic and carcinogenic being responsible of DNA damage and of several cancer-promoting effects (Dellarco, 1988; Seitz and Stickel, 2010). Accordingly, acetaldehyde and ethanol are two of the compounds for which the most comprehensive evidence on epidemiology and mechanisms of carcinogenesis is accessible. In the relationship between alcohol consumption and development of different forms of cancer, the impact of the risk of developing this pathology mostly depends on alcohol consumption (Shield et al., 2013) and even a moderate drinking has been shown to cause cancer (Bagnardi et al., 2013). Different hypothesis have been proposed to explain how ethanol and acetaldehyde may cause or contribute to carcinogenesis, the main mechanism being attributable to the metabolism of ethanol into the carcinogenic, and DNA binding, acetaldehyde (Seitz and Stickel, 2007). Accordingly, humans deficient in mitochondrial ALDH2 present an increased risk of developing malignant tumours of the upper digestive tract (Lachenmeier and Salaspuro, 2017). Likewise, ethanol may also be metabolized into acetaldehyde by cytochrome CYP2E1, a process that produces radical oxygen species (ROS) that may lead to lipid peroxidation and to the formation of mutagenic adducts (Pflaum et al., 2016). Additionally, acetaldehyde may also lead to DNA hypomethylation, which changes the expression of oncogenes and tumour-suppression genes (Seitz and Stickel, 2007; Pflaum et al., 2016). Finally, in this regard, recent research from Lachenmeier and Salaspuro (2017) reported that many of previous animal toxicology-based risk assessments might have underestimated the risk of acetaldehyde toxicity. Interestingly, buccal tablets slowly releasing L-cysteine, a semi-essential amino acid, are able to reduce or remove microbially-formed carcinogenic acetaldehyde from saliva during ethanol intake. Indeed, L-cysteine binds covalently acetaldehyde producing a stable compound (Salaspuro et al., 2002).

Another critical issue related to the neurobiological basis of the central effects of ethanol refers to the increasing evidence of other biologically active compounds (adducts), which are obtained after acetaldehyde's reaction with endogenous monoamines and appear responsible of ethanol's effects. As regards these adducts, the properties of salsolinol (formed when acetaldehyde binds to DA) as well as its potential role in the neurobiological properties of ethanol have been recently re-evaluated not only in light of the reinforcing properties of ethanol (Hipólito et al., 2012; Deehan et al., 2013) but also in light of the ability of salsolinol itself to affect ethanol intake (Quintanilla et al., 2014), locomotor activity (Hipólito et al., 2010; Quintanilla et al., 2014), conditioned place preference (CPP; Matsuzawa et al., 2000; Hipólito et al., 2011) and to exert neurotoxicity (Hernández et al., 2016).

In this regard, while many studies link Parkinson's disease with exposure to endogenous salsolinol (Tieu, 2011), this molecule has recently also been suggested as responsible for inducing experimental enteric neurodegeneration in rats (Kurnik et al., 2015).

THE ISSUE OF ACETALDEHYDE DETERMINATION

During the last years, several studies attempted to measure acetaldehyde in blood and brain following the systemic administration of either ethanol or acetaldehyde itself in order

to correlate plasma to brain concentrations. In particular, while some studies have described detection of brain acetaldehyde after peripheral ethanol (Kiessling, 1962; Sippel, 1974; Tabakoff et al., 1976; Eriksson and Sippel, 1977; Westcott et al., 1980; Hamby-Mason et al., 1997; Peana et al., 2008, 2010b) or acetaldehyde administration (Heap et al., 1995; Ward et al., 1997; Quertemont et al., 2004; Plescia et al., 2015), others reported failure to detect it after administration of either ethanol (Sippel, 1974; Eriksson and Sippel, 1977; Jamal et al., 2007) or acetaldehyde itself (Peana et al., 2010a). Indeed, increases of acetate but not acetaldehyde were detected in human plasma after ethanol intake (Puig and Fox, 1984; Sarkola et al., 2002). These controversial results could be associated with a number of critical confounding factors that overall still limit the reliable detection of this compound. In fact, acetaldehyde formation in the brain is still subject to speculation due to the lack of a specific method able to accurately and directly assay its levels. One of these limitations is certainly represented by the fact that ALDH is more abundantly expressed with respect to the catalase-H_2O_2 (Zimatkin et al., 1992). Indeed, the most efficient isoform of this dehydrogenase, ALDH2, rapidly metabolizes acetaldehyde to acetate (Deitrich, 2004; Deng and Deitrich, 2008). Moreover, considering the factors that may interfere with acetaldehyde's assessments in the brain, another aspect that should be taken into account is that acetaldehyde possesses a short elimination half-life and is a highly reactive electrophilic chemical that, thereby, is able to bind to nucleophilic structures to give condensation products. Lastly, acetaldehyde, like other volatile compounds, can easily cross the alveolar-capillary membrane of the lungs and be eliminated by exhalation (Eriksson and Sippel, 1977; Tardif, 2007) making it plausible that also this factor may contribute significantly to the difficulty of its detection.

Overall, this evidence indicates that after systemic administration of ethanol or acetaldehyde itself, acetaldehyde concentration must increase above a certain threshold level (i.e., above the detection limit of the available analytical approaches) in order to be reliably detected in the brain.

PERIPHERAL GENERATION OF ACETALDEHYDE

The conventional view that ethanol metabolism to acetaldehyde is carried out by class I liver alcohol dehydrogenase (ADH1; Haseba and Ohno, 2010), was obtained following animal (Bradford et al., 1993a,b; Escarabajal and Aragon, 2002; Peana et al., 2008) and human (Blomstrand and Theorell, 1970; Crow and Hardman, 1989; Sarkola et al., 2002) experiments with specific inhibitors (pyrazoles) of ADH (**Figure 1**). In humans, ADH1 is further classified into three subcategories, ADH1A, 1B and 1C (all inhibited by 4-methylpyrazole, 4-MP), which are the main ADHs for the oxidation of ethanol (Hempel et al., 1984). Interestingly, also class III (ADH3) has been reported to contribute to systemic ethanol metabolism in a dose-dependent manner, thereby contributing to diminish the consequences of acute ethanol intoxication. In particular, ADH3 (the isoform belonging to class III according to the old nomenclature may participate to ethanol metabolism together with ADH1 or

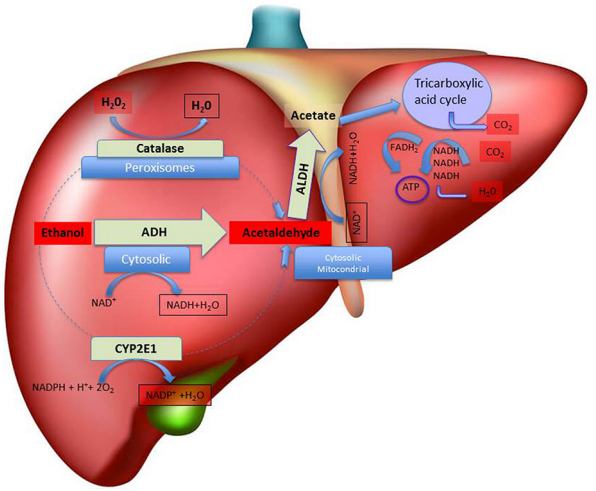

FIGURE 1 | Schematic representation of hepatic metabolism of ethanol. The figure depicts, in the liver, the sub-cellular (cytosolic, peroxisomal and mitochondrial) localization of the main pathways of ethanol oxidative metabolism to acetaldehyde and of the main pathways of ethanol by-products (acetaldehyde and acetate) disposal, with indication of the relative co-factors involved. Abbreviations: ADH, Alcohol dehydrogenase; ALDH, Aldehyde dehydrogenase; ATP, adenosine triphosphate; CYP2E1, isoform 2E1 of cytochrome P_{450}; $FADH_2$, flavin-adenine dinucleotide coenzyme in its reduced form; NAD^+, nicotinamide adenine dinucleotide coenzyme; NADPH, Nicotinamide Adenine Dinucleotide Phosphate coenzyme in its reduced form.

compensating for its reduced contribution (Haseba et al., 2003). In addition, it was suggested that chronic binge drinking might shift the key metabolic pathway from ADH1 to ADH3 (Haseba and Ohno, 2010), therefore attributing ADH3 a more critical role at high ethanol concentrations. Notably, 4-MP inhibits ADH1 but not ADH3 (Haseba and Ohno, 1997).

Besides the involvement of ADH, also other pathways may play a critical role in ethanol metabolism in the liver (Takagi et al., 1986; Zimatkin et al., 2006; Hipólito et al., 2007; Haseba and Ohno, 2010; **Figure 1**). In this regard, and after decades of attempts aimed at identifying the enzyme(s) responsible for the ADH1-independent fraction of ethanol metabolism, the research has focused on the microsomal (MEOS, mostly CYP2E1; Lieber and DeCarli, 1972; Takagi et al., 1986; Teschke and Gellert, 1986) and catalase-hydrogen peroxide (H_2O_2; Aragon et al., 1985; Handler and Thurman, 1988b; Aragon and Amit, 1992; Bradford et al., 1993a,b; Lieber, 2004) ethanol oxidizing systems, as the ones that may come into account especially when blood alcohol is high or when drinking is chronic (Sánchez-Catalán et al., 2008). Indeed, the induction of MEOS activity due to chronic ethanol consumption seems to explain the accelerated rate of ethanol metabolism observed in chronic drinkers (Pikkarainen and Lieber, 1980). Moreover, catalase and MEOS exhibit ethanol-oxidizing activities higher than that of ADH1 and both are insensitive to pyrazoles *in vitro* (Lieber and DeCarli, 1972). Interestingly, Takagi et al. (1986) have shown that 4-MP actually inhibits MEOS in deermice genetically lacking ADH, both *in vivo* and in *in vitro* concluding that MEOS plays a significant role in ethanol oxidation. However, the role of MEOS and catalase

in the consequences of systemic ethanol metabolism are yet to be fully characterized (Hipólito et al., 2007) whereas the contribution of ADH to peripheral ethanol metabolism may have been overestimated, based on some experiments where the treatment with 4-MP caused in man a very low rate of ethanol elimination (Blomstrand and Theorell, 1970), bringing to the conclusion that catalase and MEOS, and not only ADH, may be mainly responsible for ethanol's metabolism (Blomstrand et al., 1973).

The rate-limiting step in the catalase-dependent peroxidation of ethanol is that of H_2O_2 generation (Oshino et al., 1973) that can be originated at high rates also from fatty acids metabolism (Handler and Thurman, 1988a). In particular, ethanol metabolism in the liver is mediated predominantly by catalase-H_2O_2 in the fasted state (Handler and Thurman, 1988b) and it is interesting to observe that 4-MP inhibits also acyl-CoA synthase, an enzyme essential to initiate the process of fatty acid oxidation (Bradford et al., 1993a,b). Nonetheless, some authors observed that pre-treatment with catalase inhibitors does not significantly affect ethanol pharmacokinetics (bioavailability, elimination; Tampier and Mardones, 1987; Aragon et al., 1989), suggesting that catalase does not participate actively to hepatic ethanol metabolism.

Lastly, the acetaldehyde produced by the oxidation of ethanol is thereafter transformed by ALDH to acetate, which can be further metabolized through the tricarboxylic acid cycle to generate energy (**Figure 1**). ALDH, that plays an important role for determining the peripheral acetaldehyde levels, is further classified into two subcategories: ALDH1 present in the cytosol and ALDH2 present in the mitochondria (Weiner and Wang, 1994). Accordingly, high acetate but not acetaldehyde concentrations can be detected in human plasma after ethanol intake (Hernández et al., 2016). Notably, eastern Asians, because of the high prevalence of ALDH2*2 allele among those populations, may be more susceptible to the effect of ethanol (and acetaldehyde) with important public health implications that may be utilized to promote ethanol abstinence or reduce ethanol consumption.

CENTRAL GENERATION OF ACETALDEHYDE

Ethanol can easily cross the blood-brain barrier and be metabolized in the brain. However, the cellular types the blood-brain barrier is made of, endothelial cells and oligodendrocites, highly express ALDH (Zimatkin, 1991; Zimatkin et al., 1992), which metabolizes acetaldehyde to acetate, preventing the entrance of peripherally generated acetaldehyde into the brain (Eriksson and Sippel, 1977; Deitrich et al., 1978; Hipólito et al., 2007). Thus, unless the blood-brain (metabolic) barrier activity undergoes saturation (Westcott et al., 1980; Hoover and Brien, 1981; Zimatkin, 1991), acetaldehyde levels following acute ethanol administration may hardly reach the blood concentration critical to allow acetaldehyde crossing it (Tabakoff et al., 1976; Eriksson and Fukunaga, 1993) and, therefore, affecting its targets within the CNS. Otherwise, the oxidation of ethanol to acetaldehyde can occur in the brain through

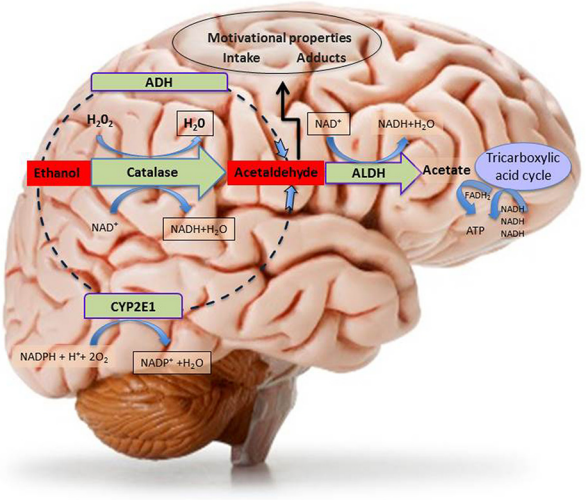

FIGURE 2 | Schematic representation of central metabolism of ethanol. The figure depicts the three main central metabolic pathways of ethanol oxidative metabolism to acetaldehyde and the main metabolic pathways of ethanol by-product (acetaldehyde and acetate) disposal, with indication of the relative co-factors involved. Abbreviations: ADH, Alcohol dehydrogenase; ALDH, Aldehyde dehydrogenase; ATP, adenosine triphosphate; CYP2E1, isoform 2E1 of cytochrome P_{450}; $FADH_2$, flavin-adenine dinucleotide in its reduced form; H_2O_2, Hydrogen peroxide; NAD^+, nicotinamide adenine dinucleotide coenzyme; NADPH, Nicotinamide Adenine Dinucleotide Phosphate coenzyme in its reduced form.

pathways that involve catalase, CYP2E1 and ADH (Hipólito et al., 2007; **Figure 2**). In particular, although under appropriate conditions the latter seems to represent a main pathway of ethanol metabolism in the liver, it has been attributed a minor contribution in the brain as indicated by biochemical (Zimatkin et al., 2006) and behavioral studies (Escarabajal and Aragon, 2002). Interestingly, a recent study, showed that ADH, whose several isoforms, such as ADH1, 3 and 4, have been found in the mammal brain (Boleda et al., 1989; Galter et al., 2003; Hipólito et al., 2007), is related to the enhancement of voluntary ethanol intake in University of Chile Bibulous (UChB) rats, bred for their high alcohol preference, after an injection into the ventral tegmental area (VTA) of a lentiviral vector encoding for ADH (Karahanian et al., 2011). Conversely, an injection into the VTA of a lentiviral vector encoding the anti-catalase short hairpin RNA (shRNA) abolished the voluntary consumption of ethanol (Karahanian et al., 2011).

Most of *in vivo* brain acetaldehyde production depends on catalase-H_2O_2 peroxidase activity (Zimatkin and Buben, 2007) and catalase seems to be expressed in all neural cells (Hipólito et al., 2007) although catalase-positive staining, resulting from immunohistochemical studies, was particularly prominent in brain areas containing aminergic neuronal bodies (Zimatkin and Lindros, 1996). Accordingly, catalase mRNA was found in a large number of neurons throughout the rat brain (Schad et al., 2003). Indeed, catalase and, to a lesser extent CYP2E1, are the main pathways of central ethanol metabolism (Aragon et al., 1992; Aragon and Amit, 1993; Zimatkin et al., 2006;

Hipólito et al., 2007, 2009; Sánchez-Catalán et al., 2008), as can be observed in rodent studies, showing that inhibitors of catalase, prevent the production of acetaldehyde (Aragon and Amit, 1992; Koechling and Amit, 1994).

Strong support to the evidence of the critical role of catalase-mediated metabolism of ethanol in its central effects was originally brought by a number of seminal studies by Aragon et al. (1985, 1989, 1992) and Aragon and Amit (1993). These authors reported the ability of catalase to oxidase ethanol in brain homogenates. They also showed the ability of 3-amino-1,2,4-triazole (3-AT), a catalase inhibitor, to prevent ethanol metabolism in these homogenates, both when directly applied to them (Aragon and Amit, 1992) and when previously administered *in vivo* to rats before homogenates preparation (Aragon and Amit, 1992). Behavioral studies further confirmed the suggestion of the critical role played by catalase in the mediation of some central effects of ethanol by showing that its potentiation (by acute lead acetate; Correa et al., 1999a) and its inhibition (by chronic lead acetate; Correa et al., 1999b) could increase and reduce, respectively, ethanol-induced locomotor activity. Behavioral studies, in addition, directly challenged the hypothesis of catalase-dependent production of brain acetaldehyde as a possible mediator of the psychopharmacological effects of ethanol. These studies showed that local intra-arcuate nucleus of the hypothalamus administration of another catalase inhibitor, sodium azide, could prevent the locomotor stimulating properties of ethanol (Sanchis-Segura et al., 2005) and that the systemic administration of catalase inhibitors could prevent both the locomotor stimulant effects of intra-substantia nigra pars reticulata (SNr) administration of ethanol (Arizzi-LaFrance et al., 2006) and the anxiolytic effects of systemically-administered ethanol (Correa et al., 2008). Further recent evidence on the role of catalase-mediated metabolism of ethanol was provided by the studies on ethanol-elicited locomotor stimulation, CPP (Ledesma and Aragon, 2012; Ledesma et al., 2012, 2013) and acquisition of ethanol oral self-administration (Peana et al., 2015). In summary, although acetaldehyde is generated locally in pharmacologically-significant amounts (Deng and Deitrich, 2008) by brain catalase, this process seems circumscribed to some specific brain nuclei (such as hypothalamus and midbrain) providing anatomical validation to the high behavioral specificity of the effects of drugs able to interfere with its enzymatic activity (Smith et al., 1997; Sanchis-Segura et al., 2005; Arizzi-LaFrance et al., 2006).

Overall, these studies support the hypothesis that brain-generated acetaldehyde promotes locomotor stimulation, CPP and ethanol drinking. Indeed, in order to increase acetaldehyde levels, cyanamide, an inhibitor of ALDH, has been suitably utilized locally in the VTA, in *in vivo* experiments in the presence of an otherwise ineffective concentration of ethanol on locomotor stimulation (Martí-Prats et al., 2013) and, upon systemic administration of ethanol, in order to increase the acetaldehyde's yield in striatal microdialysates (Jamal et al., 2007). In agreement with this idea, the administration of the ALDH2-coding vector to rats bred for their alcohol preference, decreased chronic ethanol consumption demonstrating that endowing the VTA with an augmented ability to degrade acetaldehyde greatly decreases ethanol intake (Karahanian et al., 2015). Finally, acetaldehyde oxidation is required for detoxification and it can be metabolized into acetate by ALDH that plays a crucial role in further oxidizing ethanol-derived acetaldehyde (Zimatkin et al., 2006). Lastly, the acetate produced by ALDH is metabolized through the Krebs cycle to produce energy or to provide intermediates for other molecules (Hernández et al., 2016; **Figure 2**).

The cytochrome P450 enzymes (CYP2E1) that are involved in ethanol metabolism in the liver have also been implicated in its metabolism, in particular, in mesencephalic tyrosine hydroxylase-positive neurons (Watts et al., 1998) by reducing molecular oxygen to water and thus oxidizing ethanol to acetaldehyde (**Figure 2**). Notably, the induction of CYP2E1 expression by chronic ethanol treatment has been reported in a number of brain structures including the hippocampus, cerebellum and brainstem (Zhong et al., 2012). Some authors have presented solid data suggesting the role of CYP2E1 in ethanol brain metabolism, since acetaldehyde production was decreased in mouse brain homogenates from mice with CYP2E1 genetic deficiency (Quertemont et al., 2005). Furthermore, the incubation with ethanol of brain microsomes from CYP2E1 deficient mice, results in lower levels of acetaldehyde, as compared to normal mice (Vasiliou et al., 2006), although compensatory mechanisms due to increased catalase expression in these animals should be taken into account while evaluating *in vivo* the apparent lack of effects of ethanol as compared to wild type mice (Correa et al., 2009). Moreover, as mentioned above, the expression of this enzyme is induced in response to chronic drinking (Hipólito et al., 2007; Sánchez-Catalán et al., 2008) and it may thus contribute to the increased rates of ethanol elimination in heavy drinkers (Hernández et al., 2016).

NEUROBIOLOGICAL EFFECTS OF ETHANOL AND ROLE OF ACETALDEHYDE

The research on the role of peripherally produced acetaldehyde in at least some of the central effects of ethanol provided different contributions in support of the suggestion that both ethanol on its own, and as ethanol-derived acetaldehyde, play a critical role in the reinforcing properties of ethanol. These investigations have been performed, in naïve rodents, after intragastric acetaldehyde or ethanol (passive) administration on CPP as well as on oral (operant) self-administration studies. In CPP experiments the inhibition of ADH1 (Peana et al., 2008) or catalase (Font et al., 2008) as well as the reduction of acetaldehyde bioavailability, by the use of sequestering agents, impairs the acquisition of ethanol-elicited CPP (Peana et al., 2008, 2009; Ledesma et al., 2013). Furthermore, acetaldehyde itself elicits the acquisition of CPP (Spina et al., 2010) through the activation of extracellular signal regulated kinase pathway via a DA D_1 receptor-mediated mechanism (Ibba et al., 2009; Spina et al., 2010; Vinci et al., 2010). In operant experiments, acetaldehyde was reported to be orally self-administered and, similarly to ethanol, its oral self-administration was reported to

be prevented by L-cysteine (Peana et al., 2010a), an agent that acts either as radical scavenger or as precursor of cysteine and that is also able to sequestrate acetaldehyde either peripherally or centrally. The operant oral self-administration paradigm is a preclinical model, in which animals are trained to emit a specific response for gaining the drug reinforcement (Grant and Samson, 1986; Samson et al., 1988). By these experiments, it was shown that the intraperitoneal administration of alpha lipoic acid, a radical scavenger that interferes with catalase-H_2O_2 activity (Ledesma et al., 2012), decreases maintenance, reinstatement and progressive ratio of oral operant ethanol self-administration. Likewise, L-cysteine acts also during the acquisition phase of ethanol and acetaldehyde self-administration (Peana et al., 2012, 2013b) with the acetaldehyde-binding property of cysteine being probably responsible for these effects. Likewise, Peana et al. (2015) have reported that D-penicillamine, a synthetic amino acid that strongly binds acetaldehyde, inhibits the acquisition of oral ethanol self-administration. Nonetheless, Quintanilla et al. (2016) showed that N-acetyl cysteine, a pro-drug of cysteine, fails to influence the acquisition of voluntary ethanol intake in adult female UChB rats, but greatly inhibits chronic ethanol intake. These data overall suggest that N-acetyl cysteine and L-cysteine may act by different mechanisms on the acquisition and maintenance of ethanol intake at least in part depending on experimental paradigms.

A rapidly growing body of evidence on the efficacy of radical scavengers and antioxidants such as L-cysteine (Peana et al., 2010a, 2012, 2013b), alpha lipoic acid (Ledesma et al., 2012; Peana et al., 2013a) and N-acetyl cysteine (Quintanilla et al., 2016), both on CPP experiments and in different phases (not only during the acquisition) of operant self-administration as well as on voluntary ethanol intake could be referred to the observation that a neuro-inflammatory process could be responsible of ethanol excessive taking (Montesinos et al., 2016). Indeed, the metabolism of ethanol into acetaldehyde and acetate is associated to the production of ROS that accentuate the oxidative state of cells promoting oxidative damage, neuronal injury and neurodegeneration. The oxidative balance is the result of the amount of accumulated ROS and of the activity of antioxidant enzymes. When the oxidative balance is disturbed, oxidative stress develops affecting the cell as a whole, as well as proteins, lipids and DNA. However, several defense mechanisms for reducing the deleterious effects of oxidative stress exist, and e.g., if cellular defense and repair processes fail, oxidatively damaged proteins can undergo proteasome-mediated protein degradation (Bence et al., 2001). Specifically, ethanol metabolism up-regulates the production of ROS and nitric oxide in primary cortical neurons causing blood-brain barrier dysfunction (Haorah et al., 2005, 2007, 2008). Chronic ethanol exposure has also been associated with proteasome inhibition which seems to be a key player in epigenetic mechanisms underlying alcoholism by promoting the accumulation of oxidatively damaged histones (Bardag-Gorce, 2009). Accordingly, recent findings showed that ethanol exposure reduced intracellular 20 S proteasome chymotrypsin-like activity in SH-SY5Y cells (Caputi et al., 2016) in agreement with findings obtained in the liver and the brain demonstrating that ethanol exposure decreased proteasome activity by interfering with 20 S CP and 19 S RP assembly (Bardag-Gorce, 2009; Donohue and Thomes, 2014; Erdozain et al., 2014). In addition, the CYP2E1 isoform fulfills an important role in the generation of ROS and exposure to ethanol is related to their accumulation, which may be associated to the induction of CYP2E1 in rat brain homogenates (Zhong et al., 2012). Moreover, although ALDH activity has beneficial effects, i.e., is responsible of the reduction of acetaldehyde, it also produces free radicals (Hernández et al., 2016). Notably, in the last decade, new insights into the mechanisms of the immune system response have driven research toward understanding the relationship between ethanol intake, the immune system dysregulation and its contribution in a wide range of disorders associated to ethanol exposure (Szabo and Saha, 2015), including neuro-inflammation and CNS dysfunctions (Crews et al., 2015; Montesinos et al., 2016). In this regard, it is noteworthy that antioxidant activity of human serum of patients with a diagnosis of alcohol dependence syndrome is lower than that of healthy donors (control group; Plotnikov et al., 2016).

Neuro-inflammation is associated with alcohol use disorders. Accordingly, recent work showed that the toll-like receptor 4 (TLR4) is involved in the induction of cytokines and chemokines, which promote neuro-inflammation, brain damage, behavioral and cognitive dysfunction (Pascual et al., 2015); however, it is important to note that while a selective inhibitor of these receptors was reported to decrease ethanol drinking in both ethanol-dependent and non-dependent mice (Bajo et al., 2016), other studies suggested recently that TLR4 may not be directly involved in the regulation of excessive drinking (Harris et al., 2017). Interestingly, anti-inflammatory mechanisms might also be evoked to interpret our recent observations that ethanol self-administration in Wistar rats (Peana et al., 2014) and ethanol-elicited CPP in CD-1 mice (Spina et al., 2015) were prevented by the administration of the standardized extract of the roots of *Withania somnifera* a medicinal plant renowned for its anti-inflammatory and free radical scavenger properties (Dar et al., 2015). On this line of evidence, it is worth mentioning that mesenchymal stem cells, known to reduce oxidative stress (Valle-Prieto and Conget, 2010) and to secrete anti-inflammatory cytokines (Lee et al., 2016), were able to inhibit relapse-like drinking after intracerebral administration (Israel et al., 2017).

On the other hand, chronic ethanol intake (maintenance phase of the self-administration protocols) seems to become independent of the early acetaldehyde mediated reinforcing mechanisms (Peana et al., 2015) responsible for the *first hit* (Israel et al., 2015). In this regard, the observation that the pharmacological manipulation of ethanol metabolism, by inhibition of catalase or by reduction of acetaldehyde bioavailability, does not interfere with the perpetuation (maintenance) of ethanol self-administration appears in agreement with data reported by Israel et al. (2015). To interpret these results, it was suggested that acetaldehyde could indirectly contribute to the maintenance phase of oral ethanol self-administration by the combination of two mechanisms: the first, indirect one, was hypothesized to be due to the lack of acetaldehyde itself that would make the animals to further

seek and take ethanol; the second mechanism, on the other hand, was suggested (Peana et al., 2015) to be based on the decreased metabolism of ethanol that would make available its non-metabolized fraction to act onto $GABA_A$ receptors resulting in further maintaining of ethanol self-administration. Otherwise, the alcohol relapse model based on the alcohol deprivation effect has also been widely used to assess ethanol craving and relapse. Thus, using this preclinical model, a recently published study showed that chemical inactivation of acetaldehyde, by D-penicillamine treatment in long-term ethanol experienced rats, prevents relapse into ethanol taking (Orrico et al., 2013). Moreover, the combined *therapy* of naltrexone and D-penicillamine prevents the delayed increase in ethanol consumption observed after continuous μ opioid (MOP) receptors blockade, suggesting the suitability of this combination as anti-relapse preclinical treatment (Orrico et al., 2014) and highlighting the role of acetaldehyde in the effects of ethanol. As also discussed in this review, a large number of neuropharmacological studies pointed to multiple neurochemical systems involved in the reinforcing effects of ethanol and to the interactions between ethanol and the CNS opioid signaling system, in particular (Coonfield et al., 2002; Oswald and Wand, 2004; Ghozland et al., 2005; Koob and Le Moal, 2006; Vukojević et al., 2008). Similarly, in human neuroblastoma SH-SY5Y cells, changes in the expression of the opioid receptors and the precursors of the opioid peptide ligands were observed in response to ethanol or acetaldehyde 40 mM and 0.4 mM, respectively (D'Addario et al., 2008), in agreement with the evidence that at least some of the neurochemical effects of ethanol are mediated by its first metabolite, and that, accordingly, ethanol must be metabolized into acetaldehyde to generate reward and reinforcement (Karahanian et al., 2011; Correa et al., 2012; Peana and Acquas, 2013). Furthermore, these data corroborate the hypothesis that the changes observed in the pro-enkephalin and k opioid receptors expression upon ethanol application are probably due to the action of his metabolite (D'Addario et al., 2008). In the meantime other studies correlated the epigenetic modifications with up/down regulation of genes caused by ethanol or acetaldehyde in the liver and rat brain tissue (Kim and Shukla, 2006; Shukla and Aroor, 2006; Shukla et al., 2007; Pandey et al., 2008). Notably, a temporal relationship between histone modifications and pro-dynorphin gene expression down-regulation was observed in a study conducted in human neuroblastoma cells (D'Addario et al., 2011) highlighting that ethanol or acetaldehyde exposure influences epigenetic regulation through histone acetylation, hence regulating propensity for DNA transcription at specific portions of the genome.

Ethanol and its metabolites may interfere with many biological processes, including neuronal differentiation, leading to severe brain damage and neurological disorders. In fact, the most severe ethanol-related damage, associated with a loss of neurons, was found following acute prenatal (Flentke et al., 2014) or chronic (Fernandes et al., 2002; Moulder et al., 2002; Soscia et al., 2006) pre- and post-natal exposure. Moreover, the generation of new neurons and their functional integration into the CNS is reported to be altered by ethanol (Nixon and Crews, 2002; Crews et al., 2006). In this regard, ethanol is known to affect cellular development interfering with brain-derived neurotrophic factor (BDNF; Climent et al., 2002; Sakai et al., 2005) and other neurotrophins (Moore et al., 2004; Bruns and Miller, 2007; Mooney and Miller, 2011). Studies conducted over the last decade by using *in vitro* models have enriched the information about this interplay. Thus, Hellmann et al. (2009) demonstrated that chronic ethanol exposure impaired neuronal differentiation of neuroblastoma cells and consistently impaired BDNF-mediated activation of the Mitogen-Activated Protein Kinase/Extracellular signal-Regulated Kinase (MAPK/ERK) cascade.

Together with phosphoinositide 3-kinase/protein kinase B (PI3K/AKT) pathway, MAPK/ERK cascade is activated by the growth factor midkine (MDK; Stoica et al., 2002), thus promoting cell survival and proliferation (Reiff et al., 2011). It has been hypothesized that MDK might be an ethanol-responsive gene since it protects against neuronal damage and neurodegeneration (Herradón and Pérez-García, 2014) and might regulate sensitivity to ethanol (Lasek et al., 2011). In support of this possibility, MDK expression is high in the prefrontal cortex of human alcoholics (Flatscher-Bader et al., 2005; Flatscher-Bader and Wilce, 2008), and is also elevated in the brains of mice genetically predisposed to consume high amounts of ethanol (Mulligan et al., 2006). Based on this evidence, it has been hypothesized that ethanol engages MDK and the activation of its receptor, the anaplastic lymphoma kinase (ALK). Intriguing studies, employing the neuroblastoma SH-SY5Y and IMR-32 cell lines, demonstrated that MDK and ALK are ethanol-responsive and that the activation of ALK signaling by ethanol is dependent on MDK expression. In fact, not only ethanol increased MDK and ALK gene expression, but also caused a rapid increase in the phosphorylation of ALK and ERK (He et al., 2015).

ROLE OF BRAIN ETHANOL-DERIVED ACETALDEHYDE IN THE EFFECTS OF ETHANOL

Acetaldehyde is a neuropharmacologically active substance, which has reinforcing properties *on its own* and provokes, although at lower doses/concentrations than ethanol, some behavioral responses similar to its parent compound suggestive of the fact that it could be responsible of some of its effects (Correa et al., 2012). Several authors have shown that the intracerebral administration of acetaldehyde induces locomotor stimulation (Correa et al., 2003, 2009; Arizzi-LaFrance et al., 2006; Sánchez-Catalán et al., 2009), CPP (Smith et al., 1984; Quertemont and De Witte, 2001; Quintanilla et al., 2002), conditioned taste preference (Brown et al., 1978) and self-administration (Brown et al., 1980; Myers et al., 1982; Rodd-Henricks et al., 2002). Likewise, microinjections of acetaldehyde into the posterior VTA (pVTA) increase DA release in the shell of the nucleus accumbens (Acb), measured by microdyalisis (Melis et al., 2007; Deehan et al., 2013). Moreover, electrophysiological studies have demonstrated that acetaldehyde administration stimulates the activity of VTA DA neurons

in vitro (Melis et al., 2007; Diana et al., 2008) and *in vivo* (Foddai et al., 2004; Enrico et al., 2009). However, although highly suggestive, all the above evidence does not represent unequivocal proof that acetaldehyde is the key element for the development of the neurobiological effects of ethanol (Correa et al., 2012; Israel et al., 2015).

In order to address this critical question, and to assess the role of brain ethanol-derived acetaldehyde, the strategy more commonly used has been to evaluate the consequences of the modulation of the enzymatic systems involved in brain ethanol metabolism (Hipólito et al., 2007). Indeed, the catalase activity has been the most targeted one, due to its high contribution to brain ethanol metabolism. Thus, a decreased activity of brain catalase would elicit a reduction or blockade of ethanol effects, as a consequence of decreased levels of acetaldehyde formation. Arizzi-LaFrance et al. (2006) demonstrated that the locomotor activation induced by ethanol microinjection into substantia nigra pars compacta (SNc) could be blocked with the systemic administration of sodium azide. Otherwise, intra-cerebral injection of sodium azide is able to prevent the locomotor-stimulating effects of ethanol microinjection into the hypothalamic arcuate nucleus (Pastor and Aragon, 2008) and the decreased locomotion induced by intraperitoneal administration of ethanol in rats (Sanchis-Segura et al., 2005). Moreover, the direct correlation between voluntary consumption of ethanol and brain catalase activity (Amit and Aragon, 1988; Gill et al., 1996) highlights the role of brain acetaldehyde formation. Consistent with this idea, the intra-VTA microinjection of a lentiviral vector encoding a shRNA for silencing catalase gene expression and reducing the catalase content has been shown to decrease the voluntary ethanol consumption and alcohol deprivation effect (Karahanian et al., 2011; Tampier et al., 2013). Similarly, brain ALDH activity has been modulated to increase the locomotor-stimulating effects of ethanol in rats (Spivak et al., 1987; Martí-Prats et al., 2013). Moreover, a positive correlation between voluntary ethanol consumption and brain ALDH activity (Sinclair and Lindros, 1981; Socaransky et al., 1984) has been described, also, by means of a lentiviral vector encoding for ALDH and enhancing acetaldehyde metabolism (Karahanian et al., 2015). Besides the evidence gathered by behavioral studies, the involvement of local acetaldehyde formation in the central effects of ethanol was also provided by an *in vitro* electrophysiological approach showing that local catalase inhibition, through 3-AT administration, prevents the effect of ethanol on pVTA DA neurons (Melis et al., 2007).

Several recent studies have used the acetaldehyde-sequestering agent, D-penicillamine, to assess the impact of the reduction of brain acetaldehyde levels on the development of the psychopharmacological effects of ethanol. D-penicillamine is an amino acid that interacts with acetaldehyde to form a stable adduct (Nagasawa et al., 1980), which has been detected in plasma, liver and brain following ethanol administration (Serrano et al., 2007). Moreover, intra-cisternal administration of D-penicillamine was shown to prevent the locomotor stimulation and CPP produced by intra-cisternal ethanol administration in newborn rats (Pautassi et al., 2011; March et al., 2013), whereas the intra-cerebroventricular pretreatment with D-penicillamine was reported to decrease the voluntary ethanol intake in rats (Font et al., 2006). In addition, more direct than previously described evidence has pointed to the VTA as a key brain region for the effects of ethanol-derived acetaldehyde following sequestering-agents pretreatment since the intra-VTA administration of D-penicillamine prevents the alcohol deprivation effect in Wistar rats (Orrico et al., 2013).

While the above described studies confirm that *in situ* acetaldehyde formation from ethanol is necessary to the development of the psychopharmacological effects of ethanol, the mechanism through which ethanol or acetaldehyde activate VTA DA neurons, and therefore the mesolimbic DA system, is not yet fully understood. Nowadays, it has been shown that ethanol modulates the activity of DA neurons through its interaction with several neurochemical and neuroendocrine systems including GABA, glutamate, opioid, cannabinoid and corticotropin-releasing factor systems and cytoplasmic elements (Gessa et al., 1985; Morikawa and Morrisett, 2010; D'Addario et al., 2013; Erdozain and Callado, 2014). Both ethanol and acetaldehyde are able to stimulate DA neurons through ion channels modulation (Brodie et al., 1990; Okamoto et al., 2006; Melis et al., 2007), and it has been shown, by electrophysiological approaches, that prevention of acetaldehyde formation or its inactivation leads to blockade of ethanol effects (Foddai et al., 2004; Melis et al., 2007; Enrico et al., 2009).

In particular, as to the endogenous opioid system, there is a general agreement that it is involved in the neurobiological effects of ethanol and a number of hypotheses have been proposed to explain how this interaction may take place. Several evidences support the notion that the activation of MOP receptors expressed onto VTA GABA neurons and other GABA afferents (Johnson and North, 1992; Jalabert et al., 2011; Sánchez-Catalán et al., 2014), is critically involved in the stimulation of VTA DA neurons after ethanol or acetaldehyde (Mereu and Gessa, 1985; Xiao et al., 2007; Fois and Diana, 2016). Consistent with this idea, behavioral studies have shown that the microinjection of naltrexone or β-funaltrexamine (an irreversible MOP receptor antagonist) can prevent the motor activation induced by the intra-pVTA administration of ethanol or acetaldehyde (Sánchez-Catalán et al., 2009). Moreover, such preventive effect has been also observed by using electrophysiological approaches (Xiao and Ye, 2008; Guan and Ye, 2010; Fois and Diana, 2016).

Besides the stimulating effect of ethanol or acetaldehyde into the pVTA, which seems to involve the MOP receptors and, ultimately, ethanol by-products (see below), it has been observed that ethanol on its own can activate VTA GABA neurons, eliciting inhibition of DA neurons (Steffensen et al., 2009). The reinforcing properties of ethanol would depend on the action of ethanol itself and of its metabolites, as proposed by Martí-Prats et al. (2013). Indeed, these authors demonstrated that the intra-pVTA administration of a low dose of ethanol did not induce any locomotor effect in rats, which implies a balanced effect between those of ethanol and those of its metabolites. Thus, decreased levels of ethanol-derived acetaldehyde by catalase inhibition (sodium azide) or by acetaldehyde inactivation

(D-penicillamine), converts an inactive ethanol dose into a depressive one. On the contrary, the increase of ethanol-derived acetaldehyde levels obtained by ALDH inhibition (cyanamide), converts the ethanol dose into a stimulating one. Thereby, the decrease of the ethanol-metabolized fraction (acetaldehyde and, perhaps, salsolinol) discloses the inhibitory action of the non-metabolized fraction of ethanol, whereas the increase of the metabolized fraction discloses a stimulatory effect. Following this elegant approach, Martí-Prats et al. (2015) developed further research in order to disentangle the mechanism of action of *such* ethanol non-metabolized fraction. Accordingly, $GABA_A$ antagonism (bicuculline) was able to convert an inactive ethanol dose into an active one. Moreover, bicuculline administration was also able to prevent the motor depression observed after D-penicillamine pretreatment (therefore, no motor effect was observed; Martí-Prats et al., 2013). On the other hand, the intra-pVTA administration of β-funaltrexamine induced a decrease of motor activity of the animals treated with an inactive dose of ethanol, suggesting that the consequence of MOP receptor blockade on this effect could be attributable to the blockade of the effects of the ethanol-metabolized fraction (Martí-Prats et al., 2015; **Figure 3**).

Overall, these studies provide evidence of the key role of ethanol-derived acetaldehyde, formed *in situ*, in the mechanisms underlying the psychopharmacological effects of ethanol. However, it is noteworthy that the involvement of other by-products of ethanol metabolism such as salsolinol are also a key issue to the neurobiological basis of these effects, as will be discussed in the next section.

BEYOND ACETALDEHYDE: ROLE OF SALSOLINOL

Acetaldehyde is a highly reactive compound with a very short half-life and reacts with biogenic amines to produce condensation products known as tetrahydroisoquinolines (THIQs). In the 70s, several investigators proposed the *THIQs theory of alcoholism*, pointing at these molecules as possible mediators of part of the ethanol effects on the mesolimbic system and consequently as responsible of playing an important role in alcoholism (Cohen and Collins, 1970; Davis and Walsh, 1970; Melchior and Myers, 1977; Duncan and Deitrich, 1980). Although in the late 70s and 80s, some investigators explored the role of some THIQs, including betacarbolines and tetrahydropapaveroline, on ethanol intake and DA release (Myers and Melchior, 1977; Tuomisto et al., 1982; Airaksinen et al., 1983; Myers and Robinson, 1999), no more studies have increased our knowledge in their role in alcoholism. Otherwise, the condensation product of acetaldehyde with DA, salsolinol (1-methyl-6,7-dihidroxy-1,2,3,4-tetrahydroisoquinoline, for detailed review on salsolinol formation see Hipólito et al., 2012), has been specially investigated trying to clarify its role in the neurobiological basis of alcohol addiction (**Figure 3**). When investigating the intriguing relationship between ethanol and salsolinol four main questions arise: (i) is salsolinol produced in pharmacologically significant concentrations after ethanol intake?; (ii) does salsolinol activate the DA mesolimbic pathway to induce alcohol use disorders -related behaviors?; if so, (iii) how is salsolinol activating this system?; and (iv) is salsolinol necessary for ethanol to exert its activating effect on the dopaminergic mesolimbic system?

As to the first question, the issue of salsolinol formation in the brain after ethanol administration is still unclear. In fact, beginning from the first evidences of salsolinol formation in the brain of rodents after ethanol administration brought in the mid 70 by Collins and Bigdeli (1975), different laboratories have obtained controversial data. Actually, some of these reports show an increase of salsolinol levels (Myers et al., 1985; Matsubara et al., 1987; Jamal et al., 2003a,b,c; Starkey et al., 2006; Rojkovicova et al., 2008) while others show no alterations in the brain after different protocols of ethanol administration (O'Neill and Rahwan, 1977; Baum et al., 1999; Haber et al., 1999; Lee et al., 2010). Moreover, the investigations reporting increased brain salsolinol upon chronic ethanol intake or chronic ethanol administration, were performed in rats allowed to access *ad libitum* lab chow either before and throughout the study (Sjöquist et al., 1982). Interestingly, when such rat studies were repeated using chronic ethanol intake in DOPA- and salsolinol-free liquid diets, no changes were found of endogenous brain salsolinol concentrations (Collins et al., 1990). However, when the ethanol-liquid diets were supplemented with DOPA, salsolinol levels were raised (Collins et al., 1990) suggesting that, upon prolonged ethanol intake, elevations of endogenous salsolinol concentrations, as well as of those of other THIQs, seem to depend on a number of factors including the brain region investigated, the duration of intake and the associated dietary constituents (Lee et al., 2010). The differences observed in the data from these studies have been extensively summarized and analyzed in a previous review on this specific topic (Hipólito et al., 2012). Notably, in this regard, only one report, in the last 5 years has added more knowledge to this issue. In this study, authors detected an increase in salsolinol levels in fetal rat brain after an alcoholization protocol from GD8 to GD20 (Mao et al., 2013). However, similarly to the studies mentioned previously, there is still a lack of a direct demonstration that may provide us a precise correlation between the ethanol load and the concentration of salsolinol achieved in the brain tissue. In summary, convincing data, clearly demonstrating brain (neuronal or glial) salsolinol formation in ethanol-treated and/or ethanol-consuming animals, are still missing.

As to the second question, in spite of the uncertainties mentioned above, the latest investigations on the psychopharmacological effects of salsolinol appear in support of the *THIQs theory of alcoholism*. Throughout the last 15 years, different laboratories have successfully shown that salsolinol exerts, on the mesolimbic system, effects similar to ethanol, suggesting that at least some of the effects of ethanol may be mediated by salsolinol. These latest neurochemical and behavioral studies have shed a bit of light on the previous controversial data where an effect or a lack of salsolinol's effect on the dopaminergic system had been reported. The most recent data, obtained with the intracerebral administration of low doses (0.3–30 μg) of salsolinol, revealed that it robustly increases motor activity and produces motor sensitization

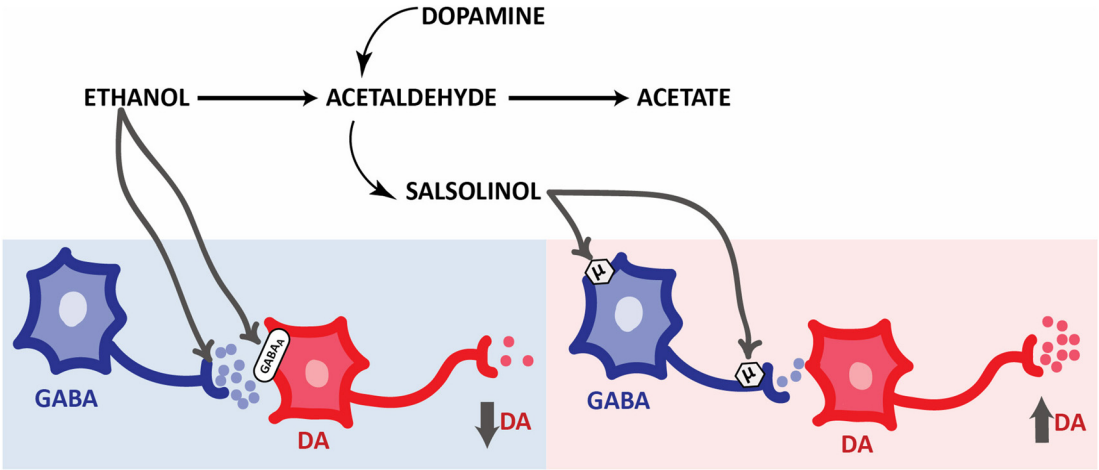

FIGURE 3 | Opposite responses elicited by ethanol and its derivatives on the activity of mesolimbic dopaminergic system. Simplified schematic representation of the effects of ethanol metabolites on pVTA DA neurons. Ethanol evokes an inhibition of pVTA DA neurons through presynaptic (Weiner and Valenzuela, 2006) and postsynaptic GABAergic mechanisms. On the other hand, salsolinol induces an excitation of pVTA DA neurons through MOP receptors activation located in the soma and terminals of GABA neurons. Abbreviations: DA, dopamine. GABA, γ-aminobutiric acid. $GABA_A$, GABA receptors type A. μ, MOP receptors.

(Quintanilla et al., 2014, 2016); this is in contrast with those studies where salsolinol, administered at high doses (100–300 μg intracranial or 10–100 mg i.p.), was reported to induce no change of motor behavior (Antkiewicz-Michaluk et al., 2000a; Vetulani et al., 2001). Indeed, Hipólito et al. (2011) and Quintanilla et al. (2016) have shown that the administration of 30 pmol of salsolinol directly into the rats pVTA increases motor activity and induces motor sensitization, being R-SAL the active stereoisomer (Quintanilla et al., 2016). These data are similar, in terms of profile and activation magnitude, to the previously reported effects of intra-pVTA administration of ethanol and acetaldehyde on motor activity (see "Central Generation of Acetaldehyde" Section for discussion on this aspect and Sánchez-Catalán et al., 2009) but with the striking difference that salsolinol is ∼100 times more potent than ethanol and acetaldehyde. Very interestingly, the pVTA seems also to be a key brain area for salsolinol reinforcing effects. In a first study, using the intracranial self-administration procedure (ICSA) in rats, Rodd et al. (2008) showed that salsolinol is self-administered at concentrations ranging from 0.03 to 0.3 μM/infusion directly into this brain region. Curiously these authors have used the same ICSA protocol with both ethanol and acetaldehyde as reinforcers showing that these three compounds share a similar response profile in this protocol but with different potencies (being salsolinol ≥ acetaldehyde >> ethanol; Rodd et al., 2004, 2005). Furthermore, although context association learning using salsolinol as reinforcer (i.e., as an unconditioned stimulus) was already revealed in a study using its peripheral, systemic, administration in a CPP paradigm (Matsuzawa et al., 2000), we recently demonstrated the involvement of the pVTA also in this effect (Hipólito et al., 2011). In this study, in fact, the administration of 30 pmol of salsolinol (the same dose used in the motor activity studies) into the pVTA, associated to a context, increased the time spent by the rats in that context on the test day under drug free conditions (Hipólito et al., 2011). Five years after this study, other investigators have reproduced these data also showing that R-salsolinol is the active stereoisomer responsible for CPP induction (Quintanilla et al., 2016).

In accordance with the above locomotor activity, ICSA and CPP studies, the administration of similar doses of salsolinol into the pVTA increases DA release in the Acb shell (Hipólito et al., 2011; Deehan et al., 2013) through an indirect stimulation of the pVTA DA neurons by a mechanism suggested by *ex vivo* electrophysiological studies (Xie et al., 2012) that will be discussed below.

Indeed, the Acb has also been shown to be responsive to the pharmacological action of salsolinol. Thus, salsolinol is also self-administered into the Acb shell of alcohol-preferring (P) rats (Rodd et al., 2003) reaching its maximal number of responses at 3 μM, a concentration 30 times greater than that (0.1 μM) required to obtain the maximal response when self-administered into the pVTA (Rodd et al., 2008). Notably, although these two studies were performed within the same lab and following an identical ICSA procedure, before concluding that the Acb shell is less sensitive than the pVTA to the reinforcing properties of salsolinol, it is important to note that, in the Acb shell experiments, Rodd et al. (2003) used P rats, which are genetically bred to present high levels of alcohol intake.

Moreover, the neurochemical effects of salsolinol in the Acb have been characterized by *in vivo* brain microdialysis in another study (Hipólito et al., 2009). Interestingly, in this research salsolinol showed a differential effect depending on the accumbal region studied. In fact, salsolinol reduced DA levels down to 50% from baseline when delivered into the shell whereas it increased them up to 130% when administered into the core (Hipólito et al., 2009). These regional differences in response to salsolinol were

previously specifically observed for MOP and δ opioid receptor agonists (Hipólito et al., 2008). In this study, the application of these agonists increased DA release in the Acb core but decreased it in the Acb shell with a profile and magnitude similar to that reported for salsolinol (Hipólito et al., 2009). In this regard, it is appropriate to note that in the ICSA experiments of Rodd et al. (2003), salsolinol was self-administered directly into the Acb shell, whereas these microdialysis data show that salsolinol is able to decrease DA release in the Acb shell. One possible explanation for this apparent discrepancy might be found in the different concentrations of salsolinol. In the case of the ICSA studies, the concentration of salsolinol able to induce the maximum number of administrations was 3 μM whereas that one able to induce the maximum decrease of DA was 5 μM, which, due to the recovery of the dialysis membrane (∼10%), is notably lower than the concentration of salsolinol in the self-administered solution. Another possibility relies on the different modalities of salsolinol administration followed in these experiments: microdialysis studies, in fact, show responses (collected every 20 min) upon the acute application of salsolinol whereas ICSA studies use repeated and more prolonged applications of salsolinol (several sessions). Hence, based on the rate of salsolinol self-administration more than one dose of salsolinol would be administered in 20 min and this observation makes highly fallacious comparing studies with so different time scales and administration modalities.

The opioid system has been suggested to mediate the salsolinol (as well as the acetaldehyde, Peana et al., 2011) effects in the CNS. In a very early set of studies salsolinol showed to bind to opioid receptors and produce opioid-like effects (Tampier et al., 1977; Blum et al., 1978; Fertel et al., 1980; Lucchi et al., 1982). Based on these studies and on the structural similarities of salsolinol with the MOP receptor agonist morphine, most part of the neurobiological consequences deriving from the administration of salsolinol were shown to be impeded by pharmacological blockade of MOP receptors. In particular, the blockade in the pVTA of these receptors by the selective and irreversible MOP antagonist β-funaltrexamine impairs the salsolinol-elicited increase in motor activity (Hipólito et al., 2010), the acquisition of CPP after either its systemic (Matsuzawa et al., 2000) and intra-pVTA administration (Hipólito et al., 2010) as well as the increase in accumbal DA release elicited by intra-pVTA salsolinol administration (Hipólito et al., 2011). Moreover, although, unfortunately, there are no data on the effects of opioid antagonists on salsolinol self-administration, the pharmacology of salsolinol ICSA has been investigated by Rodd et al. (2003, 2005, 2008). In their studies, these authors explored the role of the dopaminergic (D_2 and D_3 subtypes) and the serotoninergic (5-HT_3 subtype) receptors in the reinforcing properties of salsolinol. The co-infusion of the D_2/D_3 receptor antagonist, sulpiride, with salsolinol completely blocked the number of self-infusions into the Acb shell (Rodd et al., 2003); similarly, although by a different mechanism, co-administration of the D_2/D_3 receptor agonist, quinpirole, significantly reduced the number of responses on the active lever to obtain the ICSA of salsolinol into the pVTA (Rodd et al., 2008). Thus, although several studies have excluded a direct interaction of salsolinol with D_1 and D_2 receptors (Antkiewicz-Michaluk et al., 2000a,b; Tóth et al., 2002; Homicsko et al., 2003; Székács et al., 2007), these data indicate that somehow DA receptors are involved in the reinforcing properties of salsolinol in the mesolimbic pathway. Moreover, in similar ICSA experiments performed to deliver salsolinol into the pVTA, Rodd et al. have also investigated the implication of 5-HT_3 receptors and showed that the application of the selective antagonist of the 5-HT_3 receptors, ICS 205, 930, significantly diminished the number of salsolinol self-administrations. The authors of this study hypothesized that the involvement of 5-HT_3 receptors may be indirect, based on previous reports that show an enhancement of serotonin extracellular levels after salsolinol administration in the rats striatum (Maruyama et al., 1992; Nakahara et al., 1994). Unfortunately, no more experiments have been done to test this possibility and characterize further the pharmacology of ICSA of salsolinol.

As to the third question, in a set of more direct experiments, Xie et al. (2012) tried to shed light on how salsolinol activates DA neurons of the mesolimbic pathway, by using the patch clamp technique. In these experiments, DA neurons were patched and action potentials (APs), as well as spontaneous inhibitory postsynaptic currents (sIPSC) from GABA inputs were recorded. Salsolinol was able to increase, dose-dependently, the number of APs/10 s and to reduce the number of sIPSC/10 s after its bath application at concentrations as low as 0.003–1.0 μM. These data confirm the ability of salsolinol to activate pVTA DA neurons through an indirect mechanism that involves a decrease of GABA release onto DA neurons. Furthermore, following the idea of MOP receptors involvement in the effects of salsolinol on mesolimbic DA system, the authors used a non-selective antagonist, naltrexone, and found that it impairs salsolinol effects on both the APs and the sIPSC. Taking into account the organization of the pVTA (Johnson and North, 1992), it seems reasonable to suggest that salsolinol is able to activate MOP receptors mainly located onto pVTA GABA neurons and other GABA afferents that control the DA neurons activity. As a consequence of the GABA neurotransmission inactivation, DA neurons (under tonic control from GABA neurons) may be activated through a disinhibition mechanism (Xie et al., 2012). Notably, this mechanism of action offers a reasonable mechanistic explanation of the neurochemical and behavioral experiments exposed above but also may give an explanation of how ethanol is able to interact with the opioid system to mediate its activating effects (Font et al., 2013). Nonetheless, there are still unresolved questions about salsolinol (i.e., the involvement of 5-HT_3 receptor), and probably salsolinol mechanism of action is much more complicated. Other possibilities, besides the MOP and the 5-HT_3 receptors, have been explored *in vitro*. After another set of electrophysiology experiments, Xie and Ye (2012) concluded that the effect of salsolinol on DA neurons involves at least three mechanisms: (i) a depolarizing action of DA neurons by itself; (ii) an activating action of MOP receptors onto GABAergic inputs; and (iii) an enhancing action of presynaptic glutamatergic transmission onto DA neurons via activation of DA D_1 receptors on the glutamatergic terminals. However, as previously discussed, this is still an open question that will need more data from different techniques to be clarified.

The final question, perhaps the most difficult to answer and at the same time the most important one, refers to the involvement of salsolinol in the neurobiological effects of ethanol. All data discussed above give support to the *THIQs theory of alcoholism* without providing a direct confirmation of the crucial role of salsolinol in the effects of ethanol. In investigating the neurobiological effects of salsolinol, others and we always administered exogenous salsolinol to observe its effects but never used ethanol to obtain salsolinol *in situ*. Obviously, the lack of knowledge about salsolinol formation from ethanol does not allow us to manipulate the metabolism as others and we did to study acetaldehyde's effects. Noteworthy, a recent study from Melis et al. (2015) has provided a direct evidence of salsolinol involvement in ethanol activating effects of DA neurons in the pVTA. In this very well designed electrophysiological study, frequency of APs was measured in DA depleted mice to allow the manipulation of the hypothesized steps of salsolinol formation from ethanol metabolism. The study by Melis et al. (2015) reported that ethanol could increase the frequency of APs of DA neurons in slices from DA depleted mice only if in the presence of exogenously added DA. Moreover, DA neurons from slices obtained from DA depleted mice that were treated with 3-AT (that prevents acetaldehyde's and, consequently, salsolinol's formation) showed no APs increases in response to ethanol. These data reveal that the formation of salsolinol from ethanol-derived acetaldehyde is necessary for the ethanol activating effects on DA neurons in the pVTA.

In conclusion, although there is a yet growing interest in the field with high expectancies of providing very critical insights on this topic, some of the questions are, unfortunately, still unanswered making the role of salsolinol in the effects of ethanol a matter of further debate in the field of alcohol addiction.

SUMMARY AND FUTURE DEVELOPMENTS

As acetaldehyde is involved in many actions of ethanol in the brain, including behavioral changes and neuronal damage, the drugs used to interfere with ethanol metabolism or reduce acetaldehyde levels may represent a valuable therapy in the management of the large number of alcohol use disorders including relapse into ethanol taking and, in combination with the existing ones, might improve the outcomes of current pharmacological therapies. Moreover, compounds endowed with anti-oxidant properties represent nowadays potentially good candidates to treat distinct phases of ethanol misuse. The neuroimmune mechanisms of ethanol and acetaldehyde offer new approaches to develop more effective pharmacotherapies to treat ethanol-related neuropathologies. This recent latter evidence could explain the efficacy of different radical scavengers and antioxidant drugs in the reduction of ethanol-, ethanol-derived acetaldehyde- and acetaldehyde-dependent effects. Moreover, as some pioneering and recent studies point to the relevance of the cortico-striatal glutamatergic transmission to the development of addiction to psychostimulant and other drugs of abuse, further research appears needed to disentangle the exact relationship between ethanol, acetaldehyde and salsolinol in this pathway, which might lead to develop new treatments.

Moreover, the latest research on the acetaldehyde-DA adduct points to salsolinol as a strategic tile in the puzzle that explains the activating effects of ethanol that may eventually lead to alcohol use disorders in some individuals. In this regard, it is noteworthy that all the potential pharmacological tools, suggested by the above evidence, may be able to prevent either salsolinol formation or salsolinol actions onto DA neurons. However, although the gathered evidence discloses potential targets of promising therapies, it also requires great caution as most part, if not all, of the data discussed in the present review were obtained upon acute administrations. In other words, since in this scenario we are still missing the data under and after chronic exposure to ethanol, acetaldehyde and salsolinol, modeling more advanced states of addiction (Belin-Rauscent et al., 2016) appears the must for future research in order to really gain understanding on which may be the real role of ethanol and its by-products in abnormal ethanol taking behaviors.

In conclusion, significant new evidence supports the role of acetaldehyde and salsolinol in many actions of ethanol in the CNS which offers new insights for the search of new targets and for the discovery of effective pharmacotherapies against the development of alcohol abuse and dependence.

AUTHOR CONTRIBUTIONS

All authors, based on their expertise, contributed to write and critically edit this review article.

FUNDING

LH funds were from the Spanish Government MICINN (PSI2016-77895-R); EA funds were from Regione Autonoma della Sardegna (RAS), CUP B71J11001480002.

REFERENCES

Airaksinen, M. M., Mähönen, M., Tuomisto, L., Peura, P., and Eriksson, C. J. (1983). Tetrahydro-beta-carbolines: effect on alcohol intake in rats. *Pharmacol. Biochem. Behav.* 18, 525–529. doi: 10.1016/0091-3057(83)90230-7

Amit, Z., and Aragon, C. M. G. (1988). Catalase activity measured in rats naive to ethanol correlates with later voluntary ethanol consumption: possible evidence for a biological marker system of ethanol intake. *Psychopharmacology* 95, 512–515. doi: 10.1007/bf00172965

Antkiewicz-Michaluk, L., Michaluk, J., Romanska, I., Papla, I., and Vetulani, J. (2000a). Antidopaminergic effects of 1,2,3,4-tetrahydroisoquinoline and salsolinol. *J. Neural. Trans.* 107, 1009–1019. doi: 10.1007/s007020070049

Antkiewicz-Michaluk, L., Romañska, I., Papla, I., Michaluk, J., Bakalarz, M., Vetulani, J., et al. (2000b). Neurochemical changes induced by acute and chronic administration of 1,2,3,4-tetrahydroisoquinoline and salsolinol in dopaminergic structures of rat brain. *Neuroscience* 96, 59–64. doi: 10.1016/s0306-4522(99)00533-3

Aragon, C. M. G., and Amit, Z. (1992). The effect of 3-amino-1,2,4-triazole on voluntary ethanol consumption: evidence for brain catalase involvement in

the mechanism of action. *Neuropharmacology* 31, 709–712. doi: 10.1016/0028-3908(92)90150-n

Aragon, C. M. G., and Amit, Z. (1993). Differences in ethanol-induced behaviors in normal and acatalasemic mice: systematic examination using a biobehavioral approach. *Pharmacol. Biochem. Behav.* 44, 547–554. doi: 10.1016/0091-3057(93)90165-p

Aragon, C. M. G., Rogan, F., and Amit, Z. (1992). Ethanol metabolism in rat brain homogenates by a catalase-H_2O_2 system. *Biochem. Pharmacol.* 44, 93–98. doi: 10.1016/0006-2952(92)90042-h

Aragon, C. M. G., Spivak, K., and Amit, Z. (1985). Blockade of ethanol induced conditioned taste aversion by 3-amino-1,2,4-triazole: evidence for catalase mediated synthesis of acetaldehyde in rat brain. *Life Sci.* 37, 2077–2084. doi: 10.1016/0024-3205(85)90579-x

Aragon, C. M. G., Spivak, K., and Amit, Z. (1989). Effects of 3-amino-1,2,4-triazole on ethanol-induced open-field activity: evidence for brain catalase mediation of ethanol's effects. *Alcohol. Clin. Exp. Res.* 13, 104–108. doi: 10.1111/j.1530-0277.1989.tb00293.x

Arizzi-LaFrance, M. N., Correa, M., Aragon, C. M. G., and Salamone, J. D. (2006). Motor stimulant effects of ethanol injected into the substantia nigra pars reticulata: importance of catalase-mediated metabolism and the role of acetaldehyde. *Neuropsychopharmacology* 31, 997–1008. doi: 10.1038/sj.npp.1300849

Bagnardi, V., Rota, M., Botteri, E., Tramacere, I., Islami, F., Fedirko, V., et al. (2013). Light alcohol drinking and cancer: a meta-analysis. *Ann. Oncol.* 24, 301–308. doi: 10.1093/annonc/mds337

Bajo, M., Montgomery, S. E., Cates, L. N., Nadav, T., Delucchi, A. M., Cheng, K., et al. (2016). Evaluation of TLR4 inhibitor, T5342126, in modulation of ethanol-drinking behavior in alcohol-dependent mice. *Alcohol Alcohol.* 51, 541–548. doi: 10.1093/alcalc/agw026

Bardag-Gorce, F. (2009). Nuclear effects of ethanol-induced proteasome inhibition in liver cells. *World J. Gastroenterol.* 15, 1163–1167. doi: 10.3748/wjg.15.1163

Baum, S. S., Hill, R., Kiianmaa, K., and Rommelspacher, H. (1999). Effect of ethanol on (R)- and (S)-salsolinol, salsoline and THP in the nucleus accumbens of AA and ANA rats. *Alcohol.* 18, 165–169. doi: 10.1016/s0741-8329(98)00080-9

Belin-Rauscent, A., Fouyssac, M., Bonci, A., and Belin, D. (2016). How preclinical models evolved to resemble diagnostic criteria of drug addiction. *Biol. Psychiatry* 79, 39–46. doi: 10.1016/j.biopsych.2015.01.004

Bence, N. F., Sampat, R. M., and Kopito, R. R. (2001). Impairment of the ubiquitin-proteasome system by protein aggregation. *Science* 292, 1552–1555. doi: 10.1126/science.292.5521.1552

Blomstrand, R., Kager, L., and Lantto, O. (1973). Studies on the ethanol-induced decrease of fatty acid oxidation in rat and human liver slices. *Life Sci.* 13, 1131–1141. doi: 10.1016/0024-3205(73)90380-9

Blomstrand, R., and Theorell, H. (1970). Inhibitory effect on ethanol oxidation in man after administration of 4-methylpyrazole. *Life Sci.* 9, 631–640. doi: 10.1016/0024-3205(70)90214-6

Blum, K., Hamilton, M. G., Hirst, M., and Wallace, J. E. (1978). Putative role of isoquinoline alkaloids in alcoholism: a link to opiates. *Alcohol. Clin. Exp. Res.* 2, 113–120. doi: 10.1111/j.1530-0277.1978.tb04710.x

Boleda, M. D., Julià, P., Moreno, A., and Parés, X. (1989). Role of extrahepatic alcohol dehydrogenase in rat ethanol metabolism. *Arch. Biochem. Biophys.* 274, 74–81. doi: 10.1016/0003-9861(89)90416-5

Bradford, B. U., Forman, D. T., and Thurman, R. G. (1993a). 4-Methylpyrazole inhibits fatty acyl coenzyme synthase and diminishes catalase-dependent alcohol metabolism: has the contribution of alcohol dehydrogenase to alcohol metabolism been previously overestimated? *Mol. Pharmacol.* 43, 115–119.

Bradford, B. U., Seed, C. B., Handler, J. A., Forman, D. T., and Thurman, R. G. (1993b). Evidence that catalase is a major pathway of ethanol oxidation *in vivo*: dose-response studies in deer mice using methanol as a selective substrate. *Arch. Biochem. Biophys.* 303, 172–176. doi: 10.1006/abbi.1993.1269

Brodie, M. S., Shefner, S. A., and Dunwiddie, T. V. (1990). Ethanol increases the firing rate of dopamine neurons of the rat ventral tegmental area *in vitro*. *Brain Res.* 508, 65–69. doi: 10.1016/0006-8993(90)91118-z

Brown, Z. W., Amit, Z., and Smith, B. (1980). Intraventricular self-administration of acetaldehyde and voluntary consumption of ethanol in rats. *Behav. Neural Biol.* 28, 150–155. doi: 10.1016/s0163-1047(80)91487-9

Brown, Z. W., Amit, Z., Smith, B., and Rockman, G. E. (1978). Differential effects on conditioned taste aversion learning with peripherally and centrally administered acetaldehyde. *Neuropharmacology* 17, 931–935. doi: 10.1016/0028-3908(78)90134-x

Bruns, M. B., and Miller, M. W. (2007). Neurotrophin ligand-receptor systems in somatosensory cortex of adult rat are affected by repeated episodes of ethanol. *Exp. Neurol.* 204, 680–692. doi: 10.1016/j.expneurol.2006.12.022

Caputi, F. F., Carboni, L., Mazza, D., Candeletti, S., and Romualdi, P. (2016). Cocaine and ethanol target 26S proteasome activity and gene expression in neuroblastoma cells. *Drug Alcohol Depend.* 161, 265–275. doi: 10.1016/j.drugalcdep.2016.02.012

Chevens, L. C. (1953). Antabuse addiction. *Br. Med. J.* 1, 1450–1451. doi: 10.1136/bmj.1.4825.1450-c

Climent, E., Pascual, M., Renau-Piqueras, J., and Guerri, C. (2002). Ethanol exposure enhances cell death in the developing cerebral cortex: role of brain-derived neurotrophic factor and its signaling pathways. *J. Neurosci. Res.* 68, 213–225. doi: 10.1002/jnr.10208

Cohen, G., and Collins, M. (1970). Alkaloids from catecholamines in adrenal tissue: possible role in alcoholism. *Science* 167, 1749–1751. doi: 10.1126/science.167.3926.1749

Collins, M. A., Ung-Chhun, N., Cheng, B. Y., and Pronger, D. (1990). Brain and plasma tetrahydroisoquinolines in rats: effects of chronic ethanol intake and diet. *J. Neurochem.* 55, 1507–1514. doi: 10.1111/j.1471-4159.1990.tb04932.x

Collins, M. A., and Bigdeli, M. G. (1975). Tetrahydroisoquinolines *in vivo*. I. Rat brain formation of salsolinol, a condensation product of dopamine and acetaldehyde, under certain conditions during ethanol intoxication. *Life Sci.* 16, 585–601.

Coonfield, D. L., Hill, K. G., Kaczmarek, H. J., Ferraro, F. M., and Kiefer, S. W. (2002). Low doses of naltrexone reduce palatability and consumption of ethanol in outbred rats. *Alcohol* 26, 43–47. doi: 10.1016/s0741-8329(01)00180-x

Correa, M., Arizzi, M. N., Betz, A., Mingote, S., and Salamone, J. D. (2003). Open field locomotor effects in rats after intraventricular injections of ethanol and the ethanol metabolites acetaldehyde and acetate. *Brain Res. Bull.* 62, 197–202. doi: 10.1016/j.brainresbull.2003.09.013

Correa, M., Arizzi-Lafrance, M. N., and Salamone, J. D. (2009). Infusions of acetaldehyde into the arcuate nucleus of the hypothalamus induce motor activity in rats. *Life Sci.* 84, 321–327. doi: 10.1016/j.lfs.2008.12.013

Correa, M., Manrique, H. M., Font, L., Escrig, M. A., and Aragon, C. M. G. (2008). Reduction in the anxiolytic effects of ethanol by centrally formed acetaldehyde: the role of catalase inhibitors and acetaldehyde-sequestering agents. *Psychopharmacology* 200, 455–464. doi: 10.1007/s00213-008-1219-3

Correa, M., Miquel, M., Sanchis-Segura, C., and Aragon, C. M. G. (1999a). Acute lead acetate adAcute lead acetate administration potentiate-induced locomotor activity in mice: the role of brain catalase. *Alcohol. Clin. Exp. Res.* 23, 799–805. doi: 10.1097/00000374-199905000-00006

Correa, M., Miquel, M., Sanchis-Segura, C., and Aragon, C. M. G. (1999b). Effects of chronic lead administration on ethanol-induced locomotor and brain catalase activity. *Alcohol* 19, 43–49. doi: 10.1016/s0741-8329(99)00023-3

Correa, M., Salamone, J. D., Segovia, K. N., Pardo, M., Longoni, R., Spina, L., et al. (2012). Piecing together the puzzle of acetaldehyde as a neuroactive agent. *Neurosci. Biobehav. Rev.* 36, 404–430. doi: 10.1016/j.neubiorev.2011.07.009

Crews, F. T., Mdzinarishvili, A., Kim, D., He, J., and Nixon, K. (2006). Neurogenesis in adolescent brain is potently inhibited by ethanol. *Neuroscience* 137, 437–445. doi: 10.1016/j.neuroscience.2005.08.090

Crews, F. T., Sarkar, D. K., Qin, L., Zou, J., Boyadjieva, N., and Vetreno, R. P. (2015). Neuroimmune function and the consequences of alcohol exposure. *Alcohol Res.* 37, 331–351,

Crow, K. E., and Hardman, M. J. (1989). "Regulation of rates of ethanol metabolism," in *Regulation, Enzymology, and Metabolites of Ethanol, Human Metabolism of Alcohol*, eds K. E. Crow and R. D. Batt (Boca Raton, FL: CRC Press), 3–16.

D'Addario, C., Caputi, F. F., Rimondini, R., Gandolfi, O., Del Borrello, E., Candeletti, S., et al. (2013). Different alcohol exposures induce selective alterations on the expression of dynorphin and nociceptin systems related genes in rat brain. *Addict. Biol.* 18, 425–433. doi: 10.1111/j.1369-1600.2011.00326.x

D'Addario, C., Johansson, S., Candeletti, S., Romualdi, P., Ögren, S. O., Terenius, L., et al. (2011). Ethanol and acetaldehyde exposure induces specific epigenetic modifications in the prodynorphin gene promoter in a human neuroblastoma cell line. *FASEB J.* 25, 1069–1075. doi: 10.1096/fj.10-168534

D'Addario, C., Ming, Y., Ogren, S. O., and Terenius, L. (2008). The role of acetaldehyde in mediating effects of alcohol on expression of endogenous opioid system genes in a neuroblastoma cell line. *FASEB J.* 22, 662–670. doi: 10.1096/fj.07-8346com

Dar, N. J., Hamid, A., and Ahmad, M. (2015). Pharmacologic overview of Withania somnifera, the Indian Ginseng. *Cell. Mol. Life Sci.* 72, 4445–4460. doi: 10.1007/s00018-015-2012-1

Davis, V. E., and Walsh, M. J. (1970). Alcohol, amines, and alkaloids: a possible biochemical basis for alcohol addiction. *Science* 167, 1005–1007. doi: 10.1126/science.167.3920.1005

Deehan, G. A., Hauser, S. R., Wilden, J. A., Truitt, W. A., and Rodd, Z. A. (2013). Elucidating the biological basis for the reinforcing actions of alcohol in the mesolimbic dopamine system: the role of active metabolites of alcohol. *Front. Behav. Neurosci.* 7:104. doi: 10.3389/fnbeh.2013.00104

Deitrich, R. (2004). Acetaldehyde: déjà vu du jour. *J. Stud. Alcohol.* 65, 557–572. doi: 10.15288/jsa.2004.65.557

Deitrich, R. (2011). Ethanol as a prodrug: brain metabolism of ethanol mediates its reinforcing effects-a commentary. *Alcohol. Clin. Exp. Res.* 35, 581–583. doi: 10.1111/j.1530-0277.2011.01454.x

Deitrich, R. A., Bludeau, P., Roper, M., and Schmuck, J. (1978). Induction of aldehyde dehydrogenases. *Biochem. Pharmacol.* 27, 2343–2347. doi: 10.1016/0006-2952(78)90142-9

Dellarco, V. L. (1988). Mutagenicity assessment of acetaldehyde. *Mutat. Res.* 195, 1–20. doi: 10.1016/0165-1110(88)90013-9

Deng, X. S., and Deitrich, R. A. (2008). Putative role of brain acetaldehyde in ethanol addiction. *Clinurr. Drug Abuse Rev.* 1, 3–8. doi: 10.2174/1874473710801010003

Diana, M., Peana, A. T., Sirca, D., Lintas, A., Melis, M., and Enrico, P. (2008). Crucial role of acetaldehyde in alcohol activation of the mesolimbic dopamine system. *Ann. N Y Acad. Sci.* 1139, 307–317. doi: 10.1196/annals.1432.009

Donohue, T. M. Jr., and Thomes, P. G. (2014). Ethanol-induced oxidant stress modulates hepatic autophagy and proteasome activity. *Redox Biol.* 3, 29–39. doi: 10.1016/j.redox.2014.10.006

Duncan, C., and Deitrich, R. A. (1980). A critical evaluation of tetrahydroisoquinoline induced ethanol preference in rats. *Pharmacol. Biochem. Behav.* 13, 265–281. doi: 10.1016/0091-3057(80)90083-0

Enrico, P., Sirca, D., Mereu, M., Peana, A. T., Lintas, A., Golosio, A., et al. (2009). Acetaldehyde sequestering prevents ethanol-induced stimulation of mesolimbic dopamine transmission. *Drug Alcohol Depend.* 100, 265–271. doi: 10.1016/j.drugalcdep.2008.10.010

Erdozain, A. M., and Callado, L. F. (2014). Neurobiological alterations in alcohol addiction: a review. *Adicciones* 26, 360–370.

Erdozain, A. M., Morentin, B., Bedford, L., King, E., Tooth, D., Brewer, C., et al. (2014). Alcohol-related brain damage in humans. *PLoS One* 9:e93586. doi: 10.1093/med/9780199696758.003.0051

Eriksson, C. J. P. (2001). The role of acetaldehyde in the actions of alcohol (update 2000). *Alcohol. Clin. Exp. Res.* 25, 15S–32S. doi: 10.1111/j.1530-0277.2001.tb02369.x

Eriksson, C. J., and Fukunaga, T. (1993). Human blood acetaldehyde (update 1992). *Alcohol Alcohol.* 2, 9–25.

Eriksson, C. J., and Sippel, H. W. (1977). The distribution and metabolism of acetaldehyde in rats during ethanol oxidation-I. The distribution of acetaldehyde in liver, brain, blood and breath. *Biochem. Pharmacol.* 26, 241–247. doi: 10.1016/0006-2952(77)90310-0

Escarabajal, M. D., and Aragon, C. M. G. (2002). Concurrent administration of diethyldithiocarbamate and 4-methylpyrazole enhances ethanol-induced locomotor activity: the role of brain ALDH. *Psychopharmacology* 160, 339–343. doi: 10.1007/s00213-001-0991-0

Fernandes, P. A., Ribeiro, A. M., Pereira, R. F., Marra, H. L., and Pittella, J. E. (2002). Chronic ethanol intake and ageing effects on cortical and basal forebrain cholinergic parameters: morphometric and biochemical studies. *Addict. Biol.* 7, 29–36. doi: 10.1080/13556210120010057 1

Fertel, R. H., Greenwald, J. E., Schwarz, R., Wong, L., and Bianchine, J. (1980). Opiate receptor binding and analgesic effects of the tetrahydroisoquinolines salsolinol and tetrahydropapaveroline. *Res. Commun. Chem. Pathol. Pharmacol.* 27, 3–16.

Flatscher-Bader, T., Van der Brug, M., Hwang, J. W., Gochee, P. A., Matsumoto, I., Niwa, S., et al. (2005). Alcohol-responsive genes in the frontal cortex and nucleus accumbens of human alcoholics. *J. Neurochem.* 93, 359–370. doi: 10.1111/j.1471-4159.2004.03021.x

Flatscher-Bader, T., and Wilce, P. A. (2008). Impact of alcohol abuse on protein expression of midkine and excitatory amino acid transporter 1 in the human prefrontal cortex. *Alcohol. Clin. Exp. Res.* 32, 1849–1858. doi: 10.1111/j.1530-0277.2008.00754.x

Flentke, G. R., Garic, A., Hernandez, M., and Smith, S. M. (2014). CaMKII represses transcriptionally active β-catenin to mediate acute ethanol neurodegeneration and can phosphorylate β-catenin. *J. Neurochem.* 128, 523–535. doi: 10.1111/jnc.12464

Foddai, M., Dosia, G., Spiga, S., and Diana, M. (2004). Acetaldehyde increases dopaminergic neuronal activity in the VTA. *Neuropsychopharmacology* 29, 530–536. doi: 10.1038/sj.npp.1300326

Fois, G. R., and Diana, M. (2016). Opioid antagonists block acetaldehyde-induced increments in dopamine neurons activity. *Drug Alcohol Depend.* 158, 172–176. doi: 10.1016/j.drugalcdep.2015.11.013

Font, L., Aragon, C. M. G., and Miquel, M. (2006). Voluntary ethanol consumption decreases after the inactivation of central acetaldehyde by D-penicillamine. *Behav. Brain Res.* 171, 78–86. doi: 10.1016/j.bbr.2006.03.020

Font, L., Luján, M. Á., and Pastor, R. (2013). Involvement of the endogenous opioid system in the psychopharmacological actions of ethanol: the role of acetaldehyde. *Front. Behav. Neurosci.* 7:93. doi: 10.3389/fnbeh.2013.00093

Font, L., Miquel, M., and Aragon, C. M. G. (2008). Involvement of brain catalase activity in the acquisition of ethanol-induced conditioned place preference. *Physiol. Behav.* 93, 733–741. doi: 10.1016/j.physbeh.2007.11.026

Galter, D., Carmine, A., Buervenich, S., Duester, G., and Olson, L. (2003). Distribution of classI, III and IV alcohol dehydrogenase mRNAs in the adult rat, mouse and human brain. *Eur. J. Biochem.* 270, 1316–1326. doi: 10.1046/j.1432-1033.2003.03502.x

Gessa, G. L., Muntoni, F., Collu, M., Vargiu, L., and Mereu, G. (1985). Low doses of ethanol activate dopaminergic neurons in the ventral tegmental area. *Brain Res.* 348, 201–203. doi: 10.1016/0006-8993(85)90381-6

Ghozland, S., Chu, K., Kieffer, B. L., and Roberts, A. J. (2005). Lack of stimulant and anxiolytic-like effects of ethanol and accelerated development of ethanol dependence in mu-opioid receptor knockout mice. *Neuropharmacology* 49, 493–501. doi: 10.1016/j.neuropharm.2005.04.006

Gill, K., Liu, Y., and Deitrich, R. A. (1996). Voluntary alcohol consumption in BXD recombinant inbred mice: relationship to alcohol metabolism. *Alcohol. Clin. Exp. Res.* 20, 185–190. doi: 10.1111/j.1530-0277.1996.tb01063.x

Grant, K. A., and Samson, H. H. (1986). The induction of oral ethanol self-administration by contingent ethanol delivery. *Drug Alcohol Depend.* 16, 361–368. doi: 10.1016/0376-8716(86)90069-4

Guan, Y. Z., and Ye, J.-H. (2010). Ethanol blocks long-term potentiation of GABAergic synapses in the ventral tegmental area involving μ-opioid receptors. *Neuropsychopharmacology* 35, 1841–1849. doi: 10.1038/npp.2010.51

Haber, H., Dumaual, N., Bare, D. J., Melzig, M. F., McBride, W. F., Lumeng, L., et al. (1999). The quantitative determination of R- and S-salsolinol in the striatum and adrenal gland of rats selectively bred for disparate alcohol drinking. *Addict. Biol.* 4, 181–189. doi: 10.1080/13556219971687

Hamby-Mason, R., Chen, J. J., Schenker, S., Perez, A., and Henderson, G. I. (1997). Catalase mediates acetaldehyde formation from ethanol in fetal and neonatal rat brain. *Alcohol. Clin. Exp. Res.* 21, 1063–1072. doi: 10.1097/00000374-199709000-00020

Handler, J. A., and Thurman, R. G. (1988a). Catalase-dependent ethanol oxidation in perfused rat liver Requirement for fatty-acid-stimulated H_2O_2 production by peroxisomes. *Eur. J. Biochem.* 176, 477–484. doi: 10.1111/j.1432-1033.1988.tb14305.x

Handler, J. A., and Thurman, R. G. (1988b). Hepatic ethanol metabolism is mediated predominantly by catalase-H_2O_2 in the fasted state. *FEBS Lett.* 238, 139–141. doi: 10.1016/0014-5793(88)80243-6

Haorah, J., Knipe, B., Leibhart, J., Ghorpade, A., and Persidsky, Y. (2005). Alcohol-induced oxidative stress in brain endothelial cells causes blood-brain barrier dysfunction. *J. Leukoc. Biol.* 78, 1223–1232. doi: 10.1189/jlb.0605340

Haorah, J., Ramirez, S. H., Floreani, N., Gorantla, S., Morsey, B., and Persidsky, Y. (2008). Mechanism of alcohol-induced oxidative stress and neuronal injury. *Free Radic. Biol. Med.* 45, 1542–1550. doi: 10.1016/j.freeradbiomed.2008.08.030

Haorah, J., Ramirez, S. H., Schall, K., Smith, D., Pandya, R., and Persidsky, Y. (2007). Oxidative stress activates protein tyrosine kinase and matrix metalloproteinases leading to blood-brain barrier dysfunction. *J. Neurochem.* 101, 566–576. doi: 10.1111/j.1471-4159.2006.04393.x

Harris, R. A., Bajo, M., Bell, R. L., Blednov, Y. A., Varodayan, F. P., Truitt, J. M., et al. (2017). Genetic and pharmacologic manipulation of TLR4 has minimal impact on ethanol consumption in rodents. *J. Neurosci.* 37, 1139–1155. doi: 10.1523/jneurosci.2002-16.2016

Haseba, T., and Ohno, Y. (1997). Assay methods of alcohol metabolizing enzymes for the research on enzymatic mechanism of alcohol metabolism. *Nippon Rinnshou.* 55, 665–673. doi: 10.3390/ijerph7031076

Haseba, T., and Ohno, Y. (2010). A new view of alcohol metabolism and alcoholism—role of the high-K_m Class III alcohol dehydrogenase (ADH3). *Int. J. Environ. Res. Public Health* 7, 1076–1092. doi: 10.3390/ijerph7031076

Haseba, T., Tomita, Y., Kurosu, M., and Ohno, Y. (2003). Dose and time changes in liver alcohol dehydrogenase (ADH) activity during acute alcohol intoxication involve not only class I but also class III ADH and govern elimination rate of blood ethanol. *Legal Med.* 5, 202–211. doi: 10.1016/s1344-6223(03)00080-4

He, D., Chen, H., Muramatsu, H., and Lasek, A. W. (2015). Ethanol activates midkine and anaplastic lymphoma kinase signaling in neuroblastoma cells and in the brain. *J. Neurochem.* 135, 508–521. doi: 10.1111/jnc.13252

Heap, L., Ward, R. J., Abiaka, C., Dexter, D., Lawlor, M., Pratt, O., et al. (1995). The influence of brain acetaldehyde on oxidative status, dopamine metabolism and visual discrimination task. *Biochem. Pharmacol.* 50, 263–270. doi: 10.1016/0006-2952(94)00539-x

Hellmann, J., Rommelspacher, H., and Wernicke, C. (2009). Long term ethanol exposure impairs neuronal differentiation of human neuroblastoma cells involving neurotrophin-mediated intracellular signaling and in particular protein kinase C. *Alcohol. Clin. Exp. Res.* 33, 538–550. doi: 10.1111/j.1530-0277.2008.00867.x

Hempel, J., Bühler, R., Kaiser, R., Holmquist, B., de Zalenski, C., von Wartburg, J. P., et al. (1984). Human liver alcohol dehydrogenase 1. The primary structure of the β 1 β 1 isoenzyme. *Eur. J. Biochem.* 145, 437–445.

Hernández, J. A., López-Sánchez, R. C., and Rendón-Ramírez, A. (2016). Lipids and oxidative stress associated with ethanol-induced neurological damage. *Oxid. Med. Cell. Longev.* 2016:1543809. doi: 10.1155/2016/1543809

Herradón, G., and Pérez-García, C. (2014). Targeting midkine and pleiotrophin signalling pathways in addiction and neurodegenerative disorders: recent progress and perspectives. *Br. J. Pharmacol.* 171, 837–848. doi: 10.1111/bph.12312

Hipólito, L., Martí-Prats, L., Sánchez-Catalán, M. J., Polache, A., and Granero, L. (2011). Inductio of conditioned place preference and dopamine release by salsolinol in posterior VTA of rats: involvement of μ-opioid receptors. *Neurochem. Int.* 59, 559–562. doi: 10.1016/j.neuint.2011.04.014

Hipólito, L., Sánchez-Catalán, M. J., Granero, L., and Polache, A. (2009). Local salsolinol modulates dopamine extracellular levels from rat nucleus accumbens: shell/core differences. *Neurochem. Int.* 55, 187–192. doi: 10.1016/j.neuint.2009.02.014

Hipólito, L., Sánchez-Catalán, M. J., Martí-Prats, L., Granero, L., and Polache, A. (2012). Revisiting the controversial role of salsolinol in the neurobiological effects ofethanol: old and new vistas. *Neurosci. Biobehav. Rev.* 36, 362–378. doi: 10.1016/j.neubiorev.2011.07.007

Hipólito, L., Sanchez, M. J., Polache, A., and Granero, L. (2007). Brain metabolism of ethanol and alcoholism: an update. *Curr. Drug Metab.* 8, 716–727. doi: 10.2174/138920007782109797

Hipólito, L., Sánchez-Catalán, M. J., Zanolini, I., Polache, A., and Granero, L. (2008). Shell/core differences in mu- and delta-opioid receptor modulation of dopamine efflux in nucleus accumbens. *Neuropharmacology* 55, 183–189. doi: 10.1016/j.neuropharm.2008.05.012

Hipólito, L., Sánchez-Catalán, M. J., Zornoza, T., Polache, A., and Granero, L. (2010). Locomotor stimulant effects of acute and repeated intrategmental injections of salsolinol in rats: role of mu-opioid receptors. *Psychopharmacology* 209, 1–11. doi: 10.1007/s00213-009-1751-9

Homicsko, K. G., Kertesz, I., Radnai, B., Toth, B. E., Toth, G., Fulop, F., et al. (2003). Binding site of salsolinol: its properties in different regions of the brain and the pituitary gland of the rat. *Neurochem. Int.* 42, 19–26. doi: 10.1016/s0197-0186(02)00063-3

Hoover, D. J., and Brien, J. F. (1981). Acetaldehyde concentration in rat blood and brain during the calcium carbimide-ethanol interaction. *Can. J. Physiol. Pharmacol.* 59, 65–70. doi: 10.1139/y81-011

Ibba, F., Vinci, S., Spiga, S., Peana, A. T., Assaretti, A. R., Spina, L., et al. (2009). Ethanol-induced extracellular signal regulated kinase: role of dopamine D1 receptors. *Alcohol Clin. Exp. Res.* 33, 858–867. doi: 10.1111/j.1530-0277.2009.00907.x

Israel, Y., Ezquer, F., Quintanilla, M. E., Morales, P., Ezquer, M., and Herrera-Marschitz, M. (2017). Intracerebral stem cell administration inhibits relapse-like alcohol drinking in rats. *Alcohol Alcohol* 52, 1–4. doi: 10.1093/alcalc/agw068

Israel, Y., Quintanilla, M. E., Karahanian, E., Rivera-Meza, M., and Herrera-Marschitz, M. (2015). The "first hit" toward alcohol reinforcement: role of ethanol metabolites. *Alcohol. Clin. Exp. Res.* 39, 776–786. doi: 10.1111/acer.12709

Jalabert, M., Bourdy, R., Courtin, J., Veinante, P., Manzoni, O. J., Barrot, M., et al. (2011). Neuronal circuits underlying acute morphine action on dopamine neurons. *Proc. Natl. Acad. Sci. U S A* 108, 16446–16450. doi: 10.1073/pnas.1105418108

Jamal, M., Ameno, K., Ameno, S., Okada, N., and Ijiri, I. (2003a). Effect of different doses of cyanamide on striatal salsolinol formation after ethanol treatment. *Legal Med.* 5, S79–S82. doi: 10.1016/s1344-6223(02)00083-4

Jamal, M., Ameno, K., Ameno, S., Okada, N., and Ijiri, I. (2003b). *In vivo* study of salsolinol produced by a high concentration of acetaldehyde in the striatum and nucleus accumbens of free-moving rats. *Alcohol. Clin. Exp. Res.* 27, 79S–84S. doi: 10.1097/01.ALC.0000078617.33026.AD

Jamal, M., Ameno, K., Kubota, T., Ameno, S., Zhang, X., Kumihashi, M., et al. (2003c). *In vivo* formation of salsolinol induced by high acetaldehyde concentration in rat striatum employing microdyalisis. *Alcohol Alcohol.* 38, 197–201. doi: 10.1093/alcalc/agg056

Jamal, M., Ameno, K., Ikuo, U., Kumihashi, M., Wang, W., and Ijiri, I. (2007). Ethanol and acetaldehyde: *in vivo* quantitation and effects on cholinergic function in rat brain. *Novartis Found. Symp.* 285, 137–141.

Johnson, S. W., and North, R. A. (1992). Opioids excite dopamine neurons by hyperpolarization of local interneurons. *J. Neurosci.* 12, 483–488.

Karahanian, E., Quintanilla, M. E., Tampier, L., Rivera-Meza, M., Bustamante, D., Gonzalez-Lira, V., et al. (2011). Ethanol as a prodrug: brain metabolism of ethanol mediates its reinforcing effects. *Alcohol. Clin. Exp. Res.* 35, 606–612. doi: 10.1111/j.1530-0277.2011.01454.x

Karahanian, E., Rivera-Meza, M., Tampier, L., Quintanilla, M. E., Herrera-Marschitz, M., and Israel, Y. (2015). Long-term inhibition of ethanol intake by the administration of an aldehyde dehydrogenase-2 (ALDH2)-coding lentiviral vector into the ventral tegmental area of rats. *Addict. Biol.* 20, 336–344. doi: 10.1111/adb.12130

Kiessling, K. H. (1962). The occurrence of acetaldehyde in various parts of rat brain after alcohol injection and its effect on pyruvate oxidation. *Exp. Cell. Res.* 27, 367–368. doi: 10.1016/0014-4827(62)90248-3

Kim, J. S., and Shukla, S. D. (2006). Acute *in vivo* effect of ethanol (binge drinking) on histone H3 modifications in rat tissues. *Alcohol Alcohol.* 41, 126–132. doi: 10.1093/alcalc/agh248

Koechling, U. M., and Amit, Z. (1994). Effects of 3-amino-1,2,4-triazole on brain catalase in the mediation of ethanol consumption in mice. *Alcohol* 11, 235–239. doi: 10.1016/0741-8329(94)90036-1

Koob, G. F., and Le Moal, M. (2006). *Neurobiology of Addiction.* London: Academic Press.

Kurnik, M., Gil, K., Gajda, M., Thor, P., and Bugajski, A. (2015). Neuropathic alterations of the myenteric plexus neurons following subacute intraperitoneal administration of salsolinol. *Folia Histochem. Cytobiol.* 53, 49–61. doi: 10.5603/fhc.a2015.0010

Lachenmeier, D. W., and Salaspuro, M. (2017). ALDH2-deficiency as genetic epidemiologic and biochemical model for the carcinogenicity of acetaldehyde. *Regul. Toxicol. Pharmacol.* 86, 128–136. doi: 10.1016/j.yrtph.2017.02.024

Lasek, A. W., Lim, J., Kliethermes, C. L., Berger, K. H., Joslyn, G., Brush, G., et al. (2011). An evolutionary conserved role for anaplastic lymphoma kinase in behavioral responses to ethanol. *PLoS One* 6:e22636. doi: 10.1371/journal.pone.0022636

Ledesma, J. C., and Aragon, C. M. G. (2012). Acquisition and reconditioning of ethanol-induced conditioned place preference in mice is blocked by the H_2O_2 scavenger alpha lipoic acid. *Psychopharmacology* 226, 673–685. doi: 10.1007/s00213-012-2831-9

Ledesma, J. C., Font, L., and Aragon, C. M. G. (2012). The H_2O_2 scavenger ebselen decreases ethanol-induced locomotor stimulation in mice. *Drug Alcohol Depend.* 124, 42–49. doi: 10.1016/j.drugalcdep.2011.12.003

Ledesma, J. C., Font, L., Baliño, P., and Aragon, C. M. (2013). Modulation of ethanol-induced conditioned place preference in mice by 3-amino-1,2,4-triazole and D-penicillamine depends on ethanol dose and number of conditioning trials. *Psychopharmacology* 230, 557–568. doi: 10.1007/s00213-013-3177-7

Lee, H. J., Oh, S. H., Jang, H. W., Kwon, J. H., Lee, K. J., Kim, C. H., et al. (2016). Long-term effects of bone marrow-derived mesenchymal stem cells in dextran sulfate sodium-induced murine chronic colitis. *Gut Liver* 10, 412–419. doi: 10.5009/gnl15229

Lee, J., Ramchandani, V. A., Hamazaki, K., Engleman, E. A., McBride, W. J., Li, T. K., et al. (2010). A critical evaluation of influence of ethanol and diet on salsolinol enantiomers in humans and rats. *Alcohol. Clin. Exp. Res.* 34, 242–250. doi: 10.1111/j.1530-0277.2009.01087.x

Lieber, C. S. (2004). Alcoholic fatty liver: its pathogenesis and mechanism of progression to inflammation and fibrosis. *Alcohol* 34, 9–19. doi: 10.1016/j.alcohol.2004.07.008

Lieber, C. S., and DeCarli, L. M. (1972). The role of the hepatic microsomal ethanol oxidizing system (MEOS) for ethanol metabolism *in vivo*. *J. Pharmacol. Exp. Ther.* 181, 279–287.

Lucchi, L., Bosio, A., Spano, P. F., and Trabucchi, M. (1982). Action of ethanol and salsolinol on opiate receptor function. *Brain Res.* 232, 506–510. doi: 10.1016/0006-8993(82)90297-9

Mao, J., Ma, H., Xu, Y., Su, Y., Zhu, H., Wang, R., et al. (2013). Increased levels of monoamine-derived potential neurotoxins in fetal rat brain exposed to ethanol. *Neurochem. Res.* 38, 356–363. doi: 10.1007/s11064-012-0926-7

March, S. M., Abate, P., Spear, N. E., and Molina, J. C. (2013). The role of acetaldehyde in ethanol reinforcement assessed by pavlovian conditioning in newborn rats. *Psychopharmacology* 226, 491–499. doi: 10.1007/s00213-012-2920-9

Martí-Prats, L., Orrico, A., Polache, A., and Granero, L. (2015). Dual motor responses elicited by ethanol in the posterior VTA: consequences of the blockade of μ-opioid receptors. *J. Psychopharmacol.* 29, 1029–1034. doi: 10.1177/0269881115598337

Martí-Prats, L., Sánchez-Catalán, M. J., Orrico, A., Zornoza, T., Polache, A., and Granero, L. (2013). Opposite motor responses elicited by ethanol in the posterior VTA: the role of acetaldehyde and the non-metabolized fraction of ethanol. *Neuropharmacology* 72, 204–214. doi: 10.1016/j.neuropharm.2013.04.047

Maruyama, W., Nakahara, D., Ota, M., Takahashi, T., Takahashi, A., Nagatsu, T., et al. (1992). N-methylation of dopamine-derived 6,7-dihydroxy-1,2,3,4-tetrahydroisoquinoline, (R)-salsolinol, in rat brains: *in vivo* microdialysis study. *J. Neurochem.* 59, 395–400. doi: 10.1111/j.1471-4159.1992.tb09384.x

Matsubara, K., Fukushima, S., and Fukui, Y. (1987). A systematic regional study of brain salsolinol levels during and immediately following chronic ethanol ingestion in rats. *Brain Res.* 413, 336–343. doi: 10.1016/0006-8993(87)91025-0

Matsuzawa, S., Suzuki, T., and Misawa, M. (2000). Involvement of μ-opioid receptor in the salsolinol-associated place preference in rats exposed to conditioned fear stress. *Alcohol. Clin. Exp. Res.* 24, 366–372. doi: 10.1111/j.1530-0277.2000.tb04624.x

McBride, W. J., Li, T. K., Deitrich, R. A., Zimatkin, S., Smith, B. R., and Rodd-Henricks, Z. A. (2002). Involvement of acetaldehyde in alcohol addiction. *Alcohol. Clin. Exp. Res.* 26, 114–119. doi: 10.1111/j.1530-0277.2002.tb02439.x

Melchior, C. L., and Myers, R. D. (1977). Preference for alcohol evoked by tetrahydropapaveroline (THP) chronically infused in the cerebral ventricle of the rat. *Pharmacol. Biochem. Behav.* 7, 19–35. doi: 10.1016/0091-3057(77)90006-5

Melis, M., Carboni, E., Caboni, P., and Acquas, E. (2015). Key role of salsolinol in ethanol actions on dopamine neuronal activity of the posterior ventral tegmental area. *Addict. Biol.* 20, 182–193. doi: 10.1111/adb.12097

Melis, M., Enrico, P., Peana, A. T., and Diana, M. (2007). Acetaldehyde mediates alcohol activation of the mesolimbic dopamine system. *Eur. J. Neurosci.* 26, 2824–2833. doi: 10.1111/j.1460-9568.2007.05887.x

Mereu, G., and Gessa, G. L. (1985). Low doses of ethanol inhibit the firing of neurons in the substantia nigra, pars reticulata: a GABAergic effect? *Brain Res.* 360, 325–330. doi: 10.1016/0006-8993(85)91249-1

Montesinos, J., Alfonso-Loeches, S., and Guerri, C. (2016). Impact of the innate immune response in the actions of ethanol on the central nervous system. *Alcohol. Clin. Exp. Res.* 40, 2260–2270. doi: 10.1111/acer.13208

Mooney, S. M., and Miller, M. W. (2011). Role of neurotrophins on postnatal neurogenesis in the thalamus: prenatal exposure to ethanol. *Neuroscience* 179, 256–266. doi: 10.1016/j.neuroscience.2011.01.046

Moore, D. B., Madorsky, I., Paiva, M., and Barrow Heaton, M. (2004). Ethanol exposure alters neurotrophin receptor expression in the rat central nervous system: effects of neonatal exposure. *J. Neurobiol.* 60, 114–126. doi: 10.1002/neu.20010

Morikawa, H., and Morrisett, R. A. (2010). Ethanol action on dopaminergic neurons in the ventral tegmental area: interaction with intrinsic ion channels and neurotransmitter inputs. *Int. Rev. Neurobiol.* 91, 235–288. doi: 10.1016/S0074-7742(10)91008-8

Moulder, K. L., Fu, T., Melbostad, H., Cormier, R. J., Isenberg, K. E., Zorumski, C. F., et al. (2002). Ethanol-induced death of postnatal hippocampal neurons. *Neurobiol. Dis.* 10, 396–409. doi: 10.1006/nbdi.2002.0523

Mulligan, M. K., Ponomarev, I., Hitzemann, R. J., Belknap, J. K., Tabakoff, B., Harris, R. A., et al. (2006). Toward understanding the genetics of alcohol drinking through transcriptome meta-analysis. *Proc. Natl. Acad. Sci. U S A* 103, 6368–6373. doi: 10.1073/pnas.0510188103

Myers, R. D., and Melchior, C. L. (1977). Differential actions on voluntary alcohol intake of tetrahydroisoquinolines or a β-carboline infused chronically in the ventricle of the rat. *Pharmacol. Biochem. Behav.* 7, 381–392. doi: 10.1016/0091-3057(77)90235-0

Myers, R. D., and Robinson, D. E. (1999). Tetrahydropapaveroline injected in the ventral tegmental area shifts dopamine efflux differentially in the shell and core of nucleus accumbens in high-ethanol-preferring (HEP) rats. *Alcohol* 18, 83–90. doi: 10.1016/s0741-8329(99)00008-7

Myers, W. D., Mackenzie, L., Ng, K. T., Singer, G., Smythe, G. A., and Duncan, M. W. (1985). Salsolinol and dopamine in rat medial basal hypothalamus after chronic ethanol exposure. *Life Sci.* 36, 309–314. doi: 10.1016/0024-3205(85)90115-8

Myers, W. D., Ng, K. T., and Singer, G. (1982). Intravenous self-administration of acetaldehyde in the rat as a function of schedule, food deprivation and photoperiod. *Pharmacol. Biochem. Behav.* 17, 807–811. doi: 10.1016/0091-3057(82)90364-1

Nagasawa, H. T., Elberling, J. A., and DeMaster, E. G. (1980). Structural requirements for the sequestration of metabolically generated acetaldehyde. *J. Med. Chem.* 23, 140–143. doi: 10.1002/chin.198026137

Nakahara, D., Maruyama, W., Hashiguti, H., and Naoi, M. (1994). Characterization of the *in vivo* action of *(R)*-salsolinol, an endogenous metabolite of alcohol, on serotonin and dopamine metabolism: a microdialysis study. *Brain. Res.* 644, 226–232. doi: 10.1016/0006-8993(94)91684-5

Nixon, K., and Crews, F. T. (2002). Binge ethanol exposure decreases neurogenesis in adult rat hippocampus. *J. Neurochem.* 83, 1087–1093. doi: 10.1046/j.1471-4159.2002.01214.x

O'Neill, P. J., and Rahwan, R. G. (1977). Absence of formation of brain salsolinol in ethanoldependent mice. *J. Pharmacol. Exp. Ther.* 200, 306–313.

Okamoto, T., Harnett, M. T., and Morikawa, H. (2006). Hyperpolarization-activated cation current (ih) is an ethanol target in midbrain dopamine neurons of mice. *J. Neurophysiol.* 95, 619–626. doi: 10.1152/jn.00682.2005

Orrico, A., Hipólito, L., Sánchez-Catalán, M. J., Martí-Prats, L., Zornoza, T., Granero, L., et al. (2013). Efficacy of D-penicillamine, a sequestering acetaldehyde agent, in the prevention of alcohol relapse-like drinking in rats. *Psychopharmacology* 228, 563–575. doi: 10.1007/s00213-013-3065-1

Orrico, A., Martí-Prats, L., Cano-Cebrián, M. J., Granero, L., Polache, A., and Zornoza, T. (2014). Improved effect of the combination naltrexone/D-penicillamine in the prevention of alcohol relapse-like drinking in rats. *J. Psychopharmacol.* 28, 76–81. doi: 10.1177/0269881113515063

Oshino, N., Oshino, R., and Chance, B. (1973). The characteristics of the "peroxidatic" reaction of catalase in ethanol oxidation. *Biochem. J.* 131, 555–563. doi: 10.1042/bj1310555

Oswald, L. M., and Wand, G. S. (2004). Opioids and alcoholism. *Physiol. Behav.* 81, 339–358. doi: 10.1016/j.physbeh.2004.02.008

Pandey, S. C., Zhang, H., Ugale, R., Prakash, A., Xu, T., and Misra, K. (2008). Effector immediate-early gene arc in the amygdala plays a critical role in alcoholism. *J. Neurosci.* 28, 2589–2600. doi: 10.1523/jneurosci.4752-07.2008

Pascual, M., Baliño, P., Aragon, C. M. G., and Guerri, C. (2015). Cytokines and chemokines as biomarkers of ethanol-induced neuroinflammation and anxiety-related behavior: role of TLR4 and TLR2. *Neuropharmacology* 89, 352–359. doi: 10.1016/j.neuropharm.2014.10.014

Pastor, R., and Aragon, C. M. G. (2008). Ethanol injected into the hypothalamic arcuate nucleus induces behavioral stimulation in rats: an effect prevented by catalase inhibition and naltrexone. *Behav. Pharmacol.* 19, 698–705. doi: 10.1097/fbp.0b013e328315ecd7

Pautassi, R. M., Nizhnikov, M. E., Fabio, M. C., and Spear, N. E. (2011). An acetaldehyde-sequestering agent inhibits appetitive reinforcement and behavioral stimulation induced by ethanol in preweanling rats. *Pharmacol. Biochem. Behav.* 97, 462–469. doi: 10.1016/j.pbb.2010.10.005

Peana, A. T., and Acquas, E. (2013). Behavioral and biochemical evidence of the role of acetaldehyde in the motivational effects of ethanol. *Front. Behav. Neurosci.* 7:86. doi: 10.3389/fnbeh.2013.00086

Peana, A. T., Assaretti, A. R., Muggironi, G., Enrico, P., and Diana, M. (2009). Reduction of ethanol-derived acetaldehyde induced motivational properties by L-cysteine. *Alcohol. Clin. Exp. Res.* 33, 43–48. doi: 10.1111/j.1530-0277.2008.00809.x

Peana, A. T., Enrico, P., Assaretti, A. R., Pulighe, E., Muggironi, G., Nieddu, M., et al. (2008). Key role of ethanol-derived acetaldehyde in the motivational properties induced by intragastric ethanol: a conditioned place preference study in the rat. *Alcohol. Clin. Exp. Res.* 32, 249–258. doi: 10.1111/j.1530-0277.2007.00574.x

Peana, A. T., Giugliano, V., Rosas, M., Sabariego, M., and Acquas, E. (2013a). Effects of L-cysteine on reinstatement of ethanol-seeking behavior and on reinstatement-elicited extracellular signal-regulated kinase phosphorylation in the rat nucleus accumbens shell. *Alcohol. Clin. Exp. Res.* 37, E329–E337. doi: 10.1111/j.1530-0277.2012.01877.x

Peana, A. T., Muggironi, G., Fois, G., and Diana, M. (2013b). Alpha- lipoic acid reduces ethanol self- administration in rats. *Alcohol. Clin. Exp. Res.* 37, 1816–1822. doi: 10.1111/acer.12169

Peana, A. T., Muggironi, G., Calvisi, G., Enrico, P., Mereu, M., Nieddu, M., et al. (2010a). L-cysteine reduces oral ethanol self-administration and reinstatement of ethanol-drinking behavior in rats. *Pharmacol. Biochem. Behav.* 94, 431–437. doi: 10.1016/j.pbb.2009.10.005

Peana, A. T., Muggironi, G., and Diana, M. (2010b). Acetaldehyde motivational effects; a study on oral self-administration behavior. *Front. Psychiatry* 1:23. doi: 10.3389/fpsyt.2010.00023

Peana, A. T., Muggironi, G., Fois, G. R., Zinellu, M., Sirca, D., and Diana, M. (2012). Effect of (L)-cysteine on acetaldehyde self-administration. *Alcohol* 46, 489–497. doi: 10.1016/j.alcohol.2011.10.004

Peana, A. T., Muggironi, G., Fois, G. R., Zinellu, M., Vinci, S., and Acquas, E. (2011). Effect of opioid receptor blockade on acetaldehyde self-administration and ERK phosphorylation in the rat nucleus accumbens. *Alcohol* 45, 773–783. doi: 10.1016/j.alcohol.2011.06.003

Peana, A. T., Muggironi, G., Spina, L., Rosas, M., Kasture, S. B., Cotti, E., et al. (2014). Effects of Withania somnifera on oral ethanol self-administration in rats. *Behav. Pharmacol.* 25, 618–628. doi: 10.1097/fbp.0000000000000078

Peana, A. T., Porcheddu, V., Bennardini, F., Carta, A., Rosas, M., and Acquas, E. (2015). Role of ethanol-derived acetaldehyde in operant oral self-administration of ethanol in rats. *Psychopharmacology* 232, 4269–4276. doi: 10.1007/s00213-015-4049-0

Peana, A. T., Rosas, M., Porru, S., and Acquas, E. (2016). From Ethanol to Salsolinol: role of Ethanol metabolites in the effects of Ethanol. *J. Exp. Neurosci.* 10, 137–146. doi: 10.4137/jen.s25099

Pflaum, T., Hausler, T., Baumung, C., Ackermann, S., Kuballa, T., Rehm, J., et al. (2016). Carcinogenic compounds in alcoholic beverages: an update. *Arch. Toxicol.* 90, 2349–2367. doi: 10.1007/s00204-016-1770-3

Pikkarainen, P. H., and Lieber, C. S. (1980). Concentration dependency of ethanol elimination rates in baboons: effect of chronic alcohol consumption. *Alcohol. Clin. Exp. Res.* 4, 40–43. doi: 10.1111/j.1530-0277.1980.tb04789.x

Plescia, F., Brancato, A., Venniro, M., Maniaci, G., Cannizzaro, E., Sutera, F. M., et al. (2015). Acetaldehyde self-administration by a two-bottle choice paradigm: consequences on emotional reactivity, spatial learning, and memory. *Alcohol* 49, 139–148. doi: 10.1016/j.alcohol.2015.01.002

Plotnikov, E., Korotkova, E., Voronova, O., Sazhina, N., Petrova, E., Artamonov, A., et al. (2016). Comparative investigation of antioxidant activity of human serum blood by amperometric, voltammetric and chemiluminescent methods. *Arch. Med. Sci.* 12, 1071–1076. doi: 10.5114/aoms.2015.50234

Puig, J. G., and Fox, I. H. (1984). Ethanol-induced activation of adenine nucleotide turnover. Evidence for a role of acetate. *J. Clin. Invest.* 74, 936–941. doi: 10.1172/jci111512

Quertemont, E., and De Witte, P. (2001). Conditioned stimulus preference after acetaldehyde but not ethanol injections. *Pharmacol. Biochem. Behav.* 68, 449–454. doi: 10.1016/s0091-3057(00)00486-x

Quertemont, E., Eriksson, C. J., Zimatkin, S. M., Pronko, P. S., Diana, M., Pisano, M., et al. (2005). Is ethanol a pro-drug? Acetaldehyde contribution to brain ethanol effects. *Alcohol. Clin. Exp. Res.* 29, 1514–1521. doi: 10.1097/01.alc.0000175015.51329.45

Quertemont, E., Tambour, S., Bernaerts, P., Zimatkin, S. M., and Tirelli, E. (2004). Behavioral characterization of acetaldehyde in C57BL/6J mice: locomotor, hypnotic, anxiolytic, and amnesic effects. *Psychopharmacology* 177, 84–92. doi: 10.1007/s00213-004-1911-x

Quintanilla, M. E., Callejas, O., and Tampier, L. (2002). Aversion to acetaldehyde: differences in low-alcohol-drinking (UChA) and high-alcohol-drinking (UChB) rats. *Alcohol* 26, 69–74. doi: 10.1016/S0741-8329(01)00197-5

Quintanilla, M. E., Rivera-Meza, M., Berrios-Cárcamo, P. A., Bustamante, D., Buscaglia, M., Morales, P., et al. (2014). Salsolinol, free of isosalsolinol, exerts ethanol-like motivational/sensitization effects leading to increases in ethanol intake. *Alcohol* 48, 551–559. doi: 10.1016/j.alcohol.2014.07.003

Quintanilla, M. E., Rivera-Meza, M., Berríos-Cárcamo, P., Cassels, B. K., Herrera-Marschitz, M., and Israel, Y. (2016). (*R*)-Salsolinol, a product of ethanol metabolism, stereospecifically induces behavioral sensitization and leads to excessive alcohol intake. *Addict. Biol.* 21, 1063–1071. doi: 10.1111/adb.12268

Reiff, T., Huber, L., Kramer, M., Delattre, O., Janoueix-Lerosey, I., and Rohrer, H. (2011). Midkine and Alk signaling in sympathetic neuron proliferation and neuroblastoma predisposition. *Development* 138, 4699–4708. doi: 10.1242/dev.072157

Rodd, Z. A., Bell, R. L., Zhang, Y., Goldstein, A., Zaffaroni, A., McBride, W. J., et al. (2003). Salsolinol produces reinforcing effects in the nucleus accumbens shell of alcohol-preferring (P) rats. *Alcohol. Clin. Exp. Res.* 27, 440–449. doi: 10.1097/01.alc.0000056612.89957.b4

Rodd, Z. A., Bell, R. L., Zhang, Y., Murphy, J. M., Goldstein, A., Zaffaroni, A., et al. (2005). Regional heterogeneity for the intracranial self-administration of ethanol and acetaldehyde within the ventral tegmental area of alcohol-preferring (P) rats: involvement of dopamine and serotonin. *Neuropsychopharmacology* 30, 330–338. doi: 10.1038/sj.npp.1300561

Rodd, Z. A., Melendez, R. I., Bell, R. L., Kuc, K. A., Zhang, Y., Murphy, J. M., et al. (2004). Intracranial self-administration of ethanol within the ventral

tegmental area of male wistar rats: evidence for involvement of dopamine neurons. *J. Neurosci.* 24, 1050–1057. doi: 10.1523/jneurosci.1319-03.2004

Rodd, Z. A., Oster, S. M., Ding, Z. M., Toalston, J. E., Deehan, G., Bell, R. L., et al. (2008). The reinforcing properties of salsolinol in the ventral tegmental area: evidence for regional heterogeneity and the involvement of serotonin and dopamine. *Alcohol. Clin. Exp. Res.* 32, 230–239. doi: 10.1111/j.1530-0277.2007.00572.x

Rodd-Henricks, Z. A., Melendez, R. I., Zaffaroni, A., Goldstein, A., McBride, W. J., and Li, T. K. (2002). The reinforcing effects of acetaldehyde in the posterior ventral tegmental area of alcohol-preferring rats. *Pharmacol. Biochem. Behav.* 72, 55–64. doi: 10.1016/s0091-3057(01)00733-x

Rojkovicova, T., Mechref, Y., Starkey, J. A., Wu, G., Bell, R. L., McBride, W. J., et al. (2008). Quantitative chiral analysis of salsolinol in different brain regions of rats genetically predisposed to alcoholism. *J. Chromatogr. B Analyt. Technol. Biomed. Life Sci.* 863, 206–214. doi: 10.1016/j.jchromb.2008.01.016

Sakai, R., Ukai, W., Sohma, H., Hashimoto, E., Yamamoto, M., Ikeda, H., et al. (2005). Attenuation of brain derived neurotrophic factor (BDNF) by ethanol and cytoprotective effect of exogenous BDNF against ethanol damage in neuronal cells. *J. Neural. Transm.* 112, 1005–1013. doi: 10.1007/s00702-004-0246-4

Salaspuro, M. (2011). Acetaldehyde and gastric cancer. *J. Dig. Dis.* 12, 51–59. doi: 10.1111/j.1751-2980.2011.00480.x

Salaspuro, V., Hietala, J., Kaihovaara, P., Pihlajarinne, L., Marvola, M., and Salaspuro, M. (2002). Removal of acetaldehyde from saliva by a slow-release buccal tablet of L-cysteine. *Int. J. Cancer* 97, 361–364. doi: 10.1002/ijc.1620

Samson, H. H., Pfeffer, A. O., and Tolliver, G. A. (1988). Oral ethanol self-administration in rats: models of alcohol-seeking behavior. *Alcohol. Clin. Exp. Res.* 12, 591–598. doi: 10.1111/j.1530-0277.1988.tb00248.x

Sánchez-Catalán, M. J., Hipólito, L., Guerri, C., Granero, L., and Polache, A. (2008). Distribution and differential induction of CYP2E1 by ethanol and acetone in the mesocorticolimbic system of rat. *Alcohol Alcohol* 43, 401–407. doi: 10.1093/alcalc/agn012

Sánchez-Catalán, M. J., Hipólito, L., Zornoza, T., Polache, A., and Granero, L. (2009). Motor stimulant effects of ethanol and acetaldehyde injected into the posterior ventral tegmental area of rats: role of opioid receptors. *Psychopharmacology* 204, 641–653. doi: 10.1007/s00213-009-1495-6

Sánchez-Catalán, M. J., Kaufling, J., Georges, F., Veinante, P., and Barrot, M. (2014). The anterio-posterior heterogeneity of the ventral tegmental area. *Neuroscience* 282, 198–216. doi: 10.1016/j.neuroscience.2014.09.025

Sanchis-Segura, C., Correa, M., Miquel, M., and Aragon, C. M. G. (2005). Catalase inhibition in the Arcuate nucleus blocks ethanol effects on the locomotor activity of rats. *Neurosci. Lett.* 376, 66–70. doi: 10.1016/j.neulet.2004.11.025

Sarkola, T., Iles, M. R., Kohlenberg-Mueller, K., and Eriksson, C. J. P. (2002). Ethanol, acetaldehyde, acetate and lactate levels after alcohol intake in white men and women: effect of 4-methylpyrazole. *Alcohol. Clin. Exp. Res.* 26, 239–245. doi: 10.1177/002215540305100606

Schad, A., Fahimi, H. D., Völkl, A., and Baumgart, E. (2003). Expression of catalase mRNA and protein in adult rat brain: detection by nonradioactive *in situ* hybridization with signal amplification by catalyzed reporter deposition (ISH-CARD) and immunohistochemistry (IHC)/immunofluorescence (IF). *J. Histochem. Cytochem.* 51, 751–760. doi: 10.1177/002215540305100606

Seitz, H. K., and Stickel, F. (2007). Molecular mechanisms of alcohol-mediated carcinogenesis. *Nat. Rev. Cancer* 7, 599–612. doi: 10.1038/nrc2191

Seitz, H. K., and Stickel, F. (2010). Acetaldehyde as an underestimated risk factor for cancer development: role of genetics in ethanol metabolism. *Genes Nutr.* 5, 121–128. doi: 10.1007/s12263-009-0154-1

Sjöquist, B., Liljequist, S., and Engel, J. (1982). Increased salsolinol levels in rat striatum and limbic forebrain following chronic ethanol treatment. *J. Neurochem.* 39, 259–262. doi: 10.1111/j.1471-4159.1982.tb04730.x

Serrano, E., Pozo, O. J., Beltrán, J., Hernández, F., Font, L., Miquel, M., et al. (2007). Liquid chromatography/tandem mass spectrometry determination of (*4S,2RS*)-2,5,5-trimethylthiazolidine- 4-carboxylic acid, a stable adduct formed between D-(-)-penicillamine and acetaldehyde (main biological metabolite of ethanol), in plasma, liver and brain rat tissues. *Rapid Commun. Mass Spectrom.* 21, 1221–1229. doi: 10.1002/rcm.2951

Shield, K. D., Gmel, G., Kehoe-Chan, T., Dawson, D. A., Grant, B. F., and Rehm, J. (2013). Mortality and potential years of life lost attributable to alcohol consumption by race and sex in the United States in 2005. *PLoS One* 8: e51923. doi: 10.1371/journal.pone.0051923

Shukla, S. D., and Aroor, A. R. (2006). Epigenetic effects of ethanol on liver and gastrointestinal injury. *World J. Gastroenterol.* 12, 5265–5271. doi: 10.3748/wjg.v12.i33.5265

Shukla, S. D., Lee, Y. L., Park, P., and Aroor, A. R. (2007). Acetaldehyde alters MAP kinase signalling and epigenetic histone modifications in hepatocytes. *Novartis Found. Symp.* 285, 217–224. doi: 10.1002/9780470511848.ch1

Sinclair, J. D., and Lindros, K. O. (1981). Suppression of alcohol drinking with brain aldehyde dehydrogenase inhibition. *Pharmacol. Biochem. Behav.* 14, 377–383. doi: 10.1016/0091-3057(81)90405-6

Sippel, H. W. (1974). The acetaldehyde content in rat brain during ethanol metabolism. *J. Neurochem.* 23, 451–452. doi: 10.1111/j.1471-4159.1974.tb04380.x

Smith, B. R., Aragon, C. M. G., and Amit, Z. (1997). Catalase and the production of brain acetaldehyde: a possible mediator of the psychopharmacological effects of ethanol. *Addict. Biol.* 2, 277–290. doi: 10.1080/13556219772570

Smith, B. R., Amit, Z., and Splawinsky, J. (1984). Conditioned place preference induced by intraventricular infusions of acetaldehyde. *Alcohol* 1, 193–195.

Socaransky, S. M., Aragon, C. M. G., Amit, Z., and Blander, A. (1984). Higher correlation of ethanol consumption with brain than liver aldehyde dehydrogenase in three strains of rats. *Psychopharmacology* 84, 250–253. doi: 10.1007/bf00427454

Soscia, S. J., Tong, M., Xu, X. J., Cohen, A. C., Chu, J., Wands, J. R., et al. (2006). Chronic gestational exposure to ethanol causes insulin and IGF resistance and impairs acetylcholine homeostasis in the brain. *Cell. Mol. Life Sci.* 63, 2039–2056. doi: 10.1007/s00018-006-6208-2

Spina, L., Longoni, R., Rosas, M., Collu, M., Peana, A. T., Espa, E., et al. (2015). *Withania somnifera* Dunal (Indian ginseng) impairs acquisition and expression of ethanol-elicited conditioned place preference and conditioned place aversion. *J. Psychopharmacol.* 29, 1191–1199. doi: 10.1177/0269881115600132

Spina, L., Longoni, R., Vinci, S., Ibba, F., Peana, A. T., Muggironi, G., et al. (2010). Role of dopamine D_1 receptors and extracellular signal regulated kinase in the motivational properties of acetaldehyde as assessed by place preference conditioning. *Alcohol. Clin. Exp. Res.* 34, 607–616. doi: 10.1111/j.1530-0277.2009.01129.x

Spivak, K., Aragon, C. M. G., and Amit, Z. (1987). Alterations in brain aldehyde dehydrogenase activity modify the locomotor effects produced by ethanol in rats. *Alcohol Drug Res.* 7, 481–491.

Starkey, J. A., Mechref, Y., Muzikar, J., McBride, W. J., and Novotny, M. V. (2006). Determination of salsolinol and related catecholamines through on-line preconcentration and liquid chromatography/atmospheric pressure photoionization mass spectrometry. *Anal. Chem.* 78, 3342–3347. doi: 10.1021/ac051863j

Steffensen, S. C., Walton, C. H., Hansen, D. M., Yorgason, J. T., Gallegos, R. A., and Criado, J. R. (2009). Contingent and non-contingent effects of low-dose ethanol on GABA neuron activity in the ventral tegmental area. *Pharmacol. Biochem. Behav.* 92, 68–75. doi: 10.1016/j.pbb.2008.10.012

Stoica, G. E., Kuo, A., Powers, C., Bowden, E. T., Sale, E. B., Riegel, A. T., et al. (2002). Midkine binds to anaplastic lymphoma kinase (ALK) and acts as a growth factor for different cell types. *J. Biol. Chem.* 277, 35990–36008. doi: 10.1074/jbc.m205749200

Szabo, G., and Saha, B. (2015). Alcohol's effect on host defense. *Alcohol Res.* 37, 159–170.

Székács, D., Bodnár, I., Mravec, B., Kvetnansky, R., Vizi, E. S., Nagy, G. M., et al. (2007). The peripheral noradrenergic terminal as possible site of action of salsolinol as prolactoliberin. *Neurochem. Int.* 50, 427–434. doi: 10.1016/j.neuint.2006.10.001

Tabakoff, B., Anderson, R. A., and Ritzmann, R. F. (1976). Brain acetaldehyde after ethanol administration. *Biochem. Pharmacol.* 25, 1305–1309. doi: 10.1016/0006-2952(76)90094-0

Takagi, T., Alderman, J., Gellert, J., and Lieber, C. S. (1986). Assessment of the role of non-ADH ethanol oxidation *in vivo* and in hepatocytes from deermice. *Biochem. Pharmacol.* 35, 3601–3606. doi: 10.1016/0006-2952(86)90632-5

Tampier, L., Alpers, H. S., and Davis, V. E. (1977). Influence of catecholamine-derived alkaloids and beta-adrenergic blocking agents on stereospecific binding of 3H-naloxone. *Res. Commun. Chem. Pathol. Pharmacol.* 17, 731–734.

Tampier, L., and Mardones, J. (1987). Absence of effect of 3-amino-1,2,4-triazole pretreatment on blood ethanol levels after oral administration, in rats. *Alcohol* 4, 73–74. doi: 10.1016/0741-8329(87)90064-4

Tampier, L., Quintanilla, M. E., Karahanian, E., Rivera-Meza, M., Herrera-Marschitz, M., and Israel, Y. (2013). The alcohol deprivation effect: marked inhibition by anticatalase gene administration into the ventral tegmental area in rats. *Alcohol. Clin. Exp. Res.* 37, 1278–1285. doi: 10.1111/acer.12101

Tardif, R. (2007). The determination of acetaldehyde in exhaled breath. *Novartis Found. Symp.* 285, 125–133; discussion 133–136, 198–199. doi: 10.1002/9780470511848.ch9

Teschke, R., and Gellert, J. (1986). Hepatic microsomal ethanol-oxidizing system (MEOS): metabolic aspects and clinical implications. *Alc. Clin. Exp. Res.* 10, 20S–32S. doi: 10.1111/j.1530-0277.1986.tb05176.x

Tieu, K. (2011). A guide to neurotoxic animal models of Parkinson's disease. *Cold Spring Harb. Perspect. Med.* 1:a009316. doi: 10.1016/b978-012088382-0/50014-1

Tóth, B. E., Bodnár, I., Homicskóo, K. G., Fülöp, F., Fekete, M. I., and Nagy, G. M. (2002). Physiological role of salsolinol: its hypophysiotrophic function in the regulation of pituitary prolactin secretion. *Neurotoxicol. Teratol* 24, 655–666. doi: 10.1016/S0892-0362(02)00216-7

Tuomisto, L., Airaksinen, M. M., Peura, P., and Eriksson, C. J. (1982). Alcohol drinking in the rat: increases following intracerebroventricular treatment with tetrahydro-β-carbolines. *Pharmacol. Biochem. Behav.* 17, 831–836. doi: 10.1016/0091-3057(82)90367-7

Valle-Prieto, A., and Conget, P. A. (2010). Human mesenchymal stem cells efficiently manage oxidative stress. *Stem Cells Dev.* 19, 1885–1893. doi: 10.1089/scd.2010.0093

Vasiliou, V., Ziegler, T. L., Bludeau, P., Petersen, D. R., Gonzalez, F. J., and Deitrich, R. A. (2006). CYP2E1 and catalase influence ethanol sensitivity in the central nervous system. *Pharmacogenet. Genomics* 16, 51–58. doi: 10.1097/01.fpc.0000182777.95555.56

Vetulani, J., Nalepa, I., Antkiewicz-Michaluk, L., and Sansone, M. (2001). Opposite effect of simple tetrahydroisoquinolines on amphetamine- and morphine-stimulated locomotor activity in mice. *J. Neural. Trans.* 108, 513–526. doi: 10.1007/s007020170053

Vinci, S., Ibba, F., Longoni, R., Spina, L., Spiga, S., and Acquas, E. (2010). Acetaldehyde elicits ERK phosphorylation in the rat nucleus accumbens and extended amygdala. *Synapse* 64, 916–927. doi: 10.1002/syn.20811

Vukojević, V., Ming, Y., D'Addario, C., Rigler, R., Johansson, B., and Terenius, L. (2008). Ethanol/naltrexone interactions at the mu-opioid receptor. CLSM/FCS study in live cells. *PLoS One* 3:e4008. doi: 10.1371/journal.pone.0004008

Ward, R. J., Colantuoni, C., Dahchour, A., Quertemont, E., and De Witte, P. (1997). Acetaldehyde-induced changes in monoamine and amino acid extracellular microdialysate content of the nucleus accumbens. *Neuropharmacology* 36, 225–232. doi: 10.1016/s0028-3908(97)00007-5

Watts, P. M., Riedl, A. G., Douek, D. C., Edwards, R. J., Boobis, A. R., Jenner, P., et al. (1998). Co-localization of P450 enzymes in the rat substantia nigra with tyrosine hydroxylase. *Neuroscience* 86, 511–519. doi: 10.1016/s0306-4522(97)00649-0

Weiner, H., and Wang, X. (1994). Aldehyde dehydrogenase and acetaldehyde metabolism. *Alcohol Alcohol.* 2, 141–145.

Weiner, J. L., and Valenzuela, C. F. (2006). Ethanol modulation of GABAergic transmission: the view from the slice. *Pharmacol. Ther.* 111, 533–554. doi: 10.1016/j.pharmthera.2005.11.002

Westcott, J. Y., Weiner, H., Shultz, J., and Myers, R. D. (1980). *In vivo* acetaldehyde in the brain of the rat treated with ethanol. *Biochem. Pharmacol.* 29, 411–417. doi: 10.1016/0006-2952(80)90521-3

Xiao, C., and Ye, J. H. (2008). Ethanol dually modulates GABAergic synaptic transmission onto dopaminergic neurons in ventral tegmental area: role of mu-opioid receptors. *Neuroscience* 153, 240–248. doi: 10.1016/j.neuroscience.2008.01.040

Xiao, C., Zhang, J., Krnjević, K., and Ye, J. H. (2007). Effects of ethanol on midbrain neurons: role of opioid receptors. *Alcohol. Clin. Exp. Res.* 31, 1106–1113. doi: 10.1111/j.1530-0277.2007.00405.x

Xie, G., Hipólito, L., Zuo, W., Polache, A., Granero, L., and Krnjevic, K. (2012). Salsolinol stimulates dopamine neurons in slices of posterior ventral tegmental area indirectly by activating μ-opioid receptors. *J. Pharmacol. Exp. Ther.* 341, 43–50. doi: 10.1124/jpet.111.186833

Xie, G., and Ye, J.-H. (2012). Salsolinol facilitates glutamatergic transmission to dopamine neurons in the posterior ventral tegmental area of rats. *PLoS One* 7:e36716. doi: 10.1371/journal.pone.0036716

Zhong, Y., Dong, G., Luo, H., Cao, J., Wang, C., Wu, J., et al. (2012). Induction of brain CYP2E1 by chronic ethanol treatment and related oxidative stress in hippocampus, cerebellum and brainstem. *Toxicology* 302, 275–284. doi: 10.1016/j.tox.2012.08.009

Zimatkin, S. M. (1991). Histochemical study of aldehyde dehydrogenase in the rat CNS. *J. Neurochem.* 56, 1–11. doi: 10.1111/j.1471-4159.1991.tb02555.x

Zimatkin, S. M., and Buben, A. L. (2007). Ethanol oxidation in the living brain. *Alcohol Alcohol.* 42, 529–532. doi: 10.1093/alcalc/agm059

Zimatkin, S. M., and Lindros, K. O. (1996). Distribution of catalase in rat brain: aminergic neurons as possible targets for ethanol effects. *Alcohol Alcohol.* 31, 167–174. doi: 10.1093/oxfordjournals.alcalc.a008128

Zimatkin, S. M., Pronko, S. P., Vasiliou, V., Gonzalez, F. J., and Deitrich, R. A. (2006). Enzymatic mechanisms of ethanol oxidation in the brain. *Alcohol. Clin. Exp. Res.* 30, 1500–1505. doi: 10.1111/j.1530-0277.2006.00181.x

Zimatkin, S. M., Rout, U. K., Koivusalo, M., Bühler, R., and Lindros, K. O. (1992). Regional distribution of low-Km mitochondrial aldehyde dehydrogenase in the rat central nervous system. *Alcohol. Clin. Exp. Res.* 16, 1162–1167. doi: 10.1111/j.1530-0277.1992.tb00713.x

Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

frontiers in Behavioral Neuroscience

ORIGINAL RESEARCH
published: 23 January 2017
doi: 10.3389/fnbeh.2016.00253

Racemic Salsolinol and its Enantiomers Act as Agonists of the μ-Opioid Receptor by Activating the Gi Protein-Adenylate Cyclase Pathway

*Pablo Berríos-Cárcamo[1,2], María E. Quintanilla[1], Mario Herrera-Marschitz[1], Vasilis Vasiliou[2], Gerald Zapata-Torres[3] and Mario Rivera-Meza[4]**

[1]*Program of Molecular and Clinical Pharmacology, Faculty of Medicine, Institute of Biomedical Sciences, University of Chile, Santiago, Chile,* [2]*Department of Environmental Health Sciences, Yale School of Public Health, New Haven, CT, USA,* [3]*Department of Analytical and Inorganic Chemistry, Faculty of Chemical and Pharmaceutical Sciences, University of Chile, Santiago, Chile,* [4]*Department of Pharmacological and Toxicological Chemistry, Faculty of Chemical and Pharmaceutical Sciences, University of Chile, Santiago, Chile*

Background: Several studies have shown that the ethanol-derived metabolite salsolinol (SAL) can activate the mesolimbic system, suggesting that SAL is the active molecule mediating the rewarding effects of ethanol. *In vitro* and *in vivo* studies suggest that SAL exerts its action on neuron excitability through a mechanism involving opioid neurotransmission. However, there is no direct pharmacologic evidence showing that SAL activates opioid receptors.

Methods: The ability of racemic (R/S)-SAL, and its stereoisomers (R)-SAL and (S)-SAL, to activate the μ-opioid receptor was tested in cell-based (light-emitting) receptor assays. To further characterizing the interaction of SAL stereoisomers with the μ-opioid receptor, a molecular docking study was performed using the crystal structure of the μ-opioid receptor.

Results: This study shows that SAL activates the μ-opioid receptor by the classical G protein-adenylate cyclase pathway with an half-maximal effective concentration (EC_{50}) of 2×10^{-5} M. The agonist action of SAL was fully blocked by the μ-opioid antagonist naltrexone. The EC_{50} for the purified stereoisomers (R)-SAL and (S)-SAL were 6×10^{-4} M and 9×10^{-6} M respectively. It was found that the action of racemic SAL on the μ-opioid receptor did not promote the recruitment of β-arrestin. Molecular docking studies showed that the interaction of (R)- and (S)-SAL with the μ-opioid receptor is similar to that predicted for the agonist morphine.

Conclusions: It is shown that (R)-SAL and (S)-SAL are agonists of the μ-opioid receptor. (S)-SAL is a more potent agonist than the (R)-SAL stereoisomer. *In silico* analysis predicts a morphine-like interaction between (R)- and (S)-SAL with the μ-opioid receptor. These results suggest that an opioid action of SAL or its enantiomers is involved in the rewarding effects of ethanol.

Keywords: salsolinol, μ-opioid receptor, naltrexone, β-arrestin, molecular docking

OPEN ACCESS

Edited by:
Merce Correa,
Jaume I University, Spain

Reviewed by:
Elio Acquas,
University of Cagliari, Italy
Lucia Hipolito,
University of Valencia, Spain

****Correspondence:***
Mario Rivera-Meza
mario.rivera@ciq.uchile.cl

Received: *25 October 2016*
Accepted: *28 December 2016*
Published: *23 January 2017*

Citation:
Berríos-Cárcamo P, Quintanilla ME, Herrera-Marschitz M, Vasiliou V, Zapata-Torres G and Rivera-Meza M (2017) Racemic Salsolinol and its Enantiomers Act as Agonists of the μ-Opioid Receptor by Activating the Gi Protein-Adenylate Cyclase Pathway. Front. Behav. Neurosci. 10:253. doi: 10.3389/fnbeh.2016.00253

INTRODUCTION

The mechanisms underlying the addictive properties of ethanol are still not fully understood since specific molecular targets explaining its pharmacological actions have not yet been identified. Unlike other drugs of abuse such as morphine, cocaine or nicotine that elicit their effects at micromolar blood concentrations (10^{-6} M; Jeffcoat et al., 1989; Glare and Walsh, 1991; Benowitz and Jacob, 1993), the pharmacological effects of ethanol are evident only at millimolar blood levels (10^{-3} M; Holford, 1987). The low potency of ethanol could be attributed to its molecular simplicity, hampering its binding with high affinity to any type of receptor. Nevertheless, a number of studies have shown that acetaldehyde, the primary metabolite of ethanol in the brain, is self-administered intracranially by rats at micromolar concentrations showing strong motivational and reinforcing effects (Rodd et al., 2005). Indeed, ethanol metabolism to acetaldehyde in the brain is required to exert ethanol's reinforcing actions (Karahanian et al., 2011, 2015; Quintanilla et al., 2012; Israel et al., 2015; Peana et al., 2016).

In the brain, ethanol-derived acetaldehyde can condense non-enzymatically with dopamine to generate racemic (R/S)-salsolinol (SAL, 1-methyl-1,2,3,4-tetrahydro-6,7-dihydroxy-isoquinoline; Melchior and Collins, 1982). Studies by Rommelspacher et al. (1995) showed that levels of SAL in the blood of alcoholics were higher than those found in non-alcoholic individuals. The exposure of healthy individuals to an acute dose of ethanol results in an increase of SAL concentrations in blood and urine (Haber et al., 1996). Animal studies have shown that chronic ethanol administration to rats results in a significant increase of SAL levels in dopamine-rich areas of the brain such as striatum, limbic forebrain and hypothalamus (Sjöquist et al., 1982; Matsubara et al., 1987), yielding increased amounts of SAL (Rojkovicova et al., 2008). Behavioral studies performed in rats have shown that SAL administration results in major increases in voluntary ethanol intake (Myers and Melchior, 1977; Quintanilla et al., 2014, 2016), locomotor activity (Hipólito et al., 2010; Quintanilla et al., 2014) and conditioned place preference (Matsuzawa et al., 2000; Quintanilla et al., 2014). Microdialysis studies have shown that microinjections of SAL into the ventral tegmental area (VTA) of rats result in an increased release of dopamine in the nucleus accumbens (Deehan et al., 2013), a common hallmark shared by different drugs of abuse, including ethanol (Di Chiara and Imperato, 1988). Furthermore, SAL is self-administered intracranially by rats at concentrations ranging from 0.03 μM to 0.3 μM (Rodd et al., 2008; Deehan et al., 2013). These concentrations are 10–100 times lower than that required for acetaldehyde self-administration, suggesting that SAL is an active molecule involved in the rewarding effects of ethanol. Indeed, recent electrophysiology studies by Melis et al. (2015) showed that inhibition of dopamine synthesis by a tyrosine hydroxylase inhibitor fully abolishes the ability of ethanol (100 mM) and acetaldehyde (10 nM) to stimulate VTA dopaminergic neurons.

Experimental evidence suggests that the activating/rewarding properties of SAL are mediated by mechanisms involving μ-opioid receptors, although the levels at which this effect occurs is not known. Studies by Matsuzawa et al. (2000) showed that place preference conditioning elicited by SAL is significantly attenuated by the concomitant administration of the selective μ-opioid receptor antagonist β-funaltrexamine. Similar results were obtained by Hipólito et al. (2010), who found that the administration of β-funaltrexamine reduces the increase in locomotor activity elicited by the intra-VTA administration of SAL to rats. Recent studies by Quintanilla et al. (2014) showed that repeated systemic administration of racemic SAL (10 mg/kg, i.p.) led to a marked increase in voluntary ethanol intake in rats. This sensitization to ethanol intake was fully blocked by the concomitant intra-VTA administration of the opioid antagonist naltrexone, suggesting that the VTA is the primary site of action of SAL and its mechanism of action mediated by opioid receptors. It has been proposed that SAL could bind to μ-opioid receptors on GABAergic neurons of the VTA, inducing hyperpolarization, resulting in disinhibition and therefore activation of nearby dopaminergic neurons (Xie et al., 2013), resembling the action of opioid drugs (Johnson and North, 1992). In line with this view, electrophysiological studies performed in VTA-containing brain slices showed that SAL increased the firing of dopamine neurons by a μ-opioid/GABAergic combined mechanism, since the activating effects of SAL were blocked by both the μ-opioid antagonist naltrexone and the $GABA_A$ receptor antagonist gabazine (Xie et al., 2012).

Despite early reports suggesting that SAL (10^{-8} to 10^{-3} M) can bind to opioid receptors (Fertel et al., 1980; Lucchi et al., 1982), there are no specific studies aimed at determining the intrinsic activity of SAL on μ-opioid receptors. We report here a light emission cell-based receptor assays describing the effect of SAL on μ-opioid receptors, based on the activation of the canonical signaling pathway associated to G protein. We also assessed the capacity of SAL to activate the G protein-independent signaling pathway associated with β-arrestin recruitment. We extended these *in vitro* studies to assess the action of the SAL stereoisomers (R)-SAL and (S)-SAL. To further understand the interaction of SAL with the μ-opioid receptor, we also investigated on molecular docking analyses, comparing (R)-SAL vs. (S)-SAL on their ability to bind to the active site of the mouse μ-opioid receptor, whose crystal structure was recently published (Huang et al., 2015).

MATERIALS AND METHODS

Materials

Racemic SAL was purchased from Santa Cruz Biotechnology (Dallas, TX, USA), naltrexone was from Alfa Aesar (Ward Hill, MA, USA). Ammonium acetate was from Merck (Darmstadt, Germany) and triethylamine was from Sigma (St. Louis, MO, USA).

Separation and Purification of (R) and (S)-Salsolinol

(R)-SAL and (S)-SAL were separated from the racemic solution by high-pressure liquid chromatography (HPLC) as described previously (Quintanilla et al., 2016). Briefly, a solution of (R/S)-

SAL was injected into a NUCLEODEX β-cyclodextrin-modified column (Macherey-Nagel, Düren, Germany) kept at 20°C. The column was coupled to a LC-4C BAS amperometric detector (ED) set to a potential of 700 mV. The mobile phase, composed of volatile 100 mM ammonium acetate and 10 mM triethylamine (pH 4.0), was injected at a flow rate of 0.40 mL/min. Previous reports indicate that (S)-SAL enantiomer is the first to be eluted following similar chromatographic conditions (Deng et al., 1995; Naoi et al., 1996; Tóth et al., 2001; Quan et al., 2005; Rojkovicova et al., 2008; Lee et al., 2010). Once (R/S)-SAL was injected into the HPLC, the enantiomers were separated and collected according to their corresponding elution time (electrochemical detector disconnected). To check their purity, samples were reinjected onto the HPLC system. Each purified fraction was lyophilized at −54°C for 9 h for mobile phase elimination and dissolved in HCl 10^{-5} M, (pH 5.0). The concentration of purified samples was determined either by HPLC-ED or by absorbance at 290 nm, using a calibration curve with (R/S)-SAL as a standard. Samples were stored at −20°C in amber microtubes.

Activation of μ-Opioid Receptors through the Gi Protein-Signaling Pathway

The intrinsic activity of the ligands was studied using commercially cell-based assays, composed by recombinant CHO-K1 cells that overexpress only the human μ-opioid receptor, detecting the levels of second messengers that reflect the activation of this receptor. To assess the activation of the μ-opioid receptor through the recruitment of G protein signaling pathway, we used the cyclic adenosine monophosphate (cAMP) Hunter® eXpress G protein-coupled receptor (GPCR) Assay (DiscoverX, Freemont, CA, USA) following the manufacturer instructions. In this system, the endogenous cAMP competes with an exogenous cAMP coupled to a truncated β-galactosidase fragment (provided by the assay) for binding to a cAMP antibody. Only the unbound exogenous cAMP-β-galactosidase fragment binds to a complementary β-galactosidase fragment to form the active enzyme. The activity of β-galactosidase, measured by adding a chemiluminescent substrate, reflects proportionally the levels of cellular cAMP. (R)-SAL, (S)-SAL and (R/S)-SAL, dissolved in HCl 10^{-5} M, (pH 5.0) and including the adenylate cyclase activator forskolin (20 μM), were incubated at concentrations ranging from 1×10^{-8} M to 1×10^{-3} M with the CHO-K1 cells for 30 min. Morphine was incubated at concentrations ranging from 3×10^{-9} M to 1×10^{-4} M under the same conditions. Control cells were incubated only with forskolin. The resulting luminescence was measured with a microplate reader Synergy HT (Biotek, Winooski, VT, USA) or a microplate reader SpectraMax M3 (Molecular Devices, Sunnyvale, CA, USA). The experiment was repeated three times in duplicate for (R)-SAL, (S)-SAL and (R/S)-SAL and two times in duplicate for morphine.

In a subsequent experiment, the activation of the μ-opioid receptor by racemic SAL (150 μM) was studied after the addition of different concentrations (3×10^{-10} M to 10^{-5} M) of the antagonist naltrexone to the recombinant cells, dissolved in assay buffer, 30 min before to the addition of SAL. All measurements were expressed as relative luminescence to the controls with no ligand added. Each concentration of naltrexone was assayed once in duplicate. The antagonistic action of naltrexone was selective for μ-opioid receptor since the recombinant cells used in the study only overexpress this type of opioid receptor.

Activation of μ-Opioid Receptors through the β-Arrestin Signaling Pathway

To assess the activation of the μ-opioid receptor through the recruitment of the β-arrestin signaling pathway, we used a PathHunter® eXpress β-Arrestin GPCR chemiluminescent assay (DiscoverX) following the manufacturer instructions. In this system, the human μ-opioid receptor is fused to a small fragment of β-galactosidase (PK) and co-expressed in cells expressing a fusion protein of β-arrestin and the complementary fragment of β-galactosidase (EA). Activation of the μ-opioid receptor stimulates binding of β-arrestin to the PK-tagged receptor allowing the complementation of the two enzyme fragments of β-galactosidase. This action leads to an activation of the enzyme that can be measured using a chemiluminescent reagent resulting in luminescence, which is proportional to the recruitment of β-arrestin. DADLE ([D-Ala^2, D-Leu^5]-Enkephalin), (R/S)-SAL and morphine (dissolved in HCl 10^{-5} M, pH 5.0), were incubated at concentrations ranging from 1×10^{-8} to 1×10^{-3} M with the cells for 30 min. The experiment was repeated three times in duplicate for each concentration of ligand. The resulting luminescence was determined with a microplate reader Synergy HT (Biotek).

Molecular Docking

Molecular docking simulations were performed using the crystal structure of the mouse μ-opioid receptor bound to the morphinan high-affinity agonist BU72 (Neilan et al., 2004), obtained from the protein data bank as a PDB file (ID: 5C1M; Huang et al., 2015). For the simulations, water molecules near the receptor were conserved, but BU72 and any other co-crystallized molecules were removed. After that, polar hydrogens were added to the protein coordinates (not resolved in the crystal structure). The studied molecules, morphine, (R)-SAL and (S)-SAL, were prepared and optimized to their energy minima using SPARTAN 10. Docking simulations were performed using Autodock Vina (Trott and Olson, 2010), which renders the ligand fully flexible, while to the target protein a rigid structure. The search space was 24 Å^3 around a side-chain oxygen of Asp147, regarded as a critical residue for the binding of ligands to this receptor (Li et al., 1999; Manglik et al., 2012; Shim et al., 2013; Huang et al., 2015). The searching exhaustiveness was 800 (default 8). Ligand-protein interactions analyses and 3D figures were prepared using PyMOL (DeLano, 2002).

Statistical Analyses

The half-maximal effective concentration (EC_{50}) for each ligand and half-maximal inhibitory concentration (IC_{50}) for naltrexone in the antagonist assay were determined by correlating the

data to a non-linear equation of ligand concentration (log M) vs. response by three parameters. The fitting of the data to the correlation was assessed by its corresponding coefficient of determination (R^2), using GraphPad Prism (San Diego, CA, USA).

RESULTS

Activation of μ-Opioid Receptors by Racemic SAL through the Gi Protein-Signaling Pathway

To study the capacity of SAL to activate the μ-opioid receptor, we used a commercial assay based in CHO-K1 cells expressing the human μ-opioid receptor. Since the μ-opioid receptor is coupled to an inhibitory G protein (Gi), its activation by an agonist results in a reduction of intracellular levels of cAMP. **Figure 1** shows the relative intracellular cAMP levels, measured as a chemiluminescent signal, elicited by the action of different concentrations of racemic SAL and the μ-opioid agonist morphine. Results showed that SAL is effective to activate the μ-opioid receptor through the G protein-signaling pathway but at lower potency compared to morphine. An EC_{50} for racemic SAL was 2×10^{-5} M ($R^2 = 0.86$), while the EC_{50} for morphine was 4×10^{-9} M ($R^2 = 0.96$).

Following the pharmacodynamic characterization of SAL on the μ-opioid receptor, the effect of different concentrations (1×10^{-5} M to 3×10^{-10} M) of the antagonist naltrexone was determined on the activation of the μ-opioid receptor elicited by racemic SAL at a concentration of 1.5×10^{-4} M. As it is seen in **Figure 1**, this SAL concentration is able to elicit 80% of the maximal response in this cell-based assay. **Figure 2** shows that naltrexone antagonizes, in a dose-dependent fashion, the ability of racemic SAL to activate the μ-opioid receptor and to reduce intracellular cAMP levels. A complete antagonism of the SAL action is reached at naltrexone concentrations of 10^{-8} M. A non-linear fit analysis of the data revealed that naltrexone showed an IC_{50} of 1×10^{-9} M ($R^2 = 0.85$).

Separation and Purification of (R) and (S)-Salsolinol

The enantiomers (R)-SAL and (S)-SAL were separated and purified from racemic (R/S)-SAL by HPLC. **Figure 3A** shows the retention time of the enantiomers: (S)-SAL = 6.7 min and (R)-SAL = 8.3 min, in chromatographs showing equal areas for (S)-SAL and (R)-SAL in a sample containing racemic (R/S)-SAL. **Figures 3B,C** show the chromatograms obtained for purified (S)-SAL and (R)-SAL respectively. The quantification of the area under the curve for (R)-SAL and (S)-SAL showed that both stereoisomers were 99% pure.

Activation of μ-Opioid Receptors by (R)- and (S)-SAL through the Gi Protein-Signaling Pathway

The capacity of the purified enantiomers (R)-SAL and (S)-SAL to activate the μ-opioid receptor (G protein signaling pathway) was assayed using the same methodology used for assaying racemic SAL. **Figure 4** shows the changes in cAMP levels, measured as a chemiluminescent signal, elicited

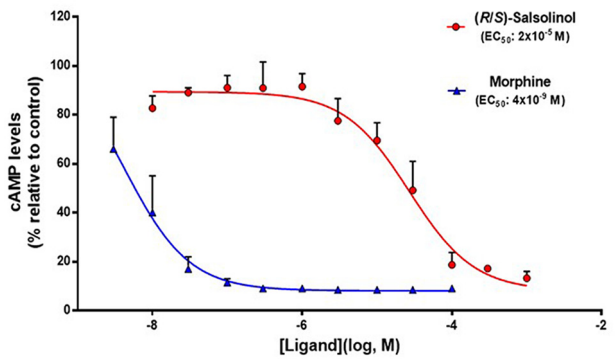

FIGURE 1 | Salsolinol (SAL) acts as an agonist of μ-opioid receptors. Functional assay of μ-opioid receptor dose-response activation by morphine and racemic (R/S)-SAL. Each ligand was assayed three times in duplicate using concentrations ranged from 1×10^{-8} M to 1×10^{-3} for (R/S)-SAL, and 3×10^{-9} M to 1×10^{-4} M for morphine. Values are expressed as the means of cyclic adenosine monophosphate (cAMP) levels (percentage relative de control) ± SEM. The luminescence is proportional to the intracellular cAMP levels (induced by forskolin); therefore, a decrease in cAMP levels signals the activation of the μ-opioid receptor, which is coupled to a inhibitory G protein (Gi). Half-maximal effective concentration (EC_{50}) corresponds to the concentration of ligand eliciting 50% of the maximal response. (R/S)-SAL curve: degrees of freedom, 26 (four points excluded as outliers, at [Ligand](log, M) = −3, −3.5, −7, −7.5); $R^2 = 0.8809$. Morphine curve: degrees of freedom, 17; $R^2 = 0.8825$.

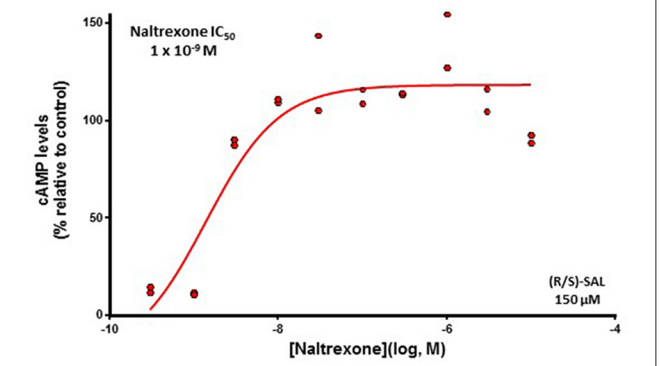

FIGURE 2 | The action of racemic SAL is fully blocked by the μ-opioid receptor antagonist naltrexone. Levels of cAMP (percentage relative to control) detected after incubation with racemic (R/S)-SAL 150 μM in the presence of different concentrations of the antagonist naltrexone (1×10^{-5} M to 3×10^{-10} M) are shown. The activation of the μ-opioid receptor by the addition of the (R/S)-SAL 150 μM was assayed 30 min after the addition of the antagonist. This concentration of (R/S)-SAL elicits 80% of the maximal response of the system. The antagonism of the inhibitory action of SAL on μ-opioid receptor results in an increase of intracellular cAMP levels. The shown results are from one experiment performed in duplicate and each point represents one of the two replicates for each concentration of antagonist. Half-maximal inhibitory concentration (IC_{50}) corresponds to the concentration of antagonist reducing 50% the maximal response to the agonist. Degrees of freedom, 7; $R^2 = 0.8550$.

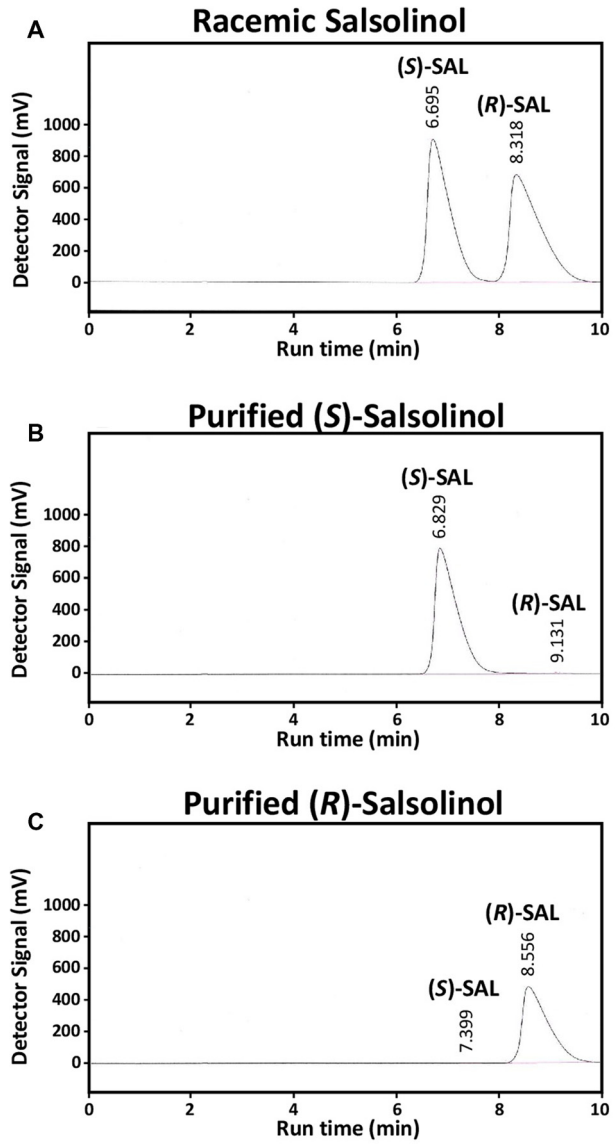

FIGURE 3 | Purification of (R)-SAL and (S)-SAL from racemic (R/S)-SAL. High-pressure liquid chromatography (HPLC) chromatographs of **(A)** racemic (R/S)-SAL 1×10^{-2} M (corresponding to 5×10^{-3} M of each enantiomer), **(B)** purified (S)-SAL 4×10^{-3} M (non-detectable levels of (R)-SAL) and **(C)** purified (R)-SAL 3×10^{-3} M (non-detectable levels of (S)-SAL). The volume of each sample was 50 μL, and the flow rate was 0.8 ml/min. The concentrations of the enantiomers in the purified solutions were determined using racemic (R/S)-SAL as standard.

by different concentrations (10^{-3} M to 10^{-8} M) of (R)-SAL and (S)-SAL. Results showed that both enantiomers were effective in activating the μ-opioid receptor, displaying a similar efficacy (capacity to reduce intracellular cAMP levels) but a higher potency (lower concentration needed to activate the receptor) for (S)-SAL compared to (R)-SAL. The EC_{50} for (S)-SAL (9×10^{-6} M, $R^2 = 0.81$) was 50 times lower than the EC_{50} calculated for (R)-SAL (6×10^{-4} M, $R^2 = 0.71$).

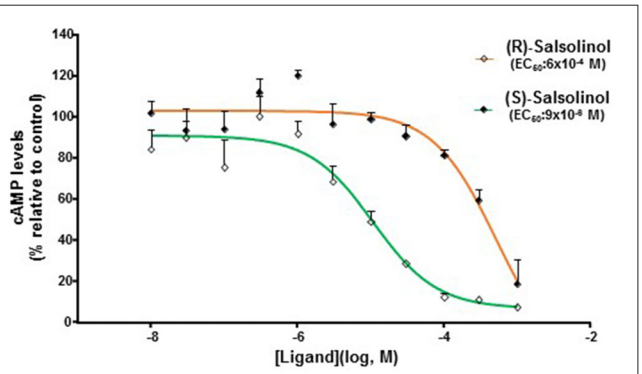

FIGURE 4 | (R)-SAL and (S)-SAL stereoisomers act as agonists on μ-opioid receptor. Functional assay of the μ-opioid receptor dose-response activation by (R)-SAL and (S)-SAL. Each ligand was assayed three times, each concentration, in duplicate using concentrations ranging from 1×10^{-8} M to 1×10^{-3} M. Values are expressed as mean cAMP levels (percentage relative de control) ± SEM. The luminescence is proportional to the intracellular cAMP levels (induced by forskolin); therefore, a decrease in cAMP levels signals the activation of the μ-opioid receptor, which is coupled to a Gi protein. EC_{50} corresponds to the concentration of ligand eliciting 50% of the maximal response. (R)-SAL curve: degrees of freedom, 28 (two points excluded as outliers, at [Ligand](log, M) = −7, −7.5); $R^2 = 0.8267$. (S)-SAL curve: degrees of freedom, 28 (two points excluded as outliers, at [Ligand](log, M) = −7, −7.5); $R^2 = 0.9072$.

Activation of μ-Opioid Receptors by Racemic SAL through the β-Arrestin Signaling Pathway

Several opioid agonists can activate with different efficacies the G protein-independent signaling pathway led by the recruitment of β-arrestin. This action of opioid agonists has been correlated with their capacity to induce internalization/recycling of μ-opioid receptors (see Williams et al., 2013). For this reason, we studied the effect of different concentrations of racemic SAL and the μ-opioid agonists DADLE and morphine on CHO-K1 cells engineered to express the μ-opioid receptor and to detect the recruitment of β-arrestin in response to such agonists.

Figure 5 shows that racemic SAL was, at all tested concentrations, unable to activate the β-arrestin signaling pathway upon its binding on the μ-opioid receptor. In contrast, DADLE showed the highest efficacy in activating the β-arrestin signaling pathway on μ-opioid receptors, displaying an EC_{50} of 1×10^{-6} M ($R^2 = 0.98$). Results also showed that morphine activates the β-arrestin pathway upon its action on μ-opioid receptors ($EC_{50} = 2 \times 10^{-6}$, $R^2 = 0.99$) but displaying a lower efficacy (50%) compared to DADLE. Since racemic SAL was inactive on the β-arrestin signaling pathway, no studies with (R)-SAL and (S)-SAL were conducted on β-arrestin recruitment determination (see "Discussion" Section).

Molecular Docking of (R)-SAL and (S)-SAL Enantiomers on the μ-Opioid Receptor

To further support the pharmacodynamics findings, molecular docking analyses of morphine, (R)-SAL and (S)-SAL on the

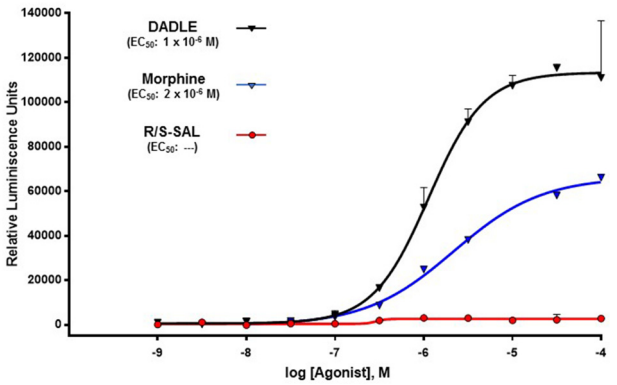

FIGURE 5 | The action of racemic SAL on the μ-opioid receptor does not induce the recruitment of β-arrestin. The recruitment of β-arrestin in response to morphine, [DAla^2, D-Leu^5]-Enkephalin (DADLE), and racemic SAL was measured using a PathHunter® eXpress β-Arrestin G protein-coupled receptor (GPCR) Assay (DiscoverX). DADLE and SAL were assayed in duplicate using concentrations ranged within 1×10^{-9} to 1×10^{-4}. Data for morphine correspond to a single assay using concentrations ranged within 1×10^{-8} to 1×10^{-4} M for morphine. Values are expressed as the mean of relative luminescence units (RLU) ± SEM. The luminescence is directly proportional to the recruitment of β-arrestin. EC_{50} corresponds to the concentration of ligand eliciting 50% of the maximal response. DADLE curve: degrees of freedom, 18; $R^2 = 0.9838$. Morphine curve: degrees of freedom, 6; $R^2 = 0.9956$.

coordinates of the binding site of the crystallized mouse μ-opioid receptor were performed. Molecular docking studies started by searching the spatial orientations (pose) of morphine, (R)-SAL and (S)-SAL allowing: (i) the lowest minimum global energy (score) of the ligand-receptor interaction; and (ii) the shorter interaction distance (<4 Å) between the amino group of the ligand and one of the side-chain oxygens of the Asp147 (N-Asp147) residue of the mouse μ-opioid receptor. This interaction has been reported as an important feature for opioid agonist activity (Li et al., 1999; Shim et al., 2013; Huang et al., 2015). The scores and N-Asp147 distances for nine different poses of morphine, (R)-SAL and (S)-SAL, hierarchized according to their minimum score, are showed in **Table 1**. Morphine displayed the lowest scores among the three tested ligands, consistent with its higher potency for the activation of the μ-opioid receptor. The docking scores for SAL enantiomers were higher than those of by morphine; however, slightly more favorable parameters were found for (S)-SAL, which also showed better scores in all the poses than (R)-SAL.

According to crystallographic studies reported by Shim et al. (2013) and Huang et al. (2015), the residues of the binding site of the μ-opioid receptor that are important for the binding of agonists are Asp147, Tyr148, Met151, Val236, Trp293, Ile296, His297, Val300, Trp318, Ile322 and Tyr326. **Figure 6** shows a molecular representation that highlights the interactions of morphine, (R)-SAL and (S)-SAL, arranged accordingly to their best pose, with the aforementioned amino acidic residues. **Figure 6B** shows that morphine establishes a salt bridge with Asp147 residue, forms hydrogen bonds with Tyr148 and, through two water molecules, with His297. The hydrophobic surface of morphine also binds to the hydrophobic domain of the binding site, formed by Val300, Ile296 and Ile322. **Figures 6C,D** show the molecular interactions predicted for (R)-SAL and (S)-SAL, respectively. Since SAL enantiomers are smaller molecules compared to morphine, they establish fewer interactions with the receptor. **Figure 6C** shows that (R)-SAL forms a salt bridge with Asp147 and, unlike morphine, the orientation of its hydroxyl groups allows a direct hydrophobic bond with His297. The hydrophobic surface of (R)-SAL also interacts with Ile296 and Met151. **Figure 6D** shows that the binding of (S)-SAL with the μ-opioid receptor is very similar to the one showed by (R)-SAL, predicting interactions with Asp147 and His297, and hydrophobic interactions with Ile296 and Met151. However, the spatial orientation of the methyl group, that defines the difference between the two SAL enantiomers, contributes to an additional interaction of (S)-SAL with Tyr148, which is not present in the simulation for the (R)-SAL enantiomer.

DISCUSSION

The findings of this study support the hypothesis that SAL acts as an agonist on the μ-opioid receptor. Overall, we found that SAL is effective in activating the μ-opioid receptor, showing an EC_{50} of 2×10^{-5} M. The agonist activity of SAL on the μ-opioid receptor was fully blocked by the μ-opioid antagonist naltrexone. Purified (R)-SAL and (S)-SAL stereoisomers showed to be effective in activating the μ-opioid receptor, displaying an EC_{50} of 6×10^{-4} M and 9×10^{-6} M respectively. In agreement with these results, molecular docking simulations predicted a morphine-like interaction of (R)-SAL and (S)-SAL stereoisomers with the μ-opioid receptor and a favored interaction for the (S)-SAL stereoisomer.

The assays aimed at determining the intrinsic activity of SAL showed that SAL is, as the full agonist morphine, effective in activating the μ-opioid receptor, since both compounds display nearly the same ability to inhibit the generation of intracellular cAMP (as shown by a reduction in chemiluminiscence in the present cell study). However, a marked difference was observed between morphine and SAL on their potency, since the EC_{50} of SAL was 5000-fold higher than that of morphine. These results are in agreement with early radioligand binding studies performed in rat striatum that showed that SAL can displace the μ-opioid receptor agonist [met]-enkephalin with an IC_{50} of 10 μM (1×10^{-5} M; Lucchi et al., 1982). Recent electrophysiological studies in slices of posterior VTA have shown that incubation with SAL (10^{-8} M to 10^{-6} M) results in an increased firing of dopamine neurons. These stimulatory effects of SAL on dopaminergic neurons are abolished by the addition of naltrexone (Xie et al., 2012). As proposed by Xie et al. (2013), SAL can indirectly activate dopaminergic neurons in the VTA through the inhibition of local GABAergic interneurons controlling the activity of dopaminergic neurons, playing a key role on the rewarding responses to SAL exposure. Recent *in vivo* studies have

TABLE 1 | Docking parameters for morphine, (R)-SAL and (S)-SAL on the binding site of the mouse μ-opioid receptor.

Pose	Morphine		(*R*)-SAL		(*S*)-SAL	
	Score (kcal/mol)	N-O:Asp (Å)	Score (kcal/mol)	N-O:Asp (Å)	Score (kcal/mol)	N-O:Asp (Å)
1	−9.7	2.9	−7.5	3.0	−7.7	3.1
2	−8.7	6.1	−6.6	3.7	−6.7	5.5
3	−8.6	5.0	−6.5	6.2	−6.6	3.3
4	−8.4	7.2	−6.5	6.6	−6.5	5.7
5	−8.4	2.8	−6.5	6.3	−6.5	3.2
6	−8.1	5.5	−6.4	3.2	−6.4	8.4
7	−7.4	5.4	−6.4	6.0	−6.3	5.8
8	−6.8	7.2	−6.4	7.8	−6.3	7.6
9	−6.8	3.4	−6.2	8.0	−6.2	6.6

Docking scores and Ligand:N-O:Asp147 distances for the poses obtained for morphine, (R)-SAL and (S)-SAL on the binding site of the mouse μ-opioid receptor. The table shows the scores and N-Asp147 distances for nine different poses of morphine, (R)-SAL, (S)-SAL hierarchized according to its minimum score. Poses and its corresponding score were determined using the Autodock Vina software. Scores are expressed as kcal/mol. The predicted distances between the amino group of the ligand and the α-carboxylic acid group of Asp147 (Ligand:N-O:Asp147) were determined using the PyMOL software. Ligand:N-O:Asp147 distances are expressed in Angstroms.

determined the effects of SAL on the activity of dopaminergic neurons in the VTA. Microdialysis studies in Wistar rats have shown that the intra-VTA administration of SAL at concentrations ranging within 10^{-7} M to 10^{-4} M can elicit a marked increase of dopamine release in the nucleus accumbens (Hipólito et al., 2011; Deehan et al., 2013). In addition, the microinjection of SAL into the VTA of Wistar rats at concentrations of 150 μM (15×10^{-5}M) resulted in a significant induction of locomotor activity (Hipólito et al., 2010), conditioned place preference (Hipólito et al., 2011) and ethanol intake (Quintanilla et al., 2014). These results show that the concentrations at which SAL exerts its neurochemical and behavioral effects in the VTA are similar to those able to activate μ-opioid receptors in the present *in vitro* study ($EC_{50} = 2 \times 10^{-5}$ M).

In support of an agonistic action of SAL on μ-opioid receptor, we found that the opioid antagonist naltrexone was able to block almost completely the action of SAL with an IC_{50} of 1×10^{-9} M. These values of IC_{50} for naltrexone are in agreement with previous reports regarding the potency of naltrexone as an antagonist of the μ-opioid receptor (Hahn et al., 1985; Michel et al., 1985). These findings are also in line with evidence showing that SAL effects are blocked by μ-opioid receptor antagonists; e.g., conditioned place preference (Matsuzawa et al., 2000); *in vitro* dopaminergic neurons activation (Xie et al., 2012); dopamine release in the nucleus accumbens (Hipólito et al., 2011); locomotor activation (Hipólito et al., 2010) and increased ethanol consumption (Quintanilla et al., 2014). An alternative mechanism by which SAL could activate opioid transmission is by increasing the release of endogenous opioids, such as β-endorphins. However, *in vitro* studies on primary cultures of hypothalamic neurons have shown that SAL, contrary to ethanol and acetaldehyde, is unable to stimulate the secretion of β-endorphins (Reddy and Sarkar, 1993).

The classic signaling pathway associated to the agonist-mediated activation of GPCRs involves the activation of the corresponding heterotrimeric G protein and phosphorylation of intracellular domains of the GPCR by G protein-coupled receptor kinases (GRKs), which results in rapid desensitization of GPCR to agonist action. Upon phosphorylation of GPCRs, a conformational change greatly increases its affinity to β-arrestin, a protein involved in receptor endocytosis processes and activation of signaling pathways leading to different cellular responses. After arrestin-mediated endocytosis, GPCR can be either degraded or dephosphorylated and recycled to the membrane surface as functional receptors (resensitization; See Williams et al., 2013). For some GPCRs, including μ-opioid receptors, there are agonists displaying a differential efficacy (biased agonists) for recruiting β-arrestin proteins (Kenakin, 2011). In this study, we found that SAL acts as a biased agonist on μ-opioid receptors, since it activates efficiently the signaling pathway led by Gi protein, but not the recruitment of β-arrestin. Interestingly, it has been proposed that agonists that do not efficiently recruit β-arrestin, and therefore are not able to promote an effective receptor endocytosis, may have an increased potential to produce tolerance and dependence due to a reduction of functional receptors (Whistler et al., 1999; Finn and Whistler, 2001).

Taking into account the relatively low potency showed by (R/S)-SAL in activating the μ-opioid receptor ($EC_{50} = 2 \times 10^{-5}$ M), the question that arises is if the concentration of SAL generated in the brain after ethanol consumption is enough to generate an opioid response. Despite several animal studies showing that chronic administration of ethanol increases SAL levels in the hypothalamus and the striatum (Sjöquist et al., 1982; Myers et al., 1985; Matsubara et al., 1987), there are no studies showing the actual concentrations of SAL generated in the brain by acute pharmacological doses of ethanol. Brain microdialysis studies by Jamal et al. (2003) showed that SAL is only detectable at nanomolar levels in the striatum of rats whether ethanol administration is preceded by a pre-treatment with the aldehyde dehydrogenase inhibitor cyanamide, thus increasing acetaldehyde levels. These results indicate that *in vivo* formation of SAL is highly dependent on acetaldehyde levels, occurring only in dopamine-rich areas

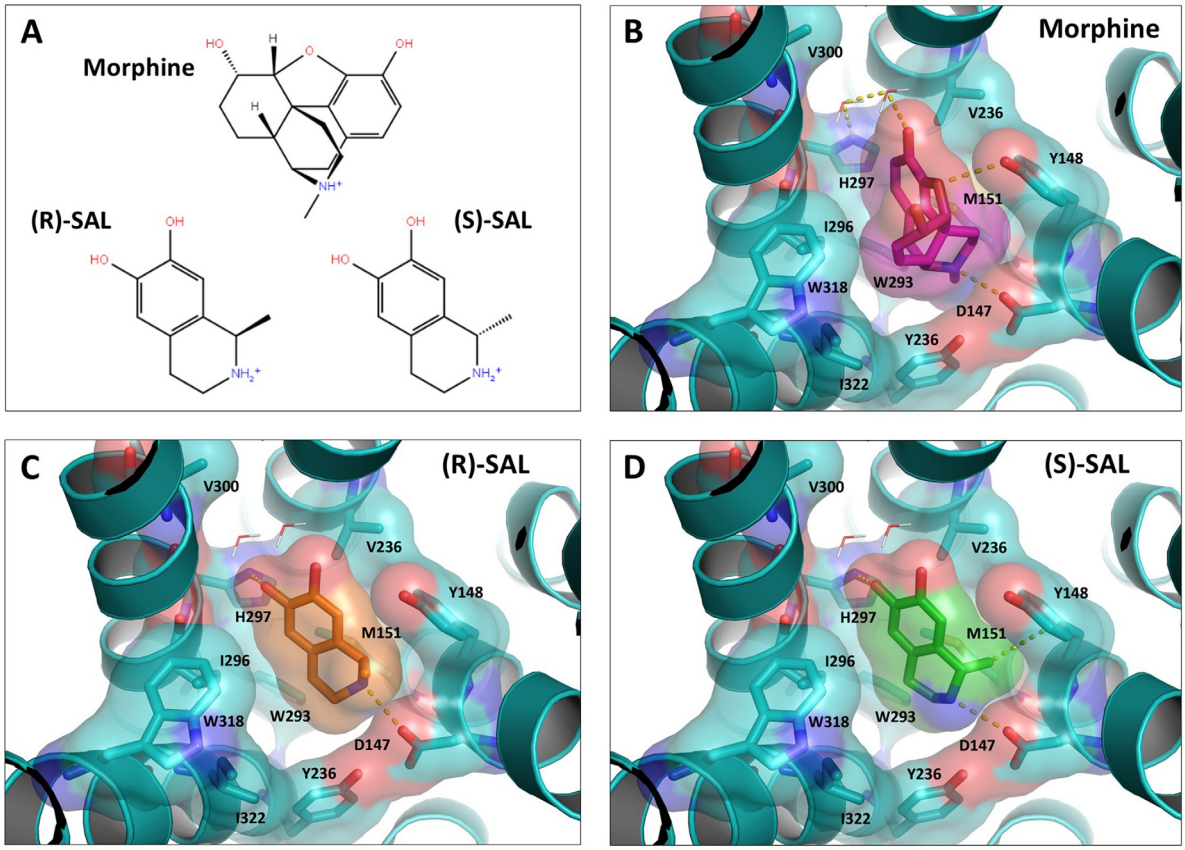

FIGURE 6 | (R)-SAL and (S)-SAL stereoisomers showed a morphine-like interaction with the binding site of the μ-opioid receptor. Molecular docking analyses of morphine, (R)-SAL and (S)-SAL on the coordinates of the crystallized mouse μ-opioid receptor were performed. **(A)** Chemical structure of the molecules studied: morphine, (*R*)-SAL and (*S*)-SAL. Hydroxyl groups are represented in red and amino groups are represented in blue. **(B–D)** Best docking fits of the three studied molecules on the binding site of the μ-opioid receptor. The molecular surface of each molecule and the receptor is represented. A dotted line highlights the main interactions of the ligands with the binding site of the μ-opioid receptor.

of the brain. Since catalase is the main enzyme responsible for the metabolism of ethanol to acetaldehyde in the brain, a heterogeneous distribution of catalase may allow the generation of site-specific accumulations of acetaldehyde, yielding high local concentrations of SAL (see Hipólito et al., 2007). In agreement to this, studies by Brannan et al. (1981) showed that catalase activity displays a regional distribution in the rat brain, with high activity in the midbrain, a dopamine-rich area that contains the VTA, deeply involved in the rewarding effects of ethanol.

An alternative ethanol-independent route of SAL biosynthesis in the brain has been proposed, involving the condensation of dopamine with pyruvic acid to yield an intermediate metabolite (salsolinol-1-carboxylic acid), which can be converted in SAL through an enzymatic oxidative decarboxylation (Naoi et al., 1996). However, experimental evidence regarding the identity and properties of the enzymes involved in this suggested biosynthetic pathway are still lacking.

The analysis of the actions of the (R) and (S) stereoisomers of SAL showed that both molecules act as full agonists of the μ-opioid receptor, with (S)-SAL being 50-fold more potent than (R)-SAL. In line with a putative role of (S)-SAL on the actions of ethanol, human studies by Rommelspacher et al. (1995) showed that (S)-SAL levels in the plasma of alcoholics are 100-fold higher than that in non-alcoholics, while (R)-SAL levels in the plasma of alcoholics are only 2-fold higher than that in non-alcoholic subjects. An animal study performed in alcohol-preferring P rats showed that after 8 weeks of chronic ethanol consumption, there was a 2-fold increase in (S)-SAL levels in the midbrain, whereas a 1.6-fold increase was detected in (R)-SAL levels (Rojkovicova et al., 2008). In contrast to these findings, a recent behavioral study by Quintanilla et al. (2016) found that (R)-SAL was the only enantiomer capable of inducing conditioned place preference after its intracerebral infusion into the VTA of rats. The reasons for these inconsistencies are unknown, but a possible explanation would be the existence of different molecular targets for (R)-SAL or its *in vivo* metabolism. Furthermore, there are studies showing that SAL increases monoaminergic transmission by inhibition of monoamine oxidase (MAO) activity in the brain (Naoi et al., 2004) and by inhibition of monoamine reuptake

(Alpers et al., 1975). Recent studies have also shown that SAL could interact with other receptors as a monoamine derivative. Electrophysiological studies by Xie and Ye (2012) showed that the stimulant effect of SAL on dopaminergic neurons could be attenuated by the D1 receptor antagonist, SKF83566. In fact, intracerebral self-administration of SAL in alcohol-preferring P rats is significantly reduced by the co-administration of the dopamine $D_{2,3}$ agonist quinpirole or the serotonin 5-HT_3 antagonist ICS-205, 930 (Rodd et al., 2008). It is also possible that both (R)-and (S)-SAL could present different affinities for other opioid receptors such as the kappa and delta subtypes.

The higher potency showed by (S)-SAL is also supported by molecular docking studies in which the binding of each stereoisomer to the μ-opioid receptor was simulated and analyzed. As shown in **Table 1**, in spite of the similarity of the two enantiomers, (S)-SAL docking poses had lower scores (predicted binding energy) than (R)-SAL in general, and also when comparing best poses, which could account for its higher experimental agonist potency. The low score of (S)-SAL can be explained by the interaction of its chiral methyl group with the receptor binding site, specifically with the Tyr148 (**Figure 6D**), filling a cavity that is empty for (R)-SAL (**Figure 6C**). To our knowledge SAL (179 Da) is the smallest molecule described to date showing an opioid full agonism. A possible limitation for the present docking study is that computational simulations were performed using the crystal structure of the mouse μ-opioid receptor instead of the human μ-opioid receptor used in the cell-based assays. However, an overall comparison of the amino acid sequence shows a 94% of homology between the mouse and human receptors. A near complete homology (>99%) is obtained if the comparison only considers the domains comprising the binding site of the receptor.

Overall, it is shown that racemic (R/S)-SAL and its stereoisomers (R)-SAL and (S)-SAL are agonists of the μ-opioid receptor, (S)-SAL being more potent than (R)-SAL. The *in silico* study also shows that the interactions of (R)-SAL and (S)-SAL with the binding-site of the μ-opioid receptor are analogous to that shown by morphine. Further studies of the action of SAL on μ-opioid receptor variants (e.g., A118G variant) or other opioid receptor subtypes (e.g., delta or kappa) would provide additional evidence of the role of SAL in the development of alcohol addiction.

AUTHOR CONTRIBUTIONS

PBC, MR-M, and GZ-T conceived and designed the experiments, analyzed the data. PB-C performed the experiments. MR-M, PB-C, MH-M, MEQ, and VV wrote or contributed to the writing of the manuscript.

FUNDING

This work was supported by Fondo Nacional de Desarrollo Científico y Tecnológico (FONDECYT) grants #11130241 (MR-M), and #1120079 (MH-M); Comisión Nacional de Investigación Científica y Tecnológica (CONICYT) PCHA/Doctorado Nacional/2013-21130865 (PB-C); National Institutes of Health (NIH) grants AA022057 (VV), and AA021724 (VV); Millennium Institute BNI P09-015-F (MH-M; MR-M).

ACKNOWLEDGMENTS

We thank Dr. Yedy Israel and Catalina Salinas for critical review of the manuscript.

REFERENCES

Alpers, H. S., McLaughlin, B. R., Nix, W. M., and Davis, V. E. (1975). Inhibition of catecholamine uptake and retention in synaptosomal preparations by tetrahydroisoquinoline and tetrahydroprotoberberine alkaloids. *Biochem. Pharmacol.* 24, 1391–1396. doi: 10.1016/0006-2952(75)90361-5

Benowitz, N. L., and Jacob, P. (1993). Nicotine and cotinine elimination pharmacokinetics in smokers and nonsmokers. *Clin. Pharmacol. Ther.* 53, 316–323. doi: 10.1038/clpt.1993.27

Brannan, T. S., Maker, H. S., and Raes, I. P. (1981). Regional distribution of catalase in the adult rat brain. *J. Neurochem.* 36, 307–309. doi: 10.1111/j.1471-4159.1981.tb02411.x

Di Chiara, G., and Imperato, A. (1988). Drugs abused by humans preferentially increase synaptic dopamine concentrations in the mesolimbic system of freely moving rats. *Proc. Natl. Acad. Sci. U S A* 85, 5274–5278. doi: 10.1073/pnas.85.14.5274

Deehan, G. A. Jr., Engleman, E. A., Ding, Z. M., McBride, W. J., and Rodd, Z. A. (2013). Microinjections of acetaldehyde or salsolinol into the posterior ventral tegmental area increase dopamine release in the nucleus accumbens shell. *Alcohol. Clin. Exp. Res.* 37, 722–729. doi: 10.1111/acer.12034

DeLano, W. L. (2002). The PyMOL molecular graphics system. Available online at: http://www.pymol.org. Accessed on June 10, 2016.

Deng, Y., Maruyama, W., Dostert, P., Takahashi, T., Kawai, M., and Naoi, M. (1995). Determination of the (R)- and (S)-enantiomers of salsolinol and N-methylsalsolinol by use of a chiral high-performance liquid chromatographic column. *J. Chromatogr. B Biomed. Appl.* 670, 47–54. doi: 10.1016/0378-4347(95)00136-7

Fertel, R. H., Greenwald, J. E., Schwarz, R., Wong, L., and Bianchine, J. (1980). Opiate receptor binding and analgesic effects of the tetrahydroisoquinolines salsolinol and tetrahydropapaveroline. *Res. Commun. Chem. Pathol. Pharmacol.* 27, 3–16.

Finn, A. K., and Whistler, J. L. (2001). Endocytosis of the mu opioid receptor reduces tolerance and a cellular hallmark of opiate withdrawal. *Neuron* 32, 829–839. doi: 10.1016/s0896-6273(01)00517-7

Glare, P. A., and Walsh, T. D. (1991). Clinical pharmacokinetics of m orphine. *Ther. Drug Monit.* 13, 1–23. doi: 10.1097/00007691-199101000-00001

Haber, H., Winkler, A., Putscher, I., Henklein, P., Baeger, I., Georgi, M., et al. (1996). Plasma and urine salsolinol in humans: effect of acute ethanol intake on the enantiomeric composition of salsolinol. *Alcohol. Clin. Exp. Res.* 20, 87–92. doi: 10.1111/j.1530-0277.1996.tb01049.x

Hahn, E. F., Nishimura, S., Goodman, R. R., and Pasternak, G. W. (1985). Irreversible opiate agonists and antagonists. II. Evidence against a bivalent mechanism of action for opiate azines and diacylhydrazones. *J. Pharmacol. Exp. Ther.* 235, 839–845.

Hipólito, L., Martí-Prats, L., Sánchez-Catalán, M. J., Polache, A., and Granero, L. (2011). Induction of conditioned place preference and dopamine release by salsolinol in posterior VTA of rats: involvement of μ-opioid receptors. *Neurochem. Int.* 59, 559–562. doi: 10.1016/j.neuint.2011.04.014

Hipólito, L., Sánchez, M. J., Polache, A., and Granero, L. (2007). Brain metabolism of ethanol and alcoholism: an update. *Curr. Drug Metab.* 8, 716–727. doi: 10.2174/138920007782109797

Hipólito, L., Sánchez-Catalán, M. J., Zornoza, T., Polache, A., and Granero, L. (2010). Locomotor stimulant effects of acute and repeated intrategmental injections of salsolinol in rats: role of μ-opioid receptors. *Psychopharmacology* 209, 1–11. doi: 10.1007/s00213-009-1751-9

Holford, N. H. (1987). Clinical pharmacokinetics of ethanol. *Clin. Pharmacokinet.* 13, 273–292. doi: 10.2165/00003088-198713050-00001

Huang, W., Manglik, A., Venkatakrishnan, A. J., Laeremans, T., Feinberg, E. N., Sanborn, A. L., et al. (2015). Structural insights into μ-opioid receptor activation. *Nature* 524, 315–321. doi: 10.1038/nature14886

Israel, Y., Quintanilla, M. E., Karahanian, E., Rivera-Meza, M., and Herrera-Marschitz, M. (2015). The "first hit" toward alcohol reinforcement: role of ethanol metabolites. *Alcohol. Clin. Exp. Res.* 39, 776–786. doi: 10.1111/acer.12709

Jamal, M., Ameno, K., Ameno, S., Okada, N., and Ijiri, I. (2003). *In vivo* study of salsolinol produced by a high concentration of acetaldehyde in the striatum and nucleus accumbens of free-moving rats. *Alcohol. Clin. Exp. Res.* 27, 79S–84S. doi: 10.1097/01.alc.0000078617.33026.ad

Jeffcoat, A. R., Perez-Reyes, M., Hill, J. M., Sadler, B. M., and Cook, C. E. (1989). Cocaine disposition in humans after intravenous injection, nasal insufflation (snorting), or smoking. *Drug Metab. Dispos.* 17, 153–159.

Johnson, S. W., and North, R. A. (1992). Opioids excite dopamine neurons by hyperpolarization of local interneurons. *J. Neurosci.* 12, 483–488.

Karahanian, E., Quintanilla, M. E., Tampier, L., Rivera-Meza, M., Bustamante, D., Gonzalez-Lira, V., et al. (2011). Ethanol as a prodrug: brain metabolism of ethanol mediates its reinforcing effects. *Alcohol. Clin. Exp. Res.* 35, 606–612. doi: 10.1111/j.1530-0277.2011.01439.x

Karahanian, E., Rivera-Meza, M., Tampier, L., Quintanilla, M. E., Herrera-Marschitz, M., and Israel, Y. (2015). Long-term inhibition of ethanol intake by the administration of an aldehyde dehydrogenase-2 (ALDH2)-coding lentiviral vector into the ventral tegmental area of rats. *Addict. Biol.* 20, 336–344. doi: 10.1111/adb.12130

Kenakin, T. (2011). Functional selectivity and biased receptor signaling. *J. Pharmacol. Exp. Ther.* 336, 296–302. doi: 10.1124/jpet.110.173948

Lee, J., Ramchandani, V. A., Hamazaki, K., Engleman, E. A., McBride, W. J., Li, T. K., et al. (2010). A critical evaluation of influence of ethanol and diet on salsolinol enantiomers in humans and rats. *Alcohol. Clin. Exp. Res.* 34, 242–250. doi: 10.1111/j.1530-0277.2009.01087.x

Li, J.-G., Chen, C., Yin, J., Rice, K., Zhang, Y., Matecka, D., et al. (1999). ASP147 in the third transmembrane helix of the rat mu opioid receptor forms ion-pairing with morphine and naltrexone. *Life Sci.* 65, 175–185. doi: 10.1016/s0024-3205(99)00234-9

Lucchi, L., Bosio, A., Spano, P. F., and Trabucchi, M. (1982). Action of ethanol and salsolinol on opiate receptor function. *Brain Res.* 232, 506–510. doi: 10.1016/0006-8993(82)90297-9

Manglik, A., Kruse, A. C., Kobilka, T. S., Thian, F. S., Mathiesen, J. M., Sunahara, R. K., et al. (2012). Crystal structure of the μ-opioid receptor bound to a morphinan antagonist. *Nature* 485, 321–326. doi: 10.1038/nature10954

Matsubara, K., Fukushima, S., and Fukui, Y. (1987). A systematic regional study of brain salsolinol levels during and immediately following chronic ethanol ingestion in rats. *Brain Res.* 413, 336–343. doi: 10.1016/0006-8993(87)91025-0

Matsuzawa, S., Suzuki, T., and Misawa, M. (2000). Involvement of μ-opioid receptor in the salsolinol-associated place preference in rats exposed to conditioned fear stress. *Alcohol. Clin. Exp. Res.* 24, 366–372. doi: 10.1097/00000374-200003000-00020

Melchior, C., and Collins, M. A. (1982). The route and significance of endogenous synthesis of alkaloids in animals. *Crit. Rev. Toxicol.* 9, 313–356. doi: 10.3109/10408448209037496

Melis, M., Carboni, E., Caboni, P., and Acquas, E. (2015). Key role of salsolinol in ethanol actions on dopamine neuronal activity of the posterior ventral tegmental area. *Addict. Biol.* 20, 182–193. doi: 10.1111/adb.12097

Michel, M. E., Bolger, G., and Weismann, B. A. (1985). Binding of a new opiate antagonist, nalmefene, to rat brain membranes. *Methods Find. Exp. Clin. Pharmacol.* 7, 175–177.

Myers, R. D., and Melchior, C. L. (1977). Differential actions on voluntary alcohol intake by tetrahydroisoquinolines or a beta-carboline infused chronically in the ventricle of the rat. *Pharmacol. Biochem. Behav.* 7, 381–392. doi: 10.1016/0091-3057(77)90235-0

Myers, W. D., Ng, K. T., Singer, G., Smythe, G. A., and Duncan, M. W. (1985). Dopamine and salsolinol levels in rat hypothalami and striatum after schedule-induced self-injection (SISI) of ethanol and acetaldehyde. *Brain Res.* 358, 122–128. doi: 10.1016/0006-8993(85)90955-2

Naoi, M., Maruyama, W., Dostert, P., Kohda, K., and Kaiya, T. (1996). A novel enzyme enantio-selectively synthesizes (R) salsolinol, a precursor of a dopaminergic neurotoxin, N-methyl(R)salsolinol. *Neurosci. Lett.* 212, 183–186. doi: 10.1016/0304-3940(96)12807-x

Naoi, M., Maruyama, W., and Nagy, G. M. (2004). Dopamine-derived salsolinol derivatives as endogenous monoamine oxidase inhibitors: occurrence, metabolism and function in human brains. *Neurotoxicology* 25, 193–204. doi: 10.1016/s0161-813x(03)00099-8

Neilan, C. L., Husbands, S. M., Breeden, S., Ko, M. C., Aceto, M. D., Lewis, J. W., et al. (2004). Characterization of the complex morphinan derivative BU72 as a high efficacy, long-lasting mu-opioid receptor agonist. *Eur. J. Pharmacol.* 499, 107–116. doi: 10.1016/j.ejphar.2004.07.097

Peana, A. T., Rosas, M., Porru, S., and Acquas, E. (2016). From ethanol to salsolinol: role of ethanol metabolites in the effects of ethanol. *J. Exp. Neurosci.* 10, 137–146. doi: 10.4137/jen.s25099

Quan, Z., Song, Y., Peters, G., Shenwu, M., Sheng, Y., Hwang, H. M., et al. (2005). Chiral CE separation of dopamine-derived neurotoxins. *Anal. Sci.* 21, 115–119. doi: 10.2116/analsci.21.115

Quintanilla, M. E., Rivera-Meza, M., Berríos-Cárcamo, P. A., Bustamante, D., Buscaglia, M., Morales, P., et al. (2014). Salsolinol, free of isosalsolinol, exerts ethanol-like motivational/sensitization effects leading to increases in ethanol intake. *Alcohol* 48, 551–559. doi: 10.1016/j.alcohol.2014.07.003

Quintanilla, M. E., Rivera-Meza, M., Berríos-Cárcamo, P., Cassels, B. K., Herrera-Marschitz, M., and Israel, Y. (2016). (*R*)-salsolinol, a product of ethanol metabolism, stereospecifically induces behavioral sensitization and leads to excessive alcohol intake. *Addict. Biol.* 21, 1063–1071. doi: 10.1111/adb.12268

Quintanilla, M. E., Tampier, L., Karahanian, E., Rivera-Meza, M., Herrera-Marschitz, M., and Israel, Y. (2012). Reward and relapse: complete gene-induced dissociation in an animal model of alcohol dependence. *Alcohol. Clin. Exp. Res.* 36, 517–522. doi: 10.1111/j.1530-0277.2011.01606.x

Reddy, B. V., and Sarkar, D. K. (1993). Effect of alcohol, acetaldehyde and salsolinol on β-endorphin secretion from the hypothalamic neurons in primary cultures. *Alcohol. Clin. Exp. Res.* 17, 1261–1267. doi: 10.1111/j.1530-0277.1993.tb05239.x

Rodd, Z. A., Bell, R. L., Zhang, Y., Murphy, J. M., Goldstein, A., Zaffaroni, A., et al. (2005). Regional heterogeneity for the intracranial self-administration of ethanol and acetaldehyde within the ventral tegmental area of alcohol-preferring (P) rats: involvement of dopamine and serotonin. *Neuropsychopharmacology* 30, 330–338. doi: 10.1038/sj.npp.1300561

Rodd, Z. A., Oster, S. M., Ding, Z. M., Toalston, J. E., Deehan, G., Bell, R. L., et al. (2008). The reinforcing properties of salsolinol in the ventral tegmental area: evidence for regional heterogeneity and the involvement of serotonin and dopamine. *Alcohol. Clin. Exp. Res.* 32, 230–239. doi: 10.1111/j.1530-0277.2007.00572.x

Rojkovicova, T., Mechref, Y., Starkey, J. A., Wu, G., Bell, R. L., McBride, W. J., et al. (2008). Quantitative chiral analysis of salsolinol in different brain regions of rats genetically predisposed to alcoholism. *J. Chromatogr. B Analyt. Technol. Biomed. Life Sci.* 863, 206–214. doi: 10.1016/j.jchromb.2008.01.016

Rommelspacher, H., Sllström Baum, S., Dufeu, P., and Schmidt, L. G. (1995). Determination of (R)- and (S)-salsolinol sulfate and dopamine sulfate levels in plasma of nonalcoholics and alcoholics. *Alcohol* 12, 309–315. doi: 10.1016/0741-8329(95)00004-b

Shim, J., Coop, A., and MacKerell, A. D.Jr. (2013). Molecular details of the activation of the μ opioid receptor. *J. Phys. Chem. B* 117, 7907–7917. doi: 10.1021/jp404238n

Sjöquist, B., Liljequist, S., and Engel, J. (1982). Increased salsolinol levels in rat striatum and limbic forebrain following chronic ethanol

treatment. *J. Neurochem.* 39, 259–262. doi: 10.1111/j.1471-4159.1982.tb04730.x

Tóth, B. E., Homicskó, K., Radnai, B., Maruyama, W., DeMaria, J. E., Vecsernyés, M., et al. (2001). Salsolinol is a putative endogenous neuro-intermediate lobe prolactin-releasing factor. *J. Neuroendocrinol.* 13, 1042–1050. doi: 10.1046/j.1365-2826.2001.00725.x

Trott, O., and Olson, A. J. (2010). AutoDock Vina: improving the speed and accuracy of docking with a new scoring function, efficient optimization, and multithreading. *J. Comput. Chem.* 31, 455–461. doi: 10.1002/jcc.21334

Whistler, J. L., Chuang, H. H., Chu, P., Jan, L. Y., and von Zastrow, M. (1999). Functional dissociation of mu opioid receptor signaling and endocytosis: implications for the biology of opiate tolerance and addiction. *Neuron* 23, 737–746. doi: 10.1016/s0896-6273(01)80032-5

Williams, J. T., Ingram, S. L., Henderson, G., Chavkin, C., von Zastrow, M., Schulz, S., et al. (2013). Regulation of μ-opioid receptors: desensitization, phosphorylation, internalization, and tolerance. *Pharmacol. Rev.* 65, 223–254. doi: 10.1124/pr.112.005942

Xie, G., Hipólito, L., Zuo, W., Polache, A., Granero, L., Krnjevic, K., et al. (2012). Salsolinol stimulates dopamine neurons in slices of posterior ventral tegmental area indirectly by activating μ-opioid receptors. *J. Pharmacol. Exp. Ther.* 341, 43–50. doi: 10.1124/jpet.111.186833

Xie, G., Krnjevic, K., and Ye, J. H. (2013). Salsolinol modulation of dopamine neurons. *Front. Behav. Neurosci.* 7:52. doi: 10.3389/fnbeh.2013.00052

Xie, G., and Ye, J.-H. (2012). Salsolinol facilitates glutamatergic transmission to dopamine neurons in the posterior ventral tegmental area of rats. *PLoS One* 7:e36716. doi: 10.1371/journal.pone.0036716

Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

frontiers in Behavioral Neuroscience

MINI REVIEW
published: 30 May 2017
doi: 10.3389/fnbeh.2017.00097

On the Accuracy of *In Vivo* Ethanol and Acetaldehyde Monitoring, a Key Tile in *the Puzzle of Acetaldehyde as a Neuroactive Agent*

Paolo Enrico[1]* *and Marco Diana*[2]

[1] *Department of Biomedical Sciences, University of Sassari, Sassari, Italy,* [2] *'G. Minardi' Cognitive Neuroscience Laboratory, Department of Chemistry and Pharmacy, University of Sassari, Sassari, Italy*

Over the last 20 years researchers have explored the postulated role of acetaldehyde (ACD) as a mediator of some of the actions of ethanol (EtOH) in the central nervous system (CNS). However, efforts have been hampered mainly by the difficulty of directly measuring *in vivo* EtOH and ACD levels in the CNS and thus, our knowledge is based on indirect evidences. Although technically challenging, the development of reliable methods for *in vivo* measurement of ACD and EtOH is of paramount importance to solve the "*puzzle of acetaldehyde as a neuroactive agent.*" In this short review we discuss the recent advances on brain EtOH pharmacokinetic and state-of-the-art available techniques that could be used for *in vivo* detect EtOH and ACD both non-invasively (magnetic resonance spectroscopy), and invasively (microdialysis and biosensors). Among the different *in vivo* sampling techniques described, particular emphasis is paid to the field of enzyme-based amperometric biosensors. Biosensors have gained much attention in recent years for their ability to online monitor biological signals *in vivo*, and several micro- and nano-structured devices have been successfully used for *in vivo* studies. Owing to their high temporal and spatial resolution, biosensors could provide the adequate technology for studying *in vivo* EtOH pharmacokinetic.

Keywords: ethanol, acetaldehyde, magnetic resonance spectroscopy, microdialysis, biosensors

OPEN ACCESS

Edited by:
Merce Correa,
Jaume I University, Spain

Reviewed by:
María José Sánchez-Catalán,
Universitat de València, Spain
Mario Rivera-Meza,
Universidad de Chile, Chile

***Correspondence:**
Paolo Enrico
enrico@uniss.it

Received: *25 January 2017*
Accepted: *09 May 2017*
Published: *30 May 2017*

Citation:
Enrico P and Diana M (2017) On the Accuracy of In Vivo Ethanol and Acetaldehyde Monitoring, a Key Tile in the Puzzle of Acetaldehyde as a Neuroactive Agent. Front. Behav. Neurosci. 11:97. doi: 10.3389/fnbeh.2017.00097

INTRODUCTION

Acetaldehyde (ACD) is a naturally occurring compound, found in several fruits and vegetables as well as in tobacco smoke and fermented alcoholic beverages (Cao et al., 2007).

In the last decades many attempts have been made to quantify brain EtOH and ACD, in order to correlate their concentrations with behavior (Correa et al., 2012; Israel et al., 2015). So far this line of research has yielded conflicting results, mostly due to discrepancy and controversy with quantitative measures of brain EtOH and ACD.

In this brief review we offer an overview of the recent advances on brain EtOH pharmacokinetic and discuss the state-of-the-art of available techniques for *in vivo* EtOH and ACD study.

EtOH METABOLISM IN THE BRAIN

Since EtOH readily enters the brain, *in situ* synthesis has been long postulated as a plausible source of brain ACD (Cohen et al., 1980). It is now demonstrated that the brain tissue contains all of the main EtOH metabolizing enzymes: alcohol dehydrogenase (ADH), cytochrome P450 2E1 (CYP2E1), and catalase; however, their relative role in metabolizing EtOH into ACD is still debated.

Alcohol dehydrogenase is a zinc-containing enzyme localized in the cytosol, it has broad substrate specificity (many primary or secondary alcohols) and is found in highest amount in the liver. However, since ADH is not uniformly expressed in the brain tissue, its real contribution to local ACD levels in discrete brain areas could have been underestimated and may deserve more detailed evaluation (Bühler et al., 1983; Kerr et al., 1989; Mori et al., 2000).

Cytochrome P450s are a family of heme enzymes mainly located in the endoplasmic reticulum and in mitochondria. CYP2E1 is the P450 family with the highest activity for oxidizing EtOH to ACD, and is widely expressed in the human and rodent brain (Tindberg and Ingelman-Sundberg, 1996; Sánchez-Catalán et al., 2008; Ferguson and Tyndale, 2011). CYP2E1 has been shown to metabolize EtOH in both neurons and astrocytes at a rate of 0.00051 μmol/min/g, and CYP2E1 pharmacological inhibition significantly reduces ACD formation in rat brain homogenates incubated with EtOH (Hansson et al., 1990; Gill et al., 1992; Warner and Gustafsson, 1994; Zimatkin et al., 2006). Further, reduced ACD brain levels have been shown in transgenic KO CYP2E1 mice after incubation with EtOH, relative to their wild-type counterparts (Ziegler et al., 2006). CYP2E1 activity has been accounted for a 20% fraction of brain EtOH oxidation, and it may represents a major adaptive response to chronic EtOH consumption as shown in a recent *in vivo* study on EtOH-induced locomotion (Hansson et al., 1990; Heit et al., 2013; Ledesma et al., 2014). Further, *in vitro* evidences in KO CYP2E1, acatalasemic and double mutants (KO CYP2E1 and acatalasemic) mice, suggest that CYP2E1 function may be linked to catalase-mediated EtOH oxidation by increasing the availability of H_2O_2 (Halliwell, 2006; Zimatkin et al., 2006; Deng and Ra, 2008).

Catalase, a heme containing enzyme, is found in the peroxisomal fraction of the cell and can oxidize EtOH as shown in reaction 1.

$$(1)\ CH_3CH_2OH + H_2O_2 \rightarrow CH_3CHO + 2H_2O$$

Recent results also show that 3-amino-1,2,4-triazole (3AT) administration impair the acquisition of operant EtOH self-administration in the rat (Peana et al., 2015). However, 3AT has been shown to cause a non-specific effects on behavior and therefore other procedures have been used to inhibit catalase-mediated ACD formation (Rotzinger et al., 1994; Tampier et al., 1995).

A valuable method for *in vivo* studying the involvement of catalase in brain ACD formation is based on the use of lentiviral vectors coding for an anticatalase shRNA (RNAi precursor), which allows for efficient (up to 75%) inhibition of catalase activity (Karahanian et al., 2011). This technique appears also of particular interest because by allowing localized inhibition of catalase activity, it may be used to precisely pinpoint those brain areas involved in the psychopharmacological effects of EtOH (Israel et al., 2015). In fact, it has been shown that administration of an anticalatase vector into the ventral tegmental area (area which plays a key role in the neurobiological basis of addiction, VTA), significantly reduced EtOH consumption and EtOH stimulated dopamine release in its projection fields (in particular the nucleus accumbens shell) (Karahanian et al., 2011; Israel et al., 2012; Quintanilla et al., 2012). Another study show that anticatalase vectors administration in the VTA can efficiently inhibit EtOH intake following deprivation (Tampier et al., 2013).

IN VIVO EtOH AND ACD DETECTION: NON-INVASIVE APPROACHES

Magnetic Resonance Spectroscopy

Magnetic resonance spectroscopy (MRS) is a non-invasive analytical technique used to provide a measure of *in vivo* brain biochemistry (Soares and Law, 2009; Strózik-Kotlorz, 2014; Buonocore and Maddock, 2015). *In vivo* MRS can be performed with common clinical magnetic resonance imaging equipments and since EtOH methyl protons can be detected (Sammi et al., 2000), MRS has been largely used to measure *in vivo* brain EtOH levels in both humans and laboratory animals (Hanstock et al., 1990; Kaufman et al., 1994; Rooney et al., 2000; Zahr et al., 2010). However, magnetic transfer evidences have clearly shown the *in vivo* presence of a free, observable EtOH pool and a membrane-associated EtOH pool that escapes direct detection (Fein and Meyerhoff, 2000; Nagel and Kroenke, 2008). Therefore, since a (possibly significant) fraction of brain EtOH content cannot be measured by MRS, this technique must be considered only for qualitative measurements.

Ethanol oxidative metabolism has been studied with MRS, after ^{13}C-labeled EtOH administration (Xiang and Shen, 2011; Wang et al., 2013a,b).The results show that ^{13}C nuclei from ^{13}C-labeled EtOH are incorporated into multiple metabolites including glutamate, glutamine, and aspartate, but no significant conversion of EtOH into ACD in the brain could be evidenced.

Despite the low sensitivity and temporal resolution, MRS still provides an opportunity for *in vivo* qualitative study of the effects of EtOH in the brain (Nagel and Kroenke, 2008; Niciu and Mason, 2014). MRS is fundamental for human studies allowing the dynamic evaluation of EtOH effects, and providing an important framework for comparing experimental results in humans and animal models (Cifuentes Castro et al., 2014). Further, since magnetic resonance images can be obtained concurrently with spectroscopic data, MRS also provides valuable structural informations (Alger, 2010; Befroy and Shulman, 2011).

IN VIVO EtOH AND ACD DETECTION: INVASIVE APPROACHES

Microdialysis

Microdialysis is *de facto* the gold-standard *in vivo* sampling technique for the central nervous system (CNS), allowing the analysis of several molecules in cerebro spinal fluid (CSF) based on their diffusion across a semi-permeable membrane (Chefer et al., 2009; Kennedy, 2013). Despite its popularity, microdialysis is not free of limitations (Westerink, 1995), some of which are particularly relevant for EtOH and ACD *in vivo* measurement. In particular, due to low probe recovery, the concentrations of substances in the dialysate only partially reflect true tissue concentrations and thus, analytes present at very low concentrations are difficult to detect. This issue could be also worsened by the fact that some compounds may be adsorbed by the dialysis membrane further decreasing probe recovery (Buttler et al., 1996). It is well-known that microdialysis has poor time resolution and therefore is not suitable for studying events that change in short time intervals. Another problem is the effect of tissue damage secondary to probe implantation; although microdialysis probes have been miniaturized in time, alterations in tissue metabolism cannot be neglected (Borland et al., 2005; Carson et al., 2015).

Despite all technical shortcomings, the advantages of using microdialysis for *in vivo* monitoring of brain neurochemistry are clear. Microdialysis is a well-known and widely reproduced technique, sampling can be performed on freely moving subjects, and long-term studies can be carried out with minimal influence on the brain tissue physiology (Westerink, 1995; Chefer et al., 2009; Kennedy, 2013). On these bases, several authors used brain microdialysis to study EtOH and ACD *in vivo* (Yoshimoto and Komura, 1993; Jamal et al., 2003, 2007, 2015). However, this approach yielded only limited results, mostly due to the various technical and analytical issues which specifically impair the usefulness of microdialysis for EtOH and (especially) ACD *in vivo* monitoring.

High Performance Liquid Chromatography (HPLC)

High performance liquid chromatography (HPLC)-based analytical methods are largely employed in microdialysis studies (Cheng et al., 2009; Guihen and O'Connor, 2009). With regard to EtOH and ACD determination HPLC-based methods appear particularly suitable, since samples are not heated during analysis and thus heat sensitive or volatile compounds (such as EtOH and ACD) can be efficiently separated. Several protocols for EtOH analysis with HPLC have been developed using flame ionization detection (Yarita et al., 2002), ultraviolet detection after conversion to acetaldehyde-phenylhydrazone (Pellegrino et al., 1999), indirect photometric detection (Takeuchi et al., 1988), and enzymatic assay (Kristoffersen and Smith-Kielland, 2005; Peris et al., 2006). An optimized HPLC-based protocol for ACD determination in biological samples after derivatization with dinitrophenylhydrazine (Vogel et al., 2000) and diode array detector is also available (Guan et al., 2011).

Gas Chromatography (GC)

Gas chromatography (GC) is an efficient analytical technique for separating volatile species in complex samples, and several GC-based protocols have been developed for the detection of EtOH and ACD in biological matrices.

Several detector types can be used in conjunction with GC for EtOH and ACD detection; the most efficient protocols available have been developed mainly using mass spectrometry (GC–MS) (Heit et al., 2016) or flame ionization (GC-FID) (Chun et al., 2016), alone or in combination (Tiscione et al., 2011).

Gas chromatography with headspace extraction and mass spectrometry or flame ionization detection is the most reliable and sensitive technique available for EtOH and ACD detection in microdialysates (Xiao et al., 2014; Heit et al., 2016). Indeed, owing to their robustness and reliability, GC-based EtOH analysis are the gold standard technique for blood alcohol concentration measurement in forensic and toxicological laboratories (Cordell et al., 2013; Xiao et al., 2014; Goullé and Guerbet, 2015).

Fluorimetry

A new fluorimetry-based analytical method for EtOH and ACD has been recently published (Zachut et al., 2016). Although not specifically developed for EtOH and ACD detection in brain dialysates, this technique appears quite compatible with microdialysis; in particular: small sample volume, no sample pre-processing, simple methodology, relatively inexpensive laboratory equipment. Further, the limits of detection of the technique are reported to be comparable with the performance of GC methods.

Biosensors

A biosensor can be defined as "a self-contained analytical device that combines a biological component with a physicochemical device for the detection of an analyte of biological importance" (Hasan et al., 2014). Biosensors typically consist of two key components: (1) a biological recognition element to detect the analyte; (2) a transducer to convert the biological response into a convenient output signal.

Among the different devices available, amperometric enzyme-based biosensors (AEBs) are increasingly employed in *in vivo* brain monitoring (Thévenot et al., 2001; Weltin et al., 2016). In fact, miniature (active surface – 1 mm, diameter – 150 μm) AEBs implantation induces reduced tissue damage, allows for real-time monitoring, with high sensitivity and specificity for analytes which cannot be studied with microdialysis (Timmerman and Westerink, 1997; Sirca et al., 2014). Another important feature of biosensors is the possibility of associating the implanted device to a telemetric system, allowing experiments in freely moving subjects (Olivo et al., 2011).

Amperometric enzyme-based biosensors are mainly based on enzymes that belong to two classes: oxidases and dehydrogenases; in their most common implementation the enzyme is linked on the transducer surface and the output signal is generated by measuring the electroactive by-products of enzymatic reaction.

In recent years several AEBs for EtOH detection have been developed, based on both alcohol oxidase (AOx) or dehydrogenase (ADH).

Alcohol oxidase catalyzes the oxidation of aliphatic, low molecular weight alcohols to their respective aldehydes using molecular oxygen (O_2) as the electron acceptor and flavin-adenine dinucleotide (FAD) as cofactor (reactions 2 and 3).

$$(2)\ \text{R-CH}_2\text{OH} + \text{AOx/FAD} \rightarrow \text{R-CHO} + \text{AOx/FADH}_2$$

$$(3)\ \text{AOx/FADH}_2 + \text{O}_2 \rightarrow \text{AOx/FAD} + \text{H}_2\text{O}_2$$

$$(4)\ \text{H}_2\text{O}_2 \rightarrow \text{O}_2 + 2\text{H}^+ + 2\text{e}^-$$

The hydrogen peroxide produced by reaction 3 can be directly detected at the transducer surface of AOx-based AEBs (reaction 4). However, the high anodic potential needed to oxidize H_2O_2 poses a problem of Faradaic interference due to the presence of other compounds (such as ascorbic acid and uric acid) physiologically present in high concentrations in the CSF, which are also oxidized at the same potential (Belluzo et al., 2008). The use of a bi-enzyme AEB is a common way to circumvent this problem. In fact, by coupling a peroxidase [usually horseradish peroxidase (HRP)] to AOx it is possible to indirectly monitor EtOH-derived H_2O_2 at low working potentials reducing interfering signals (Vijayakumar et al., 1996; Azevedo et al., 2005).

$$(5)\ \text{H}_2\text{O}_2 + 2\text{H}^+ + \text{HRP}^- \rightarrow 2\text{H}_2\text{O} + \text{HRP}^+$$

The HRP^+/HRP^- redox couple (reaction 5) is used as the sensing scheme at the transducer surface of AOx/HRP-based AEBs.

Alcohol dehydrogenase catalyzes the reversible oxidation of primary aliphatic and aromatic alcohols using nicotinamide-adenine dinucleotide (NAD) as cofactor (reaction 6).

$$(6)\ \text{R-CH}_2\text{OH} + \text{ADH/NAD}^+ \rightarrow \text{R-CHO} + \text{ADH/NADH} + \text{H}^+$$

$$(7)\ \text{NADH} \rightarrow \text{NAD}^+ + \text{H}^+ + 2\text{e}^-$$

The most common way to monitor an ADH-catalyzed reaction is by using the NAD^+/NADH redox couple (reaction 7) as the sensing scheme at the transducer surface of ADH-based AEBs (Lorenzo et al., 1998).

Acetaldehyde biosensors developed so far are based on ALDH, which catalyzes the oxidation of biogenic and xenobiotic aldehydes (including ACD) into acetate using NAD as cofactor (reaction 8).

$$(8)\ \text{R-CHO} + \text{ALDH/NAD}^+ \rightarrow \text{R-COOH} + \text{ALDH/NADH} + \text{H}^+$$

$$(9)\ \text{NADH} \rightarrow \text{NAD} + \text{H}^+ + 2\text{e}^-$$

The ALDH-catalyzed reaction is monitored by using the NAD+/NADH redox couple (reaction 9) as the sensing scheme at the transducer surface of ALDH-based AEBs (Lorenzo et al., 1998).

Acetaldehyde biosensors have been mostly developed for toxicological and industrial purposes and therefore their biological applicative potential is much less characterized, when compared with EtOH AEBs. However the available evidence show that these devices can efficiently detect ACD in the μM range *in vitro*, with high time resolution and substrate specificity (Noguer and Marty, 1997; Noguer et al., 2001; Avramescu et al., 2002; Yao and Handa, 2003; Ghica et al., 2007).

Although the development of an adequate biosensor technology for *in vivo* EtOH and ACD detection is still in its infancy, the available evidence clearly show that this approach holds tremendous technological potential. In fact the prototypical properties of biosensors including high spatial and temporal resolution together with high sensitivity and specificity, render these devices the best candidates for *in vivo* accurate EtOH and ACD detection.

Several AEBs for *in vivo* EtOH determination are already commercially available; however since *in vivo* biosensors use is not deprived of drawbacks, the fundamentals of this technology are to be well-understood in order to obtain reproducible results (Vigneshvar et al., 2015; Weltin et al., 2016). In particular, the interactions of the implanted AEB with the biological environment may severely affect its bioanalytical performances via metabolic biofouling, electrode passivation, or biodegradation. Metabolic biofouling is probably the most important problem being able to quickly alter sensitivity, limit of detection, and linear response of the implanted device (Gifford et al., 2006; Kotanen et al., 2012). Unfortunately, biocompatibility-based issues cannot be easily circumvented and adequate pre- and post-calibration procedures are needed in order to properly evaluate *in vivo* AEBs measurements (Wilson and Gifford, 2004; Wahono et al., 2012). However it is expected that the forthcoming generation of biosensors, either based on nanoscale or polymeric materials, will greatly help reducing biocompatibility issues (Nichols et al., 2013; Weltin et al., 2014; Saxena and Das, 2016).

CONCLUSION

The many attempts to quantify ACD in the brain have yielded conflicting results, mainly because of the inadequacy of the available analytical techniques. Thus, it is clear that in order to solve the puzzle of ACD as a neuroactive agent we need to use adequate analytical tools, fostering their improvement, while discarding the most problematic approaches.

Spectroscopic techniques have proved to be useful for studying *in vivo* brain EtOH kinetics, in both humans and experimental animals, but ACD measurement remains outside MRS analytical scope. Nevertheless, owing to its absolute non-invasive nature MRS provides a great opportunity for *in vivo* qualitative study of the effects of EtOH in the intact brain.

Brain microdialysis is a well-known sampling technique for *in vivo* applications. However, it is now clear that many of the features that made microdialysis so successful for *in vivo* monitoring of several neurochemicals, are of limited use when coming to *in vivo* EtOH and (especially) ACD analysis. Its invasiveness together with the low temporal resolution, and the necessity of complex analytical procedures, represent the most important problems.

Biosensors are the emerging tool for the preclinical *in vivo* study of neurochemistry. When compared to microdialysis the main advantages of AEBs are represented by their reduced invasiveness, high time resolution, and the possibility to detect analytes which cannot be studied with microdialysis. In the case of EtOH monitoring, AOX-based AEBs have proved to be capable of *in vitro* and *in vivo* detecting concentrations of EtOH in the μM range. ALDH-based ACD AEBs have not been applied for *in vivo* ACD detection yet, however *in vitro* data strongly suggest that these devices may represent the most promising opportunity for *in vivo* brain ACD detection.

AUTHOR CONTRIBUTIONS

PE: performed literature analysis and data collection, wrote manuscript and acted as corresponding author. MD: supervised development of work, helped in data interpretation and manuscript evaluation and editing.

REFERENCES

Alger, J. R. (2010). Quantitative proton magnetic resonance spectroscopy and spectroscopic imaging of the brain: a didactic review. *Top. Magn. Reson. Imaging* 21, 115–128. doi: 10.1097/RMR.0b013e31821e568f

Avramescu, A., Noguer, T., Avramescu, M., and Marty, J.-L. (2002). Screen-printed biosensors for the control of wine quality based on lactate and acetaldehyde determination. *Anal. Chim. Acta* 458, 203–213. doi: 10.1016/S0003-2670(01)01580-X

Azevedo, A. M., Prazeres, D. M. F., Cabral, J. M. S., and Fonseca, L. P. (2005). Ethanol biosensors based on alcohol oxidase. *Biosens. Bioelectron.* 21, 235–247. doi: 10.1016/j.bios.2004.09.030

Befroy, D. E., and Shulman, G. I. (2011). Magnetic resonance spectroscopy studies of human metabolism. *Diabetes Metab. Res. Rev.* 60, 1361–1369. doi: 10.2337/db09-0916

Belluzo, M. S., Ribone, M. E., and Lagier, C. M. (2008). Assembling amperometric biosensors for clinical diagnostics. *Sensors* 8, 1366–1399. doi: 10.3390/s8031366

Borland, L. M., Shi, G., Yang, H., and Michael, A. C. (2005). Voltammetric study of extracellular dopamine near microdialysis probes acutely implanted in the striatum of the anesthetized rat. *J. Neurosci. Methods* 146, 149–158. doi: 10.1016/j.jneumeth.2005.02.002

Bühler, R., Pestalozzi, D., Hess, M., and Von Wartburg, J. P. (1983). Immunohistochemical localization of alcohol dehydrogenase in human kidney, endocrine organs and brain. *Pharmacol. Biochem. Behav.* 18(Suppl. 1), 55–59. doi: 10.1016/0091-3057(83)90147-8

Buonocore, M. H., and Maddock, R. J. (2015). Magnetic resonance spectroscopy of the brain: a review of physical principles and technical methods. *Rev. Neurosci.* 26, 609–632. doi: 10.1515/revneuro-2015-0010

Buttler, T., Nilsson, C., Gorton, L., Marko-Varga, G., and Laurell, T. (1996). Membrane characterisation and performance of microdialysis probes intended for use as bioprocess sampling units. *J. Chromatogr. A* 725, 41–56. doi: 10.1016/0021-9673(95)01120-X

Cao, J., Belluzzi, J. D., Loughlin, S. E., Keyler, D. E., Pentel, P. R., and Leslie, F. M. (2007). Acetaldehyde, a major constituent of tobacco smoke, enhances behavioral, endocrine, and neuronal responses to nicotine in adolescent and adult rats. *Neuropsychopharmacology* 32, 2025–2035. doi: 10.1038/sj.npp.1301327

Carson, B. P., McCormack, W. G., Conway, C., Cooke, J., Saunders, J., O'Connor, W. T., et al. (2015). An in vivo microdialysis characterization of the transient changes in the interstitial dialysate concentration of metabolites and cytokines in human skeletal muscle in response to insertion of a microdialysis probe. *Cytokine* 71, 327–333. doi: 10.1016/j.cyto.2014.10.022

Chefer, V. I., Thompson, A. C., Zapata, A., and Shippenberg, T. S. (2009). Overview of brain microdialysis. *Curr. Protoc. Neurosci.* Chapter 7:Unit7.1. doi: 10.1002/0471142301.ns0701s47

Cheng, G.-W., Hsu, K.-C., Lee, C.-F., Wu, H.-L., and Huang, Y.-L. (2009). On-line microdialysis coupled with liquid chromatography for biomedical analysis. *J. Chromatogr. Sci.* 47, 624–630. doi: 10.1093/chromsci/47.8.624

Chun, H.-J., Poklis, J. L., Poklis, A., and Wolf, C. E. (2016). Development and validation of a method for alcohol analysis in brain tissue by headspace gas chromatography with flame ionization detector. *J. Anal. Toxicol.* 40, 653–658. doi: 10.1093/jat/bkw075

Cifuentes Castro, V. H., López Valenzuela, C. L., Salazar Sánchez, J. C., Peña, K. P., López Pérez, S. J., Ibarra, J. O., et al. (2014). An update of the classical and novel methods used for measuring fast neurotransmitters during normal and brain altered function. *Curr. Neuropharmacol.* 12, 490–508. doi: 10.2174/1570159X13666141223223657

Cohen, G., Sinet, P. M., and Heikkila, R. (1980). Ethanol oxidation by rat brain in vivo. *Alcohol. Clin. Exp. Res.* 4, 366–370. doi: 10.1111/j.1530-0277.1980.tb04833.x

Cordell, R. L., Pandya, H., Hubbard, M., Turner, M. A., and Monks, P. S. (2013). GC-MS analysis of ethanol and other volatile compounds in micro-volume blood samples—quantifying neonatal exposure. *Anal. Bioanal. Chem.* 405, 4139–4147. doi: 10.1007/s00216-013-6809-1

Correa, M., Salamone, J. D., Segovia, K. N., Pardo, M., Longoni R., Spina L., et al. (2012). Piecing together the puzzle of acetaldehyde as a neuroactive agent. *Neurosci. Biobehav. Rev.* 36, 404–430. doi: 10.1016/j.neubiorev.2011.07.009

Deng, X.-S., and Ra, D. (2008). Putative role of brain acetaldehyde in ethanol addiction. *Curr. Drug Abuse Rev.* 1, 3–8. doi: 10.2174/1874473710801010003

Fein, G., and Meyerhoff, D. J. (2000). Ethanol in human brain by magnetic resonance spectroscopy: correlation with blood and breath levels, relaxation, and magnetization transfer. *Alcohol. Clin. Exp. Res.* 24, 1227–1235. doi: 10.1111/j.1530-0277.2000.tb02088.x

Ferguson, C. S., and Tyndale, R. F. (2011). Cytochrome P450 enzymes in the brain: emerging evidence of biological significance. *Trends Pharmacol. Sci.* 32, 708–714. doi: 10.1016/j.tips.2011.08.005

Ghica, M. E., Pauliukaite, R., Marchand, N., Devic, E., and Brett, C. M. A. (2007). An improved biosensor for acetaldehyde determination using a bienzymatic strategy at poly(neutral red) modified carbon film electrodes. *Anal. Chim. Acta* 591, 80–86. doi: 10.1016/j.aca.2007.03.047

Gifford, R., Kehoe, J. J., Barnes, S. L., Kornilayev, B. A., Alterman, M. A., and Wilson, G. S. (2006). Protein interactions with subcutaneously implanted biosensors. *Biomaterials* 27, 2587–2598. doi: 10.1016/j.biomaterials.2005.11.033

Gill, K., Menez, J. F., Lucas, D., and Deitrich, R. A. (1992). Enzymatic production of acetaldehyde from ethanol in rat brain tissue. *Alcohol. Clin. Exp. Res.* 16, 910–915. doi: 10.1111/j.1530-0277.1992.tb01892.x

Goullé, J.-P., and Guerbet, M. (2015). [Pharmacokinetics, metabolism, and analytical methods of ethanol]. *Ann. Pharm. Fr.* 73, 313–322. doi: 10.1016/j.pharma.2015.03.003

Guan, X., Rubin, E., and Anni, H. (2011). An optimized method for the measurement of acetaldehyde by high-performance liquid chromatography. *Alcohol. Clin. Exp. Res.* 36, 398–405. doi: 10.1111/j.1530-0277.2011.01612.x

Guihen, E., and O'Connor, W. T. (2009). Current separation and detection methods in microdialysis the drive towards sensitivity and speed. *Electrophoresis* 30, 2062–2075. doi: 10.1002/elps.200900039

Halliwell, B. (2006). Oxidative stress and neurodegeneration: where are we now? *J. Neurochem.* 97, 1634–1658. doi: 10.1111/j.1471-4159.2006.03907.x

Hansson, T., Tindberg, N., Ingelman-Sundberg, M., and Köhler, C. (1990). Regional distribution of ethanol-inducible cytochrome P450 IIE1 in the rat central nervous system. *Neuroscience* 34, 451–463. doi: 10.1016/0306-4522(90)90154-V

Hanstock, C. C., Rothman, D. L., Shulman, R. G., Novotny, E. J., Petroff, O. A., and Prichard, J. W. (1990). Measurement of ethanol in the human brain using NMR spectroscopy. *J. Stud. Alcohol* 51, 104–107. doi: 10.15288/jsa.1990.51.104

Hasan, A., Nurunnabi, M., Morshed, M., Paul, A., Polini, A., Kuila, T., et al. (2014). Recent advances in application of biosensors in tissue engineering. *Biomed Res. Int.* 2014:307519. doi: 10.1155/2014/307519

Heit, C., Dong, H., Chen, Y., Thompson, D. C., Deitrich, R. A., and Vasiliou, V. K. (2013). "The role of CYP2E1 in alcohol metabolism and sensitivity in the central nervous system," in *Cytochrome P450 2E1: Its Role in Disease and Drug Metabolism Subcellular Biochemistry*, ed. A. Dey (Dordrecht: Springer), 235–247. doi: 10.1007/978-94-007-5881-0_8

Heit, C., Eriksson, P., Thompson, D. C., Charkoftaki, G., and Fritz, K. S. (2016). Quantification of neural ethanol and acetaldehyde using headspace GC-MS. *Alcohol. Clin. Exp. Res.* 40, 1825–1831. doi: 10.1111/acer.13156

Israel, Y., Quintanilla, M. E., Karahanian, E., Rivera-Meza, M., and Herrera-Marschitz, M. (2015). The "first hit" toward alcohol reinforcement: role of ethanol metabolites. *Alcohol. Clin. Exp. Res.* 39, 776–786. doi: 10.1111/acer.12709

Israel, Y., Rivera-Meza, M., Karahanian, E., Quintanilla, M. E., Tampier, L., Morales, P., et al. (2012). Gene specific modifications unravel ethanol and acetaldehyde actions. *Front. Behav. Neurosci.* 7:80. doi: 10.3389/fnbeh.2013.00080

Jamal, M., Ameno, K., Kumihashi, M., Ameno, S., Kubota, T., Wang, W., et al. (2003). Microdialysis for the determination of acetaldehyde and ethanol concentrations in the striatum of freely moving rats. *J. Chromatogr. B Analyt. Technol. Biomed. Life Sci.* 798, 155–158. doi: 10.1016/j.jchromb.2003.09.015

Jamal, M., Ameno, K., Tanaka, N., Ito, A., Takakura, A., Kumihashi, M., et al. (2015). Ethanol and acetaldehyde after intraperitoneal administration to Aldh2-knockout mice-reflection in blood and brain levels. *Neurochem. Res.* 41, 1029–1034. doi: 10.1007/s11064-015-1788-6

Jamal, M., Ameno, K., Uekita, I., Kumihashi, M., Wang, W., and Ijiri, I. (2007). Catalase mediates acetaldehyde formation in the striatum of free-moving rats. *Neurotoxicology* 28, 1245–1248. doi: 10.1016/j.neuro.2007.05.002

Karahanian, E., Quintanilla, M. E., Tampier, L., Rivera-Meza, M., Bustamante, D., Gonzalez-Lira, V., et al. (2011). Ethanol as a prodrug: brain metabolism of ethanol mediates its reinforcing effects. *Alcohol. Clin. Exp. Res.* 35, 606–612. doi: 10.1111/j.1530-0277.2011.01439.x

Kaufman, M. J., Chiu, T.-M., Mendelson, J. H., Woods, B. T., Mello, N. K., Lukas, S. E., et al. (1994). *In vivo* proton magnetic resonance spectroscopy of alcohol in rhesus monkey brain. *Magn. Resonan. Imaging* 12, 1245–1253. doi: 10.1016/0730-725X(94)90089-A

Kennedy, R. T. (2013). Emerging trends in in vivo neurochemical monitoring by microdialysis. *Curr. Opin. Chem. Biol.* 17, 860–867. doi: 10.1016/j.cbpa.2013.06.012

Kerr, J. T., Maxwell, D. S., and Crabb, D. W. (1989). Immunocytochemistry of alcohol dehydrogenase in the rat central nervous system. *Alcohol. Clin. Exp. Res.* 13, 730–736. doi: 10.1111/j.1530-0277.1989.tb00412.x

Kotanen, C. N., Moussy, F. G., Carrara, S., and Guiseppi-Elie, A. (2012). Implantable enzyme amperometric biosensors. *Biosens. Bioelectron.* 35, 14–26. doi: 10.1016/j.bios.2012.03.016

Kristoffersen, L., and Smith-Kielland, A. (2005). An automated alcohol dehydrogenase method for ethanol quantification in urine and whole blood. *J. Anal. Toxicol.* 29, 387–389. doi: 10.1093/jat/29.5.387

Ledesma, J. C., Miquel, M., Pascual, M., Guerri, C., and Aragon, C. M. G. (2014). Induction of brain cytochrome P450 2E1 boosts the locomotor-stimulating effects of ethanol in mice. *Neuropharmacology* 85, 36–44. doi: 10.1016/j.neuropharm.2014.05.018

Lorenzo, E., Pariente, F., Hernàndez, L., Tobalina, F., Darder, M., Wu, Q., et al. (1998). Analytical strategies for amperometric biosensors based on chemically modified electrodes. *Biosens. Bioelectron.* 13, 319–332. doi: 10.1016/S0956-5663(97)00138-3

Mori, O., Haseba, T., Kameyama, K., Shimizu, H., Kudoh, M., Ohaki, O., et al. (2000). Histological distribution of class III alcohol dehydrogenase in human brain. *Brain Res.* 852, 186–190. doi: 10.1016/S0006-8993(99)02201-5

Nagel, B. J., and Kroenke, C. D. (2008). The use of magnetic resonance spectroscopy and magnetic resonance imaging in alcohol research. *Alcohol Res. Health* 31, 243–246.

Nichols, S. P., Koh, A., Storm, W. L., Shin, J. H., and Schoenfisch, M. H. (2013). Biocompatible materials for continuous glucose monitoring devices. *Chem. Rev.* 113, 2528–2549. doi: 10.1021/cr300387j

Niciu, M. J., and Mason, G. F. (2014). Neuroimaging in alcohol and drug dependence. *Curr. Behav. Neurosci. Rep.* 1, 45–54. doi: 10.1007/s40473-013-0005-7

Noguer, T., and Marty, J. L. (1997). Reagentless sensors for acetaldehyde. *Anal. Lett.* 30, 1069–1080. doi: 10.1016/j.talanta.2008.04.040

Noguer, T., Tencaliec, A., Calas-Blanchard, C., Avramescu, A., and Marty, J.-L. (2001). Interference-free biosensor based on screen-printing technology and sol-gel immobilization for determination of acetaldehyde in wine. *J. AOAC Int.* 85, 1382–1389.

Olivo, J., Carrara, S., and De Micheli, G. (2011). Energy harvesting and remote powering for implantable biosensors. *IEEE Sens. J.* 11, 1573–1586. doi: 10.1109/JSEN.2010.2085042

Peana, A. T., Porcheddu, V., Bennardini, F., Carta, A., Rosas, M., and Acquas, E. (2015). Role of ethanol-derived acetaldehyde in operant oral self-administration of ethanol in rats. *Psychopharmacology* 232, 4269–4276. doi: 10.1007/s00213-015-4049-0

Pellegrino, S., Bruno, F. S., and Petrarulo, M. (1999). Liquid chromatographic determination of ethyl alcohol in body fluids. *J. Chromatogr. B Biomed. Sci. Appl.* 729, 103–110. doi: 10.1016/S0378-4347(99)00131-0

Peris, J., Zharikova, A., Li, Z., Lingis, M., MacNeill, M., Wu, M. T., et al. (2006). Brain ethanol levels in rats after voluntary ethanol consumption using a sweetened gelatin vehicle. *Pharmacol. Biochem. Behav.* 85, 562–568. doi: 10.1016/j.pbb.2006.10.010

Quintanilla, M. E., Tampier, L., Karahanian, E., Rivera-Meza, M., Herrera-Marschitz, M., and Israel, Y. (2012). Reward and relapse: complete gene-induced dissociation in an animal model of alcohol dependence. *Alcohol. Clin. Exp. Res.* 36, 517–522. doi: 10.1111/j.1530-0277.2011.01606.x

Rooney, W. D., Lee, J. H., Li, X., Wang, G. J., Franceschi, D., Springer, C. S., et al. (2000). 4.0 T water proton T1 relaxation times in normal human brain and during acute ethanol intoxication. *Alcohol. Clin. Exp. Res.* 24, 830–836. doi: 10.1111/j.1530-0277.2000.tb02062.x

Rotzinger, S., Smith, B. R., and Amit, Z. (1994). Catalase inhibition attenuates the acquisition of ethanol and saccharin-quinine consumption in laboratory rats. *Behav. Pharmacol.* 5, 203–209. doi: 10.1097/00008877-199404000-00012

Sammi, M. K., Pan, J. W., Telang, F. W., Schuhlein, D., Molina, P. E., Volkow, N. D., et al. (2000). Measurements of human brain ethanol T2 by spectroscopic imaging at 4T. *Magn. Reson. Med.* 44, 35–40. doi: 10.1002/1522-2594(200007)44:1<35::AID-MRM7<3.0.CO;2-G

Sánchez-Catalán, M. J., Hipólito, L., Guerri, C., Granero, L., and Polache, A. (2008). Distribution and differential induction of CYP2E1 by ethanol and acetone in the mesocorticolimbic system of rat. *Alcohol Alcohol.* 43, 401–407. doi: 10.1093/alcalc/agn012

Saxena, U., and Das, A. B. (2016). Nanomaterials towards fabrication of cholesterol biosensors: Key roles and design approaches. *Biosens. Bioelectron.* 75, 196–205. doi: 10.1016/j.bios.2015.08.042

Sirca, D., Vardeu, A., Pinna, M., Diana, M., and Enrico, P. (2014). A robust, state-of-the-art amperometric microbiosensor for glutamate detection. *Biosens. Bioelectron.* 61C, 526–531. doi: 10.1016/j.bios.2014.04.054

Soares, D. P., and Law, M. (2009). Magnetic resonance spectroscopy of the brain: review of metabolites and clinical applications. *Clin. Radiol.* 64, 12–21. doi: 10.1016/j.crad.2008.07.002

Strózik-Kotlorz, D. (2014). Magnetic resonance spectroscopy of the human brain. *Phys. Part. Nuclei* 45, 347–348. doi: 10.1134/S1063779614010997

Takeuchi, T., Murase, K., and Ishii, D. (1988). Determination of alcohols in alcoholic beverages by micro high-performance liquid chromatography with indirect photometric detection. *J. Chromatogr.* 445, 139–144. doi: 10.1016/S0021-9673(01)84515-5

Tampier, L., Quintanilla, M. E., Karahanian, E., Rivera-Meza, M., Herrera-Marschitz, M., and Israel, Y. (2013). The alcohol deprivation effect: marked inhibition by anticatalase gene administration into the ventral tegmental area in rats. *Alcohol. Clin. Exp. Res.* 37, 1278–1285. doi: 10.1111/acer.12101

Tampier, L., Quintanilla, M. E., and Mardones, J. (1995). Effects of aminotriazole on ethanol, water, and food intake and on brain catalase in UChA and UChB rats. *Alcohol* 12, 341–344. doi: 10.1016/0741-8329(95)00014-I

Thévenot, D. R., Toth, K., Durst, R. A., and Wilson, G. S. (2001). Electrochemical biosensors: recommended definitions and classification. *Biosens. Bioelectron.* 16, 121–131. doi: 10.1016/S0956-5663(01)00115-4

Timmerman, W., and Westerink, B. H. (1997). Brain microdialysis of GABA and glutamate: what does it signify? *Synapse* 27, 242–261.

Tindberg, N., and Ingelman-Sundberg, M. (1996). Expression, catalytic activity, and inducibility of cytochrome P450 2E1 (CYP2E1) in the rat central nervous

system. *J. Neurochem.* 67, 2066–2073. doi: 10.1046/j.1471-4159.1996.67052066.x

Tiscione, N. B., Alford, I., Yeatman, D. T., and Shan, X. (2011). Ethanol analysis by headspace gas chromatography with simultaneous flame-ionization and mass spectrometry detection. *J. Anal. Toxicol.* 35, 501–511. doi: 10.1093/anatox/35.7.501

Vigneshvar, S., Sudhakumari, C. C., Senthilkumaran, B., and Prakash, H. (2015). Recent advances in biosensor technology for potential applications - An overview. *Front. Bioeng. Biotechnol.* 4:11. doi: 10.3389/fbioe.2016.00011

Vijayakumar, A. R., Csöregi, E., Heller, A., and Gorton, L. (1996). Alcohol biosensors based on coupled oxidase-peroxidase systems. *Anal. Chim. Acta* 327, 223–234. doi: 10.1016/0003-2670(96)00093-1

Vogel, M., Büldt, A., and Karst, U. (2000). Hydrazine reagents as derivatizing agents in environmental analysis–a critical review. *Fresenius J. Anal. Chem.* 366, 781–791. doi: 10.1007/s002160051572

Wahono, N., Qin, S., Oomen, P., Cremers, T. I. F., de Vries, M. G., and Westerink, B. H. C. (2012). Evaluation of permselective membranes for optimization of intracerebral amperometric glutamate biosensors. *Biosens. Bioelectron.* 33, 260–266. doi: 10.1016/j.bios.2012.01.019

Wang, J., Du, H., Jiang, L., Ma, X., de Graaf, R. A., Behar, K. L., et al. (2013a). Oxidation of ethanol in the rat brain and effects associated with chronic ethanol exposure. *Proc. Natl. Acad. Sci. U.S.A.* 110, 14444–14449. doi: 10.1073/pnas.1306011110

Wang, J., Du, H., Ma, X., Pittman, B., Castracane, L., Li, T.-K., et al. (2013b). Metabolic products of [2-(13) C]ethanol in the rat brain after chronic ethanol exposure. *J. Neurochem.* 127, 353–364. doi: 10.1111/jnc.12405

Warner, M., and Gustafsson, J. A. (1994). Effect of ethanol on cytochrome P450 in the rat brain. *Proc. Natl. Acad. Sci. U.S.A.* 91, 1019–1023. doi: 10.1073/pnas.91.3.1019

Weltin, A., Kieninger, J., Enderle, B., Gellner, A.-K., Fritsch, B., and Urban, G. A. (2014). Polymer-based, flexible glutamate and lactate microsensors for in vivo applications. *Biosens. Bioelectron.* 61, 192–199. doi: 10.1016/j.bios.2014.05.014

Weltin, A., Kieninger, J., and Urban, G. A. (2016). Microfabricated, amperometric, enzyme-based biosensors for in vivo applications. *Anal. Bioanal. Chem.* 408, 4503–4521. doi: 10.1007/s00216-016-9420-4

Westerink, B. H. (1995). Brain microdialysis and its application for the study of animal behaviour. *Behav. Brain Res.* 70, 103–124. doi: 10.1016/0166-4328(95)80001-8

Wilson, G. S., and Gifford, R. (2004). Biosensors for real-time in vivo measurements. *Biosens. Bioelectron.* 20, 2388–2403. doi: 10.1016/j.bios.2004.12.003

Xiang, Y., and Shen, J. (2011). *In vivo* detection of intermediate metabolic products of [1-13C]ethanol in the brain using 13C MRS. *NMR Biomed.* 24, 1054–1062. doi: 10.1002/nbm.1653

Xiao, H.-T., He, L., Tong, R.-S., Yu, J.-Y., Chen, L., Zou, J., et al. (2014). Rapid and sensitive headspace gas chromatography-mass spectrometry method for the analysis of ethanol in the whole blood. *J. Clin. Lab. Anal.* 28, 386–390. doi: 10.1002/jcla.21698

Yao, T., and Handa, S. (2003). Electroanalytical properties of aldehyde biosensors with a hybrid-membrane composed of an enzyme film and a redox Os-polymer film. *Anal. Sci.* 19, 767–770. doi: 10.2116/analsci.19.767

Yarita, T., Nakajima, R., Otsuka, S., Ihara, T. A., Takatsu, A., and Shibukawa, M. (2002). Determination of ethanol in alcoholic beverages by high-performance liquid chromatography-flame ionization detection using pure water as mobile phase. *J. Chromatogr. A* 976, 387–391. doi: 10.1016/S0021-9673(02)00942-1

Yoshimoto, K., and Komura, S. (1993). Monitoring of ethanol levels in the rat nucleus accumbens by brain microdialysis. *Alcohol Alcohol.* 28, 171–174.

Zachut, M., Shapiro, F., and Silanikove, N. (2016). Detecting ethanol and acetaldehyde by simple and ultrasensitive fluorimetric methods in compound foods. *Food Chem.* 201, 270–274. doi: 10.1016/j.foodchem.2016.01.079

Zahr, N. M., Mayer, D., Rohlfing, T., Hasak, M. P., Hsu, O., Vinco, S., et al. (2010). Brain injury and recovery following binge ethanol: evidence from in vivo magnetic resonance spectroscopy. *Biol. Psychiatry* 67, 846–854. doi: 10.1016/j.biopsych.2009.10.028

Ziegler, T. L., Bludeau, P., Petersen, D. R., Gonzalez, F. J., and Deitrich, R. A. (2006). CYP2E1 and catalase influence ethanol sensitivity in the central nervous system. *Pharmacogenet. Genomics* 16, 51–58. doi: 10.1097/01.fpc.0000182777.95555.56

Zimatkin, S. M., Pronko, S. P., Gonzalez, F. J., and Deitrich, R. A. (2006). Enzymatic mechanisms of ethanol oxidation in the brain. *Alcohol. Clin. Exp. Res.* 30, 1500–1505. doi: 10.1111/j.1530-0277.2006.00181.x

Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

frontiers in Behavioral Neuroscience

ORIGINAL RESEARCH
published: 31 January 2017
doi: 10.3389/fnbeh.2017.00014

The Role of Acetaldehyde in the Increased Acceptance of Ethanol after Prenatal Ethanol Exposure

*Mirari Gaztañaga[1], Asier Angulo-Alcalde[1], Norman E. Spear[2] and M. Gabriela Chotro[1]**

[1]Departamento de Procesos Psicológicos Básicos y su Desarrollo, Facultad de Psicología, University of the Basque Country UPV/EHU, Donostia-San Sebastián, Gipuzkoa, Spain, [2]Department of Psychology, Centre for Development and Behavioral Neuroscience, Binghamton University, Binghamton, NY, USA

Recent studies show that acetaldehyde, the first metabolite in the oxidation of ethanol, can be responsible for both, the appetitive and the aversive effects produced by ethanol intoxication. More specifically, it has been hypothesized that acetaldehyde produced in the periphery by the liver is responsible for the aversive effects of ethanol, while the appetitive effects relate to the acetaldehyde produced centrally through the catalase system. On the other hand, from studies in our and other laboratories, it is known that ethanol exposure during the last gestational days (GD) consistently enhances the postnatal acceptance of ethanol when measured during early ontogeny in the rat. This increased liking of ethanol is a conditioned appetitive response acquired by the fetus by the association of ethanol's flavor and an appetitive reinforcer. Although this reinforcer has not yet been fully identified, one possibility points to acetaldehyde produced centrally in the fetus as a likely candidate. This hypothesis is supported by data showing that very early in the rat's ontogeny brain catalases are functional, while the liver's enzymatic system is still immature. In this study, rat dams were administered on GD 17–20 with water or ethanol, together with an acetaldehyde-sequestering agent (D-penicillamine). The offspring's responses to ethanol was then assessed at different postnatal stages with procedures adequate for each developmental stage: on day 1, using the "odor crawling locomotion test" to measure ethanol's odor attractiveness; on day 5, in an operant conditioning procedure with ethanol as the reinforcer; and on day 14 in an ethanol intake test. Results show that the absence of acetaldehyde during prenatal ethanol exposure impeded the observation of the increased acceptance of ethanol at any age. This seems to confirm the crucial role of acetaldehyde as a reinforcer in the appetitive learning occurring during prenatal ethanol exposure.

Keywords: prenatal ethanol, acetaldehyde, odor attractiveness, ethanol intake, operant conditioning, infant rat

Edited by:
Merce Correa,
Jaume I University, Spain

Reviewed by:
Mark Stanton,
University of Delaware, USA
Paula Abate,
Instituto de Investigacion Medica Mercedes y Martin Ferreyra, Argentina

***Correspondence:**
M. Gabriela Chotro
g.chotro@ehu.eus

Received: *07 December 2016*
Accepted: *16 January 2017*
Published: *31 January 2017*

Citation:
Gaztañaga M, Angulo-Alcalde A, Spear NE and Chotro MG (2017) The Role of Acetaldehyde in the Increased Acceptance of Ethanol after Prenatal Ethanol Exposure. Front. Behav. Neurosci. 11:14. doi: 10.3389/fnbeh.2017.00014

INTRODUCTION

As in most altricial mammals, the near-term fetus of the rat has the capacity to perceive chemosensory stimuli present in its environment, as well as to respond to such stimuli and to modify this response as a function of experience, i.e., it has the ability to learn about these stimuli (Pedersen et al., 1986; Smotherman and Robinson, 1988; Mickley et al., 2000). Clear evidence exists about prenatal learning with chemosensory stimuli, from relatively simple forms of learning

such as habituation and sensitization, to appetitive and aversive Pavlovian conditioning (Stickrod et al., 1982; Smotherman and Robinson, 1992; Chotro and Spear, 1997; Mickley et al., 2014). Considering these fetal capacities, along with the fact that the fetus can be exposed in the amniotic environment to chemosensory stimuli derived from the maternal diet, learning about those stimuli is expected to regularly occur. This prenatal learning has been shown to play an important role in the establishment and control of postnatal feeding and social behaviors in rats and other mammals (Robinson and Méndez-Gallardo, 2010).

One of the substances delivered to the fetus and amniotic fluid through the maternal diet is ethanol, which in addition to its pharmacological effects, it has a distinctive flavor (i.e., the integration of gustatory, olfactory and trigeminal or irritant components). When the pregnant mother consumes ethanol, this relatively small molecule passes directly through the placenta, reaching the fetal blood at similar levels to those found in maternal plasma (Szeto, 1989; Hayashi et al., 1991). From fetal circulation, ethanol is eliminated primarily through maternal metabolism, and it accumulates in the amniotic fluid, reaching higher levels than in maternal blood and taking longer to be eliminated (Guerri and Sanchis, 1985; Hayashi et al., 1991). Hence, after maternal ethanol ingestion, the fetus is exposed to the pharmacological effects of the drug as well as its chemosensory properties. Many studies with rodents have demonstrated that ethanol exposure during the entire gestation induces increased intake of ethanol after birth (Chotro et al., 2007). This effect has also been reliably found when the drug is administered exclusively during the final days of pregnancy, on gestational days (GD) 17–20 (for example, Domínguez et al., 1998; Chotro and Arias, 2003) or even during GD 19–20 (Díaz-Cenzano and Chotro, 2010; Díaz-Cenzano et al., 2014). It has also been demonstrated that the effect of increased ethanol intake is accompanied by an enhanced palatability of the flavor of ethanol (Arias and Chotro, 2005a,b). In addition, it has been found that the studied effect is mediated primarily by the endogenous opioid system (Chotro and Arias, 2003; Arias and Chotro, 2005a; Youngentob et al., 2012). At this point, we are thus able to conclude that after maternal ethanol ingestion, the rat fetus acquires a conditioned response to the chemosensory properties of ethanol, associating these properties with an appetitive reinforcer whose effects are mediated by the endogenous opioid system. Nevertheless, since the identity of the reinforcer activating the opioid system was unclear, this has been investigated by examining the role of two potential candidates: the amniotic fluid and its component "KIF" which stimulates the fetal kappa opioid-receptor system (Robinson and Méndez-Gallardo, 2010), or the pharmacological effects of ethanol on the mu-opioid receptor system. The results of those studies prompted us to discard the proposed effects of the amniotic fluid on the opioid system as the positive reinforcer; the pharmacological effects of ethanol on the mu-opioid receptor system were instead found to be crucial for the observation of the increased acceptance of ethanol after its prenatal exposure (Gaztañaga et al., 2015).

Having confirmed this possibility, the question arose as to whether the actual reinforcer was ethanol itself, or the effect of its first metabolite, acetaldehyde. Based on a growing body of literature highlighting the importance of acetaldehyde as the active molecule underpinning most of the pharmacological and behavioral effects of ethanol, we decided to investigate the role of this metabolite in the effect of postnatal enhanced preference for ethanol after prenatal ethanol exposure. In both humans and rats it is well documented that, following its consumption, ethanol is converted into acetaldehyde, both peripherally and centrally. Peripherally (predominantly in the liver) there are two main enzymatic oxidative systems that convert ethanol into acetaldehyde: the principal way is through ethanol dehydrogenase (ADH), and the second involves the cytochrome P450 2E1 (CYP2E1; Hipólito et al., 2007). In the brain, however, the system responsible for generating the majority of acetaldehyde from ethanol (approximately 60%) is the catalase system, even though CYP2E1 is also centrally active, producing around 20% of acetaldehyde (Zimatkin et al., 2006; Hipólito et al., 2007). Several studies in which the enzymatic production and degradation of acetaldehyde was manipulated in adult rats, demonstrated the role of acetaldehyde in the pharmacological and behavioral effects of ethanol. On the basis of these results, it was deduced that peripherally and centrally produced acetaldehyde has distinct and opposing behavioral effects. Acetaldehyde in the peripheral circuit has primarily aversive consequences (Quertemont and Tambour, 2004), whereas in the brain it appears to exert reinforcing effects (Wall et al., 1992; Hahn et al., 2006; for a complete review see Correa et al., 2012). Thus, the balance between acetaldehyde in the periphery and in the brain after ethanol ingestion would determine the observed effects of ethanol intoxication, and would therefore modulate the acceptance and consumption of this drug.

The few studies conducted during the early ontogeny of the rat have shown that acetaldehyde is produced in the newborn brain by the catalase system (Hamby-Mason et al., 1997) and is responsible for the reinforcing effects of ethanol when administered centrally to the rat neonate (Nizhnikov et al., 2007; March et al., 2013). It has also been shown that catalase activity in the fetal and neonatal brain is 4.5 times higher than in adults (Hamby-Mason et al., 1997). On the other hand, due to the practical absence of ADH in the fetal liver, the fetus does not produce peripheral acetaldehyde, and elimination of ethanol critically depends on the maternal metabolism (Hayashi et al., 1991; Boleda et al., 1992). In addition, the placenta protects the fetus from peripheral acetaldehyde produced by the mother's liver, particularly after ingestion of low to moderate doses of ethanol (Guerri and Sanchis, 1985; Hayashi et al., 1991). This could explain previous results in which an ethanol aversion was observed in pregnant dams administered with a relatively high ethanol dose (3 g/kg) while the offspring showed the opposite, i.e., increased acceptance and liking of ethanol's flavor (Chotro et al., 2009).

Taken together, these findings support the hypothesis that after maternal consumption of ethanol the fetus is exposed

to the chemosensory aspects of ethanol together with the reinforcing effects of central acetaldehyde, in the absence of the potentially aversive effects of peripheral acetaldehyde. This would promote the prenatal appetitive learning that results in postnatal enhanced ethanol acceptance, and consequently, this appetitive response would not be observed in the absence of prenatal acetaldehyde. This hypothesis has been tested in three experiments, by administering to the pregnant dam an acetaldehyde-sequestering agent (D-penicillamine), together with ethanol, and testing the offspring at various postnatal stages (postnatal days, PD 1, 5 and 14) using different procedures according to the developmental capacities of the pups.

MATERIALS AND METHODS

Subjects

In all experiments, Sprague-Dawley pregnant rats and their offspring were used. The subjects from Experiments 1 and 3 were born and reared in the vivarium of the University of the Basque Country UPV/EHU, Spain. The conditions of the colony room were 12-h light/12-h dark illumination cycle (light onset at 8:00 am), with controlled temperature (21–23°C) and humidity (50–60%). Female adult rats were time-mated to provide subjects for all experiments, and the presence of sperm in vaginal smears was considered as GD 0. Pregnant females were housed in pairs in maternity cages, with access to food and filtered tap water, and remained undisturbed until the beginning of the treatments on GD 17. The dams received treatments from GD 17 to GD 20, and were then housed individually, where they remained undisturbed for parturition (GD 22). The maternity cages were checked daily for births, from 9:00–14:00, and if positive, this was considered as postnatal day 0 (PD 0).

Experiment 2 was conducted in the Centre for Development and Behavioral Neuroscience, Department of Psychology, Binghamton University, Binghamton, NY, USA. Conditions of the vivarium and laboratory (AAALAC-accredited facility, Binghamton University, Binghamton, NY, USA) were similar to those described for the Spanish facilities.

The number of pups employed in each experiment was as follows: for Experiment 1, 144 1-day old pups derived from 24 litters; for Experiment 2, 160 5-day old pups derived from 20 litters; and for Experiment 3, 40 14-day old pups derived from 20 litters.

For Experiments 1 and 3, European regulations for the care and treatment of experimental animals were followed, and procedures were controlled and approved by the "Ethics and Animal Care Committee" at the University of Basque Country UPV/EHU (CEBA) and the Diputación Foral de Guipuzkoa, Spain, in compliance with the European Communities Council Directive (86/609/EEC). Experiment 2 was approved by the Binghamton University Institutional Review Committee for the Use of Animal Subjects and was in compliance with the NIH Guide for the Care and Use of Laboratory Animals (National Institutes of Health, 1996).

Procedures

Prenatal Treatments

In the three experiments of this study, pregnant rats were treated once per day from GD 17 to GD 20. There were two prenatal treatments: Prenatal DP and Prenatal EtOH, which consisted of a subcutaneous injection of D-penicillamine (DP) or saline, followed by an intragastric administration of ethanol or water, respectively. In Experiment 1 (but not in Experiments 2 and 3) a third substance (vanilla, administered intragastrically) served as a further control, based on previous results showing that vanilla prenatal exposure increases attraction for this odor on PD 1, but not on PD 5 or 14 (Gaztañaga et al., 2015). However, in order to maintain consistency between experiments, the variable was still referred to as "Prenatal EtOH".

On each treatment day the dams were removed from their home cages, marked on the tail for identification, and weighed. After being weighed, all the rats received a subcutaneous injection in the area of the neck, of either D-penicillamine (50 mg/kg) or saline (0.9% NaCl in distilled water). In all cases the volume of injection was equivalent to 0.7 μl/g of a solution of 7.5 g of D-penicillamine in 100 ml of saline. The D-penicillamine dose was selected based on previous studies in adults and infant rats in which the reinforcing effects of ethanol were effectively reduced (Font et al., 2006; Pautassi et al., 2011). Thirty minutes later the dams received an intragastric administration of the corresponding substance: ethanol, water (or vanilla, In experiment 1). The intragastric administration was performed using a 15-cm length of polyethylene tubing (PE-50 Clay Adams, Parsippany, NJ, USA) attached to a 10 ml syringe with a 24-gauge needle. The tubing was gently inserted through the mouth and slowly pushed into the stomach. The entire procedure took approximately 15 s per rat. The ethanol dose administered was 2 g/kg and resulted from the administration of a volume equivalent to 0.015 ml/g of a 16.8% v/v ethanol solution in filtered water. The control dams received a similar volume of filtered water. In Experiment 1, vanilla was administered in a 50-mg/kg dose of a solution of 500 mg% of vanillin (Sigma Aldrich) in filtered water; the administered volume was equivalent to 0.01 ml/g of body weight. After each day of treatment the dams were returned to their home-cages. Following the final treatment (GD 20) the dams remained undisturbed for parturition.

Postnatal Tests

The postnatal behavior of the pups was evaluated at different ages using a range of techniques appropriate for each developmental stage: odor-induced crawling locomotion test on PD 1 (Experiment 1), operant conditioning on PD 5 (Experiment 2), and an intake test on PD 14 (Experiment 3).

Odor-induced crawling locomotion test (Experiment 1)

Crawling is a very unique behavior that is only displayed shortly after birth. This technique was used as a measure of performance exclusively in PD1 neonates, and was adapted from the procedure described by Mendez-Gallardo and Robinson (2014). A female and a male from each litter were tested with only one odor

of the motor activity rates of the subjects. This allowed us to familiarize subjects with the reinforcer whilst minimally stimulating head and body movements. The only difference between the training and extinction session was that the infusion pump was turned off and the subjects did not receive the solution when P subjects touched the sensor, but the number of sensor contacts was recorded. Substances infused intraorally were either an ethanol solution (6% v/v in filtered water) or a saccharin solution (0.05 mg % v/v in filtered water). The concentration of these substances was selected from a previous study in which optimal learning curves at this early age were obtained with these parameters (Miranda-Morales et al., 2014).

Intake test on PD 14 (Experiment 3)

A female and a male from each litter were evaluated on two consecutive intake tests, one of water and one of ethanol, both separated by a 1-h interval. At the beginning of the procedure pups were separated from their mother, marked on the tail for identification, and cannulated following the procedure described previously. After cannulation, the subjects were grouped according to litter in heated holding chambers (15 × 8 × 15 cm) for 1 h before the test. A few minutes before the test, the pups' bladders were voided by gently brushing the anogenital area, and body weights were then registered. The pups were then tested in individual clear plastic chambers (8 × 8 × 25 cm). Each subject's intraoral cannula was connected using a polyethylene tube PE-50 to the syringes placed in an automated pump (KDS Scientific). This pump was scheduled to administer the different fluids at a rate of 0.1 ml/min per infusion for 15 min (i.e., 1.5 ml of the given substance) with a continuous flow. In all cases pups could either consume or reject the infused fluid during the test. At the end of the water test, post-infusion weights were registered and pups returned to the holding chambers. One hour later these procedures were repeated for the ethanol intake test. Intake of water and ethanol was calculated using pre and post-infusion body weights and expressed as a percentage of body weight gained (% BWG). At the end of the procedure, the cannulas were removed and the pups were returned to their home cages.

Data Analysis

The data from each experiment were analyzed using factorial ANOVAs, and significant main effects and interactions between variables were further explored with Duncan's *post hoc* tests. The experimental design for each experiment is described in the results section. The alpha level was set *a priori* at $p < 0.05$ for all analyses.

RESULTS

Experiment 1. Odor-Induced Crawling Locomotion Test

In this first experiment newborn rats were tested for their attraction to ethanol odor as a function of their prenatal experience with ethanol and the concomitant presence of acetaldehyde. The 3 × 2 × 3 factorial design for this experiment resulted in 18 groups defined by the Prenatal EtOH (ethanol, vanilla, or water), the Prenatal DP (DP or saline), and the Test odor (ethanol, vanilla, or water). The factorial ANOVA conducted on the test data revealed significant effects of Prenatal EtOH $F_{(2,126)} = 14.55$, $p < 0.001$, and Test odor $F_{(2,126)} = 28.73$, $p < 0.001$, as well as an interaction between these two variables $F_{(4,126)} = 24.03$, $p < 0.001$, and between Prenatal EtOH and Prenatal DP $F_{(2,126)} = 6.43$, $p < 0.005$. Of more interest, however, for the aim of this study was the significant three-way interaction Prenatal EtOH × Prenatal DP × Test odor, $F_{(4,126)} = 5.90$, $p < 0.001$. The *post hoc* analysis of this interaction revealed that Group ethanol-saline-ethanol was more attracted to the ethanol odor than water-saline-ethanol and vanilla-saline-ethanol groups, as well as the Group ethanol-DP-ethanol, which did not display any attraction to ethanol odor. This suggests that D-penicillamine treatment impeded the observation of the increased acceptance for ethanol after its prenatal exposure. These analyses also revealed that Groups vanilla-saline-vanilla and vanilla-DP-vanilla did not differ from each other, but both crawled for longer towards the vanilla odor than their corresponding controls (water-saline-vanilla, ethanol-saline-vanilla, water-DP-vanilla or ethanol-DP-vanilla). This indicates that prenatal exposure to vanilla induced an enhanced attraction to this odor immediately after birth, an effect that was not modified by the prenatal treatment with D-penicillamine (**Figure 1**).

Experiment 2. Operant Conditioning on PD 5

The experimental design resulted in eight groups defined by Prenatal EtOH (ethanol or water), Prenatal DP (DP or saline), and Conditioning (P or Y). The resulting groups were referred to as: ethanol-DP-P, ethanol-DP-Y, ethanol-saline-P, ethanol-saline-Y, water-DP-P, water-DP-Y, water-saline-P, and water-saline-Y. Half of the pups in each group were tested with saccharin as the reinforcer and the other half with ethanol. The dependent variable analyzed was total number of sensor touches. The data obtained in both training and extinction sessions with both test substances (saccharin and ethanol) were analyzed separately with 4 factorial ANOVAs (2 × 2 × 2).

The ANOVA with the data from the training with saccharin as the reinforcer indicated significant main effects of Prenatal EtOH, $F_{(1,72)} = 5.60$, $p < 0.05$; Prenatal DP $F_{(1,72)} = 5.23$, $p < 0.05$, and Conditioning $F_{(1,72)} = 6.84$, $p < 0.05$. Although no interactions between these variables were observed (data not shown). Means ± SEM: ethanol-DP-P, 3.50 ± 0.50; ethanol-DP-Y, 1.63 ± 0.60; ethanol-saline-P, 6.40 ± 1.54; ethanol-saline-Y, 2.90 ± 0.95; water-DP-P, 2.40 ± 0.65; water-DP-Y, 1.44 ± 0.53; water-saline-P, 2.42 ± 0.47; and water-saline-Y, 2.62 ± 0.66. *Post hoc* tests revealed that pups from mothers treated with water responded less than pups from ethanol treated dams. Also that groups treated with DP responded less than groups treated with saline. Finally, Paired subjects responded more than their respective Yoked controls, independent from

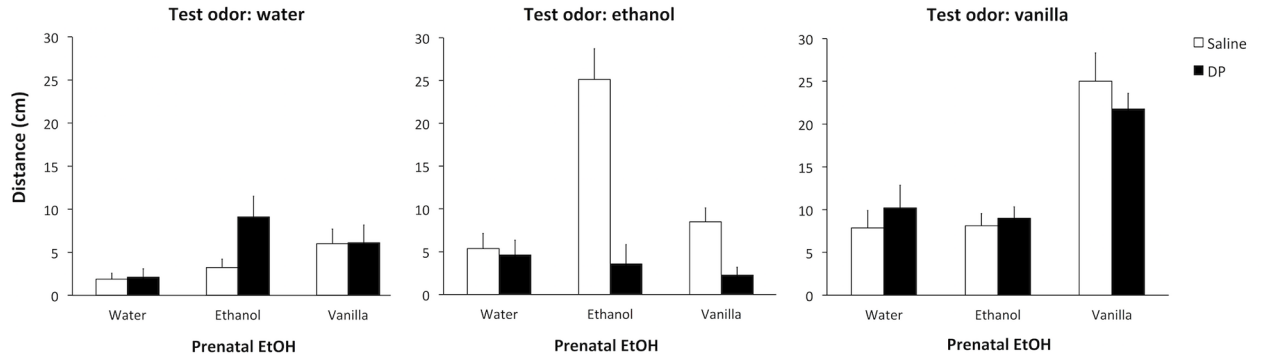

FIGURE 1 | Distance crawled (cm) towards water as a function of Prenatal DP (saline or DP), Prenatal EtOH (water, ethanol or vanilla) and the Test odor (water, ethanol or vanilla).

both prenatal treatments. During the extinction phase with saccharin neither significant main effects nor interactions between variables were observed. Means ± SEM: ethanol-DP-P, 0.75 ± 0.42; ethanol-DP-Y, 1.50 ± 0.57; ethanol-saline-P, 2.50 ± 0.83; ethanol-saline-Y, 2.00 ± 1.13; water-DP-P, 0.90 ± 0.28; water-DP-Y, 2.00 ± 0.88; water-saline-P, 0.75 ± 0.35; and water-saline-Y, 0.77 ± 0.36.

The factorial ANOVA on the data from the training session with ethanol as the reinforcer (**Figure 2A**) revealed significant main effects of Prenatal DP $F_{(1,72)} = 10.73$, $p < 0.001$, and Conditioning $F_{(1,72)} = 26.50$, $p < 0.001$. The following interactions were also significant: Prenatal DP × Conditioning $F_{(1,72)} = 13.13$, $p < 0.001$; Prenatal EtOH × Conditioning $F_{(1,72)} = 7.49$, $p < 0.001$; and Prenatal EtOH × Prenatal DP × Conditioning $F_{(1,72)} = 5.69$, $p < 0.01$. Subsequent analyses of this 3-way interaction revealed that ethanol-saline-P subjects responded significantly more when ethanol was the reinforcer than their ethanol-saline-Y controls ($p < 0.001$), and also responded significantly more than Groups water-saline-P, and ethanol-DP-P. In the extinction phase with ethanol (**Figure 2B**), the ANOVA revealed a significant effect of Prenatal EtOH $F_{(1,72)} = 8.26$, $p < 0.005$, Prenatal DP $F_{(1,72)} = 5.45$, $p < 0.05$, Conditioning $F_{(1,72)} = 8.95$, $p < 0.005$, as well as the significant interactions Prenatal EtOH × Conditioning $F_{(1,72)} = 4.42$ $p < 0.05$, Prenatal DP × Conditioning $F_{(1,72)} = 15.04$, $p < 0.001$, and a three-way interaction between all variables $F_{(1,72)} = 3.36$, $p < 0.05$. When analyzing this interaction, a similar pattern of results to those described for the training session was obtained. All of these results indicate that sequestering acetaldehyde during prenatal ethanol exposure reduces the reinforcing properties of ethanol in an operant learning task on PD 5.

Experiment 3: Intake Test on PD 14

The experimental design resulted in four groups defined by Prenatal EtOH (ethanol or water) and Prenatal DP (DP or saline): ethanol-DP, ethanol-saline, water-DP and water-saline. The subjects were first tested with water and an hour later with ethanol. A factorial ANOVA (2 × 2) conducted on the data from the water intake test revealed no significant differences between groups (data not shown). Means ± SEM: ethanol-DP, 1.71 ± 0.21; ethanol-saline, 1.46 ± 0.21; water-DP, 1.16 ± 0.20; and water-saline, 1.55 ± 0.13.

However, with the ethanol intake data a significant effect of Prenatal DP $F_{(1,36)} = 10.67$, $p < 0.002$ was found, along with an interaction between Prenatal EtOH and Prenatal DP $F_{(1,36)} = 6.76$, $p < 0.05$. *Post hoc* analyses revealed that subjects from Group ethanol-saline consumed significantly more ethanol than those from Groups water-saline or ethanol-DP. Subjects from the latter group consumed the same amount of ethanol as the control groups water-DP and water-saline (**Figure 3**). These results also suggest that the D-penicillamine treatment reduced the enhanced acceptance for ethanol observed after ethanol prenatal exposure.

DISCUSSION

The present study demonstrates that pups prenatally exposed to ethanol show an increased attraction for the odor of this substance on PD 1, a facilitated acquisition of the operant appetitive conditioned response when ethanol flavor was the reinforcer on PD 5, and an increased consumption of ethanol on PD 14. Further—and more interesting for the aims of this study—these effects were not observed when ethanol was administered together with D-penicillamine, a result that confirms our hypothesis. In particular, we have found that after prenatal ethanol exposure in the absence of acetaldehyde, the ethanol odor did not become particularly attractive for PD 1 neonates. In addition, the ethanol flavor was not able to serve as a reinforcer in an operant conditioning paradigm on PD 5, with no observed increase intake of ethanol on PD 14. These results indicate that the enhanced acceptance of ethanol observed after prenatal exposure is abolished when acetaldehyde is sequestered, thus suggesting that acetaldehyde is vital for the reinforcing effects of ethanol and therefore for the acquisition of a prenatal appetitive conditioned response.

These conclusions are in agreement with studies by Quertemont and Tambour (2004) and Karahanian et al. (2011) in which they highlight the essential role of ethanol's first metabolite in the reinforcing effects of the drug. Further, the results are in

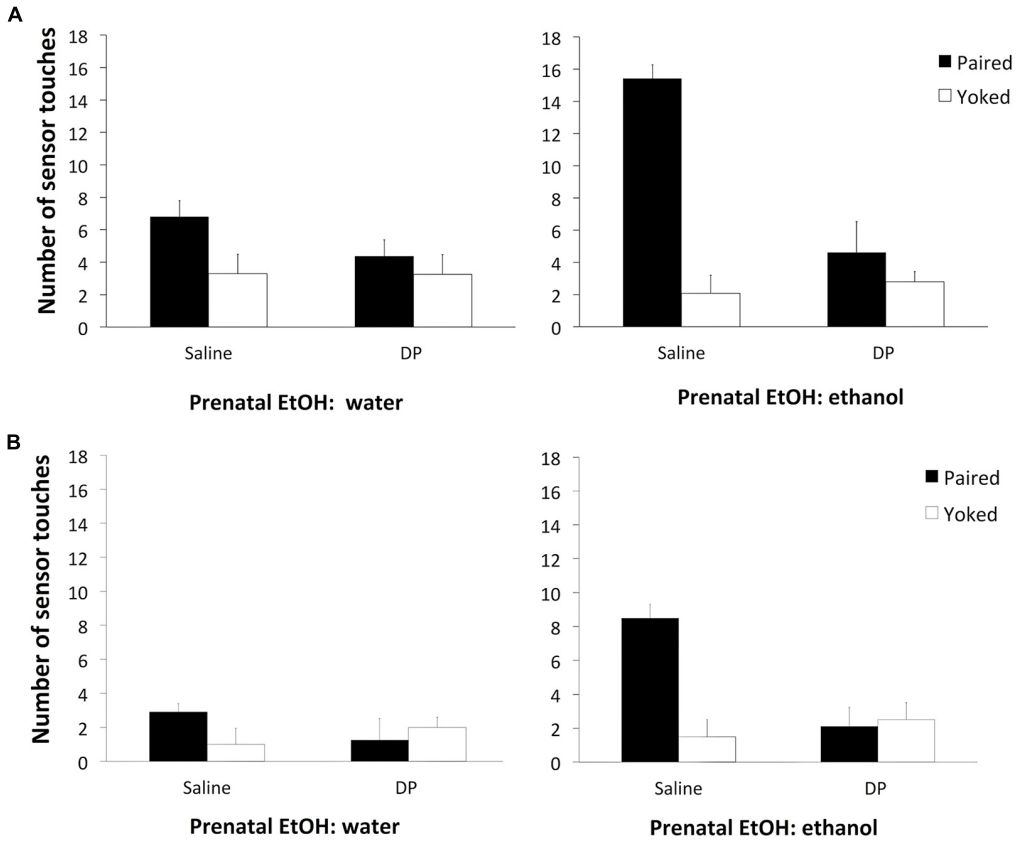

FIGURE 2 | (A) Total number of responses (sensor touches) during the training session as a function of Prenatal DP (saline or DP) and Conditioning (P or Y). The left-hand panel displays the data for subjects receiving water prenatally; the right-hand panel for subjects that received prenatal ethanol. **(B)** Total number of responses (sensor touches) during the extinction session as a function of Prenatal DP (saline or DP) and Conditioning (P or Y). The left-hand panel displays the data for subjects receiving water prenatally; the right-hand panel for subjects that received prenatal ethanol.

accordance with other studies conducted with adult rats (using a variety of behavioral measures) in which D-penicillamine was used to sequester acetaldehyde. For instance, voluntary drinking of ethanol is decreased by the administration of this drug (Font et al., 2006), operant ethanol self-administration is reduced (Peana et al., 2015), ethanol relapse-like drinking is prevented (Martí-Prats et al., 2015), and anxiolytic effects produced by moderate doses of ethanol are abolished (Correa et al., 2008). If we focus on studies with infant or neonatal rats, very few have analyzed the role of acetaldehyde. The most

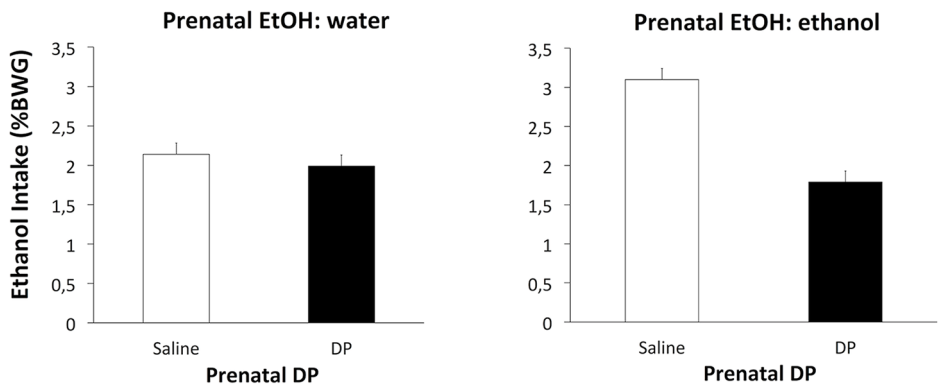

FIGURE 3 | Mean intake (% BWG) of ethanol as a function of Prenatal DP (saline or DP). The left-hand panel displays the data for subjects receiving water prenatally; the right-hand panel for subjects that received prenatal ethanol.

recent studies have shown that acetaldehyde may act as an unconditioned stimulus in the same manner as ethanol, and that D-penicillamine abolishes conditioned responses acquired with both ethanol and acetaldehyde as the US (Pautassi et al., 2011; March et al., 2013). The results of those experiments confirm the relevance of centrally produced acetaldehyde, as opposed to peripheral acetaldehyde. As mentioned in the "Introduction" Section, within the fetal context, due to the hepatic immaturity of the developing fetus, ethanol reaching the fetus from the maternal diet is metabolized into acetaldehyde only in the fetus brain by catalases, and the hepatic ADH enzymes supposedly produce no peripheral acetaldehyde. Further, it is important to recall that the acetaldehyde produced in the mother's liver does not cross the placenta (at least with the moderate ethanol doses used here). In sum, the only acetaldehyde experienced by the fetus after prenatal ethanol administration to the mother is that produced in the brain by the catalase system. Interestingly, this is precisely the central acetaldehyde that has been shown to have reinforcing effects in both adult and in neonate rats (Quertemont and Tambour, 2004; Karahanian et al., 2011; Pautassi et al., 2011; March et al., 2013). Given the fact that in our studies acetaldehyde was not directly administered, but was instead derived from ethanol, the possibility exists that the sequestering drug would eliminate acetaldehyde, whilst ethanol would still be present in the amniotic fluid and the fetus' body for a longer time until its complete metabolization and/or elimination. In this highly probable case, the complete absence of a postnatal response to the ethanol flavor (no increased acceptance at any age) observed in our experiments, may indicate that acetaldehyde is the main, if not the only, prenatal reinforcer responsible for the effect studied here. However, this needs to be further investigated by directly manipulating the presence of either substance (ethanol or acetaldehyde) possibly through the enzymes involved in each step of the ethanol metabolic chain.

Further studies should also investigate the connection between prenatal acetaldehyde and the stimulation of the fetal opioid system, which undoubtedly mediates the reinforcing effects of ethanol in both infancy and prenatal stages (Díaz-Cenzano et al., 2014; Gaztañaga et al., 2015). In adult rats the opioid system has been demonstrated to mediate the reinforcing effects of acetaldehyde attributed to ethanol (Peana et al., 2010; Correa et al., 2012). In addition it has recently been demonstrated that the stimulation of the mesolimbic dopaminergic system induced by acetaldehyde is mediated by the endogenous opioid system (Fois and Diana, 2016). Based on these findings in adults, and with the knowledge that the fetal dopamine and opioid systems are functional, it could be inferred that similar mechanisms were acting for the reinforcing aspects of ethanol and acetaldehyde in the near-term fetus.

It may also be of interest to mention some other outcomes of Experiment 1, particularly when comparing the effects of prenatal exposure to ethanol and vanilla. Pups prenatally exposed to vanilla—either alone or with D-penicillamine—showed an increased attraction to the odor of vanilla in comparison with water or ethanol exposed subjects. This is interpreted as the result of familiarization with the odor experienced in the amniotic fluid. However, pups administered prenatally with ethanol and D-penicillamine, i.e., those that have supposedly experienced ethanol's chemosensory properties in the amniotic fluid in the absence of a reinforcer, did not show an increased attraction for its odor compared with the other groups. This lack of attraction for the ethanol odor following its mere exposure (familiarization) may reflect the response to the irritant and hence aversive component of this odor, which possibly needs even more exposure trials to become less aversive. In fact, previous data from this laboratory have shown that in infant rats familiarization with the flavor of ethanol, among other stimuli, resulted in sensitization to the aversive chemosensory properties of this substance (Díaz-Cenzano and Chotro, 2010).

In addition, this set of findings constitutes the first step in a promising line of enquiry to determine the role played by each of the elements involved in the neurobehavioral chain between the exposure to prenatal ethanol and the increased acceptance and liking of this substance at various postnatal stages. This knowledge would allow for manipulating the prenatal appetitive memories generated during ethanol exposure, and could thus help to prevent the effects related to early ethanol initiation and ethanol abuse.

AUTHOR CONTRIBUTIONS

MG and AA-A contributed equally to this work participating in all steps of this investigation and the writing of the manuscript. MGC and NES also contributed in all aspects to this work and manuscript.

FUNDING

This research was supported by grants from MEC, Spain (PSI2011-24231 and PSI2012-38019), and from the Basque Government (IT-694-13).

ACKNOWLEDGMENTS

We would like to thank Dr. Michelle Symonds for her valuable assistance reviewing the manuscript, as well as to Teri Tanenhaus and SGiker for personal and technical support.

REFERENCES

Arias, C., and Chotro, M. G. (2005a). Increased palatability of ethanol after prenatal ethanol exposure is mediated by the opioid system. *Pharmacol. Biochem. Behav.* 82, 434–442. doi: 10.1016/j.pbb.2005.09.015

Arias, C., and Chotro, M. G. (2005b). Increased preference for ethanol in the infant rat after prenatal ethanol exposure, expressed on intake and taste reactivity tests. *Alcohol. Clin. Exp. Res.* 29, 337–346. doi: 10.1097/01.alc.0000156115.35817.21

Arias, C., Spear, N. E., Molina, J. C., Molina, A., and Molina, J. C. (2007). Rapid acquisition of operant conditioning in 5-day-old rat pups: a new technique

articulating suckling-related motor activity and milk reinforcement. *Dev. Psychobiol.* 49, 576–588. doi: 10.1002/dev.20236

Boleda, M. D., Farrés, J., Guerri, C., and Parés, X. (1992). Alcohol dehydrogenase isoenzymes in rat development: effect of maternal ethanol consumption. *Biochem. Pharmacol.* 43, 1555–1561. doi: 10.1016/0006-2952(92)90214-4

Chotro, M. G., and Arias, C. (2003). Prenatal exposure to ethanol increases ethanol consumption: a conditioned response? *Alcohol* 30, 19–28. doi: 10.1016/s0741-8329(03)00037-5

Chotro, M. G., Arias, C., and Laviola, G. (2007). Increased ethanol intake after prenatal ethanol exposure: studies with animals. *Neurosci. Biobehav. Rev.* 31, 181–191. doi: 10.1016/j.neubiorev.2006.06.021

Chotro, M. G., Arias, C., and Spear, N. E. (2009). Binge ethanol exposure in late gestation induces ethanol aversion in the dam but enhances ethanol intake in the offspring and affects their postnatal learning about ethanol. *Alcohol* 43, 453–463. doi: 10.1016/j.alcohol.2009.08.001

Correa, M., Manrique, H. M., Font, L., Escrig, M. A., and Aragon, C. M. G. (2008). Reduction in the anxiolytic effects of ethanol by centrally formed acetaldehyde: the role of catalase inhibitors and acetaldehyde-sequestering agents. *Psychopharmacology* 200, 455–464. doi: 10.1007/s00213-008-1219-3

Correa, M., Salamone, J. D., Segovia, K. N., Pardo, M., Longoni, R., Spina, L., et al. (2012). Piecing together the puzzle of acetaldehyde as a neuroactive agent. *Neurosci. Biobehav. Rev.* 36, 404–430. doi: 10.1016/j.neubiorev.2011.07.009

Chotro, M. G., and Spear, N. E. (1997). Repeated exposure to moderate doses of alcohol in the rat fetus: evidence of sensitization to toxic and chemosensory aspects of alcohol. *Alcohol. Clin. Exp. Res.* 21, 360–367. doi: 10.1097/00000374-199704000-00027

Díaz-Cenzano, E., and Chotro, M. G. (2010). Prenatal binge ethanol exposure on gestation days 19–20, but not on days 17–18, increases postnatal ethanol acceptance in rats. *Behav. Neurosci.* 124, 362–369. doi: 10.1037/a0019482

Díaz-Cenzano, E., Gaztañaga, M., and Gabriela Chotro, M. (2014). Exposure to ethanol on prenatal days 19–20 increases ethanol intake and palatability in the infant rat: involvement of kappa and mu opioid receptors. *Dev. Psychobiol.* 56, 1167–1178. doi: 0.1002/dev.21162

Domínguez, H. D., López, M. F., and Molina, J. C. (1998). Neonatal responsiveness to alcohol odor and infant alcohol intake as a function of alcohol experience during late gestation. *Alcohol* 16, 109–117. doi: 10.1016/s0741-8329(97)00169-9

Fois, G. R., and Diana, M. (2016). Opioid antagonists block acetaldehyde-induced increments in dopamine neurons activity. *Drug Alcohol Depend.* 158, 172–176. doi: 10.1016/j.drugalcdep.2015.11.013

Font, L., Aragon, C. M., and Miquel, M. (2006). Voluntary ethanol consumption decreases after the inactivation of central acetaldehyde by d-penicillamine. *Behav. Brain Res.* 171, 78–86. doi: 10.1016/j.bbr.2006.03.020

Gaztañaga, M., Aranda-Fernández, P. E., and Chotro, M. G. (2015). Prenatal exposure to vanilla or alcohol induces crawling after these odors in the neonate rat: the role of mu and kappa opioid receptor systems. *Physiol. Behav.* 148, 58–64. doi: 10.1016/j.physbeh.2014.12.046

Guerri, C., and Sanchis, R. (1985). Acetaldehyde and alcohol levels in pregnant rats and their fetuses. *Alcohol* 2, 267–270. doi: 10.1016/0741-8329(85)90057-6

Hahn, C. Y., Huang, S. Y., Ko, H. C., Hsieh, C. H., Lee, I. H., Yeh, T. L., et al. (2006). Acetaldehyde involvement in positive and negative alcohol expectancies in han Chinese persons with alcoholism. *Arch. Gen. Psychiatry* 63, 817–823. doi: 10.1001/archpsyc.63.7.817

Hamby-Mason, R., Chen, J. J., Schenker, S., Perez, A., and Henderson, G. I. (1997). Catalase mediates acetaldehyde formation from ethanol in fetal and neonatal rat brain. *Alcohol. Clin. Exp. Res.* 21, 1063–1072. doi: 10.1097/00000374-199709000-00020

Hayashi, M., Shimazaki, Y., Kamata, S., Kakiichi, N., and Ikeda, M. (1991). Disposition of ethanol and acetaldehyde in maternal blood, fetal blood and amniotic fluid of near-term pregnant rats. *Bull. Environ. Contam. Toxicol.* 47, 184–189. doi: 10.1007/bf01688638

Hipólito, L., Sánchez, M. J., Polache, A., and Granero, L. (2007). Brain metabolism of ethanol and alcoholism: an update. *Curr. Drug Metab.* 8, 716–727. doi: 10.2174/138920007782109797

Karahanian, E., Quintanilla, M. E., Tampier, L., Rivera-Meza, M., Bustamante, D., González-Lira, V., et al. (2011). Ethanol as a prodrug: brain metabolism of ethanol mediates its reinforcing effects. *Alcohol. Clin. Exp. Res.* 35, 606–612. doi: 10.1111/j.1530-0277.2011.01439.x

March, S. M., Abate, P., and Molina, J. C. (2013). Acetaldehyde involvement in ethanol's postabsortive effects during early ontogeny. *Front. Behav. Neurosci.* 7:70. doi: 10.3389/fnbeh.2013.00070

Martí-Prats, L., Zornoza, T., López-Moreno, J. A., Granero, L., and Polache, A. (2015). Acetaldehyde sequestration by d-penicillamine prevents ethanol relapse-like drinking in rats: evidence from an operant self-administration paradigm. *Psychopharmacology* 232, 3597–3606. doi: 10.1007/s00213-015-4011-1

Mendez-Gallardo, V., and Robinson, S. R. (2014). Odor-induced crawling locomotion in the newborn rat: effects of amniotic fluid and milk. *Dev. Psychobiol.* 56, 327–339. doi: 10.1002/dev.21102

Mickley, G. A., Hoxha, Z., DiSorbo, A., Wilson, G. N., Remus, J. L., Biesan, O., et al. (2014). Latent inhibition of a conditioned taste aversion in fetal rats. *Dev. Psychobiol.* 56, 435–447. doi: 10.1002/dev.21110

Mickley, G. A., Remmers-Roeber, D. R., Crouse, C., Walker, C., and Dengler, C. (2000). Detection of novelty by perinatal rats. *Physiol. Behav.* 70, 217–225. doi: 10.1016/s0031-9384(00)00229-8

Miranda-Morales, R. S., Nizhnikov, M. E., and Spear, N. E. (2014). Prenatal exposure to ethanol during late gestation facilitates operant self-administration of the drug in 5-day-old rats. *Alcohol* 48, 19–23. doi: 10.1016/j.alcohol.2013.11.001

Nizhnikov, M. E., Molina, J. C., and Spear, N. E. (2007). Central reinforcing effects of ethanol are blocked by catalase inhibition. *Alcohol* 41, 525–534. doi: 10.1016/j.alcohol.2007.08.006

Pautassi, R. M., Nizhnikov, M. E., Fabio, M. C., and Spear, N. E. (2011). An acetaldehyde-sequestering agent inhibits appetitive reinforcement and behavioral stimulation induced by ethanol in preweanling rats. *Pharmacol. Biochem. Behav.* 97, 462–469. doi: 10.1016/j.pbb.2010.10.005

Peana, A. T., Muggironi, G., and Diana, M. (2010). Acetaldehyde-reinforcing effects: a study on oral self-administration behavior. *Front. Psychiatry* 1:23. doi: 10.3389/fpsyt.2010.00023

Peana, A. T., Porcheddu, V., Bennardini, F., Carta, A., Rosas, M., and Acquas, E. (2015). Role of ethanol-derived acetaldehyde in operant oral self-administration of ethanol in rats. *Psychopharmacology* 232, 4269–4276. doi: 10.1007/s00213-015-4049-0

Pedersen, P. E., Greer, C. A., and Shepherd, G. M. (1986). "Early development of olfactory function," in *Handbook of Behavioral Neurobiology. Developmental Psychobiology and Behavioral Neurobiology*, (Vol. 8), ed. E. M. Blass (New York, NY: Plenum Press), 163–204.

Quertemont, E., and Tambour, S. (2004). Is ethanol a pro-drug? The role of acetaldehyde in the central effects of ethanol. *Trends Pharmacol. Sci.* 25, 130–134. doi: 10.1016/j.tips.2004.01.001

Robinson, S. R., and Méndez-Gallardo, V. (2010). "Amniotic fluid as an extended milieu interieur," in *Handbook of Developmental Science, Behavior and Genetics*, eds K. E. Hood, C. T. Halpern, G. Greenberg and R. N. Lerner (New York, NY: Wiley Blackwell), 234–384.

Smotherman, W. P., and Robinson, S. R. (Eds.) (1988). *Behavior of the Fetus.* Caldwell, NJ: Telford Press.

Smotherman, W. P., and Robinson, S. R. (1992). Prenatal experience with milk: fetal behavior and endogenous opioid systems. *Neurosci. Biobehav. Rev.* 16, 351–364. doi: 10.1016/s0149-7634(05)80205-2

Spear, L. P., Specht, S. M., Kirstein, C. L., and Kuhn, C. M. (1989). Anterior and posterior, but not cheek, intraoral canulation procedures elevate serum corticosterone levels in neonatal rat pups. *Dev. Psychobiol.* 22, 401–411. doi: 10.1002/dev.420220407

Stickrod, G., Kimble, D. P., and Smotherman, W. P. (1982). in utero taste/odor aversion conditioning in the rat. *Physiol. Behav.* 28, 5–7. doi: 10.1016/0031-9384(82)90093-2

Szeto, H. H. (1989). Maternal-fetal pharmacokinetics and fetal dose-response relationships. *Ann. N Y Acad. Sci.* 562, 42–55. doi: 10.1111/j.1749-6632.1989.tb21006.x

Wall, T. L., Thomasson, H. R., Schuckit, M. A., and Ehlers, C. L. (1992). Subjective feelings of alcohol intoxication in Asians with genetic variations of ALDH2 alleles. *Alcohol. Clin. Exp. Res.* 16, 991–995. doi: 10.1111/j.1530-0277.1992.tb01907.x

Youngentob, S. L., Kent, P. F., and Youngentob, L. M. (2012). Gestational naltrexone ameliorates fetal ethanol exposures enhancing effect on the postnatal behavioral and neural response to ethanol. *Exp. Biol. Med. (Maywood)* 237, 1197–1208. doi: 10.1258/ebm.2012.012132

Zimatkin, S. M., Pronko, S. P., Vasiliou, V., Gonzalez, F. J., and Deitrich, R. A. (2006). Enzymatic mechanisms of ethanol oxidation in the brain. *Alcohol. Clin. Exp. Res.* 30, 1500–1505. doi: 10.1111/j.1530-0277.2006.00181.x

Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

frontiers in Behavioral Neuroscience

ORIGINAL RESEARCH
published: 21 March 2017
doi: 10.3389/fnbeh.2017.00039

Brain Acetaldehyde Exposure Impacts upon Neonatal Respiratory Plasticity and Ethanol-Related Learning in Rodents

María B. Acevedo[1], Génesis D'Aloisio[1,2], Olga B. Haymal[1] and Juan C. Molina[1,2]**

[1] Laboratorio de Alcohol, Ontogenia y Aprendizaje, Instituto de Investigación Médica Mercedes y Martín Ferreyra, Consejo Nacional de Investigaciones Científicas y Técnicas (INIMEC-CONICET), Universidad Nacional de Córdoba, Córdoba, Argentina, [2] Experimental Psychobiology Chair, Department of Psychology, Universidad Nacional de Córdoba, Córdoba, Argentina

Prior studies indicate that neonates are very sensitive to ethanol's positive reinforcing effects and to its depressant effects upon breathing. Acetaldehyde (ACD) appears to play a major role in terms of modulating early reinforcing effects of the drug. Yet, there is no pre-existing literature relative to the incidence of this metabolite upon respiratory plasticity. The present study analyzed physiological and behavioral effects of early central administrations of ethanol, acetaldehyde or vehicle. Respiration rates (breaths/min) were registered at post-natal days (PDs) 2 and 4 (post-administration time: 5, 60, or 120 min). At PD5, all pups were placed in a context (plethysmograph) where they had previously experienced the effects of central administrations and breathing patterns were recorded. Following this test, pups were evaluated using and operant conditioning procedure where ethanol or saccharin served as positive reinforcers. Body temperatures were also registered prior to drug administrations as well as at the beginning and the end of each specific evaluation. Across days, breathing responses were high at the beginning of the evaluation session and progressively declined as a function of the passage of time. At PDs 2 and 4, shortly after central administration (5 min), ACD exerted a significant depression upon respiration frequencies. At PD5, non-intoxicated pups with a prior history of ACD central administrations, exhibited a marked increase in respiratory frequencies; a result that probably indicates a conditioned compensatory response. When operant testing procedures were conducted, prior ethanol or ACD central administrations were found to reduce the reinforcing effects of ethanol. This was not the case when saccharin was employed as a reinforcer. As a whole, the results indicate a significant role of central ACD upon respiratory plasticity of the neonate and upon ethanol's reinforcing effects; phenomena that affect the physiological integrity of the immature organism and its subsequent affinity for ethanol operationalized through self-administration procedures.

Keywords: neonates, ethanol, acetaldehyde, breathing, operant learning

OPEN ACCESS

Edited by:
Merce Correa, Jaume I University, Spain

Reviewed by:
M. Yanina Pepino, Washington University School of Medicine, USA
Inmaculada Cubero, University of Almería, Spain

****Correspondence:***
María B. Acevedo
sallycabooh@gmail.com
Juan C. Molina
juancmolina2003@hotmail.com.ar

Received: *28 December 2016*
Accepted: *23 February 2017*
Published: *21 March 2017*

Citation:
Acevedo MB, D'Aloisio G, Haymal OB and Molina JC (2017) Brain Acetaldehyde Exposure Impacts upon Neonatal Respiratory Plasticity and Ethanol-Related Learning in Rodents. Front. Behav. Neurosci. 11:39. doi: 10.3389/fnbeh.2017.00039

INTRODUCTION

Low doses of ethanol (0.11 g/kg/h), combined or not with a tocolytic agent (ritodrine), have been employed to reduce the incidence of human preterm births. Under these clinical conditions, in approximately 80% of the patients, uterine contractions were suppressed and a significant number of preterm births were prevented (Schrock et al., 1989). According to this study no adverse effects of ethanol were observed. Yet, the obstetric use of ethanol, depending upon factors such as dose, frequency of exposure and fetal stage of development has been questioned due to a variety of disruptive physiological effects of the drug upon the fetus. Hypothermia, acidosis, hypercapnia, bradycardia, hypoglycemia, apneas and hypoxia are likely to occur in the developing organism exposed to ethanol (Abel, 1981; Duxbury, 2001; Abate et al., 2004). When considering the central nervous system, there are also numerous studies confirming disruptions caused by relatively infrequent and small doses of the drug upon a variety of parameters. Rat fetuses exposed to low ethanol doses (blood ethanol concentration $\leq$30 mg%) show impairments in spatial learning accompanied by alterations in hippocampal glutamate-dependent synaptic neurotransmission (Savage et al., 2002). In rhesus monkeys, moderate maternal ethanol consumption (0.6 g/kg ethanol daily) during midgestation to late gestation, induces heightened dopaminergic function (Wise, 2002). During a stage in the development of the mouse characterized by a brain growth spurt, similar to the one observed during the third gestational trimester in humans (Dobbing and Sands, 1973, 1979), a single ethanol dose (0.63 g/kg) yielding relatively low peak blood ethanol levels (57 mg%) is sufficient to trigger a significant neuroapoptosis response (Young and Olney, 2006).

Preclinical and clinical studies have indicated that relatively low ethanol doses during pregnancy are sufficient to trigger fetal sensory and learning capabilities with an impact upon later patterns of chemosensory recognition of the drug, ethanol odor and taste preference (Faas et al., 2000, 2015; Abate et al., 2008) and sensitivity to the drug's positive reinforcing effects (Nizhnikov et al., 2006). Fetal experience with ethanol generates conditioned responses derived from the association between the drug's odor and taste and its motivational properties (Abate et al., 2001; Spear and Molina, 2005; Molina et al., 2007; Cullere et al., 2015). These phenomena predispose the organism to heightened seeking and intake patterns of the drug during infancy and adolescence (Dominguez et al., 1998; Foltran et al., 2011; Fabio et al., 2013; Acevedo et al., 2017). Epidemiological studies have validated the significant association existing between fetal ethanol exposure and subsequent predisposition to seek and consume the drug (Baer et al., 1998, 2003; Griesler and Kandel, 1998; Yates et al., 1998; Alati et al., 2006).

Acetaldehyde (ACD), ethanol's principal metabolite, mainly and rapidly forms in the perinatal brain via the oxidative process of the catalase system. The activity of this enzymatic system is significantly higher during early ontogeny relative to adolescence and adulthood (Del Maestro and McDonald, 1987; Gill et al., 1992; Hamby-Mason et al., 1997). When considering ethanol's reinforcing effects, ACD formation in the brain plays a critical role (Wall et al., 1992; Hahn et al., 2006). In newborn rats, intracisternal administration of relatively low doses of ethanol (100 mg%) or of ACD (0.35 μmol) promote appetitive conditioning (Nizhnikov et al., 2007; March et al., 2013a,b). Furthermore, when considering either peripheral or central administration of ethanol, the establishment of early appetitive memories are blocked when sequestering brain ACD via the use of d-penicilamine (Pautassi et al., 2011; March et al., 2013a,b) or when inhibiting the catalase system through sodium azide (Nizhnikov et al., 2007).

Ethanol consumption during pregnancy has also been found to endanger the wellbeing of the fetus and the neonate due to its detrimental effects upon the respiratory system and its plasticity; a phenomenon that has stimulated research efforts based on the fact that fetal alcohol exposure is a risk factor for Sudden Infant Death Syndrome (Burd et al., 2004; O'Leary et al., 2013). In human and ewes, the depressant effects of the drug upon fetal breathing movements (FBMs) have been well documented (Vojcek et al., 1988; Brien and Smith, 1991) and there is evidence that maternal human consumption of only two glasses of wine during late gestation significantly suppresses fetal breathing activity (Brien and Smith, 1991; Dillner et al., 1996). In rats, chronic ethanol exposure during pregnancy, reduces brainstem-dependent respiratory rhythmic activity in the progeny and sensitizes juveniles to the depressant effects of acute ethanol upon phrenic and hypoglossal nerve activity (Dubois et al., 2006). Analogous effects in rats have been recently reported utilizing moderate levels of ethanol exposure during the last days of pregnancy or during the first days of post-natal life (Cullere et al., 2015; Macchione et al., 2016; Acevedo et al., 2017). These stages in development, in terms of brain developmental patterns, are equivalent to the 2nd and 3rd human gestational trimester; respectively (Dobbing and Sands, 1973, 1979). Indeed, we have reported that maternal intragastric (i.g.) administration of ethanol (2.0 g/kg) during gestational days (GDs) 17–20 is sufficient to sensitize the progeny to the drug's depressant effects upon respiratory rates and exacerbate the presence of apneic episodes; disruptions that occur without affecting different pulmonary morphometric parameters (Cullere et al., 2015). This sensitization process has also been observed when neonates [post-natal days (PDs) 3, 5, and 7] were peripherically administered with ethanol (i.g.: 2.0 g/kg). Furthermore, in both studies, it was observed that the explicit association between ethanol's sensory attributes and the depressant consequences of the drug resulted in conditioned isodirectional breathing responses (Macchione et al., 2016).

After systematically reviewing the pre-existing literature concerning ethanol's central effects upon breathing patterns of the perinate, we were unable to find specific literature related with possible contributions of ACD. Despite this observation, it should be noted that respiratory plasticity is linked with thermoregulatory disruptions. Indeed, prenatal or neonatal hypothermia can cause respiratory arrests (Duxbury, 2001). Among the multiple physiological consequences of ACD is the modulation of the thermoregulatory system. In mice, peripheral administration of ACD causes hypothermia (Closon et al., 2009). Nevertheless, there is certain degree of contradiction

relative to the prior statement. It has been observed that in rats the inhibition of the central catalase system following ethanol administration seems not to play a significant role in the induction of hypothermia (Aragon et al., 1991).

The first goal of the present study was based on the preceding observations: (i) the absence of specific literature related with the central role of ethanol or its first metabolite (ACD) in terms of disruptive effects upon early respiratory plasticity and (ii) a possible association existing between ethanol and/or ACD central effects leading to thermoregulatory alterations that may impact upon breathing responsiveness. To address these phenomena in perinatal rats, it was decided to employ similar central ethanol (100 mg%) and ACD (0.35 μmol) doses that have been observed to exert analogous motivational effects (Nizhnikov et al., 2007; March et al., 2013a,b). A second goal was to further analyze if central pre-exposure to the drug or its metabolite modulate subsequent seeking behavior of ethanol as a reinforcer in an operant task specifically developed for perinatal or infant rats (Arias et al., 2007; Bordner et al., 2008; March et al., 2009; Miranda-Morales et al., 2014). These goals were sequentially examined. As a first step, during PDs 2 and 4, pups were intracisternally administered with either ethanol, ACD or a phosphate buffer as a control solution and respiration rates were recorded at different post-administration times. At PD5, all pups were re-exposed to the respiratory testing chamber without receiving any specific drug. This particular strategy obeys to the fact that prior experiments have indicated that early respiratory plasticity is also dependent upon exteroceptive ambient cues originally associated with breathing pattern changes. Following this testing procedure, pups were evaluated in terms of operant responding regulated by either an ethanol solution or a sweet reinforcer (saccharin). The inclusion of this last reinforcer obeyed to the need to control for unspecific learning alterations derived from the preceding central drug administration experiences. Body temperatures, before and after each specific drug treatment or evaluation procedure, were recorded.

MATERIALS AND METHODS

Subjects

A total of 146 Wistar neonate rats, representative of 17 litters, were employed. Rats were born and reared at the vivarium of the Instituto de Investigación Médica Mercedes y Martin Ferreyra (INIMEC-CONICET-UNC, Argentina). The colony room was illuminated on a 12 h light/dark cycle (lights on: 08:00–20:00) at an ambient temperature and humidity of 22 ± 1°C and 45%, respectively. Births were daily examined and the day of parturition was considered post-natal day 0 (PD0). At PD1 each litter was randomly culled to 10 pups (5 males and 5 females, whenever possible). Throughout days pups were kept with their dams in standard cages that contained water and food *ad libitum* (ACA Nutrición, Buenos Aires, Argentina).

All experimental treatments were in accordance with the Guide for Care and Use of Laboratory Animals (National Research Council, 1996) and were approved by the Institutional Animal Care and Use Committee of our institution (CICUAL-INIMEC-CONICET-UNC). To reduce confounds between litter and treatment effects (Holson and Pearce, 1992) no more than one male and one female per litter were assigned to a given experimental condition.

General Experimental Procedures

During PDs 2 and 4, pups were removed from their maternal cages and placed in similar cages partially filled with clean corncob. Ambient temperature was kept at 31–33°C via heating pads placed beneath the cages. Pups were centrally administered with a buffer solution, ethanol or ACD (see below) and tested in a plethysmograph at post-administration time 5, 60, or 120 min. Respiratory frequencies were assessed during 5 consecutive minutes. At PD5 pups representative of each prior treatment were removed from their maternal cages and kept in pairs under the same holding conditions as in the previous experimental days. Fifteen minutes later, respiratory evaluations were performed. Following these physiological recordings, pups were subjected to a minor surgical procedure in order to implant an intraoral cannula that served to conduct operant conditioning procedures defined by saccharin or ethanol reinforcement. Body temperatures were recorded before and after each specific physiological or behavioral evaluation.

Central Drug Administration Procedures

Ethanol (100 mg%), acetaldehyde (0.35 μmol) and phosphate buffer (PB 0.1M) were administered with a 30 gauge hypodermic needle attached to a 20-cm length of polyethylene-10 tubing (PE-10 Clay Adams, Parsippany, New Yersey, USA) connected to a 50 μl gastight syringe (Hamilton, Reno Nevada, USA). Fluids (1 μl) were slowly injected (5–8 s) into the foramen magnum between the occipital bone and the first cervical vertebra, with the needle tip placed 1.5 mm depth in the cisterna magna (IC). PB 0.1M served as vehicle for ethanol and acetaldehyde solutions. The needle was kept in position for 10 s. The appearance of a small quantity of cerebrospinal fluid served to indicate the successful placement of the administrations. Similar procedures have been previously utilized in different studies (Varlinskaya et al., 1996; Petrov et al., 1998; Nizhnikov et al., 2006, 2007; March et al., 2013a,b).

Determination of Breathing Frequencies

Breathing frequencies were determinated through a whole body plethysmograph (Model 10G equipped with the software "Breath Medidor de Respiración," Itcom, Argentina). The apparatus was built to record breathing patterns of small organisms weighing between 6 and 28 g. It consists of two identical transparent and hermetic Plexiglas chambers (5 × 10 × 5 cm), that are interconnected via a polyurethane hose system. The hose system allows injection and extraction of equivalent amounts of air in both chambers in order to maintain constant and equivalent pressures. One of the chambers is used as a testing device while the other serves as a reference box in terms of flow/air pressure. The plethysmograph records air pressure/flow rate differences between the testing and reference chambers. These differences activate a pressure sensor (AWM2100 Honeywell) with the capability of recording one complete breathing event every 1 ×

10^{-7} s. The plethysmograph records the breathing response every 1.5 s. These scores are transformed to mean breaths per minute.

For each session, unrestrained awake pups were introduced into the chambers and the lids were closed. One minute after that pups were individually placed inside the chamber, respiratory responses were measured during 10 consecutive minutes. The minute of delay at the beginning of the test was used to allow air pressure stabilization in the chamber.

An air conditioner kept the room temperature at 22 ± 1°C during experimental sessions. The temperature was kept at 31–33°C (similar to their maternal nest thermal condition) inside the plethysmograph chamber through heating pads placed beneath the apparatus (Julien et al., 2010). The overall procedure has been previously used to evaluate breathing disruptions as a function of pre- and post-natal ethanol exposure (Cullere et al., 2015; Macchione et al., 2016; Acevedo et al., 2017).

Body Temperature Measurements

Body temperatures were non-invasively registered through a thermal infrared imaging camera ("Flir Exx Series," Boston FLIR System, Inc.). The temperature corresponding to the nape of the neck of each subject served as the dependent variable. Thermal measurements were taken before and after each plethysmograph recording (PDs 2, 4, and 5) as well as prior and following each operant conditioning test (PD 5).

Apparatus and Operant Conditioning Test

At PD5, and following breathing evaluations, pups were removed from their maternal cages and were intraorally implanted with a cannula (PE-10) that allowed liquid infusions (Hall, 1979; Dominguez et al., 1996; Abate et al., 2001; Cheslock et al., 2001; Arias et al., 2007; Bordner et al., 2008; Miranda-Morales et al., 2014). They remained pair-housed in holding cages for 3 h until operant procedures took place. Before commencement of the evaluation, animals were anogenitally stimulated with a cotton swab to promote urination and defecation, weighed to the nearest 0.01 g. They were then fastened inside a disposable respirator mask (3M dust, fume and mist respirator 8801 P2) through a restrictor vest, expandable enough, to allow free movements of the head and limbs. The respiration mask was tilted at 40 degrees from the floor surface supported with a cardboard box (see Arias et al., 2007 for further procedural details).

All procedures took place at a constant temperature (31–33°C) via the use of heating pads. A 40–50-cm section of polyethylene-50 tubing (PE-50 Clay Adams, Parsippany, New Yersey, USA) was connected to the end of oral cannula (PE-10) and to a 5 ml syringe (Becton Dickinson, Rutherford, NJ) with a 23-gauge needle that was filled with a specific solution and mounted in an infusion pump (KD Scientific, Model 200, Holliston, MA). The pump was set to deliver 1 μl of fluid in 1 s directly into the intraoral cavity of a given "Paired" pup and its corresponding "Yoked" control (see description below). Once evaluations begun, pups were able to gain access to intraoral infusions of 0.1% w/v saccharin or 3% v/v ethanol solution (Porta Hnos, Córdoba, Argentina; vehicle: tap water).

To this end, two same-sex and same drug-treatment pups from a single litter with similar body weights were placed in front of a touch-sensitive copper sensor (5 cm length × 1 cm width × 45 cm 1 mm thickness). The sensor was 1 cm away from their mouths and perpendicular to the floor while they remained hold inside the mask. Each time the animal touched the sensor a red light bulb lit signaling a physical contact, which resulted in an infusion pump pulse.

The apparatus was set to work with two subjects at a time: a Paired animal receiving infusions in a fixed ratio (FR) 1 schedule and a Yoked control receiving infusions in accordance to its corresponding paired pup. Each evaluation lasted 15 min. During these sessions pups received a given solution (ethanol or saccharin reinforcers) contingent upon their operant behavior (i.e., sensor contact). All pups received 4 priming pulses at the beginning of the training session, 60, 120, and 180 s. These pulses were administered independently of motor activity patterns in order to introduce the pup with the reinforcer and to minimally stimulate head and body movements. The number of sensor contacts of each Paired subject and its corresponding Yoked control were recorded. Similar procedures have been employed when analyzing early operant leaning regulated by positive reinforcers such as milk, sucrose and ethanol (Arias et al., 2007; Bordner et al., 2008; March et al., 2009; Miranda-Morales et al., 2014).

Experimental Design and Data Analysis

Body weights were analyzed using a four-way mixed analysis of variance (ANOVA). Drug treatment at PDs 2 and 4 (PB 0.1 M, ACD 0.35 μmol or ethanol 100 mg%), sex (male or female) and post-administration time (5, 60, or 120 min) served as between-group factors. Days of assessment (PDs 2, 4, and 5) served as the within-measure factor. A five-way mixed ANOVA was performed to analyze mean respiration rates at PDs 2-4 where drug treatment, sex and post-administration time served as the independent factors, while days of assessment and minutes corresponding to each specific evaluations (minutes: 1–5) represented repeated measures. At PD5 breathing patterns were analyzed through a between-within ANOVA defined by the same independent factors and repeated measures.

Thermoregulatory processes were analyzed using a five- or four-way mixed ANOVA (PDs 2, 4, and 5; respectively) where drug treatment, sex, post-administration time served as between factors while post-natal days and time of temperature recordings (before and after plethysmograph assessments) were considered as within-group variables.

Relative to the operant task, the total number of sensor contacts was considered the dependent variable. Separate ANOVAs were conducted to analyze operant performance when either saccharin or ethanol served as reinforcers. More specifically, a two-way mixed ANOVA was utilized. This inferential analysis was defined by prior drug treatments the between-group factor and conditioning status (Paired or Yoked) as the within-group factor.

Preliminary analysis relative to operant performance indicated no significant main or interaction effects when considering sex as a factor. Therefore, data were collapsed across sex for all the remaining analyses. The absence of sex effects has also been observed in prior studies when employing a variety of

reinforcers in operant conditioning tasks during early ontogeny (Bordner et al., 2008; March et al., 2009; Miranda-Morales et al., 2010, 2012a,b, 2014).

The loci of significant main effects were further analyzed with Ducan's *post-hoc* tests. A rejection criterion of $p < 0.05$ was adopted for all statistical analyses in the present study. According to the nature of the dependent variables under consideration, tests were performed using between or within error terms. Since there is no unambiguous choice of appropriate error term for *post-hoc* comparisons involving between- and within-group significant interactions (Winer, 1991), orthogonal planned comparisons were conducted when such interactions were obtained. All the statistical analyses were performed using the STATISTICA 8.0 software.

RESULTS

Body Weights across Days (PDs 2, 4, and 5)

Data corresponding to body weights across days was analyzed via a between-within ANOVA (drug treatment at PDs 2 and 4 × post-administration time × post-natal days × sex). As expected body weights progressively increased as a function of age; $F_{(2, 250)} = 3{,}897.01$, $p < 0.0001$. Duncan's *post-hoc* tests showed that pups at PD4 exhibited significantly greater body weights than those previously recorded during PD2. At PD5, weight values were significantly higher than those observed at PD4 (means ± standard errors of the means for each day were as follows: PD2, 7.25 ± 0.06 g; PD4, 10.08 ± 0.07 g and PD5, 11.41 ± 0.09 g. Body weights did not differ as a function of the other factors under consideration.

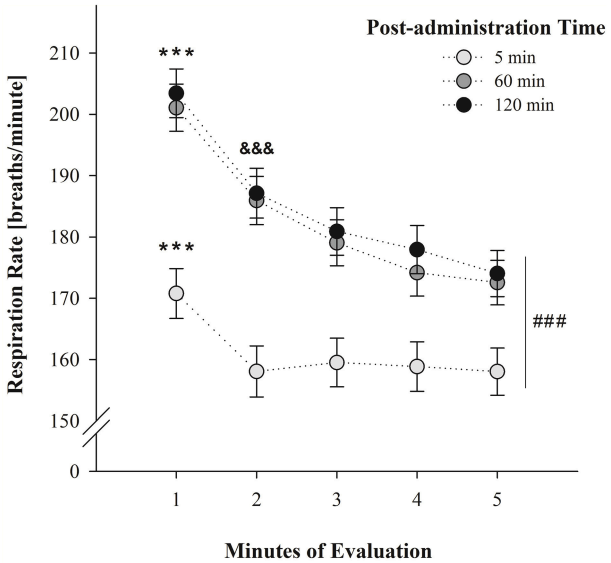

FIGURE 1 | Breathing rates (breaths/min) as a function of post-administration time (5, 60, or 120 min) and minutes of evaluation. Data have been collapsed across sex, postnatal days and drug treatment. ### Indicates significant differences between breathing scores at post-administration time 5 min relative to scores attained at post-administration time 60 and 120 min. *** Indicates significant differences between respiratory rates at minute 1 relative to the remaining minutes of evaluation; $p < 0.0001$. &&& Indicates significant differences between minute 2 and minute 5; $p < 0.0001$. Vertical lines indicate standard errors of the means (SEMs).

Breathing Frequencies during Drug Pretreatment (PDs 2 and 4) and test (PD5)

Figure 1 illustrates average breathing responses corresponding to PDs 2 and 4 as a function of post-administration time (5, 60, or 120 min) and across minutes of evaluation. Respiration rates were not significantly different across days. The between-within ANOVA [drug treatment (PB 0.1 M, ACD 0.35 μmol or ethanol 100 mg%) × sex (female or male) × post-administration time (5, 60, or 120 min) × days of assessment (PDs 2 and 4) × minutes of evaluation (1–5)] indicated significant main effects of sex $F_{(1, 125)} = 5.90$, $p = 0.0158$; post-administration time $F_{(2, 125)} = 12.27$, $p < 0.0001$; minutes of evaluation $F_{(4, 500)} = 118.49$, $p < 0.0001$ as well as significant interactions between post-administration time and minutes of evaluation $F_{(8, 500)} = 7.48$, $p < 0.0001$. Drug treatment was also found to significantly interact with post-administration time and minutes of evaluation; $F_{(16, 500)} = 2.13$, $p = 0.0063$.

According to Duncan's *post-hoc* tests, breathing frequencies were significantly higher in male than female pups (181.30 ± 2.99 and 170.91 ± 3.01 breaths/min; respectively). Relative to the significant main effects of post-administration time, minutes of evaluation, and its significant interaction at PDs 2 and 4; *post-hoc* tests showed that breathing responses were significantly lower at post-administration time 5 min relative to the scores attained at 60 and 120 min. It is likely that the stress related with the intracisternal administration of the drugs, affected breathing rates shortly after performing these procedures. At 60 and 120 min, respiratory frequencies were similar to those reported in previous experiments where a given vehicle (e.g., water) was intragastrically administered 30 min prior to the evaluation (Macchione et al., 2016; Acevedo et al., 2017)

Within each test, respiration rates decreased as a function of the progression of the test; a phenomenon probably indicative of habituation to the context. This progressive depression was particularly observed in the group of animals evaluated at 60 and 120 min. When the evaluation was conducted 5 min after drug administration pups exhibited heightened respiratory rates during the initial minute of the test relative to the remaining minutes. This interaction has been depicted in **Figure 1**.

With regard to the triple interaction involving drug treatment, post-administration time and minutes of evaluation, planned comparisons indicated significant differences in respiration rates between PB-treated and ACD-treated pups during the first minute of evaluation (**Figure 2**). This effect was only observed 5 min after administering the drug. At this point in time, ethanol-treated animals showed intermediate respiratory frequencies relative to PB- and ACD-treated pups.

At PD 5, the corresponding between-within ANOVA (drug treatment at PDs 2 and 4 × sex × post-administration time × minutes of evaluation) showed that breaths per minute significantly varied as a function of drug treatment [$F_{(2, 128)} = 4.37$, $p = 0.0146$], minutes of evaluation [$F_{(4, 512)} = 110.95$, $p <$

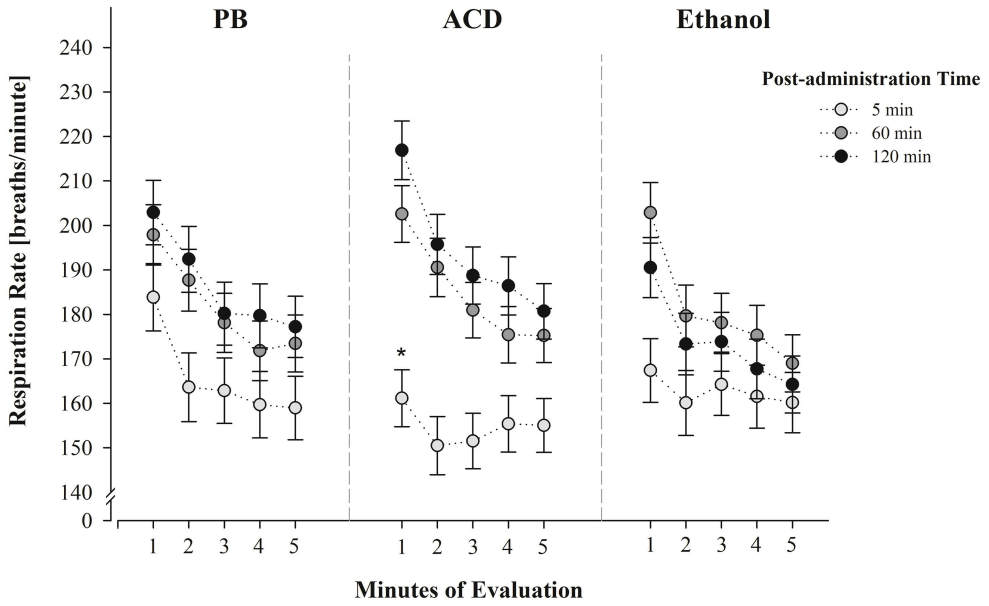

FIGURE 2 | Respiration frequencies (breaths/min) at PDs 2 and 4 as a function of drug treatment (PB, ACD or Ethanol), post-administration time (5, 60, or 120 min) and minutes of evaluation (1–5). Data have been collapsed across sex and postnatal days. * Indicates a significant difference between PB-treated and ACD-treated pups (post-administration time: 5 min.; minute of evaluation: 1 min); $p < 0.05$. Vertical lines indicate standard errors of the means (SEMs).

0.0001] and the following two-way interaction: drug treatment × minutes of evaluation [$F_{(8, 512)} = 1.99$, $p = 0.0447$].

Once, again breathing responses progressively decreased as a function of the passage of time. Moreover, the group of animals previously treated with acetaldehyde during PDs 2 and 4 exhibited significantly higher breathing frequencies relative to the control group (PB). This significant difference was observed at minutes 2, 3, 4, and 5. Breathing scores of ethanol-treated pups did not significantly differ from PB- or ACD-treated subjects throughout the evaluation. This interaction has been depicted in **Figure 3**. As can be observed ACD-treated pups exhibited during PD5 breathing patterns which were opposite to those recorded at PDs 2 and 4 at the earliest post-administration time (5 min). When tested without being administered with acetaldehyde (PD5) breathing frequencies were significantly higher than in controls while under the effects of the drug (PDs 2 and 4), respiration rates were significantly lower.

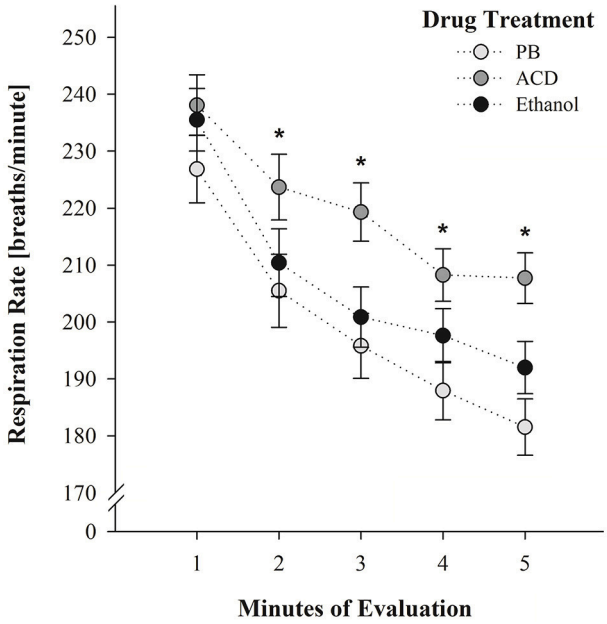

FIGURE 3 | Breathing rates (breaths/min) as a function of drug treatment (PB, ACD, or Ethanol) and minutes of evaluation at PD 5. * Indicates significant differences between ACD pups and pups pre-exposed to PB administrations. Vertical lines indicate standard errors of the means (SEMs).

Thermal Responsiveness During Drug Treatment (PDs 2 and 4) and Test (PD 5)

As stated, body temperatures of pups treated with PB 0.1M, ACD 0.35 μmol or ethanol 100 mg% were recorded immediately before and after being exposed to the plethysmograph at PDs 2 and 4 as well as at PD5 (see **Table 1**). The corresponding between-within ANOVA (drug treatment × sex × post-administration time × days of assessment x moment of recording) during PDs 2 and 4 only indicated a significant main effect of post-administration time; $F_{(2, 117)} = 92.26$, $p < 0.0001$. Thermal temperatures soon after drug treatment (5 min) were significantly lower (33.91 ± 0.12°C) than those observed at post-administration times 60 and 120 min (35.96 ± 0.11 and 35.62 ± 0.12°C; respectively) Apparently, a stress-related factor derived form intracerebral

administrations was responsible for the significant decrease in body temperature in pups tested 5 mins after the injection. At PD 5 (drug treatment × sex × post-administration time × moment of recording) no significant differences emerged when considering the main factors and the interactions between them. Notice that at this age pups were not IC administered. A similar lack of main significant effects or interactions was observed when processing body temperatures before and after the operant task.

Operant Conditioning at PD5

Figure 4 depicts the total number of sensor contacts in Paired and Yoked groups reinforced with either intraorally administered saccharin or ethanol. Separate ANOVAs were conducted to analyze learning patterns dependent upon either saccharin (0.1%) or ethanol (3%) reinforcement. In each case, tree-way mixed ANOVAs were used. This analyses were defined by drug treatment as the between factor and conditioning (Paired or Yoked) as well as minutes of evaluation as within factors.

TABLE 1 | Pup's body temperatures across days as a function of drug treatment and post-administration time.

Drug treatment at PDs 2 and 4	Post-administration Time (min)	Body temperature (°C)					
		PD2		PD4		PD5	
		Before	After	Before	After	Before	After
Phosphate buffer (PB 0.1M)	5	34.23 ± 0.31	34.12 ± 0.29	33.96 ± 0.27	34.05 ± 0.28	34.99 ± 0.27	34.77 ± 0.28
	60	35.73 ± 0.30	36.01 ± 0.28	35.81 ± 0.26	35.66 ± 0.27	34.91 ± 0.24	35.06 ± 0.25
	120	35.67 ± 0.31	35.86 ± 0.29	35.56 ± 0.27	35.40 ± 0.28	34.66 ± 0.26	34.58 ± 0.27
Acetaldehyde (0.35 μmol)	5	33.83 ± 0.28	34.12 ± 0.26	33.71 ± 0.25	33.83 ± 0.25	34.72 ± 0.24	34.57 ± 0.24
	60	36.23 ± 0.27	36.35 ± 0.25	35.98 ± 0.23	35.83 ± 0.24	35.12 ± 0.23	34.38 ± 0.24
	120	35.68 ± 0.29	35.36 ± 0.27	36.04 ± 0.26	35.49 ± 0.26	34.64 ± 0.23	34.96 ± 0.24
Ethanol (100 mg%)	5	33.83 ± 0.30	34.12 ± 0.28	33.42 ± 0.27	33.63 ± 0.28	34.43 ± 0.26	34.17 ± 0.26
	60	36.21 ± 0.26	36.07 ± 0.25	35.70 ± 0.23	35.87 ± 0.24	35.18 ± 0.23	34.65 ± 0.24
	120	35.76 ± 0.30	35.53 ± 0.28	35.78 ± 0.26	35.28 ± 0.27	34.57 ± 0.25	34.39 ± 0.25

Values are expressed as means ± SEMs. Body temperatures at PDs 2 and 4, recorded immediately after intracisternal administrations (5 min), were significantly lower than those registered at 60 or 120 min.

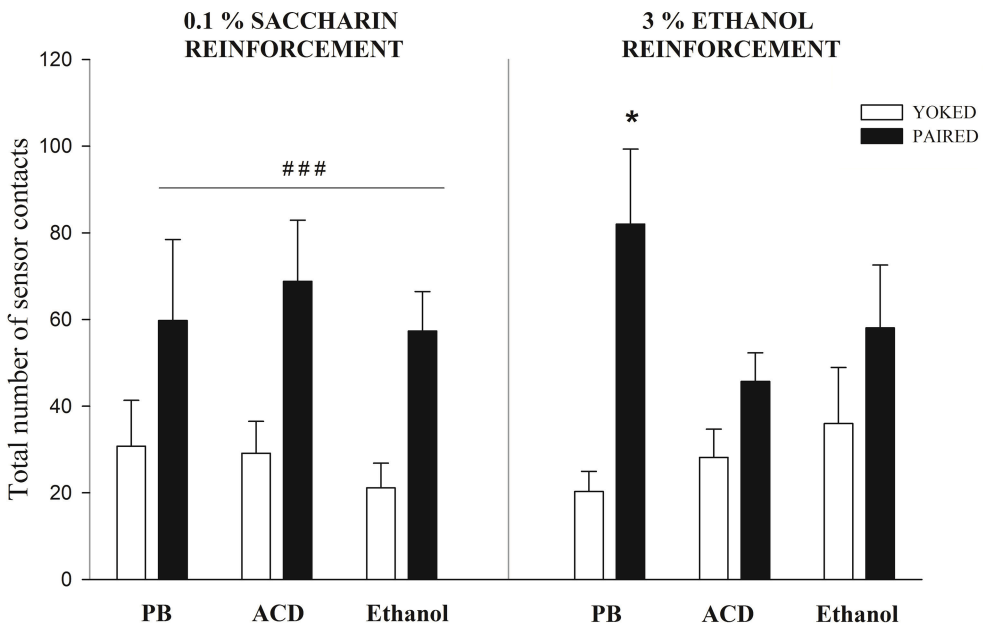

FIGURE 4 | Sensor contacts at PD5 when pups were reinforced with saccharin or ethanol. When saccharin was employed a main significant effect of conditioning (Paired vs. Yoked) was observed. ### Indicates the significantly higher level of responding of Paired pups when compared to Yoked controls; $p < 0.0001$. When ethanol served as a reinforcer, only Paired pups pre-exposed to PB exhibited significantly higher levels of sensor contacts when compared with the corresponding Yoked controls (*); $p < 0.05$. Vertical lines indicate standard errors of the means (SEMs).

When saccharin was employed as a reinforcer, the ANOVA indicated that conditioning exerted a significant main effect [$F_{(1, 28)} = 34.82$, $p < 0.0001$]. As can be observed in **Figure 4**, all Paired groups, independently from prior drug experience, showed higher operant responsiveness relative to the corresponding Yoked controls. This result is analogous to those reported when employing saccharin in older infants (Miranda-Morales et al., 2014) or when neonates are reinforced with milk (Arias et al., 2007; Bordner et al., 2008). It is interesting to note that prior drug exposure appears not to affect the learning capability of the organisms nor its overall activity. Relative to the activity rate, Yoked controls pretreated with buffer, ethanol or acetaldehyde showed similar levels of spontaneous sensor contacts.

When ethanol was employed as a reinforcer not all Paired groups differed from the corresponding Yoked controls. The ANOVA revealed a significant main effect of conditioning and a significant interaction between this factor and drug treatment [$F_{(1, 32)} = 21.75$, $p < 0.0001$ and $F_{(2, 32)} = 3.51$, $p = 0.0419$; respectively]. Planned comparisons indicated that only Paired pups treated with a PB control solution had higher sensor contacts that their corresponding Yoked controls. Pretreatment with ethanol or its metabolite appeared to decrease the reinforcement capability of ethanol. Once again, this effect cannot be attributed to motor activity differences across drug pretreatments that can contribute to the probability of sensor contacts. Relative to this issue, all Yoked groups has similar levels of activity.

DISCUSSION

As stated (see Introduction section) the present study pursued two main goals: (i) the analysis of central ethanol and ACD effects in terms of disruptive effects upon early respiratory plasticity and possible association existing between ethanol and/or ACD central effects leading to thermoregulatory alterations in neonatal rats, and (ii) the analysis of central pre-exposure to the ethanol or its metabolite effect on subsequent seeking behavior of ethanol as a reinforcer in an operant task in neonate rats (Arias et al., 2007; Bordner et al., 2008; March et al., 2009; Miranda-Morales et al., 2014).

Ethanol and ACD doses were chosen according to previous literature indicating analogous effects at least when considering the motivational properties of these drugs (Nizhnikov et al., 2006, 2007; March et al., 2013a,b). When doing so, during PDs 2 and 4, neonates exhibited a respiratory depression when administered with ACD and tested only 5 min after drug administration. This effect was clear at the beginning of the testing procedure (minute 1) relative to control pups administered with PB. Pups treated with ethanol exhibited intermediate values relative to the above mentioned groups. These effects were observed despite the fact that at this post-administration time, respiration rates were very low across groups (see **Figure 2**). Preliminary pilot studies performed with untreated animals confirmed that the mere handling of the neonates is enough to alter respiratory frequencies when evaluations are temporally close to this manipulation. The values obtained in these untreated pups were found to be almost identical to the PB controls here utilized.

It was also observed that respiratory frequencies increased at 60 and 120 min post-administration time (**Figure 1**) and neither ethanol nor ACD exerted depressant effects relative to controls. Relative to ACD, these null results may indicate that further pharmacokinetic processes (e.g., ACD conversion into acetate) partially or completely reduce brain concentrations of the metabolite (Quertemont and Didone, 2006; Zimatkin et al., 2006; Hipolito et al., 2007). The fact that ethanol was never found to produce significant respiratory decrements may be related with the dose here employed. Under the present experimental circumstances, it is not possible to determine whether the 100 mg% dose is sufficient to negatively act upon respiratory plasticity or generate, via oxidative processes, ACD levels capable of disrupting breathing patterns. Relative to this dose-related problem, and as previously stated, it is interesting to note that pups tested shortly after receiving brain ethanol administration, exhibited a trend toward a reduction in breathing frequencies relative to controls but not as profound as the group treated with ACD (**Figure 2**).

At PD5, pups were re-exposed to the testing chamber without receiving any explicit drug treatment. ACD pretreated neonates were found to show heightened respiratory frequencies relative to the remaining groups (**Figure 3**). We cannot discard the possibility that prior administrations of the metabolite disrupted the respiratory system causing hyperventilation. Nevertheless, a second hypothesis seems plausible. The effect at PD5 (high respiration rates) was opposite relative to the one observed at PDs 2 and 4 (low respiration rates). This apparent contradiction is in agreement with the establishment of learned tolerance to drugs of abuse where conditioned stimuli elicit neurally-mediated homeostatic responses that serve to reduce a specific perturbation (Woods and Ramsay, 2000). Some studies have shown that ambient cues associated with the depressant effects of ethanol appear to modulate the effects of the drug upon respiratory plasticity (Cullere et al., 2015; Macchione et al., 2016; Acevedo et al., 2017). This modulation is related with classical conditioning learning where ambient cues such as the testing environment or a specific odorant (e.g., ethanol odor perceived in the amniotic fluid or as an ambient odor) are associated with the unconditioned effect of the drug. Similar learning processes have been observed in 2-day-old mice when olfactory cues associated with maternal care resulted in heightened conditioned respiratory responses (Durand et al., 2003). When utilizing peripheral ethanol in developing rats, conditioned responses are *isodirectional* relative to the depressant effects upon respiration. In accordance with the systematic review of Eikelboom and Stewart (1982), physiological disruptions mediated by the central nervous system are associated with conditioned stimuli which later elicit *compensatory* conditioned reactivity. If the drug acts on afferent pathways of the brain, the associative process results in isodirectional conditioned responses. In agreement with their analysis and predictions based on specific feedback mechanisms, cues associated with respiratory depressions caused by peripheral (i.g.) ethanol administrations, latter elicit isodirectional learned responses (Macchione et al., 2016). As observed in the present

experiment, contextual cues associated with central-nervous-system-mediated respiratory depressions caused by ACD, cause the opposite (probably compensatory) effect.

As previously stated (see Introduction), thermoregulatory disruptions can determine or modulate respiratory depressions. When considering neonatal thermal responsiveness at PDs 2 and 4, it was clear that soon after intracisternal administrations (5 min), body temperatures were low when compared to those recorded at post-administrations time 60 or 120 min (**Table 1**). Stress-related effects of the administration procedure or even the temperature of the solutions injected into the cisterna magna are factors which can account for this phenomenon. As mentioned, at the earlier post-administration time (5 min) we also observed very low breathing frequencies; a result which argues in favor of the modulatory effects of thermoregulation upon breathing. Yet, drug treatment affected breathing but not thermal responsiveness; a result that argues in favor of early breathing disruptions caused by ACD independently from thermal alterations. At PD5, when tests were performed without any prior administration procedure, temperatures were similar across all groups but as stated, ACD pre-exposed neonates exhibited heightened respiratory rates. Once again, this phenomenon favors the hypothesis that ACD respiratory effects across the experiment were not related with temperature variations. Nevertheless, when taken into account that the administration procedure does affect thermoregulation and that all breathing tests were performed in chambers maintained at 31–32°C, a possible association between thermal and breathing disruptions should not be completely overruled.

The second major goal of the study attempted to elucidate whether prior exposure to central ethanol or ACD impacts upon operant conditioning processes where ethanol or saccharin serve as positive intraoral reinforcers. The results obtained with saccharin confirmed the rapid learning capability of neonates that has been reported when utilizing alternative sweet reinforcer or milk (Arias et al., 2007; Bordner et al., 2008; March et al., 2009). Independently of prior drug condition, pups exposed to the explicit contingency between sensor contacts and saccharin intraoral administration (Paired groups), exhibited relative to Yoked controls, a significantly higher number of responses. This pattern of results was not observed when ethanol served as a reinforcer. Once again, Paired pups pre-exposed to the buffer control solution significantly differed from the corresponding Yoked control group. As in previous studies, neonates without any specific prior drug experience rapidly learn to self-administer an ethanol solution (Bordner et al., 2008; March et al., 2009). This was not the case when neonates were centrally administered with ethanol or ACD (PDs 2 and 4) prior to the assessment of response-stimulus learning associations (PD5). When using these drugs Paired pups did not differ from Yoked controls. Furthermore, Paired pups with a prior history of ACD administrations differed from Paired pups pretreated with the buffer solution. As in the case of respiratory frequencies, Paired subjects pre-exposed to central ethanol, exhibited intermediate levels of responding relative to the two remaining drug-related Paired conditions (ACD or PB).

The results obtained with saccharin indicate that neither ethanol nor ACD pre-exposure altered learning capabilities of the neonates. Therefore, the absence of operant conditioning observed in Paired pups reinforced with ethanol that were previously treated with ethanol or ACD, argues against deleterious effects of these drugs upon the learning process itself. Two hypotheses appear pertinent when addressing the lack of significant learning in Paired subjects pre-exposed to ethanol or ACD and subsequently reinforced with ethanol. Both of them are based on the direct action of ACD in the central nervous system and the biotransformation of ethanol into its principal metabolite via the central catalase system. Favoring the possibility of rapid ethanol metabolism in the neonatal brain is the fact that catalase concentrations in the brain of the newborn rat are approximately eight times higher than in adult animals (Del Maestro and McDonald, 1987). The first hypothesis is related with prior findings concerning altered motor neonatal activity induced by central ACD administration. March et al. (2013a) reported that a single neonatal central administration of ACD (0.52 μmol) exerts a sedative effect upon motor activity. Hence, it is difficult to discard the possibility that sequential administrations of lower ACD doses (0.35 μmol) or of ethanol (100 mg%) into the brain, sensitizes the organism to later sedative of effects of ethanol administered during the operant conditioning task. Nevertheless, this hypothesis is not supported by the following observations. When focusing on the motor activity of Yoked controls (i.e., number of spontaneous sensor contacts) that also received ethanol as a function of the activity of the corresponding Paired neonates, no specific effects of prior drug treatment were detected. The second observation arguing against the motor-related hypothesis is that March et al. (2013a) also found that late prenatal exposure to ethanol generates tolerance rather than sensitization to the depressant of ACD. As stated, this metabolite is likely to be formed due to brain metabolic processes when neonates were exposed to ethanol during the operant task. Yet, when considering both ethanol and ACD pre-exposure effects upon operant performance, we cannot completely discard an alternative possibility of sensitization effects related with anxiogenic or antianxiety effects of both drugs. The arousal state involved in the acquisition of the operant response could be affected by either of these effects. Infants are sensitive to ethanol's antianxiety effects (Pautassi et al., 2007) but there is still no empirical evidence supporting a sensitization effect as a function of prior ethanol treatment during early development. On the contrary, in neonates, exposure to moderate or high ethanol doses seem to potentiate later states of anxiety (Brolese et al., 2014; Baculis et al., 2015). In adults, when considering centrally administered ACD, inhibition of the catalase system or when sequestering this metabolite, the results argue in favor of anxiogenic rather than antianxiety effects (Correa et al., 2008). Taken these considerations into account, sensitization to ethanol's or ACD's anti-anxiety effects does not seem to adequately account for the described disruptions in operant learning processes. The possibility of sensitization to anxiogenic effects of early ethanol or ACD central administration should not be discarded. The second hypothesis is related with the consequences of drug pre-exposure

upon subsequent ethanol's motivational properties. Only one conditioning trial has been utilized when central ethanol or ACD are observed to exert positive reinforcing effects in neonates (Nizhnikov et al., 2007; March et al., 2013a,b). So far we ignore if increasing the number of doses results in the recruitment of aversive properties of these psychopharmacological agents or maximizes the possibility of an unconditioned stimulus pre-exposure effect that later competes with the contingency existing between operant responses and ethanol reinforcement. In either case, subsequent ethanol positive reinforcing effects are likely to be devalued. Most importantly, it is necessary to consider that during PDs 2 and 4, these drugs were intracisternally administered. This procedure, the additional handling of the neonate and the isolation from the mother can be viewed as significant aversive stressors (Molina et al., 2000; Hofer et al., 2002; Pautassi et al., 2007). Hence, during these days the effects of the drugs were contingent with aversive events; an association that may compete with subsequent reinforcing effects of ethanol or its metabolite. Notice that whenever pre-exposure to ethanol has resulted in early sensitization to the reinforcing effects of the drug (Nizhnikov et al., 2006; Pautassi et al., 2012), the initial drug experience occurred during late prenatal life via maternal ethanol administration and without any explicit manipulation of the fetus or its natural environment. In support of the present hypothesis, studies have demonstrated that early in ontogeny, the rat is capable of associating different motivational effects of ethanol (positive reinforcing, aversive or anxiolytic) with aversive and appetitive stimuli such as citric acid and sucrose; respectively. As a result of the nature of the associations, the effects of the drug or of the alternative stimuli are reduced or potentiated (Molina et al., 1996; Pautassi et al., 2006; Cullere et al., 2014). The proposed hypothesis may also apply when considering the heightened respiratory rates (PD5) observed in neonates pre-exposed to acetaldehyde. The interoceptive effects of the metabolite (PDs 2 and 4) were experienced in a distinct context (plethysmograph chamber) immediately following intracisternal administration of the drug and while pups were isolated from the mother. At test (PD5), these pups were again placed in the context and as stated, they exhibited a significant increase in breathing frequencies. This physiological reaction, under the framework of the present hypothesis, may represent an anticipatory physiological response linked with prior experiences involving the context, the interoceptive effects of acetaldehyde and different stressors. Obviously, this hypothesis requires further investigation.

Beyond these considerations, the results of this study argue in favor of centrally mediated respiratory and motivational effects of acetaldehyde during a stage in development comparable to the 3rd human gestational trimester. To our knowledge this is the first study indicating a significant role of the metabolite upon early respiratory plasticity. This phenomenon logically requires further investigation particularly when considering the negative effects of ethanol upon the developing respiratory network (Abel, 1981; Dubois et al., 2013). In conjunction with recently conducted studies in rat fetuses and neonates (Cullere et al., 2015; Macchione et al., 2016; Acevedo et al., 2017), the present results confirm that disruptions of the developing respiratory network are attained even when employing moderate levels of exposure to ethanol, or in the present case, to acetaldehyde. Breathing depressions associated with hypoxemia and bradycardia represent a risk factor in terms of hypoxic ischemic effects upon the developing human brain (Pillekamp et al., 2007). As mentioned, fetal alcohol exposure also represents a risk factor for Sudden Infant Death Syndrome (Burd et al., 2004; O'Leary et al., 2013), a phenomenon that has driven scientific attention toward the effects of the drug upon respiratory plasticity. Hence, when considering breathing disruptions involved in hypoxic ischemic consequences upon the brain and the need to better understand factors that can potentially predispose to a devastating pathology such as the Sudden Infant Death Syndrome, the role of acetaldehyde, following ethanol exposure, deserves further scientific attention. Finally, the present study also indicates that centrally administered acetaldehyde impacts upon later ethanol self-administration patterns operationalized through operant conditioning procedures. From a general perspective, these results validate the notions that the metabolite is a neuroactive agent capable of mediating ethanol's motivational properties (Quertemont et al., 2005; Correa et al., 2012) and that it plays a significant role during early ontogeny in terms of structuring ethanol affinity (March et al., 2013a).

AUTHOR CONTRIBUTIONS

All authors (MA, GD, OH and JM) meet the following criteria: -contributed to the conception or design of the work, -contributed to the analysis and interpretation of data, -participated in the writing and revision of the draft, and -agree to be accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved.

FUNDING

This work was supported by grants from Agencia Nacional de Promoción Científica y Tecnológica (FONCyT, PICT 2011-0999 and PICT 2014-1606), SECyT and CONICET PIP 11220110100763 awarded to JM;.

REFERENCES

Abate, P., Pepino, M. Y., Spear, N. E., and Molina, J. C. (2004). Fetal learning with ethanol: correlations between maternal hypothermia during pregnancy and neonatal responsiveness to chemosensory cues of the drug. *Alcohol. Clin. Exp. Res.* 28, 805–815. doi: 10.1097/01.ALC.0000125354.15808.24

Abate, P., Pueta, M., Spear, N. E., and Molina, J. C. (2008). Fetal learning about ethanol and later ethanol responsiveness: evidence against "safe" amounts of prenatal exposure. *Exp. Biol. Med.* 233, 139–154. doi: 10.3181/0703-MR-69

Abate, P., Spear, N. E., and Molina, J. C. (2001). Fetal and infantile alcohol-mediated associative learning in the rat. *Alcohol. Clin. Exp. Res.* 25, 989–998. doi: 10.1111/j.1530-0277.2001.tb02307.x

Abel, E. L. (1981). A critical evaluation of the obstetric use of alcohol in preterm labor. *Drug Alcohol Depend.* 7, 367–378. doi: 10.1016/0376-8716(81)90051-X

Acevedo, M. B., Macchione, A. F., Anunziata, F., Haymal, O. B., and Molina, J. C. (2017). Neonatal experiences with ethanol intoxication modify respiratory and thermoregulatory plasticity and affect subsequent ethanol intake in rats. *Dev. Psychobiol.* 59, 48–59. doi: 10.1002/dev.21466

Alati, R., Al Mamun, A., Williams, G. M., O'Callaghan, M., Najman, J. M., and Bor, W. (2006). *In utero* alcohol exposure and prediction of alcohol disorders in early adulthood: a birth cohort study. *Arch. Gen. Psychiatry* 63, 1009–1016. doi: 10.1001/archpsyc.63.9.1009

Aragon, C. M., Stotland, L. M., and Amit, Z. (1991). Studies on ethanol-brain catalase interaction: evidence for central ethanol oxidation. *Alcohol. Clin. Exp. Res.* 15, 165–169. doi: 10.1111/j.1530-0277.1991.tb01848.x

Arias, C., Spear, N. E., Molina, J. C., Molina, A., and Molina, J. C. (2007). Rapid acquisition of operant conditioning in 5-day-old rat pups: a new technique articulating suckling-related motor activity and milk reinforcement. *Dev. Psychobiol.* 49, 576–588. doi: 10.1002/dev.20236

Baculis, B. C., Diaz, M. R., and Valenzuela, C. F. (2015). Third trimester-equivalent ethanol exposure increases anxiety-like behavior and glutamatergic transmission in the basolateral amygdala. *Pharmacol. Biochem. Behav.* 137, 78–85. doi: 10.1016/j.pbb.2015.08.009

Baer, J. S., Barr, H. M., Bookstein, F. L., Sampson, P. D., and Streissguth, A. P. (1998). Prenatal alcohol exposure and family history of alcoholism in the etiology of adolescent alcohol problems. *J. Stud. Alcohol* 59, 533–543. doi: 10.15288/jsa.1998.59.533

Baer, J. S., Sampson, P. D., Barr, H. M., Connor, P. D., and Streissguth, A. P. (2003). A 21-year longitudinal analysis of the effects of prenatal alcohol exposure on young adult drinking. *Arch. Gen. Psychiatry* 60, 377–385. doi: 10.1001/archpsyc.60.4.377

Bordner, K. A., Molina, J. C., and Spear, N. E. (2008). Analysis of ethanol reinforcement in 1-day-old rats: assessment through a brief and novel operant procedure. *Alcohol. Clin. Exp. Res.* 32, 580–592. doi: 10.1111/j.1530-0277.2007.00609.x

Brien, J. F., and Smith, G. N. (1991). Effects of alcohol (ethanol) on the fetus. *J. Dev. Physiol.* 15, 21–32.

Brolese, G., Lunardi, P., Broetto, N., Engelke, D. S., Lirio, F., Batassini, C., et al. (2014). Moderate prenatal alcohol exposure alters behavior and neuroglial parameters in adolescent rats. *Behav. Brain Res.* 269, 175–184. doi: 10.1016/j.bbr.2014.04.023

Burd, L., Klug, M., and Martsolf, J. (2004). Increased sibling mortality in children with fetal alcohol syndrome. *Addict. Biol.* 9, 179–186. discussion: 187–178. doi: 10.1080/13556210410001717088

Cheslock, S. J., Varlinskaya, E. I., Petrov, E. S., Silveri, M. M., Spear, L. P., and Spear, N. E. (2001). Ethanol as a reinforcer in the newborn's first suckling experience. *Alcohol. Clin. Exp. Res.* 25, 391–402. doi: 10.1111/j.1530-0277.2001.tb02226.x

Closon, C., Didone, V., Tirelli, E., and Quertemont, E. (2009). Acetaldehyde and the hypothermic effects of ethanol in mice. *Alcohol. Clin. Exp. Res.* 33, 2005–2014. doi: 10.1111/j.1530-0277.2009.01039.x

Correa, M., Manrique, H. M., Font, L., Escrig, M. A., and Aragon, C. M. (2008). Reduction in the anxiolytic effects of ethanol by centrally formed acetaldehyde: the role of catalase inhibitors and acetaldehyde-sequestering agents. *Psychopharmacology* 200, 455–464. doi: 10.1007/s00213-008-1219-3

Correa, M., Salamone, J. D., Segovia, K. N., Pardo, M., Longoni, R., Spina, L., et al. (2012). Piecing together the puzzle of acetaldehyde as a neuroactive agent. *Neurosci. Biobehav. Rev.* 36, 404–430. doi: 10.1016/j.neubiorev.2011.07.009

Cullere, M. E., Spear, N. E., and Molina, J. C. (2014). Prenatal ethanol increases sucrose reinforcement, an effect strengthened by postnatal association of ethanol and sucrose. *Alcohol* 48, 25–33. doi: 10.1016/j.alcohol.2013.11.003

Cullere, M., Macchione, A. F., Haymal, B., Paradelo, M., Langer, M. D., Spear, N. E., et al. (2015). Neonatal sensitization to ethanol-induced breathing disruptions as a function of late prenatal exposure to the drug in the rat: modulatory effects of ethanol's chemosensory cues. *Physiol. Behav.* 139, 412–422. doi: 10.1016/j.physbeh.2014.10.017

Del Maestro, R., and McDonald, W. (1987). Distribution of superoxide dismutase, glutathione peroxidase and catalase in developing rat brain. *Mech. Ageing Dev.* 41, 29–38. doi: 10.1016/0047-6374(87)90051-0

Dillner, L., Josefson, D., Karcher, H., Sheldon, T., Dorozynski, A., and Zinn, C. (1996). Alcohol–pushing the limits. *BMJ* 312, 7–9. doi: 10.1136/bmj.312.7022.7

Dobbing, J., and Sands, J. (1973). Quantitative growth and development of human brain. *Arch. Dis. Child.* 48, 757–767. doi: 10.1136/adc.48.10.757

Dobbing, J., and Sands, J. (1979). Comparative aspects of the brain growth spurt. *Early Hum. Dev.* 3, 79–83. doi: 10.1016/0378-3782(79)90022-7

Dominguez, H. D., Lopez, M. F., Chotro, M. G., and Molina, J. C. (1996). Perinatal responsiveness to alcohol's chemosensory cues as a function of prenatal alcohol administration during gestational days 17-20 in the rat. *Neurobiol. Learn. Mem.* 65, 103–112. doi: 10.1006/nlme.1996.0012

Dominguez, H. D., Lopez, M. F., and Molina, J. C. (1998). Neonatal responsiveness to alcohol odor and infant alcohol intake as a function of alcohol experience during late gestation. *Alcohol* 16, 109–117. doi: 10.1016/S0741-8329(97)00169-9

Dubois, C. J., Kervern, M., Naassila, M., and Pierrefiche, O. (2013). Chronic ethanol exposure during development: disturbances of breathing and adaptation. *Respir. Physiol. Neurobiol.* 189, 250–260. doi: 10.1016/j.resp.2013.06.015

Dubois, C. J., Naassila, M., Daoust, M., and Pierrefiche, O. (2006). Early chronic ethanol exposure in rats disturbs respiratory network activity and increases sensitivity to ethanol. *J. Physiol.* 576(Pt 1), 297–307. doi: 10.1113/jphysiol.2006.111138

Durand, E., Dauger, S., Vardon, G., Gressens, P., Gaultier, C., De Schonen, S., et al. (2003). Classical conditioning of breathing pattern after two acquisition trials in 2-day-old mice. *J. Appl. Physiol. (1985)* 94, 812–818. doi: 10.1152/japplphysiol.00488.2002

Duxbury, M. (2001). "El papel de la enfermera en neonatología," in *Neonatología: Fisiopatología y Manejo del Recién Nacido*, eds G. Avery and M. A. Flechter Hardcover (Buenos Aires: Editorial Médica Panamericana S.A), 75–115.

Eikelboom, R., and Stewart, J. (1982). Conditioning of drug-induced physiological responses. *Psychol. Rev.* 89, 507–528. doi: 10.1037/0033-295X.89.5.507

Faas, A. E., March, S. M., Moya, P. R., and Molina, J. C. (2015). Alcohol odor elicits appetitive facial expressions in human neonates prenatally exposed to the drug. *Physiol. Behav.* 148, 78–86. doi: 10.1016/j.physbeh.2015.02.031

Faas, A. E., Sponton, E. D., Moya, P. R., and Molina, J. C. (2000). Differential responsiveness to alcohol odor in human neonates: effects of maternal consumption during gestation. *Alcohol* 22, 7–17. doi: 10.1016/S0741-8329(00)00103-8

Fabio, M. C., March, S. M., Molina, J. C., Nizhnikov, M. E., Spear, N. E., and Pautassi, R. M. (2013). Prenatal ethanol exposure increases ethanol intake and reduces c-Fos expression in infralimbic cortex of adolescent rats. *Pharmacol. Biochem. Behav.* 103, 842–852. doi: 10.1016/j.pbb.2012.12.009

Foltran, F., Gregori, D., Franchin, L., Verduci, E., and Giovannini, M. (2011). Effect of alcohol consumption in prenatal life, childhood, and adolescence on child development. *Nutr. Rev.* 69, 642–659. doi: 10.1111/j.1753-4887.2011.00417.x

Gill, K., Menez, J. F., Lucas, D., and Deitrich, R. A. (1992). Enzymatic production of acetaldehyde from ethanol in rat brain tissue. *Alcohol. Clin. Exp. Res.* 16, 910–915. doi: 10.1111/j.1530-0277.1992.tb01892.x

Griesler, P. C., and Kandel, D. B. (1998). The impact of maternal drinking during and after pregnancy on the drinking of adolescent offspring. *J. Stud. Alcohol* 59, 292–304. doi: 10.15288/jsa.1998.59.292

Hahn, C. Y., Huang, S. Y., Ko, H. C., Hsieh, C. H., Lee, I. H., Yeh, T. L., et al. (2006). Acetaldehyde involvement in positive and negative alcohol expectancies in han Chinese persons with alcoholism. *Arch. Gen. Psychiatry* 63, 817–823. doi: 10.1001/archpsyc.63.7.817

Hall, W. G. (1979). Feeding and behavioral activation in infant rats. *Science* 205, 206–209. doi: 10.1126/science.451591

Hamby-Mason, R., Chen, J. J., Schenker, S., Perez, A., and Henderson, G. I. (1997). Catalase mediates acetaldehyde formation from ethanol in fetal and neonatal rat brain. *Alcohol. Clin. Exp. Res.* 21, 1063–1072. doi: 10.1111/j.1530-0277.1997.tb04255.x

Hipolito, L., Sanchez, M. J., Polache, A., and Granero, L. (2007). Brain metabolism of ethanol and alcoholism: an update. *Curr. Drug Metab.* 8, 716–727. doi: 10.2174/138920007782109797

Hofer, M. A., Shair, H. N., and Brunelli, S. A. (2002). Ultrasonic vocalizations in rat and mouse pups. *Curr. Protoc. Neurosci.* Chapter 8:Unit 8.14. doi: 10.1002/0471142301.ns0814s17

Holson, R. R., and Pearce, B. (1992). Principles and pitfalls in the analysis of prenatal treatment effects in multiparous species. *Neurotoxicol. Teratol.* 14, 221–228. doi: 10.1016/0892-0362(92)90020-B

Julien, C. A., Joseph, V., and Bairam, A. (2010). Caffeine reduces apnea frequency and enhances ventilatory long-term facilitation in rat pups raised in chronic intermittent hypoxia. *Pediatr. Res.* 68, 105–111. doi: 10.1203/PDR.0b013e3181e5bc78

Macchione, A. F., Anunziata, F., Cullere, M. E., Haymal, B. O., Spear, N., Abate, P., et al. (2016). Conditioned breathing depression during neonatal life as a function of associating ethanol odor and the drug's intoxicating effects. *Dev. Psychobiol.* 58, 670–686. doi: 10.1002/dev.21398

March, S. M., Abate, P., and Molina, J. C. (2013a). Acetaldehyde involvement in ethanol's postabsortive effects during early ontogeny. *Front. Behav. Neurosci.* 7:70. doi: 10.3389/fnbeh.2013.00070

March, S. M., Abate, P., Spear, N. E., and Molina, J. C. (2009). Fetal exposure to moderate ethanol doses: heightened operant responsiveness elicited by ethanol-related reinforcers. *Alcohol. Clin. Exp. Res.* 33, 1981–1993. doi: 10.1111/j.1530-0277.2009.01037.x

March, S. M., Abate, P., Spear, N. E., and Molina, J. C. (2013b). The role of acetaldehyde in ethanol reinforcement assessed by Pavlovian conditioning in newborn rats. *Psychopharmacology* 226, 491–499. doi: 10.1007/s00213-012-2920-9

Miranda-Morales, R. S., Molina, J. C., Spear, N. E., and Abate, P. (2010). Participation of the endogenous opioid system in the acquisition of a prenatal ethanol-related memory: effects on neonatal and preweanling responsiveness to ethanol. *Physiol. Behav.* 101, 153–160. doi: 10.1016/j.physbeh.2010.04.033

Miranda-Morales, R. S., Molina, J. C., Spear, N. E., and Abate, P. (2012a). Naloxone attenuation of ethanol-reinforced operant responding in infant rats in a re-exposure paradigm. *Psychopharmacology* 219, 235–246. doi: 10.1007/s00213-011-2402-5

Miranda-Morales, R. S., Nizhnikov, M. E., and Spear, N. E. (2014). Prenatal exposure to ethanol during late gestation facilitates operant self-administration of the drug in 5-day-old rats. *Alcohol* 48, 19–23. doi: 10.1016/j.alcohol.2013.11.001

Miranda-Morales, R. S., Spear, N. E., Nizhnikov, M. E., Molina, J. C., and Abate, P. (2012b). Role of mu, delta and kappa opioid receptors in ethanol-reinforced operant responding in infant rats. *Behav. Brain Res.* 234, 267–277. doi: 10.1016/j.bbr.2012.07.002

Molina, J. C., Bannoura, M. D., Chotro, M. G., McKinzie, D. L., Arnold, H. M., and Spear, N. E. (1996). Alcohol-mediated tactile conditioned aversions in infant rats: devaluation of conditioning through alcohol-sucrose associations. *Neurobiol. Learn. Mem.* 66, 121–132. doi: 10.1006/nlme.1996.0053

Molina, J. C., Pautassi, R. M., Truxell, E., and Spear, N. (2007). Differential motivational properties of ethanol during early ontogeny as a function of dose and postadministration time. *Alcohol* 41, 41–55. doi: 10.1016/j.alcohol.2007.01.005

Molina, J. C., Pepino, M. Y., Johnson, J., and Spear, N. E. (2000). The infant rat learns about alcohol through interaction with an intoxicated mother. *Alcohol. Clin. Exp. Res.* 24, 428–437. doi: 10.1111/j.1530-0277.2000.tb02009.x

National Research Council (1996). *Guide for the Care and Use of Laboratory Animals*. Washington, DC: National Academy Press.

Nizhnikov, M. E., Molina, J. C., and Spear, N. E. (2007). Central reinforcing effects of ethanol are blocked by catalase inhibition. *Alcohol* 41, 525–534. doi: 10.1016/j.alcohol.2007.08.006

Nizhnikov, M. E., Varlinskaya, E. I., and Spear, N. E. (2006). Reinforcing effects of central ethanol injections in newborn rat pups. *Alcohol. Clin. Exp. Res.* 30, 2089–2096. doi: 10.1111/j.1530-0277.2006.00253.x

O'Leary, C. M., Jacoby, P. J., Bartu, A., D'Antoine, H., and Bower, C. (2013). Maternal alcohol use and sudden infant death syndrome and infant mortality excluding SIDS. *Pediatrics* 131, e770–e778. doi: 10.1542/peds.2012-1907

Pautassi, R. M., Nizhnikov, M. E., Fabio, M. C., and Spear, N. E. (2011). An acetaldehyde-sequestering agent inhibits appetitive reinforcement and behavioral stimulation induced by ethanol in preweanling rats. *Pharmacol. Biochem. Behav.* 97, 462–469. doi: 10.1016/j.pbb.2010.10.005

Pautassi, R. M., Nizhnikov, M. E., Spear, N. E., and Molina, J. C. (2012). Prenatal ethanol exposure leads to greater ethanol-induced appetitive reinforcement. *Alcohol* 46, 585–593. doi: 10.1016/j.alcohol.2012.05.004

Pautassi, R. M., Nizhnikov, M., Molina, J. C., Boehm, S. L. II. and Spear, N. (2007). Differential effects of ethanol and midazolam upon the devaluation of an aversive memory in infant rats. *Alcohol* 41, 421–431. doi: 10.1016/j.alcohol.2007.05.001

Pautassi, R. M., Sanders, S., Miller, S., Spear, N., and Molina, J. C. (2006). Early ethanol's anxiolytic effects assessed through an unconditional stimulus revaluation procedure. *Alcohol. Clin. Exp. Res.* 30, 448–459. doi: 10.1111/j.1530-0277.2006.00049.x

Petrov, E. S., Varlinskaya, E. I., Becker, L. A., and Smotherman, W. P. (1998). Endogenous mu opioid systems and suckling in the neonatal rat. *Physiol. Behav.* 65, 591–599. doi: 10.1016/S0031-9384(98)00228-5

Pillekamp, F., Hermann, C., Keller, T., von Gontard, A., Kribs, A., and Roth, B. (2007). Factors influencing apnea and bradycardia of prematurity - implications for neurodevelopment. *Neonatology* 91, 155–161. doi: 10.1159/000097446

Quertemont, E., and Didone, V. (2006). Role of acetaldehyde in mediating the pharmacological and behavioral effects of alcohol. *Alcohol Res. Health* 29, 258–265.

Quertemont, E., Grant, K. A., Correa, M., Arizzi, M. N., Salamone, J. D., Tambour, S., et al. (2005). The role of acetaldehyde in the central effects of ethanol. *Alcohol. Clin. Exp. Res.* 29, 221–234. doi: 10.1097/01.ALC.0000156185.39073.D2

Savage, D. D., Becher, M., de la Torre, A. J., and Sutherland, R. J. (2002). Dose-dependent effects of prenatal ethanol exposure on synaptic plasticity and learning in mature offspring. *Alcohol. Clin. Exp. Res.* 26, 1752–1758. doi: 10.1111/j.1530-0277.2002.tb02480.x

Schrock, A., Fidi, C., Low, M., and Baumgarten, K. (1989). Low-dose ethanol for inhibition of preterm uterine activity. *Am. J. Perinatol.* 6, 191–195. doi: 10.1055/s-2007-999574

Spear, N. E., and Molina, J. C. (2005). Fetal or infantile exposure to ethanol promotes ethanol ingestion in adolescence and adulthood: a theoretical review. *Alcohol. Clin. Exp. Res.* 29, 909–929. doi: 10.1097/01.ALC.0000171046.78556.66

Varlinskaya, E. I., Petrov, E. S., and Smotherman, W. P. (1996). Classical conditioning in the fetal rat: reinforcing properties of dynorphin A (1-13). *Behav. Neurosci.* 110, 154–167. doi: 10.1037/0735-7044.110.1.154

Vojcek, L., Lampe, L. G., Princzkel, E., and Turnbull, A. C. (1988). Effects of ethanol infusion on foetal EEG and breathing movements. *Acta Physiol. Hung.* 71, 511–515.

Wall, T. L., Thomasson, H. R., Schuckit, M. A., and Ehlers, C. L. (1992). Subjective feelings of alcohol intoxication in Asians with genetic variations of ALDH2 alleles. *Alcohol. Clin. Exp. Res.* 16, 991–995. doi: 10.1111/j.1530-0277.1992.tb01907.x

Winer, B. J. (1991). *Statistical Principles in Experimental Design, 2nd Edn*. New York, NY: McGraw-Hill.

Wise, R. A. (2002). Brain reward circuitry: insights from unsensed incentives. *Neuron* 36, 229–240. doi: 10.1016/S0896-6273(02)00965-0

Woods, S. C., and Ramsay, D. S. (2000). Pavlovian influences over food and drug intake. *Behav. Brain Res.* 110, 175–182. doi: 10.1016/S0166-4328(99)00194-1

Yates, W. R., Cadoret, R. J., Troughton, E. P., Stewart, M., and Giunta, T. S. (1998). Effect of fetal alcohol exposure on adult symptoms of nicotine, alcohol, and drug dependence. *Alcohol. Clin. Exp. Res.* 22, 914–920. doi: 10.1111/j.1530-0277.1998.tb03889.x

Young, C., and Olney, J. W. (2006). Neuroapoptosis in the infant mouse brain triggered by a transient small increase in blood alcohol concentration. *Neurobiol. Dis.* 22, 548–554. doi: 10.1016/j.nbd.2005.12.015

Zimatkin, S. M., Pronko, S. P., Vasiliou, V., Gonzalez, F. J., and Deitrich, R. A. (2006). Enzymatic mechanisms of ethanol oxidation in the brain. *Alcohol. Clin. Exp. Res.* 30, 1500–1505. doi: 10.1111/j.1530-0277.2006.00181.x

Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

frontiers in Behavioral Neuroscience

ORIGINAL RESEARCH
published: 23 March 2017
doi: 10.3389/fnbeh.2017.00046

Ethanol Sensitization during Adolescence or Adulthood Induces Different Patterns of Ethanol Consumption without Affecting Ethanol Metabolism

*Priscila F. Carrara-Nascimento[1], Lucas B. Hoffmann[1], Marcos B. Contó[1], Tania Marcourakis[2] and Rosana Camarini[1]**

[1]*Laboratory of Neurochemistry and Behavioral Pharmacology, Department of Pharmacology, Institute of Biomedical Sciences, Universidade de São Paulo, São Paulo, Brazil,* [2]*Department of Clinical and Toxicological Analysis, School of Pharmaceutical Sciences, Universidade de São Paulo, São Paulo, Brazil*

In previous study, we demonstrated that ethanol preexposure may increase ethanol consumption in both adolescent and adult mice, in a two-bottle choice model. We now questioned if ethanol exposure during adolescence results in changes of consumption pattern using a three-bottle choice procedure, considering drinking-in-the-dark and alcohol deprivation effect as strategies for ethanol consumption escalation. We also analyzed aldehyde dehydrogenase (ALDH) activity as a measurement of ethanol metabolism. Adolescent and adult Swiss mice were treated with saline (SAL) or 2.0 g/kg ethanol (EtOH) during 15 days (groups: Adolescent-SAL, Adolescent-EtOH, Adult-SAL and Adult-EtOH). Five days after the last injection, mice were exposed to the three-bottle choice protocol using sucrose fading procedure (4% + sucrose vs. 8%–15% ethanol + sucrose vs. water + sucrose) for 2 h during the dark phase. Sucrose was faded out from 8% to 0%. The protocol was composed of a 6-week acquisition period, followed by four withdrawals and reexposures. Both adolescent and adult mice exhibited ethanol behavioral sensitization, although the magnitude of sensitization in adolescents was lower than in adults. Adolescent-EtOH displayed an escalation of 4% ethanol consumption during acquisition that was not observed in Adult-EtOH. Moreover, Adult-EtOH consumed less 4% ethanol throughout all the experiment and less 15% ethanol in the last reexposure period than Adolescent-EtOH. ALDH activity varied with age, in which older mice showed higher ALDH than younger ones. Ethanol pretreatment or the pattern of consumption did not have influence on ALDH activity. Our data suggest that ethanol pretreatment during adolescence but not adulthood may influence the pattern of ethanol consumption toward an escalation in ethanol consumption at low dose, without exerting an impact on ALDH activity.

Keywords: ethanol, adolescence, behavioral sensitization, voluntary ethanol consumption, aldehyde dehydrogenase, ethanol metabolism

OPEN ACCESS

Edited by:
Merce Correa, Jaume I University, Spain

Reviewed by:
Christian P. Müller, University of Erlangen-Nuremberg, Germany
José Antonio Fuentealba, Pontificia Universidad Catolica de Chile, Chile

****Correspondence:***
Rosana Camarini
camarini@icb.usp.br

Received: *19 November 2016*
Accepted: *28 February 2017*
Published: *23 March 2017*

Citation:
Carrara-Nascimento PF, Hoffmann LB, Contó MB, Marcourakis T and Camarini R (2017) Ethanol Sensitization during Adolescence or Adulthood Induces Different Patterns of Ethanol Consumption without Affecting Ethanol Metabolism. Front. Behav. Neurosci. 11:46. doi: 10.3389/fnbeh.2017.00046

INTRODUCTION

Some characteristics of the adolescence (impulsivity, risky behavior, seeking of new experiences) have been related to delayed maturation of prefrontal cortex and neurotransmitter systems as well as late development of behavioral inhibitory systems, which may render adolescents especially vulnerable to taking drugs of abuse and developing addiction (Spear, 2000; Chambers et al., 2003). Ethanol exposure during adolescence can cause dramatic neurobehavioral and neurotoxicological effects compared to exposure during adulthood, as described in humans (Grant and Dawson, 1997; De Wit et al., 2000; Ehlers et al., 2006) and rodents (Crews et al., 2000; Faria et al., 2008; Walker and Ehlers, 2009; Guerri and Pascual, 2010; Soares-Simi et al., 2013; Carrara-Nascimento et al., 2014).

The main route of ethanol elimination is the liver metabolism, where it is converted into acetaldehyde by alcohol dehydrogenase and subsequently to acetate by aldehyde dehydrogenase (ALDH). Those enzymes are responsible for the elimination of alcohol in concentrations below 20 mmol/L (Li, 1977; Lieber, 1986). The efficacy of ethanol metabolism increases with age following its systemic administration, since the blood ethanol concentration remains higher for longer time in younger rats compared to older ones (Kelly et al., 1987). In fact, liver alcohol dehydrogenase efficiency and ALDH activity varies with age (Collins et al., 1975; Hollstedt et al., 1977). Recent data of our group suggested that adult but not adolescent mice developed metabolic tolerance to increases in blood ethanol concentration induced by chronic intermittent ethanol exposure (Carrara-Nascimento et al., 2013), suggesting that the age of exposure to ethanol may also influence ethanol metabolism.

The activity of ALDH may exert some influence on ethanol consumption, since accumulation of acetaldehyde in the peripheral system induces aversive effects when accumulated in the blood (Quertemont, 2004). As an example, high alcohol-drinkers show faster acetaldehyde metabolism and are less vulnerable to its aversive effects, such as flushing, headache, tachycardia, dizziness and nausea (Quintanilla et al., 2006).

Animal models that promote motivation for alcohol seeking/intake include alcohol withdrawal periods since periods of abstinence lead to progressive increases in alcohol consumption that ultimately results in the relief of the abstinence-induced withdrawal symptoms (the so called alcohol deprivation effect—ADE; Spanagel and Hölter, 1999). Drinking in the dark (DID) paradigm is considered a binge-like model since it promotes high levels of blood ethanol concentration (Rhodes et al., 2005). Another procedure to promote increase in ethanol consumption is to preexpose the animals to the drug (Lessov et al., 2001; Camarini and Hodge, 2004; Carrara-Nascimento et al., 2014).

In the present study, we designed a protocol that includes some aspects of human alcohol addiction, such as age of first contact with ethanol, ADE and DID. We hypothesized that mice exposed to ethanol during adolescence would have higher ethanol consumption later in life. We also assessed whether these differences in ethanol consumption pattern might be related to ALDH activity in the liver.

MATERIALS AND METHODS

Animals

Adolescent (PND 28) and adult (PND 68) male Swiss mice were obtained from the Animal Facility of the Department of Pharmacology of the Institute of Biomedical Sciences at the University of São Paulo, Brazil. Mice were housed in groups of five in standard Plexiglas cages (30 cm × 20 cm × 12.5 cm) in a colony room with controlled lighting (12:12 light/dark cycle; lights on from 7:00 AM to 7:00 PM) and temperature (22 ± 2°C) conditions. Mice were allowed to adapt to the colony room for at least 7 days before the start of the experiment. Food and water were provided *ad libitum*. All procedures were approved by the Ethics Committee on Animal Use (Comitê de Ética no Uso de Animais—CEUA—Protocol #18/2013) of the Institute of Biomedical Sciences at the University of São Paulo. Animals were single housed only during the 2-h period of the ethanol consumption procedure.

Drugs

Ethanol (EtOH, 95% v/v, Merck do Brasil, Rio de Janeiro, Brazil) was diluted in 0.9% w/v sodium chloride (saline, SAL) and injected intraperitoneally (i.p.) as 20% v/v ethanol solution at a dose of 2.0 g/kg during the protocol of behavioral sensitization. Control animals received equivalent volumes of SAL.

For the voluntary ethanol consumption procedure, 95% v/v EtOH was diluted in tap water to produce EtOH solutions according to the concentrations described in **Table 1** (4, 8, 10, 12.5 and 15% ethanol v/v).

Experimental Design

The whole experimental design is shown in **Figure 1**. It involves two phases: Phase 1 (Behavioral Sensitization) and Phase 2 (Voluntary Ethanol Consumption).

Phase 1—Behavioral Sensitization

Fifty-seven mice were used for this experiment. Their locomotor activity was assessed using a cylindrical wooden-made open-field arena (40 cm diameter and 35 cm height). A video camera placed above the apparatus and connected to a computer located outside the experimental room recorded the trials. Five minutes after SAL or EtOH injections, the animals' locomotor activity (distance traveled in cm) was assessed during 5 min and quantified with Ethovision software (Noldus, Wageningen, Netherlands). The 5-min trial duration after 5 min of ethanol injection is based on previous pilot studies conducted in our laboratory and on studies showing optimal ethanol sensitization between 5 and 10 min after injection (Broadbent and Harless, 1999; Meyer and Phillips, 2007). The apparatus was cleaned with a 5% ethanol/water solution between each trial.

In order to let the animals to habituate to the injection procedure and the open-field apparatus, mice were first injected with SAL for two consecutive days prior to the treatment with

FIGURE 1 | Behavioral sensitization: on Habituation days 1 and 2 (H1 and H2) mice were treated with saline (SAL). From treatment days 1–15 (D1–D15) mice received intraperitoneal (i.p.) injections of SAL or 2 g/kg Ethanol. OF: locomotor activity assessment in the open-field. **Ethanol consumption**: 5 days after behavioral sensitization procedure, mice were exposed to voluntary ethanol consumption protocol, which consisted of an acquisition phase, followed by withdrawals and reexposures to the three-bottle choice (water, 4% ethanol and 15% ethanol).

TABLE 1 | Voluntary ethanol consumption.

Age (PND)	Experimental day	Phase	Ethanol 4% solution		Ethanol 15% solution	
			Ethanol	Sucrose	Ethanol	Sucrose
Ado: 50–54 Adu: 90–94	1–5 (period 1)	Acquisition	4%	8%	8%	8%
Ado: 57–61 Adu: 97–101	8–12 (period 2)		4%	8%	10%	8%
Ado: 64–68 Adu: 104–108	15–19 (period 3)		4%	6%	10%	6%
Ado: 71–75 Adu: 111–115	22–26 (period 4)		4%	6%	10%	6%
Ado: 78–82 Adu: 118–122	29–33 (period 5)		4%	4%	12.5%	4%
Ado: 85–89 Adu: 125–129	36–40 (period 6)		4%	2%	15%	2%
Ado: 90–103 Adu: 130–143	41–54	Withdrawal 1				
Ado: 104–106 Adu: 144–146	55–57	Reexposure 1	4%	2%	15%	2%
Ado: 107–113 Adu: 147–153	58–64	Withdrawal 2				
Ado: 114–116 Adu: 154–156	65–67	Reexposure 2	4%	2%	15%	2%
Ado: 117–123 Adu: 157–163	68–74	Withdrawal 3				
Ado: 124–126 Adu: 164–166	75–77	Reexposure 3	4%	2%	15%	2%
Ado: 127–133 Adu: 167–173	78–84	Withdrawal 4				
Ado: 134 Adu: 174	85	Reexposure 4*	4%	0	15%	0
Ado: 135–136 Adu: 175–176	86–87	Reexposure 5	4%	0	15%	0

*The protocol consisted of an acquisition phase, withdrawals and reexposures. *Quinine; Ado, Adolescent; Adu, Adult.*

EtOH. Adolescent and adult mice received an injection of SAL and were placed in the open-field apparatus to assess their locomotor activity. From the next day on, mice were treated for 15 consecutive days with i.p. injections of SAL or 2.0 g/kg of EtOH once a day. Therefore, there were four experimental groups: Adolescent-SAL (n = 15), Adolescent-EtOH (n = 14), Adult-SAL (n = 14) and Adult-EtOH (n = 14). Group names refer to age and treatment in which mice received SAL or EtOH to induce behavioral sensitization during adolescence or adulthood. It is important to emphasize that mice were preexposed i.p. to ethanol during adolescence or adulthood and the testing (consumption) was actually performed during either adulthood or young adulthood. By using this protocol, we aimed to assess ethanol consumption in adult mice preexposed to ethanol during adolescence. Locomotor activity was assessed on days 1, 8 and 15. Injections and locomotor activity assessment were always carried out between 9:00 AM and 11:30 AM. Following this first phase of the protocol, mice underwent 5 days of abstinence before being exposed to the voluntary ethanol consumption.

Phase 2—Voluntary Ethanol Consumption—Drinking in the Dark

The protocol of this phase is shown in **Table 1**.

During the Phase 1 of the experiment, one mouse from Adult-SAL and one from Adult-EtOH died.

Three hours after the lights were turned off (9:00 AM), animals had access to three-bottle choice: one water bottle and two bottles containing different ethanol concentrations, for 2 h according to the DID procedure (Rhodes et al., 2005; Crabbe et al., 2014). The voluntary ethanol consumption consisted of: *Acquisition*: we reiterate that animals belonging to Adolescent groups reached post-adolescence period during Phase 2 of this study. We used a modified sucrose fading procedure (Samson, 1986) because Swiss mice are not classified as high preferring mice. Instead, this mouse strain shows high variability of ethanol drinking patterns (Ribeiro et al., 2012). The sucrose fading procedure is used when the taste aversion to ethanol may be a problem in initiation of drinking. During acquisition phase, mice were exposed to three-bottle choice for five consecutive days followed by 2 days of abstinence. This procedure was repeated six times. The short withdrawals were included to accelerate the ethanol intake. The sucrose concentration in both ethanol bottles was gradually reduced from 8% to 2%. The ethanol concentration in one of the bottles was of 4% throughout the whole experiment, while the ethanol concentration in the other bottle was gradually increased from 8% to 15%. *Withdrawals and reexposures*: four withdrawal periods were intercalated with five reexposures. The first withdrawal lasted 14 days whilst the others lasted 7 days. This protocol was based on studies showing that a longer withdrawal may result in increased ethanol consumption in the following reexposures (Rodd-Henricks et al., 2000, 2001; Rodd et al., 2003, 2009). Each of the three first withdrawals was followed by a reexposure period (resulting in Reexposures 1, 2 and 3). Each reexposure period consisted of 2-h access to ethanol/day for three consecutive days. During these reexposures mice had access to the three-bottle choice (4% ethanol + 2% sucrose, 15% ethanol + 2% sucrose and water). On the fourth reexposure (2-h access to ethanol for 1 day) ethanol solutions were adulterated with 0.005 g/L quinine and no sucrose was added. The quinine concentration was chosen based on previous studies showing that this concentration in water creates an aversive bitter taste and reduces its intake without causing total inhibition of intake (Fachin-Scheit et al., 2006; Vendruscolo et al., 2012; Leão et al., 2015). On the next day, during the fifth reexposure (2-h access to ethanol/day for two consecutive days) the sucrose was completely faded and no quinine was added to the ethanol solutions.

The ethanol intake data from each set of days within each period of exposure of the three-bottle choice protocol was averaged and plotted as a single time point in the graph.

Ethanol intake was calculated in grams per kilogram of mice body weight (g/kg) according to the formula:

$$\text{volume consumed (mL)} \times \text{ethanol concentration in the solution} \times \text{ethanol density (g/mL)} / \text{mouse body weight (kg)}.$$

Quantification of Aldehyde Dehydrogenase (ALDH) Activity

Immediately after the last reexposure, mice were euthanized by cervical dislocation. The livers were collected, immediately frozen and kept at −80°C. Eight mice from each group were randomly chosen for enzyme analysis.

The ALDH assay was performed according to the description of the manufacturer (GWB-AXR339, Genway). Briefly, liver tissues (50 mg) were homogenized with 200 μL of ice cold buffer. The homogenates were left for 10 min on ice, centrifuged at 12,000 g for 5 min at 4°C to remove nuclei and insoluble material and the resulting supernatants were collected to be used in the assay. The principle of the colorimetric assay kit consists in the oxidation of acetaldehyde by the enzyme ALDH of the sample. The reaction generates NADH that reduces an uncolored probe into a colored product with strong absorbance at 450 nm. The samples were read in a spectrophotometer at a wavelength of 450 nm in a kinetic mode (each 2 min), picking the linear range within NADH standard curve. The activity of ALDH was determined by subtracting the values in the absence of the substrate acetaldehyde from the values in the presence of the substrate (performed in duplicates). A standard curve was performed using five distinct amounts of NADH ranging from 2 nmol to 10 nmol, and the ALDH activity was calculated as nmol of NADH released/min/mL.

Statistical Analysis

The behavioral sensitization data was analyzed with a two-way ANOVA (habituation: age × days) and three-way ANOVA (treatment: age × days × treatment) and days were used as repeated measure.

The ethanol consumption data was analyzed using a three-way ANOVA (age × treatment × time) with time as repeated measure. When necessary, three-way ANOVA for repeated measures was deconstructed into two-way ANOVAs (age × time) to evaluate age differences within each treatment. A two-way ANOVA (age × treatment) was performed to analyze reexposure 4 (quinine adulteration) and reexposure 5 (0% sucrose).

Data from the ALDH activity was analyzed using a two-way ANOVA (age × treatment).

Newman-Keuls was used for all *post hoc* comparisons.

For all analysis performed, statistical significance was considered when $p < 0.05$. We used the program STATISTICA 7 (StatSoft) to analyze the data.

RESULTS

Behavioral Sensitization

Habituation

A two-way ANOVA (age × days) for repeated measures revealed an effect of time. Locomotor activity decreased in the second day compared to the first day ($F_{(1,55)} = 37.66$; $p < 0.01$), showing habituation to the apparatus.

Repeated Ethanol Treatment

A three-way ANOVA (age × treatment × days) revealed effects of age ($F_{(1,53)}$ = 8.96, $p < 0.01$), treatment ($F_{(1,53)}$ = 22.67, $p < 0.01$), age × treatment ($F_{(1,53)}$ = 10.71, $p < 0.01$), days ($F_{(2,106)}$ = 18.81, $p < 0.01$), treatment × days ($F_{(2,106)}$ = 15.72, $p < 0.01$) and age × treatment × days interaction ($F_{(2,106)}$ = 4.35, $p < 0.05$). Mean comparisons among treatments showed that mice treated with ethanol displayed greater locomotor activity than those treated with SAL. *Post hoc* analysis of the significant age × treatment effect revealed that the locomotor activity in Adolescent-EtOH was lower than in Adult-EtOH mice. Pairwise comparisons of the significant age × treatment × days interaction showed that both adolescent and adult mice treated with ethanol displayed higher locomotor activity on days 8 and 15 as compared to day 1, revealing that they developed behavioral sensitization. On day 8, Adolescent-EtOH showed a lower locomotor activity than Adult-EtOH. The locomotor activity of all groups on Day 1 was analyzed by a two-way ANOVA (age × treatment) and revealed a tendency to hypolocomotor activity after an acute ethanol injection in adolescent mice (p = 0.08), while adult mice showed the opposite effect (p = 0.09; **Figure 2**).

Voluntary Ethanol Consumption

The results are shown in **Figure 3**.

Ethanol 4% (Figure 3A)

We first performed a repeated three-way ANOVA (age × treatment × time) considering the last period of acquisition phase (when ethanol consumption was stabilized) and reexposures (1–5) as repeated measures. There were significant effects of age ($F_{(1,51)}$ = 10.85, $p < 0.01$), treatment ($F_{(1,51)}$ = 9.36, $p < 0.01$), age × treatment ($F_{(1,51)}$ = 4.44, $p < 0.05$), time ($F_{(5,255)}$ = 37.12, $p < 0.01$), age × time ($F_{(5,255)}$ = 4.85, $p < 0.01$), but no age × treatment × time interaction. The age × treatment interaction effect showed that Adult-EtOH consumed less ethanol than the other groups (Adolescent-EtOH, Adolescent-SAL, Adult-SAL; $F_{(1,52)}$ = 4.35, $p < 0.05$). The age × time interaction effect ($F_{(5,255)}$ = 4.85, $p < 0.01$) demonstrated that Adult groups and Adolescent groups exhibited reduced ethanol consumption on the reexposures 4 (quinine adulteration) and 5 (0% sucrose) as compared to the previous periods (acquisition 6, reexposures 1, 2 and 3).

Following this analysis, we performed ANOVAs for each of the phases of ethanol consumption.

Acquisition

A three-way ANOVA for repeated measures revealed effects of age ($F_{(1,51)}$ = 10.74, $p < 0.01$), time ($F_{(5,255)}$ = 4.51, $p < 0.01$) and age × treatment × time interaction ($F_{(5,255)}$ = 2.66, $p < 0.05$). A two-way ANOVA performed to analyze SAL

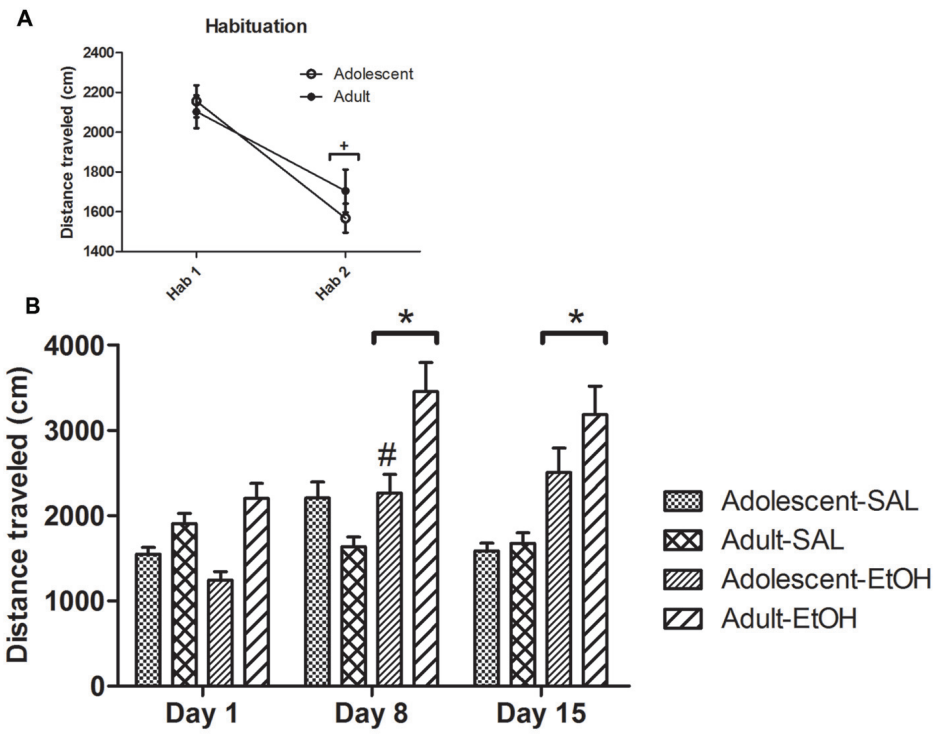

FIGURE 2 | Behavioral sensitization. The main graph **(B)** illustrates the locomotor activity of adolescent and adult mice repeatedly treated with i.p. injections of SAL or 2.0 g/kg ethanol (EtOH) during 15 consecutive days (Adolescent-SAL, n = 15; Adult-SAL, n = 14; Adolescent-EtOH, n = 14; Adult-EtOH, n = 14). The locomotor activity was assessed on Days 1, 8 and 15. The smaller graph **(A)** illustrates locomotor activity on Habituation Days 1 and 2, when all mice received SAL injections. $^{+}$H2 < H1; *Locomotor activity was higher than on Day 1; $^{\#}$Adolescent-EtOH < Adult EtOH.

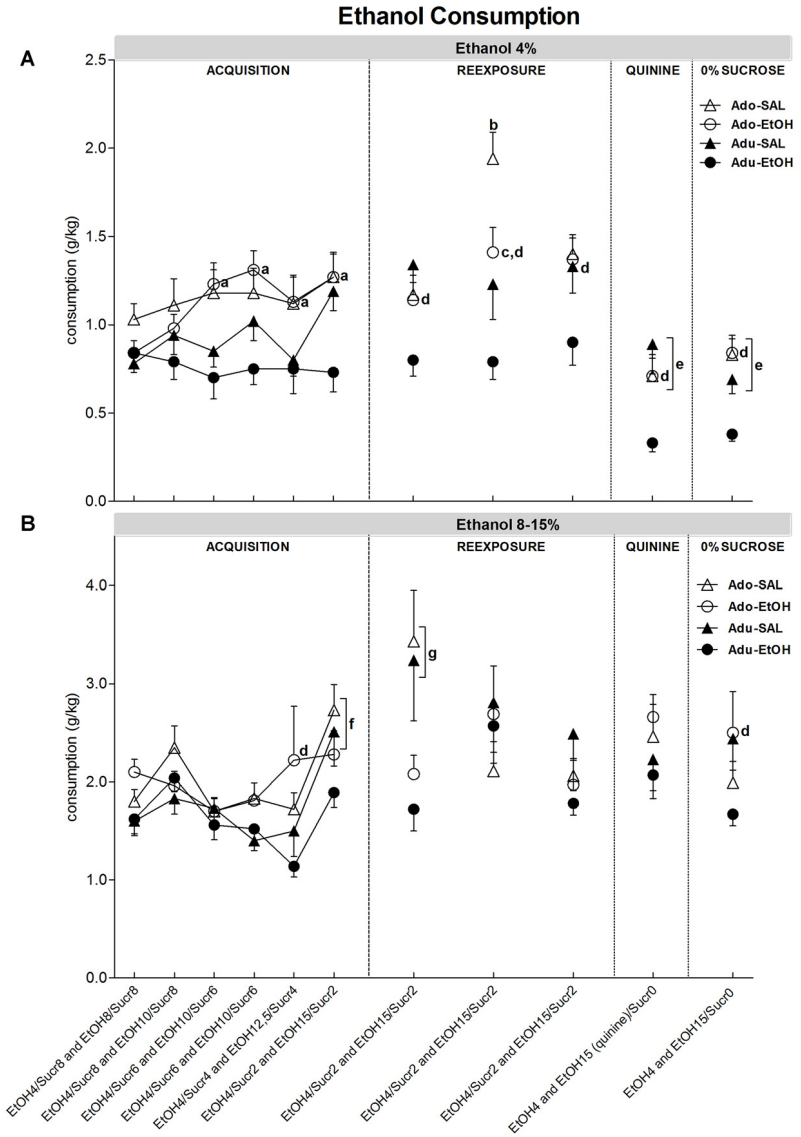

FIGURE 3 | Voluntary ethanol consumption. The figure contains graphs illustrating ethanol consumption during acquisition and reexposure periods to 4% EtOH solution **(A)** and 8%–15% EtOH solution **(B)**, as described in **Table 1**. Animals previously treated during adolescence or adulthood with SAL or EtOH were exposed to the three-bottle choice protocol: water vs. 4% EtOH vs. 8%–15% EtOH. The *x* axis shows the concentration of ethanol and sucrose in each ethanol bottle within each consumption period. The whole experimental protocol of this phase is described in **Table 1**. The letters indicate the following statistically significant differences: a = Adolescent-EtOH displayed greater 4% ethanol intake on acquisition periods 3, 4, 5 and 6 compared to period 1; b = Adolescent-SAL displayed greater 4% ethanol consumption on reexposure 2 compared to reexposures 1 and 3; c = Adolescent-EtOH displayed greater 4% ethanol consumption on reexposure 2 compared to reexposure 1; d = Adolescent-EtOH consumed more ethanol than Adult-EtOH; e = Adult and Adolescent groups exhibited reduced ethanol consumption on the reexposures 4 and 5 as compared to the previous periods (acquisition 6, reexposures 1, 2 and 3); f = Ethanol intake on period 6 was higher than on period 1; g = SAL groups showed higher EtOH intake than EtOH groups on reexposure1 (Adolescent-SAL, *n* = 15; Adult-SAL, *n* = 14; Adolescent-EtOH, *n* = 13; Adult-EtOH, *n* = 13).

groups revealed no age × time interaction. A two-way ANOVA performed to analyze EtOH groups revealed effects of age ($F_{(1,25)}$ = 10.64, $p < 0.01$) and age × time interaction ($F_{(5,125)}$ = 3.99, $p < 0.01$). *Post hoc* analysis showed that Adolescent-EtOH exhibited higher ethanol consumption compared to Adult-EtOH. Adolescent-EtOH mice but not Adult-EtOH showed escalation of 4% ethanol intake, since ethanol intake was greater on acquisition periods 3, 4, 5 and 6 compared to first acquisition period in the Adolescent-EtOH.

Reexposures

A three-way ANOVA for repeated measures used to evaluate reexposures 1, 2 and 3 revealed effects of age ($F_{(1,51)}$ = 9.47,

$p < 0.01$), treatment ($F_{(1,51)} = 8.2$, $p < 0.01$), time ($F_{(2,102)} = 6.35$, $p < 0.01$), age × time interaction ($F_{(2,102)} = 10.14$, $p < 0.01$) and age × treatment × time interaction ($F_{(2,102)} = 3.1$, $p < 0.05$). Adult-EtOH mice displayed lower ethanol intake compared to the other groups. The three-way ANOVAs were deconstructed into two-way ANOVAs to evaluate age differences within each treatment. A two-way ANOVA used to analyze the SAL groups revealed effects of time ($F_{(2,52)} = 4.05$, $p < 0.05$) and age × time interaction ($F_{(2,52)} = 7.55$, $p < 0.01$). Adolescent-SAL displayed greater 4% ethanol consumption on reexposure 2 compared to reexposures 1 and 3. A two-way ANOVA performed to analyze EtOH groups revealed effects of age ($F_{(1,25)} = 10.22$, $p < 0.01$), time ($F_{(2,50)} = 5.56$, $p < 0.05$) and age × time interaction ($F_{(2,50)} = 3.43$, $p < 0.05$). Adolescent-EtOH displayed greater 4% ethanol intake on reexposure 2 compared to reexposure 1. *Post hoc* analysis of the significant age effect revealed that Adolescent-EtOH exhibited higher ethanol intake than Adult-EtOH mice. A two-way ANOVA performed for reexposure 4 (quinine adulteration) revealed effects of treatment ($F_{(1,51)} = 5.69$, $p < 0.05$) and age × treatment interaction ($F_{(1,51)} = 5.26$, $p < 0.05$). *Post hoc* analysis showed that Adult-EtOH drank less ethanol than Adolescent-EtOH and its respective control group (Adult-SAL), and almost reached statistical significance compared to Adolescent-SAL ($p = 0.07$). Analysis of reexposure 5 (0% sucrose) by a two-way ANOVA (age × treatment) revealed that Adult-EtOH mice displayed lower ethanol intake compared to the other groups ($F_{(1,52)} = 4.25$, $p < 0.05$).

Ethanol 8%–15% (Figure 3B)

A repeated three-way ANOVA was performed considering the last period of acquisition phase and reexposures as repeated measures. A significant effect of treatment × time was found ($F_{(5,255)} = 4.23$, $p < 0.05$).

Acquisition

A three-way ANOVA for repeated measures revealed effects of age ($F_{(1,51)} = 5.19$, $p < 0.05$), time ($F_{(5,255)} = 9.93$, $p < 0.01$) and treatment × time interaction ($F_{(5,255)} = 2.32$, $p < 0.05$). No age × treatment × time interaction was found. A two-way ANOVA performed to analyze SAL groups revealed an effect of time ($F_{(5,130)} = 16.67$, $p < 0.05$). A two-way ANOVA performed to analyze EtOH groups revealed an effect of age ($F_{(1,25)} = 4.46$, $p < 0.05$). Although we have found a statistically significant age effect (Adolescent-EtOH drank more ethanol than Adult-EtOH), the difference comes only from the acquisition period 5. *Post hoc* analysis of the significant time effect showed that ethanol intake on acquisition period 6 was higher than on acquisition period 1 for both adolescent and adult SAL groups.

Reexposures

A three-way ANOVA for repeated measures used to analyze reexposures 1, 2 and 3 revealed effects of treatment ($F_{(1,51)} = 4.72$, $p < 0.05$) and time × treatment interaction ($F_{(2,102)} = 5.49$, $p < 0.01$). *Post hoc* analysis of the significant time × treatment effect showed that SAL groups drank more ethanol than EtOH groups on reexposure 1. SAL groups also showed a gradual decrease in ethanol intake over time. A two-way ANOVA used to analyze reexposure 4 (quinine adulteration) revealed no significant effect. A two-way ANOVA used to analyze reexposure 5 (0% sucrose) revealed an age × treatment interaction, in which Adult-EtOH drank less ethanol than Adolescent-EtOH ($F_{(1,51)} = 5.26$, $p < 0.05$).

ALDH Activity

Data from ALDH activity was analyzed using a two-way ANOVA (age × treatment), which showed an effect of age ($F_{(1,28)} = 4.66$, $p < 0.05$). *Post hoc* analysis showed that Adolescent groups (-SAL and -EtOH) exhibited lower ALDH activity as compared to Adult groups (-SAL and -EtOH; **Figure 4**).

DISCUSSION

The current study proposes an experimental protocol that includes risk factors for addiction (adolescence period), incentive salience (behavioral sensitization) and induction of binge-like consumption (withdrawal and reexposures during dark period) to resemble some of the aspects of the addiction in humans. We showed that both adolescent and adult mice treated with 2.0 g/kg ethanol (Adolescent-EtOH and Adult-EtOH) displayed ethanol behavioral sensitization and that adolescents were less sensitive than adults, which is in agreement with studies from our laboratory and others (Stevenson et al., 2008; Quoilin et al., 2012; Soares-Simi et al., 2013; Carrara-Nascimento et al., 2014). The most striking result is that Adolescent-EtOH but not Adult-EtOH displayed escalated amounts of 4% ethanol intake during acquisition and maintained higher ethanol intake than Adult-EtOH after repeated withdrawals and reexposures, even when ethanol solution was adulterated with quinine or

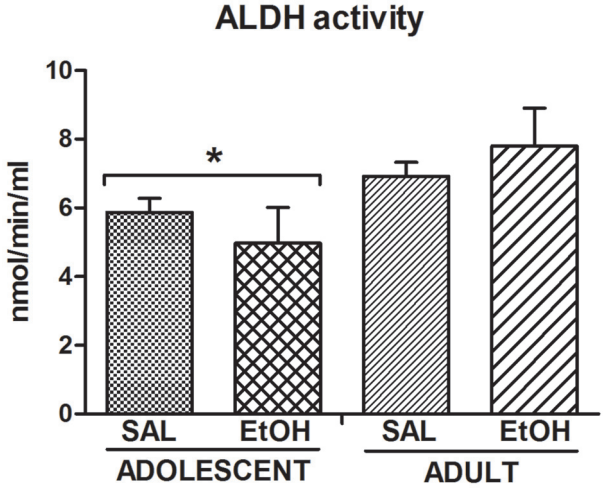

FIGURE 4 | Aldehyde dehydrogenase (ALDH) activity. The enzyme activity was measured in the liver of mice ($n = 8$ mice/group) previously treated with repeated i.p. injections of SAL or EtOH during 15 days and subsequently exposed to a voluntary ethanol consumption protocol that consisted of an acquisition phase followed by withdrawals and reexposures (**Table 1**). *Decreased enzymatic activity compared to Adults.

when sucrose was reduced to 0%. Age-related differences in 15% ethanol intake emerged only during the last reexposure (reexposure 6) after repeated withdrawals. In all phases, Adult-EtOH mice displayed lower 4% ethanol intake compared to the other groups.

Repeated cycles of withdrawals and reexposures have been used to increase ethanol consumption. In our previous study (Carrara-Nascimento et al., 2014), using behavioral sensitization paradigm as pretreatment exposure, we demonstrated that ethanol pretreated mice showed higher 10% ethanol intake when compared to SAL pretreated mice, regardless of age of preexposure or behavioral sensitization magnitude. Adolescent and adult mice exposed to chronic ethanol vapor chamber also increased ethanol intake similarly in a two-bottle choice test (Carrara-Nascimento et al., 2013).

In the current study, using three-bottle choice test, age-differences in ethanol-pretreated mice emerged when sucrose concentration was 6% in the 4% ethanol solution, in that Adolescent-EtOH consumed more sweetened 4% ethanol solution than Adult-EtOH. We could explain the gradual divergence between those groups based on the facts that: (1) low ethanol concentration may be perceived as more palatable than high concentration; (2) adolescent animals consume more sucrose than adults; (Anderson et al., 2010); and (3) adolescents present higher sensitivity to the hedonic properties of sucrose than adults (Wilmouth and Spear, 2009) and thus, they would increase their consumption because of the appetitive taste of both ethanol and sucrose. However, these explanations do not take in consideration the lack of difference in ethanol intake between Adolescent-SAL and Adult-SAL. Moreover, Maldonado et al. (2008) demonstrated that adolescent rats consumed more ethanol than adults using sweetened alcohol solutions and concluded that sucrose was not relevant to the age difference found. In the present study, the age differences in ethanol pretreated mice were maintained even when sucrose was completely faded out, suggesting that the behavioral sensitization during adolescence or adulthood may account for the age-differences in voluntary ethanol consumption. We may suggest that previous behavioral sensitization decreased ethanol intake in adult but not in adolescent mice. This might be because of the ontogeny of the dopaminergic system with an inverted U-shaped format in brain regions involved in motivation and rewarding (McCutcheon and Marinelli, 2009). Functional characteristics of the dopaminergic system during development have been implicated in distinct patterns of behavioral response to drugs between younger and older animals, such as sensitization and/or consumption (Doremus et al., 2005; Frantz et al., 2007; Camarini et al., 2008; Faria et al., 2008; Valzachi et al., 2013; Camarini and Pautassi, 2016).

Although we have not found differences to the acute simulant effects of ethanol between adolescent and adult mice in the present and previous studies (Faria et al., 2008; Carrara-Nascimento et al., 2011, 2013; Soares-Simi et al., 2013), adolescents showed lower levels of locomotor sensitization to ethanol than adults when receiving low doses of ethanol (Faria et al., 2008; Stevenson et al., 2008). Moreover, it has been demonstrated that female adolescent mice need higher ethanol doses (i.e., 4.0 g/kg) than adults to develop ethanol locomotor sensitization (Quoilin et al., 2012), suggesting ontogenic differences in ethanol-induced behavioral sensitization. However, we cannot discard the hypothesis that the lower activity in adolescents compared to adults is in fact a process of tolerance to the hypolocomotor (sedative) effect of ethanol rather than locomotor sensitization. Although Phillips et al. (1996) have shown that sensitization does not result from tolerance to the sedative effects of ethanol in BXD/Ty recombinant inbred strains, this hypothesis should be further investigated in adolescent mice.

In the present study, sensitized adolescent mice displayed an ethanol consumption pattern that differed from the sensitized adult mice, in that they showed a gradual increase in consumption of 4% ethanol solution. It seems to have an interaction between low sensitivity to behavioral sensitization and consumption of ethanol solution at low concentration. In other words, sensitized adolescent mice drunk ethanol at low concentrations in stimulant doses to reach the appetitive effects of ethanol. The availability of ethanol solution at low concentration allowed those mice to control the ethanol self-administration to reach those effects. Taking this into account, it is likely that the low predisposition to behavioral sensitization in adolescents, in fact, yield animals more prone to escalate alcohol consumption at low concentration.

It has been demonstrated that adolescent rodents are not efficient to titrate their ethanol consumption as adults (Maldonado et al., 2008). Rodents learn to titrate ethanol intake based on their previous experiences with ethanol, likely mediated by postingestional effects of ethanol (Samson et al., 2002; Czachowski et al., 2006). In the present study, preexposure to ethanol during adolescence or adulthood differentially impacted the ability of animals to titrate their ethanol consumption, in that Adult-EtOH consumed less ethanol than Adolescent-EtOH. The statistical analysis that considered all experimental phases revealed that Adult-EtOH displayed lower 4% ethanol intake compared to all the other groups. Moreover, Adult-EtOH mice also drank less 15% ethanol than Adolescent-EtOH during the last phase of the experiment (0% sucrose). Thus, we suggest that previous behavioral sensitization in adult but not in adolescent mice exerted a protective effect in adult mice towards increased ethanol intake in a model of three-bottle choice using sucrose fading procedure. Using a different protocol, we have demonstrated that preexposure to ethanol increased ethanol intake, regardless of age (Carrara-Nascimento et al., 2014). It is noteworthy that in the latter study, the protocol included only one abstinence phase.

A hypothesis to explain the steady escalation of 4% ethanol solution is through pharmacological sensitization (Zernig et al., 2007). It is likely that those mice showed a rapid escalation to reach a state of greater sensitivity to ethanol-induced sensitization, and desired stimulation levels. They also showed persisted ethanol intake throughout the five reexposure periods and higher consumption than Adult-EtOH, confirming the important role of ethanol exposure during adolescence to induce use disorders later on adulthood. It is interesting to note that when more chronic ethanol reexposures were

introduced, a significant higher increase in 15% ethanol intake in Adolescent-EtOH group compared to Adult-EtOH group also appeared, suggesting an influence of high ethanol consumption at higher concentrations in the timeline of ethanol exposure. Rodent preferences usually shift to the highest ethanol concentrations after withdrawals in three-bottle choice tests with multiple ethanol concentrations (Hölter et al., 1998; Rodd-Henricks et al., 2001; Ribeiro et al., 2012).

Even though quinine has decreased 4% ethanol consumption in both Adolescent-EtOH and Adult-EtOH mice, the age-differences were maintained, showing that motivation levels to drink were equally preserved. Interestingly, aversion to quinine taste was minimized in 15% ethanol solution. This suggests a great motivation to drink even under aversive taste or, alternatively, quinine bitter tasting was masked by the high ethanol concentration.

ADE exerted a greater effect in younger mice, since consumption of 4% ethanol increased during reexposure 2 in Adolescent-EtOH and Adolescent SAL, which was tolerated in Adolescent-SAL but not in Adolescent-EtOH. Tolerance was also observed to 15% ethanol consumption in both Adolescent-SAL and Adult-SAL. A marked difference between mice pretreated with ethanol or SAL is that non-pretreated mice, regardless of age, showed increased 15% ethanol intake during the last acquisition period and developed tolerance during subsequent reexposures. Moreover, the initial longer ADE induced a greater effect on 15% ethanol consumption in non-pretreated mice compared to sensitized mice (Adolescent-EtOH and Adult-EtOH). Although speculative, these data suggest that behavioral sensitization is not necessarily related to increased ethanol consumption. Discordant results have been reported on the correlation between behavioral sensitization and ethanol consumption. Abrahao et al. (2013) demonstrated an association between locomotor sensitization and ethanol drinking in Swiss mice. The difference between our study and theirs is the number of ethanol bottles during the test, ethanol concentration, and more important, the classification of mice receiving ethanol into "sensitized" and "nonsensitized" in their study. Other important difference in this study from ours is that during the initial phase of the self-administration protocol, animals were given forced exposure to the ethanol solution before having access to the two-bottle choice (Abrahao et al., 2013). Ribeiro et al. (2008) did not find a correlation between these two parameters. Fabio et al. (2014) showed an enhancement of ethanol consumption in adolescent, but not in adult mice, preexposed to binge ethanol intoxication, regardless development of behavioral sensitization.

Regardless of the previous treatment, we found a significant effect of age on ALDH activity, with older mice showing higher activity compared to younger ones, suggesting decreased rate of alcohol metabolism in younger mice. In support of our results, Collins et al. (1975) demonstrated age-differences in ALDH activity between mice from PND = 50–60 and PND = 95–110. We also found that exposure to ethanol during adolescence did not alter ALDH activity on adulthood, since there were no significant differences in ALDH activity between Adolescent-EtOH and Adolescent-SAL. This is a caveat in our study because all mice were exposed to ethanol during voluntary consumption. Although we lack this control, ethanol self-administration studies in rats reported no differences in the ALDH activity between those that were given ethanol compared to their controls (Amir, 1978). In humans, chronic exposure to alcohol increases acetaldehyde in the blood and decreases ALDH activity in the liver (Jenkins and Peters, 1980; Palmer and Jenkins, 1982). Interestingly, reduction in this enzyme activity is related to liver damage or excessive alcohol consumption. Aldehydes have an important role on cell signaling for apoptosis and in the pathophysiology of alcoholism (Kruman et al., 1997; Hayes et al., 2000).

In conclusion, preexposure to ethanol during adolescence may have altered ethanol-induced stimulation threshold. Behavioral sensitization during adolescence or adulthood induced different patterns of ethanol consumption, in that adult but not adolescent preexposed mice showed lower ethanol consumption, without affecting ALDH activity.

AUTHOR CONTRIBUTIONS

PFC-N and RC conceived the experiments. PFC-N and LBH conducted the behavioral experiments. PFC-N and MBC performed the aldehyde dehydrogenase activity assay. PFC-N, LBH, TM and RC analyzed the data and participated in drafting the article. RC is responsible for funding and revising the final version.

FUNDING

The authors thank Fundação de Amparo à Pesquisa do Estado de São Paulo (FAPESP; São Paulo Research Foundation) for the research funding (grant # 2012/10260-7) and for scholarship to PFC-N (grant # 2012/17228-1) and CNPq (National Council for Scientific and Technological Development) for the research funding (grant # 470070/2012-9). RC and TM are research fellows of CNPq.

REFERENCES

Abrahao, K. P., Ariwodola, O. J., Butler, T. R., Rau, A. R., Skelly, M. J., Carter, E., et al. (2013). Locomotor sensitization to ethanol impairs NMDA receptor-dependent synaptic plasticity in the nucleus accumbens and increases ethanol self-administration. *J. Neurosci.* 33, 4834–4842. doi: 10.1523/JNEUROSCI.5839-11.2013

Amir, S. (1978). Brain and liver aldehyde dehydrogenase activity and voluntary ethanol consumption by rats: relations to strain, sex, and age. *Psychopharmacology* 57, 97–102. doi: 10.1007/bf00426964

Anderson, R. I., Varlinskaya, E. I., and Spear, L. P. (2010). Ethanol-induced conditioned taste aversion in male sprague-dawley rats: impact of age and stress. *Alcohol. Clin. Exp. Res.* 34, 2106–2115. doi: 10.1111/j.1530-0277.2010.01307.x

Broadbent, J., and Harless, W. E. (1999). Differential effects of $GABA_A$ and $GABA_B$ agonists on sensitization to the locomotor stimulant effects of ethanol in DBA/2J mice. *Psychopharmacology* 141, 197–205. doi: 10.1007/s002130050825

Camarini, R., Griffin, W. C. III, Yanke, A. B., Rosalina dos Santos, B., and Olive, M. F. (2008). Effects of adolescent exposure to cocaine on locomotor

activity and extracelular dopamine and glutamate levels in nucleus accumbens of DBA/2J mice. *Brain Res.* 1193, 34–42. doi: 10.1016/j.brainres.2007.11.045

Camarini, R., and Hodge, C. W. (2004). Ethanol preexposure increases ethanol self-administration in C57BL/6J and DBA/2J mice. *Pharmacol. Biochem. Behav.* 79, 623–632. doi: 10.1016/j.pbb.2004.09.012

Camarini, R., and Pautassi, R. M. (2016). Behavioral sensitization to ethanol: neural basis and factors that influence its acquisition and expression. *Brain Res. Bull.* 125, 53–78. doi: 10.1016/j.brainresbull.2016.04.006

Carrara-Nascimento, P. F., Griffin, W. C. III., Pastrello, D. M., Olive, M. F, and Camarini, R. (2011). Changes in extracellular levels of glutamate in the nucleus accumbens after ethanol-induced behavioral sensitization in adolescent and adult mice. *Alcohol* 45, 451–460. doi: 10.1016/j.alcohol.2011.01.002

Carrara-Nascimento, P. F., Lopez, M. F., Becker, H. C., Olive, M. F., and Camarini, R. (2013). Similar ethanol drinking in adolescent and adult C57BL/6J Mice after chronic ethanol exposure and withdrawal. *Alcohol. Clin. Exp. Res.* 37, 961–968. doi: 10.1111/acer.12056

Carrara-Nascimento, P. F., Olive, M. F., and Camarini, R. (2014). Ethanol pre-exposure during adolescence or adulthood increases ethanol intake but ethanol-induced conditioned place preference is enhanced only when pre-exposure occurs in adolescence. *Dev. Psychobiol.* 56, 36–48. doi: 10.1002/dev.21089

Chambers, R. A., Taylor, J. R., and Potenza, M. N. (2003). Developmental neurocircuitry of motivation in adolescence: a critical period of addiction vulnerability. *Am. J. Psychiatry* 160, 1041–1052. doi: 10.1176/appi.ajp.160.6.1041

Collins, A. C., Yeager, T. N., Lebsack, M. E., and Panter, S. S. (1975). Variations in alcohol metabolism: influence of sex and age. *Pharmacol. Biochem. Behav.* 3, 973–978. doi: 10.1016/0091-3057(75)90004-0

Crabbe, J. C., Metten, P., Belknap, J. K., Spence, S. E., Cameron, A. J., Schlumbohm, J. P., et al. (2014). Progress in a replicated selection for elevated blood ethanol concentrations in HDID mice. *Genes Brain Behav.* 13, 236–246. doi: 10.1111/gbb.12105

Crews, F. T., Braun, C. J., Hoplight, B., Switzer, R. C. III, and Knapp, D. J. (2000). Binge ethanol consumption causes differential brain damage in young adolescent rats compared with adult rats. *Alcohol. Clin. Exp. Res.* 24, 1712–1723. doi: 10.1097/00000374-200011000-00014

Czachowski, C. L., Prutzman, S., and DeLory, M. J. (2006). Volume and dose effects of experimenter-administered ethanol preloads on ethanol-seeking and self-administration. *Alcohol* 40, 35–40. doi: 10.1016/j.alcohol.2006.09.033

De Wit, D. J., Adlaf, E. M., Offord, D. R., and Ogborne, A. C. (2000). Age at first alcohol use: a risk factor for the development of alcohol disorders. *Am. J. Psychiatry* 157, 745–750. doi: 10.1176/appi.ajp.157.5.745

Doremus, T. L., Brunell, S. C., Rajendran, P., and Spear, L. P. (2005). Factors influencing elevated ethanol consumption in adolescent relative to adult rats. *Alcohol. Clin. Exp. Res.* 29, 1796–1808. doi: 10.1097/01.alc.0000183007.65998.aa

Ehlers, C. L., Slutske, W. S., Gilder, D. A., Lau, P., and Wilhelmsen, K. C. (2006). Age at first intoxication and alcohol use disorders in Southwest California Indians. *Alcohol. Clin. Exp. Res.* 30, 1856–1865. doi: 10.1111/j.1530-0277.2006.00222.x

Fabio, M. C., Nizhnikov, M. E., Spear, N. E., and Pautassi, R. M. (2014). Binge ethanol intoxication heightens subsequent ethanol intake in adolescent, but not adult, rats. *Dev. Psychobiol.* 56, 574–583. doi: 10.1002/dev.21101

Fachin-Scheit, D. J., Frozino Ribeiro, A., Pigatto, G., Oliveira Goeldner, F., and Boerngen de Lacerda, R. (2006). Development of a mouse model of ethanol addiction: naltrexone efficacy in reducing consumption but not craving. *J. Neural Transm. (Vienna)* 113, 1305–1321. doi: 10.1007/s00702-005-0416-z

Faria, R. R., Lima Rueda, A. V., Sayuri, C., Soares, S. L., Malta, M. B., Carrara-Nascimento, P. F., et al. (2008). Environmental modulation of ethanol-induced locomotor activity: correlation with neuronal activity in distinct brain regions of adolescent and adult Swiss mice. *Brain Res.* 1239, 127–140. doi: 10.1016/j.brainres.2008.08.056

Frantz, K. J., O'Dell, L. E., and Parsons, L. H. (2007). Behavioral and neurochemical responses to cocaine in periadolescent and adult rats. *Neuropsychopharmacology* 32, 625–637. doi: 10.1038/sj.npp.1301130

Grant, B. F., and Dawson, D. A. (1997). Age at onset of alcohol use and its association with DSM-IV alcohol abuse and dependence: results from the national longitudinal alcohol epidemiologic survey. *J. Subst. Abuse* 9, 103–110. doi: 10.1016/s0899-3289(97)90009-2

Guerri, C., and Pascual, M. (2010). Mechanisms involved in the neurotoxic, cognitive, and neurobehavioral effects of alcohol consumption during adolescence. *Alcohol* 44, 15–26. doi: 10.1016/j.alcohol.2009.10.003

Hayes, R. L., Szweda, L., Pickin, K., Welker, M. E., and Townsend, A. J. (2000). Structure-activity relationship for growth inhibition and induction of apoptosis by 4-hydroxynonenal in Raw 264.7 cells. *Mol. Pharmacol.* 58, 788–794. doi: 10.1124/mol.58.4.788

Hollstedt, C., Olsson, O., and Rydberg, U. (1977). The effect of alcohol on the developing organism. Genetical, teratological and physiological aspects. *Med. Biol.* 55, 1–14.

Hölter, S. M., Engelmann, M., Kirschke, C., Liebsch, G., Landgraf, R., and Spanagel, R. (1998). Long-term ethanol self-administration with repeated ethanol deprivation episodes changes ethanol drinking pattern and increases anxiety-related behavior during ethanol deprivation in rats. *Behav. Pharmacol.* 9, 41–48.

Jenkins, W. J., and Peters, T. J. (1980). Selectively reduced hepatic acetaldehyde dehydrogenase in alcoholics. *Lancet* 1, 628–629. doi: 10.1016/s0140-6736(80)91121-6

Kelly, S. J., Bonthius, D. J., and West, J. R. (1987). Developmental changes in alcohol pharmacokinetics in rats. *Alcohol. Clin. Exp. Res.* 11, 281–286. doi: 10.1111/j.1530-0277.1987.tb01308.x

Kruman, I., Bruce-Keller, A. J., Bredesen, D., Waeg, G., and Mattson, M. P. (1997). Evidence that 4-hydroxynonenal mediates oxidative stress-induced neuronal apoptosis. *J. Neurosci.* 17, 5089–5100.

Leão, R. M., Cruz, F. C., Vendruscolo, L. F., de Guglielmo, G., Logrip, M. L., Planeta, C. S., et al. (2015). Chronic nicotine activates stress/reward-related brain regions and facilitates the transition to compulsive alcohol drinking. *J. Neurosci.* 35, 6241–6253. doi: 10.1523/JNEUROSCI.3302-14.2015

Lessov, C. N., Palmer, A. A., Quick, E. A., and Phillips, T. J. (2001). Voluntary ethanol drinking in C57BL/6J and DBA/2J mice before and after sensitization to the locomotor stimulant effects of ethanol. *Psychopharmacology* 155, 91–99. doi: 10.1007/s002130100699

Li, T.-K. (1977). Enzymology of human alcohol metabolism. *Adv. Enzymol. Relat. Areas Mol. Biol.* 45, 427–483. doi: 10.1002/9780470122907.ch6

Lieber, C. S. (1986). "Alcohol and the liver," in *The Liver Annual* (Vol. 5), eds I. M. Arias, M. Frenkel and J. H. P. Wilson (Amsterdam: Elsevier Science Publishers), 116–159.

Maldonado, A. M., Finkbeiner, L. M., Alipour, K. K., and Kirstein, C. L. (2008). Voluntary ethanol consumption differs in adolescent and adult male rats using a modified sucrose-fading paradigm. *Alcohol. Clin. Exp. Res.* 32, 1574–1582. doi: 10.1111/j.1530-0277.2008.00733.x

McCutcheon, J. E., and Marinelli, M. (2009). Age matters. *Eur. J. Neurosci.* 29, 997–1014. doi: 10.1111/j.1460-9568.2009.06648.x

Meyer, P. J., and Phillips, T. J. (2007). Behavioral sensitization to ethanol does not result in cross-sensitization to NMDA receptor antagonists. *Psychopharmacology* 195, 103–115. doi: 10.1007/s00213-007-0871-3

Palmer, K. R., and Jenkins, W. J. (1982). Impaired acetaldehyde oxidation in alcoholics. *Gut.* 23, 729–733. doi: 10.1136/gut.23.9.729

Phillips, T. J., Lessov, C. N., Harland, R. D., and Mitchell, S. R. (1996). Evaluation of potential genetic associations between ethanol tolerance and sensitization in BXD/Ty recombinant inbred mice. *J. Pharmacol. Exp. Ther.* 277, 613–623.

Quertemont, E. (2004). Genetic polymorphism in ethanol metabolism: acetaldehyde contribution to alcohol abuse and alcoholism. *Mol. Psychiatry* 9, 570–581. doi: 10.1038/sj.mp.4001497

Quintanilla, M. E., Israel, Y., Sapag, A., and Tampier, L. (2006). The UChA and UChB rat lines: metabolic and genetic differences influencing ethanol intake. *Addict. Biol.* 11, 310–323. doi: 10.1111/j.1369-1600.2006.00030.x

Quoilin, C., Didone, V., Tirelli, E., and Quertemont, E. (2012). Chronic ethanol exposure during adolescence alters the behavioral responsiveness to ethanol in adult mice. *Behav. Brain Res.* 229, 1–9. doi: 10.1016/j.bbr.2011.12.039

Rhodes, J. S., Best, K., Belknap, J. K., Finn, D. A., and Crabbe, J. C. (2005). Evaluation of a simple model of ethanol drinking to intoxication in C57BL/6J mice. *Physiol. Behav.* 84, 53–63. doi: 10.1016/j.physbeh.2004.10.007

Ribeiro, A. F., Correia, D., Torres, A. A., Boas, G. R., Rueda, A. V., Camarini, R., et al. (2012). A transcriptional study in mice with different ethanol-drinking

profiles: possible involvement of the $GABA_B$ receptor. *Pharmacol. Biochem. Behav.* 102, 224–232. doi: 10.1016/j.pbb.2012.04.010

Ribeiro, A. F., Pigatto, G., Goeldner, F. O., Lopes, J. F., and de Lacerda, R. B. (2008). Lack of relation between drug-seeking behavior in an addiction model and the expression of behavioral sensitization in response to ethanol challenge in mice. *J. Neural Transm. (Vienna)* 115, 43–54. doi: 10.1007/s00702-007-0843-0

Rodd, Z. A., Bell, R. L., Kuc, K. A., Murphy, J. M., Lumeng, L., Li, T. K., et al. (2003). Effects of repeated alcohol deprivations on operant ethanol self-administration by alcohol-preferring (P) rats. *Neuropsychopharmacology* 28, 1614–1621. doi: 10.1038/sj.npp.1300214

Rodd, Z. A., Bell, R. L., Kuc, K. A., Murphy, J. M., Lumeng, L., and McBride, W. J. (2009). Effects of concurrent access to multiple ethanol concentrations and repeated deprivations on alcohol intake of high-alcohol-drinking (HAD) rats. *Addict. Biol.* 14, 152–164. doi: 10.1111/j.1369-1600.2008.00140.x

Rodd-Henricks, Z. A., Bell, R. L., Kuc, K. A., Murphy, J. M., McBride, W. J., Lumeng, L., et al. (2001). Effects of concurrent access to multiple ethanol concentrations and repeated deprivations on alcohol intake of alcohol-preferring rats. *Alcohol. Clin. Exp. Res.* 25, 1140–1150. doi: 10.1097/00000374-200108000-00008

Rodd-Henricks, Z. A., McKinzie, D. L., Shaikh, S. R., Murphy, J. M., McBride, W. J., and Lumeng, L. (2000). Alcohol deprivation effect is prolonged in the alcohol preferring (P) rat after repeated deprivations. *Alcohol. Clin. Exp. Res.* 24, 8–16. doi: 10.1097/00000374-200001000-00002

Samson, H. H. (1986). Initiation of ethanol reinforcement using a sucrose-substitution procedure in food- and water-sated rats. *Alcohol. Clin. Exp. Res.* 10, 436–442. doi: 10.1111/j.1530-0277.1986.tb05120.x

Samson, H. H., Chappell, A., and Legg, B. (2002). Effect of self-administered alcohol or sucrose preloads on subsequent consumption in the rat. *J. Stud. Alcohol* 63, 107–113.

Soares-Simi, S. L., Pastrello, D. M., Ferreira, Z. S., Yonamine, M., Marcourakis, T., and Scavone, C. (2013). Changes in CREB activation in the prefrontal cortex and hippocampus blunt ethanol-induced behavioral sensitization in adolescent mice. *Front. Integr. Neurosci.* 7:94. doi: 10.3389/fnint.2013.00094

Spanagel, R., and Hölter, S. M. (1999). Long-term alcohol self-administration with repeated alcohol deprivation phases: an animal model of alcoholism? *Alcohol Alcohol.* 34, 231–243. doi: 10.1093/alcalc/34.2.231

Spear, L. P. (2000). The adolescent brain and age-related behavioral manifestations. *Neurosci. Biobehav. Rev.* 24, 417–463. doi: 10.1016/s0149-7634(00)00014-2

Stevenson, R. A., Besheer, J., and Hodge, C. W. (2008). Comparison of ethanol locomotor sensitization in adolescent and adult DBA/2J mice. *Psychopharmacology* 197, 361–370. doi: 10.1007/s00213-007-1038-y

Valzachi, M. C., Teodorov, E., Marcourakis, T., Bailey, A., and Camarini, R. (2013). Enhancement of behavioral sensitization, anxiety-like behavior, and hippocampal and frontal cortical CREB levels following cocaine abstinence in mice exposed to cocaine during adolescence. *PLoS One* 8:e78317. doi: 10.1371/journal.pone.0078317

Vendruscolo, L. F., Barbier, E., Schlosburg, J. E., Misra, K. K., Whitfield, T. W. Jr., and Logrip, M. L. (2012). Corticosteroid-dependent plasticity mediates compulsive alcohol drinking in rats. *J. Neurosci.* 32, 7563–7571. doi: 10.1523/JNEUROSCI.0069-12.2012

Walker, B. M., and Ehlers, C. L. (2009). Appetitive motivational experience during adolescence results in enhanced alcohol consumption during adulthood. *Behav. Neurosci.* 123, 926–935. doi: 10.1037/a0016002

Wilmouth, C. E., and Spear, L. P. (2009). Hedonic sensitivity in adolescent and adult rats: taste reactivity and voluntary sucrose consumption. *Pharmacol. Biochem. Behav.* 92, 566–573. doi: 10.1016/j.pbb.2009.02.009

Zernig, G., Ahmed, S. H., Cardinal, R. N., Morgan, D., Acquas, E., Foltin, R. W., et al. (2007). Explaining the escalation of drug use in substance dependence: models and appropriate animal laboratory tests. *Pharmacology* 80, 65–119. doi: 10.1159/000103923

Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

frontiers in Behavioral Neuroscience

ORIGINAL RESEARCH
published: 13 October 2016
doi: 10.3389/fnbeh.2016.00195

Post-weaning Environmental Enrichment, But Not Chronic Maternal Isolation, Enhanced Ethanol Intake during Periadolescence and Early Adulthood

Luciana R. Berardo[1,2], María C. Fabio[1,2†] and Ricardo M. Pautassi[1,2]*

[1] *Instituto de Investigación Médica Mercedes y Martín Ferreyra, Consejo Nacional de Investigaciones Científicas y Técnicas, Universidad Nacional de Córdoba, Córdoba, Argentina,* [2] *Facultad de Psicología, Universidad Nacional de Córdoba, Córdoba, Argentina*

OPEN ACCESS

Edited by:
Merce Correa, Jaume I University, Spain

Reviewed by:
Joerg Bock, Otto-von-Guericke University Magdeburg, Germany
Laura Lopez-Cruz, University of Cambridge, UK

***Correspondence:**
Ricardo M. Pautassi
rpautassi@gmail.com

†Present address:
María C. Fabio
Centro de Investigaciones en Química Biológica de Córdoba, Consejo Nacional de Investigaciones Científicas y Técnicas, Universidad Nacional de Córdoba, Córdoba, Argentina

Received: *02 August 2016*
Accepted: *28 September 2016*
Published: *13 October 2016*

Citation:
Berardo LR, Fabio MC and Pautassi RM (2016) Post-weaning Environmental Enrichment, But Not Chronic Maternal Isolation, Enhanced Ethanol Intake during Periadolescence and Early Adulthood. Front. Behav. Neurosci. 10:195. doi: 10.3389/fnbeh.2016.00195

This study analyzed ethanol intake in male and female Wistar rats exposed to maternal separation (MS) during infancy (postnatal days 1–21, PD1–21) and environmental enrichment (EE) during adolescence (PD 21–42). Previous work revealed that MS enhances ethanol consumption during adulthood. It is still unknown if a similar effect is found during adolescence. Several studies, in turn, have revealed that EE reverses stress experiences, and reduces ethanol consumption and reinforcement; although others reported greater ethanol intake after EE. The interactive effects between these treatments upon ethanol's effects and intake have yet to be explored. We assessed chronic ethanol intake and preference (12 two-bottle daily sessions, spread across 30 days, 1st session on PD46) in rats exposed to MS and EE. The main finding was that male – but not female – rats that had been exposed to EE consumed more ethanol than controls given standard housing, an effect that was not affected by MS. Subsequent experiments assessed several factors associated with heightened ethanol consumption in males exposed to MS and EE; namely taste aversive conditioning and hypnotic-sedative consequences of ethanol. We also measured anxiety response in the light-dark box and in the elevated plus maze tests; and exploratory patterns of novel stimuli and behaviors indicative of risk assessment and risk-taking, via a modified version of the concentric square field (CSF) test. Aversive conditioning, hypnosis and sleep time were similar in males exposed or not to EE. EE males, however, exhibited heightened exploration of novel stimuli and greater risk taking behaviors in the CSF test. It is likely that the promoting effect of EE upon ethanol intake was due to these effects upon exploratory and risk-taking behaviors.

Keywords: ethanol, wistar, maternal separation, environmental enrichment, adolescence

INTRODUCTION

Pilatti et al. (2013a) indicated lifetime prevalence of alcohol sipping or tasting in 50% (females) to 70% (males) of Argentinean children aged 8–12 years-old. Lifetime prevalence of alcohol drinking (i.e., ≥1 full drink) was 34.3%. Another study, conducted in the same city and in an older sample (mean age = 20 years) of similar sociodemographic characteristics, indicated less than 7% of abstainers, with most of the subjects reporting last month drinking and approximately half of them reporting an average of 4/5 drinks per drinking occasion, which constitutes binge drinking associated with several adverse consequences, including greater likelihood of alcohol abuse and dependence (Gruber et al., 1996; DeWit et al., 2000; Pilatti et al., 2013b). Together, these studies illustrate the pathway from initiation to sustained alcohol use that, almost normatively across cultures, takes place during late infancy and adolescence.

Epidemiological and animal research has indicated that the quality of the early (maternal and then fraternal/peer) environment is a key factor to accelerate or deter from alcohol engagement during infancy and adolescence. Subjects who experienced early life stress are more likely to begin drinking early in life (Rothman et al., 2008; Enoch, 2012) and to report stress coping as a motive for drinking during the first year of drinking (Rothman et al., 2008). Early onset of drinking, in turn, increases the risk for stress-related drinking (Dawson et al., 2007) and predicts subsequent alcohol abuse and dependence (DeWit et al., 2000). Conversely, social enrichment during adolescence reverses the social deficits observed in rats exposed to ethanol (Middleton et al., 2012) or valproic acid (Schneider et al., 2006) during pregnancy. The effects of early life environmental conditions on reactivity to ethanol can be assessed via the maternal separation (MS) (Francis and Kuhar, 2008) or the environmental enrichment (EE) experimental preparations (Rueda et al., 2012).

In the MS preparation, rats experience 180 or 360 min of maternal separation (commonly referred to as MS180 or MS360 treatments, respectively), every day from postnatal day (PD) 1 to PD14 or until weaning on PD21 (Kawakami et al., 2007). Maternally separated animals exhibit, when tested at adulthood, enhanced ethanol self-administration and greater hormonal and behavioral responsiveness to stress (Huot et al., 2001; Cruz et al., 2008) than animals reared under normal animal facility rearing (AFR) conditions. The home cage of rodents exposed to EE features several combinations of interactive objects, including tunnels, toys and running wheels that provide opportunity for voluntary physical activity. EE holds promise as a non-pharmacological alternative to reduce ethanol-induced reinforcement and intake. Exposure to EE inhibits ethanol consumption and reduces the magnitude of ethanol-(de Carvalho et al., 2010) or cocaine-induced (Solinas et al., 2009) conditioned place preference in rats. Moreover, adolescent mice exposed to EE were insensitive to the increase in motor stimulation observed after repeated and intermittent ethanol administration (i.e., ethanol-induced behavioral sensitization) (Rueda et al., 2012).

Few animal studies assessed the effects of maternal separation soon after termination of this treatment, during infancy or adolescence. These studies have reported no effect of MS upon ethanol drinking at adolescence or periadolescence, although alterations in open field activity and play behavior were observed (Arnold and Siviy, 2002). Daoura et al. (2011) found greater ethanol intake in MS360 vs. AFR animals when testing began at adulthood, but not when testing began at adolescence; whereas others (Palm et al., 2013) found no differences in ethanol consumption in adolescent, male Wistar rats, subjected to MS360 or control conditions. The effects of EE upon ethanol drinking during adolescence have not been explored. Moreover, although most of the available suggests that EE may reduce ethanol-seeking (Roman et al., 2003; Daoura et al., 2011), there are contradictory results. Long-term exposure to EE (i.e., 3 or more months) was associated with significantly greater ethanol intake in adults, genetically heterogeneous rats (Rockman et al., 1989) and in rats selected for low or high anxiety response (Fernández-Teruel et al., 2002). It is still an open question whether the promoting effects of MS upon ethanol intake are immediately evident during adolescence or whether they follow a more delayed pattern of expression, appearing only later in development, after brain maturation. Also unknown is if EE will serve as potential treatment to reduce ethanol engagement during adolescence. The interactive effects between these treatments have not yet been explored.

The present study assessed, in Wistar male and female rats, the effects of maternal separation during infancy, followed by exposure to EE throughout adolescence, on ethanol drinking during periadolescence [i.e., between PD42 and PD60, Spear, 2000] and early adulthood [i.e., between PD61 and PD72]. After establishing that EE actually enhanced male ethanol drinking (Experiment 1), subsequent experiments assessed several effects of EE likely to underlie this promoting effect. We assessed EE effects, an MS modulation, of anxiety response in an elevated plus maze (EPM), aversive effects of ethanol and sensitivity to the sedative and sleep-inducing effects of ethanol (Experiment 2). Greater anxiety may facilitate ingestion of ethanol due to the anxiolytic effects of this drug (Spanagel et al., 1995), whereas the aversive and sedative effects of ethanol serve as barriers precluding further drug seeking and taking (Spear and Varlinskaya, 2010). Treatments that ameliorate these effects may promote ethanol drinking. Experiment 3 tested the hypothesis that EE may increase ethanol drinking by exacerbating the proclivity to take risks and explore new environments.

GENERAL MATERIALS AND METHODS

Subjects

One hundred and twenty-one Wistar rats were used. Number of animals in each experiment was as follows: Experiment 1, 32 males, 32 females (derived from 8 l, four experienced AFR, four experienced daily episodes of MS); Experiment 2, 32 males (derived from 8 l, four experienced AFR, four experienced daily episodes of MS); and Experiment 3, 32 males (derived from 8 l, four experienced AFR, four experienced daily episodes of MS). These animals were born and reared at the production vivarium at INIMEC-CONICET-Universidad

Nacional de Córdoba (Córdoba, Argentina), which is kept at 22–24°C with a12 h/12 h light/dark cycle (lights on at 8:00 AM). The pregnant dams came from the regular stock of the vivarium and births were checked daily. The day of birth was considered as PD0 and on PD1 litters were culled to four females and four males. Subjects were naïve to experimental procedures in each Experiment. Unless specified, litters were housed in standard maternity cages and given *ad libitum* access to water and lab chow. The experimental protocol was reviewed and approved by Institutional Animal Care and Use Committee (CICUAL protocol No. 2014-10) and complied with the regulations of the Guide for Care and Use of Laboratory Animals (National-Research-Council, 1996).

Litter effects across experiments were controlled by including no more than one male or female per litter in any group condition and by conducting a cross-fostering procedure at PD1, after the culling and before commencement of experimental treatment. More in detail, at PD1 the dams were briefly moved to a separate, clean cage and two males and two females from a given litter were transferred to another litter, which in turn provided two males and two females to the former litter. This procedure helped avoid assigning more than one male or female from the same litter to a given experimental group. Specifically, any given litter was randomly assigned to the MS or AFR condition. Within each litter, two males (one fostered and one not fostered) were assigned (at weaning time) to the EE condition and 2 were assigned the CTRL condition. The same procedure was done for females. Had we chosen not to conduct cross-foster, we would have needed additional litters, because we could have only assigned one male and one female to the post-weaning EE and CTRL conditions. Informal observations of the dam's behavior upon their return to the homecage indicated that the pups were immediately accepted by the foster dams, which readily exhibited a normal maternal behavioral repertoire (e.g., pup retrieval, nest building, licking, and grooming of the pups).

Rearing Conditions across PDs 1–21 (Experiments 1, 2, and 3)

On PD 1, litters were randomly assigned to the AFR condition or to experience 180 (Experiments 1, 2m and 3, see **Figure 1**) min of daily MS, once daily during PD 1–21. MS followed a standardized protocol, commonly used in our lab (see Fernandez et al., 2014). At 0900 AM the pups were removed from the dam and placed, as a litter, in a room located next to the housing room, in a clean maternity cage. The cage was equipped with a heating pad that kept floor temperature at 35°C. The pups were returned with the dam at noon. The dam stayed in the homecage during the MS procedure. AFR and MS litters were exposed to a weekly change in maternal cages and beddings.

Rearing Conditions across PDs 21–42 (Experiments 1, 2, and 3)

After termination of the maternal separation session on PD21 (weaning day in most rodent breeding protocols), the animals were randomly assigned to EE or standard (control) housing (CTRL). Control rats were transferred, in same-sex groups of four, to a standard cage (60 cm length × 40 cm width × 20 cm height), and rats in the EE groups were housed in same-sex groups of four in similar, yet taller, cages (60 cm length × 40 cm width × 40 cm height) that featured two levels connected by a ramp and equipped with seven objects and toys, including ladders, cylinders, pipes, house-like objects, and a running wheel. Food was placed always in the floor, in a corner. To prevent habituation, the experimenter changed the location and composition of the objects twice a week. **Figure 2** illustrates one of these compositions. The animals were kept under EE or CTRL conditions until the morning of PD42. This is, EE was conducted throughout the juvenile and adolescent stages of development. Following recommendations from our institutional animal care committee the rats were pair-housed in same-sex couples after PD42. This recommendation takes into account the relationship between size of the homecage and weight of the animal. A succinct description of the rearing protocol can be found in **Figure 1**.

Light-Dark Box (LDB, Experiment 1) Test

In Experiment 1, animals were tested in an LDB apparatus at PD42, immediately after termination of EE exposure. This day the animals were withdrawn from the home-cage, which was still enriched for those in EE groups. The LDB featured two compartments made of high impact acrylic, one white (24.5 cm × 25 cm × 25 cm) illuminated by a 60 W white bulb lamp adjusted to generate an illumination level of 400 lux, and one black (17.5 cm × 25 cm × 25 cm) without illumination (i.e., 0 lux). A divider with an opening at floor level separated both compartments. The test began by gently placing the animal in the center of the white area, facing away from the black area. After termination of the 5-min LDB test, all animals were housed in standard cages (two animals per cage). Illumination of the apparatus was being measured via a digital lux meter (LX1010B). The following variables were measured: number of transfers between compartments, latency (s) to enter the dark compartment, time (s) spent in the white compartment and frequency of stretching behavior.

Ethanol Intake Procedures (Experiment 1)

We used a two-bottle intake procedure, described in Fabio et al. (2015), to assess ethanol intake and preference from PD46 to PD72. This period encompass periadolescence (i.e., between PD42 and PD60, Spear, 2000) and early adulthood (i.e., between PD61 and PD72). The animals went through a 4 weeks, intermittent-access ethanol intake protocol (three sessions per week starting on Monday, Wednesday, and Friday, 24 h per session; described in **Figure 1**). They were exposed to 3, 4, and 5% ethanol vs. plain water, on weeks 1–3, respectively. Animals were also exposed to 5% ethanol vs. plain water in the Monday and Wednesday sessions of week 4. On the last test session on Friday, however, the animals were "challenged" with 7.5% ethanol vs. plain water.

Animals were housed individually during the course of each 24 h test. Before and after each intake session, however,

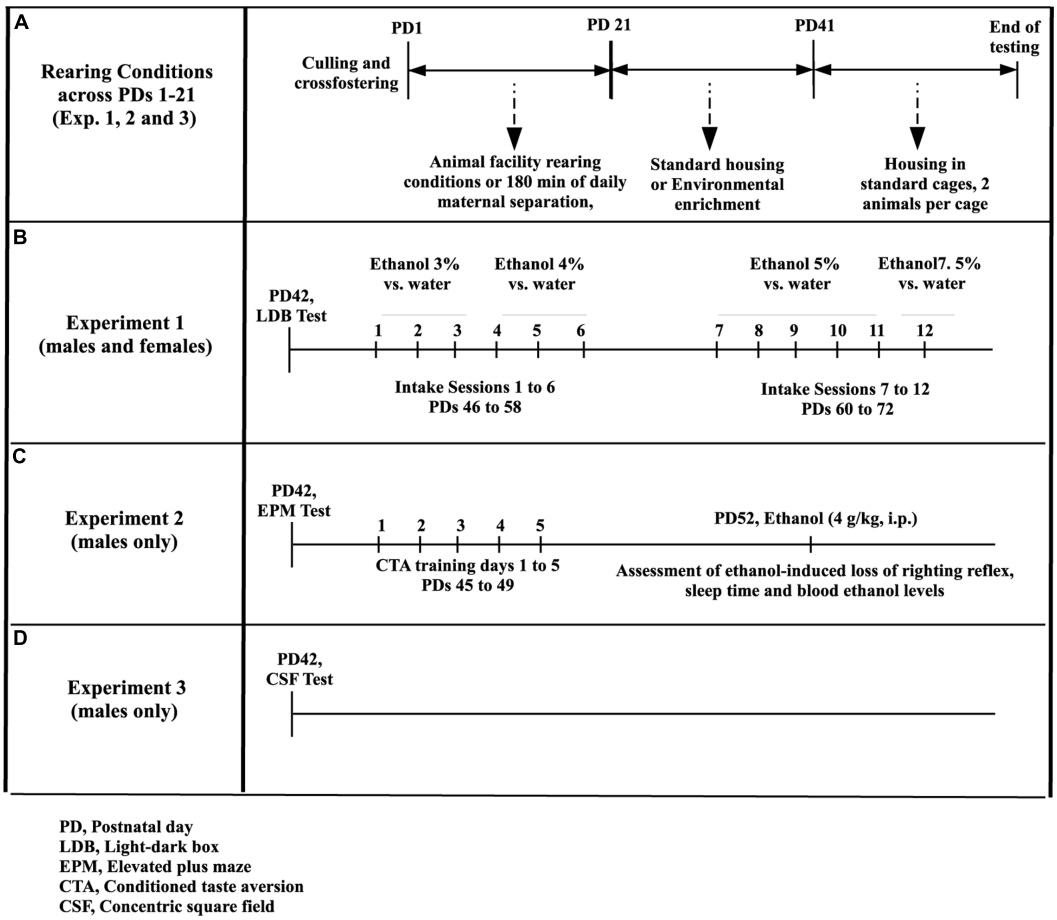

FIGURE 1 | Methods for the analysis of the effects of maternal isolation during infancy and environmental enrichment during adolescence on ethanol intake in adolescent Wistar rats. (A) From postnatal day (PD) 1 to PD21, the rats were reared under animal facility rearing conditions or were given daily episodes of maternal isolation (180 min). Between PD21 and PD42 they were given standard housing or environmental enrichment. The tests conducted on Experiments 1, 2, and 3 began on or after PD42. **(B)** The rats (males and females) were assessed for ethanol intake (Experiment 1) during 4 weeks, from PD46 to PD72. During each week animals were given three every other day, two-bottle choice tests (ethanol vs. plain water), followed by two rest days. Ethanol concentration was 3% (week 1, intake sessions 1–3), 4% (week 2, intake sessions 4–6), 5% (weeks 3 and 4, intake sessions 7–11) or 7.5% (session 12). **(C)** The rats, males only, were tested in an elevated plus-maze test at PD42. Taste conditioning, employing ethanol (2.5 g/kg, i.p.) as the unconditional stimulus, was acquired on PD47 and tested on PD49. Animals were given 4.0 g/kg ethanol (i.p.) and tested for ethanol-induced loss of righting reflex, sleep time and blood ethanol levels at PD52. **(D)** In Experiment 3, male rats exposed or not to MS during infancy and reared under EE or controls conditions during adolescence were tested on PD42, in a modified version of the concentric square field (CSF).

the animals were pair-housed in same-sex couples with *ad libitum* access to food and water. More in detail, during intake sessions, each animal was individually housed in half (i.e., 27 cm × 18.5 cm × 20 cm) of a standard homecage and separated from its conspecific by a divider made of high impact acrylic (27.5 cm × 18.5 cm). Each half of the homecage had a metal lid that accommodated food pellets and two bottles. The animals and the bottles were weighed before and after each session. These records were used to calculate ethanol intake on a gram per kilogram (g/kg) basis, and percent (%) selection of ethanol intake. Leakage was accounted for by having a bottle of ethanol and a bottle of ethanol in an empty cage, located next to the experimental cages. The readings of these bottles were subtracted from the amount of the corresponding fluid (ethanol, vehicle) registered in each cage.

Elevated-Plus Maze (EPM) Test (Experiment 2)

Experiment 1 indicated an effect of EE on ethanol intake, in males only. Experiment 2 was aimed at analyzing mechanisms underlying this effect of EE and employed only male rats. These males were exposed to MS or AFR during infancy and reared under EE or CTRL conditions during adolescence. They were submitted to a 5-min EPM test on PD42, immediately after termination of EE or CTRL housing, and before commencement of standard housing.

The EPM was made of black metal with a black Plexiglas cover and consisted of two open, unprotected arms (45 cm × 5 cm) and two closed, protected arms (45 cm length × 5 cm width × 45 cm height) that extended from a central platform (5 cm × 5 cm) elevated 50 cm above the floor. Each rat was placed in the central

FIGURE 2 | Wistar rats under environmental enrichment conditions were housed in large opaque cages (60 cm length × 40 cm width × 40 cm height) that contained seven objects and toys, including ladders, cylinders, pipes, house-like objects, and a running wheel. The photograph illustrates these conditions.

platform facing an open arm. Percentage of entries into the open arms and total number of arm entries were calculated and considered an index of anxiety response and overall exploratory behavior, respectively. The following naturalistic behaviors were recorded as well: rearing (standing on the hind limbs not in contact with a wall), stretching (propelling the body forward while keeping immovable the hind paws), sniffing (head upward with movement of the nostrils), and head-dipping (positioning the head out of the maze border and below the floor level). Grooming, defined as strokes over the nose that were eventually followed by large bilateral strokes and body licking (Arias et al., 2010), was not observed. Due to their definition, rearing and head dipping can only be performed in the open arm. These behaviors are exploratory behaviors associated with exploration of novelty (Fernández-Teruel et al., 2002; Lever et al., 2006). Stretching, indicate of risk assessment (Bailey and Crawley, 2009) was measured toward the open and closed arms (no stretching was observed toward the center area), whereas sniffing was measured in the open and closed arms, and in the center section.

Taste Aversive Conditioning (Experiment 2)

The rats were submitted to a 5-day taste aversion conditioning protocol, which began 3 days after the EPM test (see **Figure 1**). The aim was to analyze potential EE-induced modulation of the aversive effects of ethanol, which are key regulators of ethanol intake (Dyr et al., 2016). The procedure has been commonly used in our lab (Acevedo et al., 2010; Fabio et al., 2015). On PD45 (day 1), the adolescents were housed individually and given *ad libitum* access to food and water. On the morning of the next day (PD46) the bottle of water was replaced by a new bottle filled with 50% of the volume of water they had drank during the previous day. On day 3 (PD47), the animals were weighed to the nearest 0.1 g. Upon returning to the cage the water bottle had been substituted by a graded tube containing a 0.09% sodium chloride solution. Animals had free access to the solution for 30-min, then sodium chloride intake was measured and animals were immediately administered ethanol (2.5 g/kg, i.p., concentration: 21%, mixed in physiological saline, volume of administration: 0.015 ml per gram of body weight). On day 4 (PD48), the adolescents were again given 50% of the volume of water they had ingested on day 1 (corrected by the weight registered on PD48). Aversive conditioning was assessed on day 5 (PD49). On the morning of that day the water bottle was replaced by a graded tube containing a 0.09% sodium chloride solution. Intake was recorded after 30 min and expressed as milliliters consumed per 100 g of the rat (ml/100 g).

Assessment of Ethanol-Induced Loss of Righting Reflex, Sleep Time and Blood Ethanol Levels (Experiment 2)

Ethanol's sedative and sleep-inducing effects limit sustained engagement in ethanol self-administration (Spear and Swartzwelder, 2014). Experiment 2 assessed these effects 3 days after termination of the taste aversive conditioning (see **Figure 1**). All animals had been administered ethanol during the aversive conditioning, thus they were equated in terms of ethanol exposure when the test for ethanol-induced sleep began.

On PD52 the rats, were i.p., injected with ethanol (4.0 g/kg, concentration: 21%, vehicle: physiological saline, volume of administration: 0.024 ml per gram of body weight) and immediately monitored. Signs of sedations lead the experimenter to position the animal in a supine position. If the animal turned over the experimenter would put him back again in a supine position. The loss of the righting reflex was considered when the animal was not able to recuperate the prone posture three times in 30 s. The period elapsing between times of loss to time of regaining the righting reflex was considered sleep time. The animal that regained the prone posture when placed supine three times within a 30 s interval was considered recovered.

Blood trunk (2 ml) samples were obtained at recuperation through decapitation, using a capillary tube with heparin. The samples were kept at −70°C for later analysis of blood ethanol concentrations, via a Hewlett-Packard gas chromatographer (Model 5890). The vials containing the samples were incubated into a hot water bath (60°C) for 30 min and then a gas-tight syringe (Hamilton Co., Reno, NV, USA) was used to extract the volatile component of each vial, which was in turn injected into the chromatographer. The carrier gas was nitrogen (speed: 15 ml/min) and the column, oven and detector were set at 60, 150, and 250°C, respectively.

Assessment of Shelter Seeking, Exploratory and Risk Taking Behaviors (Experiment 3)

In Experiment 3, male rats exposed to AFR or MS during infancy and reared under EE or CTRL conditions during adolescence were tested (PD42) in a modified version of the concentric square field (CSF, first described by Meyerson et al., 2006). The CSF, which is usually used in adult rats (Karlsson and Roman, 2016), features a central square interconnected to several other areas by corridors. Some of the areas evoke shelter-seeking behavior, whereas others evoke exploration, risk assessment and risk taking. The front of the maze is a high-risk, brightly illuminated area with an elevated wire mesh structure that animals can climb. Compared with other tests, the CSF allows simultaneous measurement of different behavioral patterns, allowing investigating a broader behavioral profile (Roman et al., 2012). The CSF does not impose subjects a single or binary behavioral option but instead allows a graded set of exploratory activities that bridge the gap from seeking sheltered, enclosed dark spaces to seeking illuminated and elevated spaces that entail potential high-risk (Karlsson and Roman, 2016).

The CSF (48 cm × 48 cm) was made of black melamine, except for the front side wall (i.e., next to the bridge), which was made of transparent PET. The external walls were 48 cm high and the internal walls were 40 cm high. The central square (26 cm × 26 cm) gave access to three corridors (A, B, C). Corridor A led to the dark shelter (SHEL, 10 cm × 15 cm × 40 cm), which was the only enclosed section of the maze. Corridor B (18 cm × 10 cm × 48 cm) led to the challenge (CHA) area, so called because animals had to jump through a hole, elevated 10 cm from the floor, to get into it. There were two of these holes in the CHA area, one led to corridor B and the other headed to corridor C (15 cm × 10 cm). The latter corridor also allowed access to the front section of the maze, a brightly illuminated runway separated from the outside by a transparent plastic. An animal coming from the C corridor to the front area first encountered a ramp (RAMP, 12 cm × 10 cm, inclination: 20°), which led to an elevated bridge (BRIDGE, 30 cm × 10 cm). RAMP and BRIDGE were made of a hard wire mesh. Lighting conditions (lx) in the CSF arena, which were established following previous studies (Karlsson and Roman, 2016) and measured by digital luxometer (LX1010B), were as follows: SHEL: 0; CF, corridors A, B, C and CHA: 20–30; RAMP and BRIDGE: 600–650. The test lasted 20 min and was video recorded for subsequent processing via ETHOLOG 2.2 (Ottoni, 2000). Time spent and frequency of entries in each section was measured, along with frequency of nose-poking in the CHA holes.

Experimental Designs and Statistical Analysis

Experiment 1 employed a 2 (Rearing conditions during infancy: AFR or MS) × 2 (Rearing conditions during adolescence: CTRL or EE) × 2 (sex: male or female) factorial design, with eight animals per group. Animals were exposed to MS on PDs 1-21 and to EE on PDs 21–42. Anxiety responses in the LDB test (latency to exit the bright compartment, time spent in the bright compartment and number of transfer between compartments) were separately analyzed via factorial analyses of variance (ANOVAs). The dependent variables of the ethanol intake assessments [overall fluid intake (ml/100 g), and ethanol intake (g/kg and percent preference)] were examined using separate four-way mixed ANOVAs. Rearing conditions during infancy and adolescence, and Sex were the between-group factors, and Session (sessions 1–12) was the repeated measure (RM).

In Experiments 2 and 3 the rats (only males) were distributed into four groups ($n = 8$) as a function of Rearing conditions during infancy and Rearing conditions during adolescence. The anxiety responses registered during the EPM test (latency to exit from and time spent in the bright area, number of transfers between compartments) were analyzed via independent factorial ANOVAS (between factors: Rearing conditions during infancy and during adolescence). Similar ANOVAs were used to analyze latency to lose the righting reflex, ethanol-induced sleep time, blood ethanol levels at awakening time (Experiment 2) and the time spent and total number of entries in the different sections of the CSF (Experiment 3). Consumption of sodium chloride

(NaCl, ml/100 of body weight) during conditioning and testing of Experiment 2 was analyzed with a 3-way RM ANOVA, in which Rearing conditions during adolescence and adulthood served as between factors and Days of assessment as RM. A significant reduction in NaCl intake between conditioning and testing was taken as an indication of taste avoidance.

The total number of section entries is a measure of exploratory activity in the CSF, but it conflates locomotion in protected and unprotected sections of the CSF. To better understand the difference in exploration of risk areas vs. exploration of sheltered/protected areas of the apparatus, the total number of entries was split between (a) entries in risk taking/assessment areas (RAMP, CHA and BRIDGE), (b) entries in the sheltered area and in the corridor A that leads to it, and (c) entries in corridors B and C. Separate RM ANOVAs (between factors: Rearing conditions during infancy and during adolescence, within factor: Section of the apparatus) were conducted for each group of variables. Separate factorial ANOVAs analyzed time spent and frequency of entries in the central sector. Another factorial ANOVAs was used to analyze nose-poking in the CHA sector.

Significant main effects and significant interactions were scrutinized via follow-up ANOVAs, *post hoc* tests or planned comparisons. More in detail, Tukey's tests were used to scrutinize simple main effects or interaction involving "between" factors, whereas significant interactions involving RMs were analyzed through orthogonal planned comparisons. The rationale was that there is no unambiguous choice of pertinent error terms for *post hoc* comparisons involving between-by-within factors (Winer et al., 1991). The partial eta square (η_p^2) was used to estimate effect size and the alpha level was $\leq$0.05. STATISTICA 8.0 (StatSoft, Tulsa, OK, USA) was used for the respective statistical analyses. Data from 7 animals (3 in Experiment 1 and 4 in Experiment 3) were lost due to errors during the experimental procedures or the processing of the videotapes. These data were not replaced.

RESULTS

Experiment 1

Table 1 presents the data yielded by the LDB test, which was conducted at termination of the EE treatment and before the ethanol intake tests. Latency to exit the bright compartment was not affected by Sex or Rearing conditions, whereas the ANOVAs for number of transfers between compartments and for time spent in the bright compartment yielded significant main effects of Sex [$F_{(1,53)} = 5.07$, $p \leq 0.05$, $\eta_p^2 = 0.08$; $F_{(1,53)} = 4.13$, $p \leq 0.05$, $\eta_p^2 = 0.07$; respectively] and a significant interaction between Rearing conditions at infancy and at adolescence [$F_{(1,53)} = 4.14$, $p \leq 0.05$, $\eta_p^2 = 0.07$; $F_{(1,53)} = 4.91$, $p \leq 0.05$, $\eta_p^2 = 0.08$; respectively]. Females, irrespective of rearing conditions, exhibited significantly more transfers and spent significantly more time in the bright compartment than males did. The *post hoc* indicated significantly greater number of transfers in the MS-EE group than in the MS-CTRL group ($p \leq 0.05$). The *post hoc* also revealed significantly greater time spent in the bright compartment in the MS-EE group than in groups MS-CTRL or AFR-EE ($p \leq 0.05$). Stretching behaviors were significantly greater in animals exposed to MS than in AFR controls, an effect that was independent of rearing conditions during adolescence [significant main effect of rearing conditions during infancy: $F_{(1,53)} = 5.60$, $p \leq 0.05$, $\eta_p^2 = 0.10$].

Figure 3 illustrates ethanol intake patterns across groups. The analysis for absolute (g/kg) ethanol intake revealed significant main effects of Sex and Session [$F_{(1,53)} = 7.05$, $p \leq 0.05$, $\eta_p^2 = 0.12$; $F_{(11,583)} = 12.81$, $p \leq 0.001$, $\eta_p^2 = 0.19$; respectively]. The interaction between Sex, Rearing conditions at adolescence and Sessions achieved significance [$F_{(11,583)} = 1.84$, $p \leq 0.05$, $\eta_p^2 = 0.03$].

The significant three-way interaction, which is depicted in **Figure 4**, was explored via follow-up ANOVAs (Rearing condition at adolescence × Session) for each sex. The ANOVA conducted for females only revealed a significant main effect of Sessions [$F_{(11,297)} = 7.37$, $p \leq 0.001$; $\eta_p^2 = 0.21$]. The *post hoc* tests indicated significantly greater drinking scores on sessions 7–11 than in sessions 1, 2 ($p \leq 0.05$) or 12 (challenge session, $p \leq 0.001$). This pattern was not affected by the rearing conditions during infancy or adolescence.

The ANOVA for males, in turn, yielded significant main effects of Session and Rearing conditions at adolescence, [$F_{(11,286)} = 6.54$, $p \leq 0.0001$, $\eta_p^2 = 0.20$; $F_{(1,26)} = 9.75$, $p \leq 0.005$, $\eta_p^2 = 0.27$; respectively]. The interaction between these factors was significant [$F_{(11,286)} = 7.64$, $p \leq 0.0001$, $\eta_p^2 = 0.11$]. Males exposed to EE drank, regardless of whether they had been exposed to MS or not, significantly more than males in the CTRL group, from the second week of testing onward. Among males, the planned comparisons revealed significantly greater ethanol drinking (g/kg) in EE than in CTRL rats at sessions 5, 6, 7, 9, 10, 11 and also during the challenge at session 12 ($p < 0.005$, 0.001, 0.005, 0.05, 0.005, 0.005, and 0.05, respectively).

The analysis of percent ethanol preference yielded similar results to those obtained with absolute intake scores. The ANOVA revealed a significant main effect of Session [$F_{(11,583)} = 3.95$, $p \leq 0.0001$, $\eta_p^2 = 0.07$] and a significant interaction between Sex, Session and Rearing conditions at adolescence [$F_{(11.583)} = 1.87$, $p \leq 0.05$, $\eta_p^2 = 0.04$]. The follow-up ANOVA for females revealed a lack of significant main effects or significant interactions, whereas the ANOVA for males indicated significant main effects of Session and Rearing conditions at adolescence, as well as a significant interaction between these factors [$F_{(11,286)} = 3.10$, $p \leq 0.0001$, $\eta_p^2 = 0.11$; $F_{(1.26)} = 4.52$, $p \leq 0.05$, $\eta_p^2 = 0.15$; $F_{(11,286)} = 1.92$, $p \leq 0.05$, $\eta_p^2 = 0.07$, respectively]. The planned comparisons indicated, among males, significantly greater ethanol percent preference in the EE than in the AFR group at sessions 5, 6, 7, 11, and 12 ($p < 0.05$, 0.01, 0.001, 0.05, and 0.05, respectively).

The ANOVA for water consumption scores (ml/100 g of body weight, descriptive data not shown) yielded significant main effects of Sex [$F_{(1,53)} = 6.38$, $p \leq 0.05$, $\eta_p^2 = 0.11$] and Session [$F_{(11,583)} = 8.92$, $p \leq 0.001$, $\eta_p^2 = 0.14$]. The Session × Sex interaction also achieved significance [$F_{(11.583)} = 2.78$, $p \leq 0.005$, $\eta_p^2 = 0.05$]. The planned comparisons indicated that females,

TABLE 1 | Data (expressed as mean ± SEM) gathered in the light-dark box (LDB, Experiment 1) and elevated plus maze (EPM, Experiment 2) tests, and in the test for sensibility to the sedative and hypnotic effects of ethanol (Experiment 2).

	Males				Females			
	Environmental enrichment (EE) groups		Standard (Control, CTRL) housing groups		Environmental enrichment (EE) groups		Standard (Control, CTRL) housing groups	
LDB	**Maternal separation (MS)**	**AFR**	**Maternal separation (MS)**	**AFR**	**Maternal separation (MS)**	**AFR**	**Maternal separation (MS)**	**AFR**
Latency to exit the bright sector (s)	28.10 ± 7.42	28.10 ± 7.42	29.76 ± 18.90	10.41 ± 3.64	17.73 ± 5.88	37.29 ± 16.16	28.99 ± 12.38	28.00 ± 6.2
Transfers between sectors	2.43 ± 0.36	2.43 ± 0.36	1.25 ± 0.62	2.12 ± 0.51	4.75 ± 0.82 (#)	2.28 ± 0.75 (#)	2.62 ± 0.59 (#)	2.5 ± 0.57 (#)
Time in bright sector (s)	2.42 ± 0.37 (&)	2.42 ± 0.37	1.25 ± 0.62	2.62 ± 0.59	4.75 ± 0.82 (&) (#)	2.28 ± 0.74 (#)	1.25 ± 0.68 (#)	2.5 ± 0.57 (#)
Stretching (frequency)	1.86 ± 0.63 (*)	1.86 ± 0.63	1.87 ± 0.35 (*)	0.5 ± 0.27	2.12 ± 0.77 (*)	2.43 ± 0.48	1.5 ± 0.60 (*)	1.62 ± 0.98
EPM								
Stretching (open, closed arms)	0.63 ± 0.26, 3.88 ± 0.30	2.63 ± 0.68 (&), 2.75 ± 0.70	1.13 ± 0.58, 3.13 ± 0.50	0.75 ± 0.25, 3.50 ± 0.87				
Sniffing (center, open, closed arms)	3.62 ± 0.78, 4.87+/1.14, 13.00 ± 1.85 (*)	2.87 ± 0.61, 4.13+/1.11, 9.25 ± 1.77	1.5 ± 0.65, 2.75+/1.21, 14.88 ± 2.75 (*)	8.38 ± 2.51 4.00+/1.49, 8.38 ± 2.51				
Assessment of sedative effects of ethanol and BELs								
Sleep time (s)	8845.34 ± 736.44	9223.57 ± 835.85	8327.96 ± 514.17	9189.50 ± 173.91				
Loss of righting reflex (s)	916.83 ± 596.99	166.00 ± 9.18	144.6 ± 9.57	213.90 ± 21.07				
Blood ethanol levels (mg%)	360.46 ± 30.56	415.32 ± 20.01	308.42 ± 30.83	358.65 ± 30.73				

The animals, male or female Wistar rats, were exposed or not to maternal separation on PDs 1–21 and housed under environmental enrichment or standard (control) housing conditions on PDs 21–42. Tests were conducted at PD42 (EPM, LDB) or PD52 (ethanol-induced sedation and hypnosis). The pound sign (#) indicates a significant main effect of Sex on the number of transfers between sectors and on the time spent in the bright sector (LDB test). The asterisk sign (*) indicates a significant main effect of Rearing conditions during infancy on frequency of stretching (LDB test) and on sniffing (closed arms, EPM test). The ampersand sign (&) indicates that AFR-EE animals made significantly more stretching in the open arms (EPM test) than the rest of the groups.

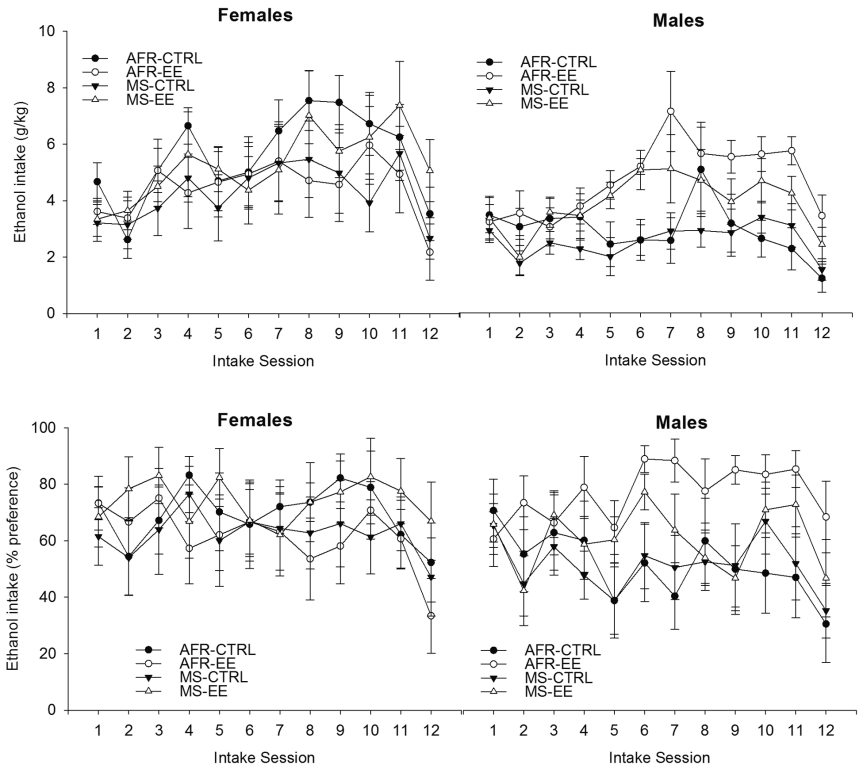

FIGURE 3 | Ethanol intake (g/kg and percent preference) (upper and lower panels, respectively) in male and female Wistar rats as a function of rearing conditions during postnatal days 1–21 (animal facility rearing or daily episodes of maternal separation, AFR and MS groups, respectively), rearing conditions during postnatal days 21 to 42 (standard control housing or environmental enrichment, CTRL and EE groups, respectively) and intake session. Two-bottle intake sessions (ethanol vs. plain water) were conducted on Monday, Wednesday and Friday (session length: 24 h), during 4 weeks. Ethanol concentration was 3% (week 1, intake sessions 1–3), 4% (week 2, intake sessions 4–6), 5% (weeks 3 and 4, intake sessions 7–11) or 7.5% (session 12). The analyses of variance (ANOVAs) indicated, for both variables, a significant interaction between Sex, Rearing conditions at adolescence and Sessions. These significant interactions are depicted in **Figure 4**. Follow-up ANOVAs (Rearing conditions at adolescence × Sessions) for each sex indicated a lack of significant main effects or significant interactions in the females; whereas the ANOVAs for males indicated greater ethanol intake and percent preference in subjects given EE than in controls at sessions 5, 6, 7, 9, 10, 11, and 12 (g/kg) or 5, 6, 7, 11, and 12 (% preference). The data are expressed as mean ± SEM.

irrespective of the rearing conditions experimented during infancy and adolescence, drank significantly more than males, at sessions 3, 8, 11, and 12 (all $p > 0.05$).

Rearing conditions at infancy did not exert a significant main effect, nor were involved in any significant interaction, in any of the variables analyzed.

Experiment 2

Maternal separation at infancy, as an individual factor, significantly reduced the percent time spent in the open arms of the EPM [$F_{(1,28)} = 4.09$, $p \leq 0.05$, $\eta_p^2 = 0.13$], without altering the total number of arm entries. The ANOVA for the latter variable revealed no significant main effects or significant interactions. The ANOVA for rearing behavior indicated a significant interaction between Rearing conditions at infancy and Rearing conditions at adolescence [$F_{(1,28)} = 4.97$, $p \leq 0.05$, $\eta_p^2 = 0.17$]. The *post hoc* revealed that rearing was significantly greater in the AFR-EE group than in the AFR-CTRL group. Rearing was measured only in the open arms.

The ANOVA for stretching revealed an interaction between Rearing conditions at infancy, Rearing conditions at adolescence and the section of the EPM where this behavior was measured [$F_{(1,28)} = 4.05$, $p \leq 0.05$, $\eta_p^2 = 0.13$]. The planned comparisons indicated that AFR-EE animals made significantly more stretching in the open arms than the rest of the groups. MS rats exhibited, irrespective of whether or not they had been given EE, significantly more sniffing than AFR animals in the closed arms, but not in the rest of sections, [significant main effect of section: $F_{(2,56)} = 40.35$, $p \leq 0.001$, $\eta_p^2 = 0.59$; significant Section × Rearing conditions at infancy interaction: $F_{(2,56)} = 3.88$, $p \leq 0.05$, $\eta_p^2 = 0.12$]. EE animals shown, irrespective of whether or not they had been given MS, a trend for greater head-dipping [main effect of EE: $F_{(1,28)} = 3.71$, $p = 0.06$, $\eta_p^2 = 0.12$]. Head-dipping was only measured in the open arms. **Figure 5** illustrates time spent in the open arms (%), total number of arm entries and frequency of rearing and head-dipping in the EPM, whereas the lower section of **Table 1** presents mean and SEM across conditions, for frequency of stretching (open, closed arms) and sniffing (center, open, closed arms).

Across groups, intake of NaCl (ml/100g) exhibited a three-fold reduction between conditioning (3.69 ± 0.28) and testing (1.13 ± .30). This reduction, suggestive of acquired taste aversion,

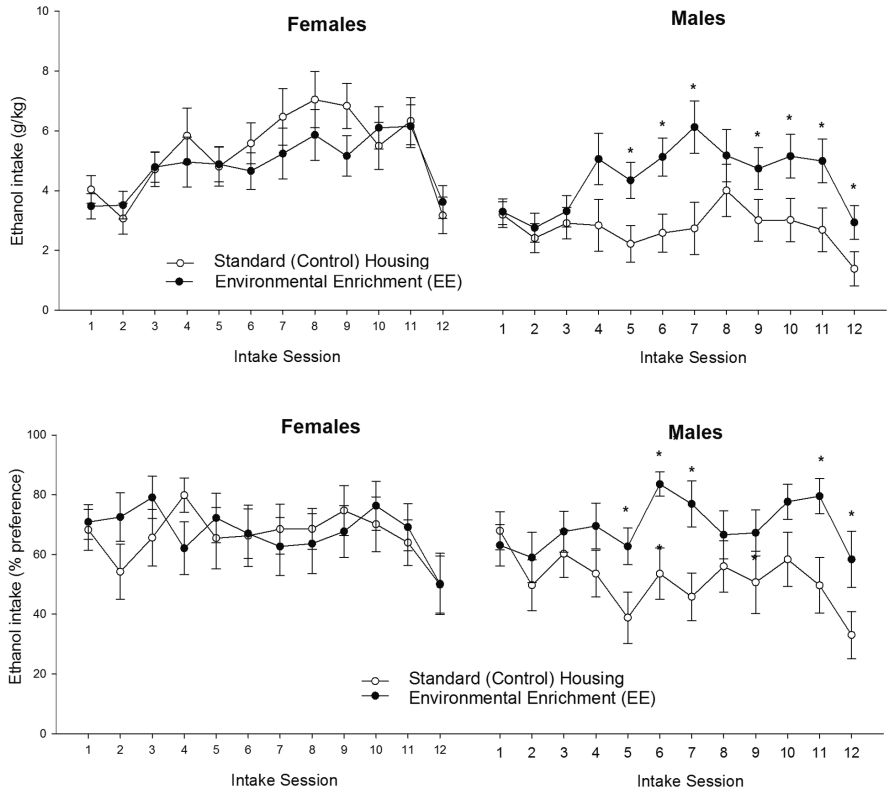

FIGURE 4 | Ethanol intake (g/kg and percent preference) (upper and lower panels, respectively) in male and female Wistar rats as a function of rearing conditions during postnatal days 21 to 42 (standard control housing or environmental enrichment, CTRL and EE groups, respectively) and intake session. The asterisk sign (*) indicates a significant difference between EE and CTRL male rats, in a given session. The data are expressed as mean ± SEM.

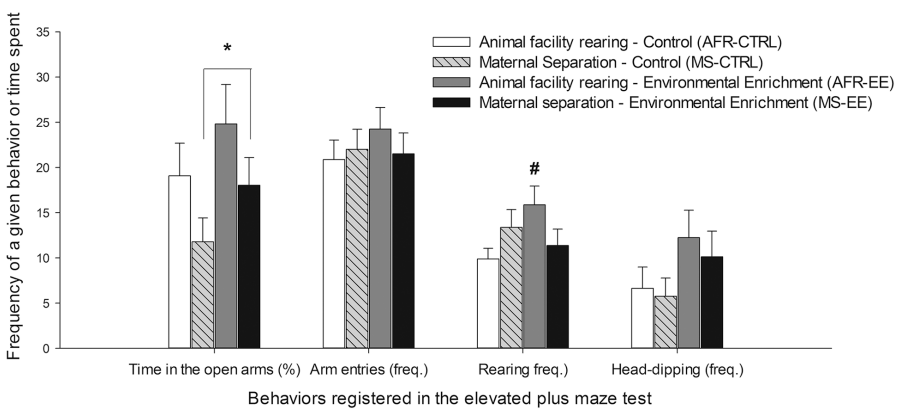

FIGURE 5 | Time spent in the open arms (%), total number of arm entries and frequency of rearing and head-dipping (expressed as mean ± SEM) in the elevated plus maze (EPM, Experiment 2) test. The animals, male Wistar rats, were exposed or not to maternal separation on postnatal days (PDs) 1–21 (AFR and MS groups, respectively) and housed under environmental enrichment or standard (control) housing conditions on PDs 21–42 (EE and CTRL groups, respectively). The tests were conducted at PD42. The asterisk sign (*) indicates that maternal separation at infancy, as an individual factor, significantly reduced the percent time spent in the open arms of the EPM. The pound sign (#) indicates that frequency of rearing was significantly greater in the AFR-EE group than in the AFR-CTRL group.

did not seem to be affected by Rearing conditions at infancy or adolescence. Mean ± SEM consumption of NaCl (ml/100 g) across groups, during conditioning and testing, were as follows: MS-CTRL 3.96 ± 0.46 and 1.31 ± 0.70, AFR-CTRL 3.37 ± 0.30 and 0.63 ± 0.40, MS-EE 3.36 ± 0.63 and 0.70 ± 0.44, AFR-EE 4.05 ± 0.81 and 1.88 ± 0.75. The ANOVA confirmed these impressions. The analysis only revealed a significant main effect of Session [$F_{(1,28)} = 74.25$, $p \leq 0.001$, $\eta_p^2 = 0.73$]. Maternal

Separation and EE did not exert a significant main effect upon intake of NaCl nor were involved in any significant interaction. Similarly, the latter factors did not significantly affect ethanol-induced sleep time, latency to lose the righting reflex after the ethanol administration or blood ethanol levels at awakening time. Mean ± SEM for these variables are in **Table 1**.

Experiment 3

Overall locomotion in the CSF remained unaffected by rearing conditions at infancy or adolescence. The ANOVA for total number of section entries indicated the lack of significant main effects or significant interactions. Mean and SEM across groups were as follows MS-CTRL 104.28 ± 4.95., AFR-CTRL 114.50 ± 14.66., MS-EE 127.71 ± 6.21., AFR-EE 101.2 ± 9.60.

Figure 6 illustrates mean number of entries and time spent (s) in the risk taking/assessment areas of the apparatus. The ANOVAs indicated, for both variables, a significant main effect of EE and Sector and a significant interaction between these factors, [Number of entries: $F_{(1,24)} = 12.23$, $p \leq 0.05$, $\eta_p^2 = 0.34$; $F_{(2,48)} = 19.5$, $p < 0.001$, $\eta_p^2 = 0.45$ and $F_{(2,48)} = 3.61$, $p \leq 0.05$, $\eta_p^2 = 0.13$, respectively; Time spent: $F_{(1,24)} = 14.02$, $p \leq .001$, $\eta_p^2 = 0.36$; $F_{(2,48)} = 9.66$, $p \leq 0.001$, $\eta_p^2 = 0.29$ and $F_{(2,48)} = 5.42$, $p \leq 0.01$, $\eta_p^2 = 0.18$, respectively]. The planned comparisons indicated that, when compared to CTRL animals (i.e., those given standard housing after the weaning), EE animals exhibited (regardless of the rearing conditions experimented during infancy) significantly greater time spent ($p < 0.005$) and number of entries ($p < 0.001$) in the CHA sector and a trend for greater number of entries an time spent in the BRIDGE (both $p = 0.07$).

The ANOVA for number of entries in the sheltered area and in the corridor A yielded a significant main effect of sector [$F_{(1,24)} = 239.76$, $p \leq 0.001$, $\eta_p^2 = 0.91$]. The rats – regardless their rearing conditions at infancy or adolescence – exhibited significantly more number of entries in the corridor than in the sheltered area. The ANOVA also indicated a borderline effect of MS stress, as an individual factor [$F_{(1,24)} = 3.69$, $p = 0.06$, $\eta_p^2 = 0.13$]. MS rats (as a group, irrespective of whether they had been exposed to EE or CTRL housing conditions during adolescence) exhibited greater number of entries into these areas than AFR rats, although this difference did not reach statistical significance. Time spent in the sheltered area was significantly greater than time spent in corridor A [$F_{(1,24)} = 11.44$, $p \leq .005$, $\eta_p^2 = 0.32$], an effect that was not significantly affected by Rearing conditions at infancy stress or at adolescence.

The CHA sector can be accessed via corridor B or corridor C, yet only corridor C allows entry into the brighter RAMP, which in turn leads to the bridge. The RM ANOVA for number of entries in these corridors yielded a significant main effect of sector [$F_{(1,24)} = 81.16$, $p \leq 0.0001$, $\eta_p^2 = 0.77$]. The interactions between MS stress and Enrichment [$F_{(1,24)} = 4.75$, $p \leq 0.05$, $\eta_p^2 = 0.17$] and between MS Stress, Enrichment and Sector [$F_{(1,24)} = 6.15$, $p \leq 0.05$, $\eta_p^2 = 0.20$] also achieved significance. The *post hoc* revealed that the number of entries into corridor B was similar between the different groups. The *post hoc* also indicated that the number of entries into corridor C was significantly greater in rats from group MS-EE than in rats from group MS-CTRL ($p < 0.05$). The ANOVA for time spent in these corridors only indicated that animals, regardless their rearing conditions during infancy or adolescence, spent significantly more time in C than in B [$F_{(1,24)} = 25.98$, $p \leq 0.001$, $\eta_p^2 = 0.52$]. These results area illustrated in **Figure 6**.

The ANOVAs for time spent and frequency of entries into the central square (see **Figure 6**), and for nose-poke into the CHA holes (data not shown) did not yield significant main effects or interactions.

DISCUSSION

The main finding of the present study was that exposure to EE throughout adolescence induced a significant increase in ethanol intake and preference during periadolescence and young adulthood.

Environmental enrichment male, but not female, rats exhibited a two-fold increase in ethanol intake when compared to counterparts given standard housing, and achieved up to 80% ethanol predilection vs. water. The sex-related difference can simply be due to the fact that female rats (Lancaster et al., 1996; Doremus et al., 2005) or mice (Lopez and Laber, 2015) often exhibit increased ethanol intake and preference than males, which in turn can impede assessment of treatments that increase ethanol predilection. In other words, it is possible that females in the present study exhibited a ceiling effect in terms of ethanol intake or preference. An important finding was that the differences in ethanol intake between EE and control males were still significant in the last testing day, when ethanol concentration was increased from 5 to 7.5%.

In the present study, the rats were exposed (or not) to daily episodes of MS (duration: 180 min), throughout infancy. The decision of using 180 min of MS, instead of 15 or 360 min, was based on a previous study (Kawakami et al., 2007) that reported faster development of ethanol-induced behavioral sensitization after MS180, but not after MS15. Behavioral sensitization is the gradual increment in the motor-stimulating effects of ethanol following repeated ethanol administration, thought to reflect the transition from controlled to problematic ethanol drinking (Camarini and Pautassi, 2016).

Chronic MS, unlike EE, did not significantly affect ethanol intake in the present study. This was not unexpected, as the few previous studies that tested ethanol intake shortly after termination of MS reported no effect of MS upon ethanol drinking at infancy, adolescence-periadolescence or early adulthood. Rats exposed to MS180 or AFR during PD1-13 exhibited no differences in ethanol intake (6%, tested via intraoral infusions Pautassi et al., 2012) at PD15. Other studies used the more conventional exposure to MS360 on PD1-21. Palm et al. (2013) reported no differences in adolescent ethanol intake between MS360 and AFR controls (only males were employed). In a second study, Daoura et al. (2011) assessed ethanol intake via an intermittent, three-bottle, test (0.0, 5, or 20% ethanol), for 5 weeks, starting on PD26 (adolescence) or PD68 (adulthood). Ethanol intake, which again was assessed in males only, was

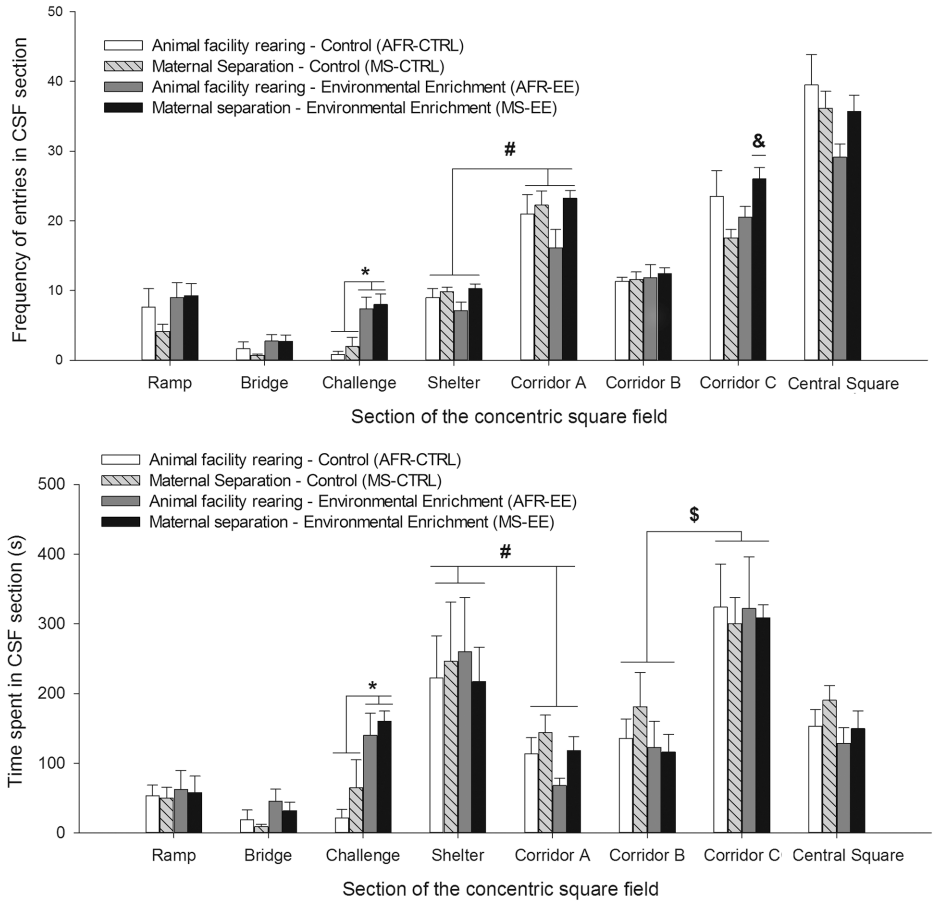

FIGURE 6 | Frequency of entries and time spent (upper and lower panels, respectively) in each section of the concentric square field, in male Wistar rats tested at postnatal day 42. The data are expressed as mean ± SEM and shown as a function of rearing conditions during postnatal days 1–21 (animal facility rearing or daily episodes of maternal separation, AFR and MS groups, respectively) and rearing conditions during postnatal days 21–42 [standard (i.e., control) housing or environmental enrichment, CTRL and EE groups, respectively]. The asterisk sign (*) indicates that EE groups exhibited, when compared to animals reared under standard (control) housing conditions (CTRL groups, irrespective of whether they had been given or not maternal separation during infancy), significantly greater time spent and number of entries in the challenge sector. The pound sign (#) indicates that the animals, regardless of the rearing conditions during infancy and adolescence, exhibited significantly greater time spent, and significantly less number of entries, in the sheltered area than in corridor A. These effects were not affected rearing conditions at infancy or adolescence. The ampersand sign (&) indicates that frequency of entries in corridor C was significantly greater in MS-EE animals than in MS-CTRL or AFR-EE counterparts. The currency ($) sign indicates that, regardless the rearing conditions during infancy and adolescence, animals of all groups spent significantly more time in corridor C than in corridor B. The data are expressed as mean ± SEM.

exacerbated in animals exposed to MS360, yet only when testing began at adulthood. Similarly, Roman et al. (2003) assessed ethanol intake in the ethanol-preferring AA strain from late adolescence - early adulthood (i.e., approximately PD77) to full adulthood (i.e., until PD120). Early maternal separation did not significantly affect ethanol intake in females. Intriguingly, the male's acceptance of ethanol was unaffected by early rearing conditions during the first 5 weeks of testing, yet after that – when the rats were in full adulthood – the MS360 group exhibited a significant increase in ethanol drinking.

Taken together, these studies (i.e., Roman et al., 2003; Daoura et al., 2011; Pautassi et al., 2012; Palm et al., 2013) and the results obtained in the present study cement the notion that, in rats, the effects of MS upon ethanol intake remain silent during infancy or adolescence and are expressed only when subjects reach full adulthood. It should be noted that it is not the case that MS was devoid of effects in the present study. Maternal separation induced significantly less exploration of the open spaces of the EMP and resulted in greater time spent in the sheltered area (and in the corridor leading to it) of the CSF. These results are consistent with the hypothesis that MS increases anxiety responses (Huot et al., 2001), yet it seems that the magnitude of this change was not substantial enough to influence ethanol drinking or, as indicated earlier, it is possible that the anxiety phenotype only affects ethanol intake at adulthood. This may be a consequence of ethanol intake being driven by different neurobiological mechanisms in adolescent vs. adults. Adolescent, but not adult, rats exhibit conditioned place preference by ethanol (Philpot et al., 2003; Pautassi et al., 2008), a result suggestive of greater ethanol-induced appetitive effects in the younger

animals. Ethanol drinking in adolescents may be driven by these appetitive effects of ethanol; whereas the anti-anxiety effects of ethanol could be more involved in drinking during adulthood. Consistent with this postulate, Samson et al. (1998) suggested that rats require protracted experience with ethanol drinking to learn about ethanol's anti-anxiety effects. Also noteworthy is that maternal separation affects a plethora of neural systems, yet the most prominent is the heightened responsiveness of the hypothalamic–pituitary–adrenal system toward subsequent stressors (Pryce et al., 2002). In Huot et al. (2001), for instance, adult rats exposed during infancy to maternal separation drank more ethanol and exhibited greater corticosterone response to airpuff startle, than non-stressed controls.

Environmental enrichment has been proposed as a non-pharmacological tool to reduce drug-induced adaptive changes (Solinas et al., 2009; de Carvalho et al., 2010). Swiss mice housed during adolescence in large-than-usual cages equipped with house-like objects, a running wheel and several tubes, were resistant to the development of ethanol-induced behavioral sensitization (Rueda et al., 2012), a behavioral proxy for the neural changes taking place during the transition from regular drug use to addiction (Camarini and Pautassi, 2016). Yet other studies provided contradictory information. Early work (Rockman et al., 1986, 1989) found greater ethanol intake after EE, although these researchers only tested ethanol intake in adulthood and after lengthy (i.e., $\geq$90 days) EE exposure. A facilitating effect of EE upon drug reactivity has also been observed with other drugs. EE exposure resulted in heightened amphetamine (Bowling and Bardo, 1994) or nicotine (Ewin et al., 2015) induced conditioned place preference.

What are the mechanisms that, in the present study, led to increased ethanol intake after EE? We analyzed, in male rats exposed to EE, sensitivity to the hypnotic-sedative effects and to the post-ingestive, aversive effects of ethanol. These effects have been suggested to serve as barriers that prevent initiation or escalation into ethanol intake, and differences in these effects have been used to explain differences in ethanol intake between adolescent and adult rats (Spear and Varlinskaya, 2010; Spear and Swartzwelder, 2014). Our hypothesis was that EE rats would be resistant to these effects, yet this was not corroborated. Ethanol induced significant flavor aversion and readily resulted in hypnosis, yet these effects were fairly similar across rearing conditions. Significant limitations of Experiment 2 were, however, the use of a single dose of ethanol and the lack of vehicle or unpaired controls in the taste conditioning procedure. This introduces the possibility that the aversion to the salty solution obeys to the lingering, toxic effects of ethanol, and perhaps differentially so across groups. Another important limitation of this study is that the animals were individually housed during the course of each ethanol intake test, and in-between tests they were again pair-housed. This repeated social isolation probably resulted in significant stress and, therefore, should be considered a factor contributing to the observed effects.

A study (Fernández-Teruel et al., 2002) found greater head-dipping and ethanol intake after EE, in a rat strain exhibiting high anxiety and low levels of novelty seeking. This points to the possibility that, in the present study, EE facilitated ethanol intake by increasing novelty-seeking. Evidence supporting this is that EE rats exhibited greater head-dipping and rearing behaviors in the EMP test, as well as more stretching in the open arm and in the center of the apparatus, than their non-enriched counterparts. Previous work suggest that head-dipping and rearing reflect novelty seeking and exploration (Fernández-Teruel et al., 2002; Lever et al., 2006), whereas stretching involves risk assessment (Bailey and Crawley, 2009). Enriched animals, although only those also exposed to MS stress, also exhibit significantly greater number of transfers and time spent in the bright compartment of the LDB, when compared to the rest of the groups. This finding represents a priming effect of MS on subsequent EE exposure, indicating that these two environmental treatments can sometimes act in an additive fashion. Perhaps more important, EE significantly increased frequency of entries and time spent in the challenge area, a risk-taking area of the CSF. The access to this area required jumping through an elevated hole. The brightly lit, open and elevated bridge was also more visited by enriched than by non-enriched rats, irrespective of the rearing conditions experimented during infancy, although this was a trend that did not achieve statistical significance. The overall number of entries in the different sections of the CSF was unaffected by EE, indicating that EE effects upon risk-taking behavior were not a by-product of unspecific changes in motor activity.

Previous studies indicate that the changes that define an enriched homecage, relative to the conditions of the standard housing, do not have to be dramatic to alter ethanol's effects or intake. Lopez and Laber (2015) found increased ethanol consumption at adulthood after chronic single housing during adolescence. This effect was inhibited by simply adding cotton nestlets to the homecage during the isolation period. The EE in the present study, on the other hand, involved a significantly larger homecage featuring new configurations of objects that kept rotating and the possibility to perform physical activity. This raises the possibility that EE effects upon ethanol intake depend on the magnitude of the stimulation provided by the environment: relatively low magnitudes of EE may inhibit ethanol intake, yet exposure to a relatively high-magnitude EE treatment may result in greater ethanol intake. Similar complex relationships have been claimed to explain the controversial effects of stress upon ethanol intake. Studies have found greater (Caplan and Puglisi, 1986), diminished (Boyce-Rustay et al., 2008), or unaltered (Ponce et al., 2004) ethanol intake after stress exposure, and some claim that these apparent disparate results could be explained by the Yerkes–Dobson law (reviewed in Miczek et al., 2008), with low stress inducing behavioral activation and promoting ethanol intake whereas high stress inducing behavioral depression and a reduced ethanol intake. Under this framework, the EE rats in this study may have perceived the EE treatment as a mild stressor. Consistent with this, it has been suggested that rats exposed to EE exhibit a mild stress-like phenotype, which may inoculate from subsequent response to more intense stressors (Crofton et al., 2015). These are, of course, just hypotheses and more work should be done to describe the effects of EE and its underlying mechanisms.

It is noteworthy, however, that compared to adults, adolescents have been described to be more reactive to stress and to stress-ethanol interactions. In a recent study we found significantly greater ethanol intake time in adolescent, but not in adult, rats given chronic restraint stress (Fernandez et al., 2016).

In summary, the present study confirmed that the effects of MS stress upon ethanol intake are not expressed during late adolescence, in spite of MS inducing other behavioral changes indicative of heightened anxiety response. Perhaps more important, animals that had been exposed to EE throughout adolescence subsequently exhibited significantly greater ethanol intake, an effect found in males only and not affected by MS. The promoting effect of EE upon ethanol intake was not related to changes in the aversive or sedative effects of ethanol, nor in ethanol's metabolism. Instead, it seems that EE heightened exploration of novel stimuli and risk-taking behaviors in the CSF test. Further studies should assess if EE may affect ethanol intake and preference via alterations in novelty-seeking and risk-taking behaviors.

AUTHOR CONTRIBUTIONS

LB run all the experiments, supervised the construction of the mazes, processed the data and wrote an initial draft of the MS. RP had the original research idea, designed the studies, supervised the running of the Experiments, conducted the statistical analyses, graphed the data and wrote the final version of the paper. MF help design the experiments along with RMP, run all the experiments, supervised the construction of the mazes and help wrote the initial draft of the paper. All authors revised the final version of the paper.

FUNDING

Funding was provided by grants PICT 2012-0436 and PIP2013-2015 to RP and by doctoral and undergraduate fellowships awarded by CONICET and CIN to MF and LB, respectively.

REFERENCES

Acevedo, M. B., Molina, J. C., Nizhnikov, M. E., Spear, N. E., and Pautassi, R. M. (2010). High ethanol dose during early adolescence induces locomotor activation and increases subsequent ethanol intake during late adolescence. *Dev. Psychobiol.* 52, 424–440. doi: 10.1002/dev.20444

Arias, C., Pautassi, R. M., Molina, J. C., and Spear, N. E. (2010). A comparison between taste avoidance and conditioned disgust reactions induced by ethanol and lithium chloride in preweanling rats. *Dev. Psychobiol.* 52, 545–557. doi: 10.1002/dev.20460

Arnold, J. L., and Siviy, S. M. (2002). Effects of neonatal handling and maternal separation on rough-and-tumble play in the rat. *Dev. Psychobiol.* 41, 205–215. doi: 10.1002/dev.10069

Bailey, K. R., and Crawley, J. N. (2009). "Anxiety-related behaviors in mice," in *Methods of Behavior Analysis in Neuroscience*, 2nd Edn, ed. J. J. Buccafusco (Boca Raton, FL: CRC Press/Taylor & Francis).

Bowling, S. L., and Bardo, M. T. (1994). Locomotor and rewarding effects of amphetamine in enriched, social, and isolate reared rats. *Pharmacol. Biochem. Behav.* 48, 459–464. doi: 10.1016/0091-3057(94)90553-3

Boyce-Rustay, J. M., Janos, A. L., and Holmes, A. (2008). Effects of chronic swim stress on EtOH-related behaviors in C57BL/6J, DBA/2J and BALB/cByJ mice. *Behav. Brain Res.* 186, 133–137. doi: 10.1016/j.bbr.2007.07.031

Camarini, R., and Pautassi, R. M. (2016). Behavioral sensitization to ethanol: neural basis and factors that influence its acquisition and expression. *Brain Res. Bull.* 125, 53–78. doi: 10.1016/j.brainresbull.2016.04.006

Caplan, M. A., and Puglisi, K. (1986). Stress and conflict conditions leading to and maintaining voluntary alcohol consumption in rats. *Pharmacol. Biochem. Behav.* 24, 271–280. doi: 10.1016/0091-3057(86)90350-3

Crofton, E. J., Zhang, Y., and Green, T. A. (2015). Inoculation stress hypothesis of environmental enrichment. *Neurosci. Biobehav. Rev.* 49, 19–31. doi: 10.1016/j.neubiorev.2014.11.017

Cruz, F. C., Quadros, I. M., Planeta Cda, S., and Miczek, K. A. (2008). Maternal separation stress in male mice: long-term increases in alcohol intake. *Psychopharmacology (Berl.)* 201, 459–468. doi: 10.1007/s00213-008-1307-4

Daoura, L., Haaker, J., and Nylander, I. (2011). Early environmental factors differentially affect voluntary ethanol consumption in adolescent and adult male rats. *Alcohol. Clin. Exp. Res.* 35, 506–515. doi: 10.1111/j.1530-0277.2010.01367.x

Dawson, D. A., Grant, B. F., and Li, T. K. (2007). Impact of age at first drink on stress-reactive drinking. *Alcohol. Clin. Exp. Res.* 31, 69–77. doi: 10.1111/j.1530-0277.2006.00265.x

de Carvalho, C. R., Pandolfo, P., Pamplona, F. A., and Takahashi, R. N. (2010). Environmental enrichment reduces the impact of novelty and motivational properties of ethanol in spontaneously hypertensive rats. *Behav. Brain Res.* 208, 231–236. doi: 10.1016/j.bbr.2009.11.043

DeWit, D. J., Adlaf, E. M., Offord, D. R., and Ogborne, A. C. (2000). Age at first alcohol use: a risk factor for the development of alcohol disorders. *Am. J. Psychiatry* 157, 745–750. doi: 10.1176/appi.ajp.157.5.745

Doremus, T. L., Brunell, S. C., Rajendran, P., and Spear, L. P. (2005). Factors influencing elevated ethanol consumption in adolescent relative to adult rats. *Alcohol. Clin. Exp. Res.* 29, 1796–1808. doi: 10.1097/01.alc.0000183007.65998.aa

Dyr, W., Wyszogrodzka, E., Paterak, J., Siwinska-Ziolkowska, A., Malkowska, A., and Polak, P. (2016). Ethanol-induced conditioned taste aversion in Warsaw Alcohol High-Preferring (WHP) and Warsaw Alcohol Low-Preferring (WLP) rats. *Alcohol* 51, 63–69. doi: 10.1016/j.alcohol.2015.11.011

Enoch, M. A. (2012). The influence of gene-environment interactions on the development of alcoholism and drug dependence. *Curr. Psychiatry Rep.* 14, 150–158. doi: 10.1007/s11920-011-0252-9

Ewin, S. E., Kangiser, M. M., and Stairs, D. J. (2015). The effects of environmental enrichment on nicotine condition place preference in male rats. *Exp. Clin. Psychopharmacol.* 23, 387–394. doi: 10.1037/pha0000024

Fabio, M. C., Macchione, A. F., Nizhnikov, M. E., and Pautassi, R. M. (2015). Prenatal ethanol increases ethanol intake throughout adolescence, alters ethanol-mediated aversive learning, and affects mu but not delta or kappa opioid receptor mRNA expression. *Eur. J. Neurosci.* 41, 1569–1579. doi: 10.1111/ejn.12913

Fernandez, M., Fabio, M. C., Nizhnikov, M. E., Spear, N. E., Abate, P., and Pautassi, R. M. (2014). Maternal isolation during the first two postnatal weeks affects novelty-induced responses and sensitivity to ethanol-induced locomotor activity during infancy. *Dev. Psychobiol.* 56, 1070–1082. doi: 10.1002/dev.21192

Fernandez, M. S., Fabio, M. C., Miranda-Morales, R. S., Virgolini, M. B., De Giovanni, L. N., Hansen, C., et al. (2016). Age-related effects of chronic restraint stress on ethanol drinking, ethanol-induced sedation, and on basal and stress-induced anxiety response. *Alcohol* 51, 89–100. doi: 10.1016/j.alcohol.2015.11.009

Fernández-Teruel, A., Driscoll, P., Gil, L., Aguilar, R., Tobena, A., and Escorihuela, R. M. (2002). Enduring effects of environmental enrichment on novelty seeking, saccharin and ethanol intake in two rat lines (RHA/Verh and RLA/Verh) differing in incentive-seeking behavior. *Pharmacol. Biochem. Behav.* 73, 225–231. doi: 10.1016/S0091-3057(02)00784-0

Francis, D. D., and Kuhar, M. J. (2008). Frequency of maternal licking and grooming correlates negatively with vulnerability to cocaine and alcohol use in rats. *Pharmacol. Biochem. Behav.* 90, 497–500. doi: 10.1016/j.pbb.2008.04.012

Gruber, E., DiClemente, R. J., Anderson, M. M., and Lodico, M. (1996). Early drinking onset and its association with alcohol use and problem behavior in late adolescence. *Prev. Med.* 25, 293–300. doi: 10.1006/pmed.1996.0059

Huot, R. L., Thrivikraman, K. V., Meaney, M. J., and Plotsky, P. M. (2001). Development of adult ethanol preference and anxiety as a consequence of neonatal maternal separation in Long Evans rats and reversal with antidepressant treatment. *Psychopharmacology (Berl.)* 158, 366–373. doi: 10.1007/s002130100701

Karlsson, O., and Roman, E. (2016). Dose-dependent effects of alcohol administration on behavioral profiles in the MCSF test. *Alcohol* 50, 51–56. doi: 10.1016/j.alcohol.2015.10.003

Kawakami, S. E., Quadros, I. M., Takahashi, S., and Suchecki, D. (2007). Long maternal separation accelerates behavioural sensitization to ethanol in female, but not in male mice. *Behav. Brain Res.* 184, 109–116. doi: 10.1016/j.bbr.2007.06.023

Lancaster, F. E., Brown, T. D., Coker, K. L., Elliott, J. A., and Wren, S. B. (1996). Sex differences in alcohol preference and drinking patterns emerge during the early postpubertal period. *Alcohol. Clin. Exp. Res.* 20, 1043–1049. doi: 10.1111/j.1530-0277.1996.tb01945.x

Lever, C., Burton, S., and O'Keefe, J. (2006). Rearing on hind legs, environmental novelty, and the hippocampal formation. *Rev. Neurosci.* 17, 111–133. doi: 10.1515/REVNEURO.2006.17.1-2.111

Lopez, M. F., and Laber, K. (2015). Impact of social isolation and enriched environment during adolescence on voluntary ethanol intake and anxiety in C57BL/6J mice. *Physiol. Behav.* 148, 151–156. doi: 10.1016/j.physbeh.2014.11.012

Meyerson, B. J., Augustsson, H., Berg, M., and Roman, E. (2006). The Concentric Square Field: a multivariate test arena for analysis of explorative strategies. *Behav. Brain Res.* 168, 100–113. doi: 10.1016/j.bbr.2005.10.020

Miczek, K. A., Yap, J. J., and Covington, H. E. (2008). Social stress, therapeutics and drug abuse: preclinical models of escalated and depressed intake. *Pharmacol. Ther.* 120, 102–128. doi: 10.1016/j.pharmthera.2008.07.006

Middleton, F. A., Varlinskaya, E. I., and Mooney, S. M. (2012). Molecular substrates of social avoidance seen following prenatal ethanol exposure and its reversal by social enrichment. *Dev. Neurosci.* 34, 115–128. doi: 10.1159/000337858

National-Research-Council (1996). *Guide for the Care and Use of Laboratory Animals*. Washington, DC: National Academy Press.

Ottoni, E. B. (2000). EthoLog 2.2: a tool for the transcription and timing of behavior observation sessions. *Behav. Res. Methods Instrum. Comput.* 32, 446–449. doi: 10.3758/BF03200814

Palm, S., Daoura, L., Roman, E., and Nylander, I. (2013). Effects of rearing conditions on behaviour and endogenous opioids in rats with alcohol access during adolescence. *PLoS ONE* 8:e76591. doi: 10.1371/journal.pone.0076591

Pautassi, R. M., Myers, M., Spear, L. P., Molina, J. C., and Spear, N. E. (2008). Adolescent but not adult rats exhibit ethanol-mediated appetitive second-order conditioning. *Alcohol. Clin. Exp. Res.* 32, 2016–2027. doi: 10.1111/j.1530-0277.2008.00789.x

Pautassi, R. M., Nizhnikov, M. E., Fabio, M. C., and Spear, N. E. (2012). Early maternal separation affects ethanol-induced conditioning in a nor-BNI insensitive manner, but does not alter ethanol-induced locomotor activity. *Pharmacol. Biochem. Behav.* 100, 630–638. doi: 10.1016/j.pbb.2011.11.005

Philpot, R. M., Badanich, K. A., and Kirstein, C. L. (2003). Place conditioning: age-related changes in the rewarding and aversive effects of alcohol. *Alcohol. Clin. Exp. Res.* 27, 593–599. doi: 10.1111/j.1530-0277.2003.tb04395.x

Pilatti, A., Godoy, J. C., Brussino, S., and Pautassi, R. M. (2013a). Underage drinking: prevalence and risk factors associated with drinking experiences among Argentinean children. *Alcohol* 47, 323–331. doi: 10.1016/j.alcohol.2013.02.001

Pilatti, A., Godoy, J. C., Brussino, S. A., and Pautassi, R. M. (2013b). Patterns of substance use among Argentinean adolescents and analysis of the effect of age at first alcohol use on substance use behaviors. *Addict. Behav.* 38, 2847–2850. doi: 10.1016/j.addbeh.2013.08.007

Ponce, L. F., Pautassi, R. M., Spear, N. E., and Molina, J. C. (2004). Nursing from an ethanol-intoxicated dam induces short- and long-term disruptions in motor performance and enhances later self-administration of the drug. *Alcohol. Clin. Exp. Res.* 28, 1039–1050. doi: 10.1097/01.alc.0000131298.32045.96

Pryce, C. R., Ruedi-Bettschen, D., Dettling, A. C., and Feldon, J. (2002). Early life stress: long-term physiological impact in rodents and primates. *News Physiol. Sci.* 17, 150–155.

Rockman, G. E., Borowski, T. B., and Glavin, G. B. (1986). The effects of environmental enrichment on voluntary ethanol consumption and stress ulcer formation in rats. *Alcohol* 3, 299–302. doi: 10.1016/0741-8329(86)90005-4

Rockman, G. E., Gibson, J. E., and Benarroch, A. (1989). Effects of environmental enrichment on voluntary ethanol intake in rats. *Pharmacol. Biochem. Behav.* 34, 487–490. doi: 10.1016/0091-3057(89)90545-5

Roman, E., Hyytia, P., and Nylander, I. (2003). Maternal separation alters acquisition of ethanol intake in male ethanol-preferring AA rats. *Alcohol. Clin. Exp. Res.* 27, 31–37. doi: 10.1097/01.ALC.0000047352.88145.80

Roman, E., Stewart, R. B., Bertholomey, M. L., Jensen, M. L., Colombo, G., Hyytia, P., et al. (2012). Behavioral profiling of multiple pairs of rats selectively bred for high and low alcohol intake using the MCSF test. *Addict. Biol.* 17, 33–46. doi: 10.1111/j.1369-1600.2011.00327.x

Rothman, E. F., Edwards, E. M., Heeren, T., and Hingson, R. W. (2008). Adverse childhood experiences predict earlier age of drinking onset: results from a representative US sample of current or former drinkers. *Pediatrics* 122, e298–e304. doi: 10.1542/peds.2007-3412

Rueda, A. V., Teixeira, A. M., Yonamine, M., and Camarini, R. (2012). Environmental enrichment blocks ethanol-induced locomotor sensitization and decreases BDNF levels in the prefrontal cortex in mice. *Addict. Biol.* 17, 736–745. doi: 10.1111/j.1369-1600.2011.00408.x

Samson, H. H., Slawecki, C. J., Sharpe, A. L., and Chappell, A. (1998). Appetitive and consummatory behaviors in the control of ethanol consumption: a measure of ethanol seeking behavior. *Alcohol. Clin. Exp. Res.* 22, 1783–1787. doi: 10.1111/j.1530-0277.1998.tb03980.x

Schneider, T., Turczak, J., and Przewlocki, R. (2006). Environmental enrichment reverses behavioral alterations in rats prenatally exposed to valproic acid: issues for a therapeutic approach in autism. *Neuropsychopharmacology* 31, 36–46. doi: 10.1038/sj.npp.1300767

Solinas, M., Thiriet, N., El Rawas, R., Lardeux, V., and Jaber, M. (2009). Environmental enrichment during early stages of life reduces the behavioral, neurochemical, and molecular effects of cocaine. *Neuropsychopharmacology* 34, 1102–1111. doi: 10.1038/npp.2008.51

Spanagel, R., Montkowski, A., Allingham, K., Stohr, T., Shoaib, M., Holsboer, F., et al. (1995). Anxiety: a potential predictor of vulnerability to the initiation of ethanol self-administration in rats. *Psychopharmacology (Berl)* 122, 369–373. doi: 10.1007/BF02246268

Spear, L. P. (2000). The adolescent brain and age-related behavioral manifestations. *Neurosci. Biobehav. Rev.* 24, 417–463. doi: 10.1016/S0149-7634(00)00014-2

Spear, L. P., and Swartzwelder, H. S. (2014). Adolescent alcohol exposure and persistence of adolescent-typical phenotypes into adulthood: a mini-review. *Neurosci. Biobehav. Rev.* 45C, 1–8. doi: 10.1016/j.neubiorev.2014.04.012

Spear, L. P., and Varlinskaya, E. I. (2010). Sensitivity to ethanol and other hedonic stimuli in an animal model of adolescence: implications for prevention science? *Dev. Psychobiol.* 52, 236–243. doi: 10.1002/dev.20457

Winer, B., Brown, D. R., and Michels, K. M. (1991). *Statistical Principles in Experimental Design*. New York, NY: McGraw Hill.

Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

frontiers in Behavioral Neuroscience

MINI REVIEW
published: 23 May 2017
doi: 10.3389/fnbeh.2017.00095

Modulation of Ethanol-Metabolizing Enzymes by Developmental Lead Exposure: Effects in Voluntary Ethanol Consumption

Miriam B. Virgolini, Mara S. Mattalloni, Paula A. Albrecht, Romina Deza-Ponzio and Liliana M. Cancela*

IFEC-CONICET, Departamento de Farmacología, Facultad de Ciencias Químicas, Universidad Nacional de Córdoba, Córdoba, Argentina

This review article provides evidence of the impact of the environmental contaminant lead (Pb) on the pattern of the motivational effects of ethanol (EtOH). To find a mechanism that explains this interaction, the focus of this review article is on central EtOH metabolism and the participating enzymes, as key factors in the modulation of brain acetaldehyde (ACD) accumulation and resulting effect on EtOH intake. Catalase (CAT) seems a good candidate for the shared mechanism between Pb and EtOH due to both its antioxidant and its brain EtOH-metabolizing properties. CAT overactivation was reported to increase EtOH consumption, while CAT blockade reduced it, and both scenarios were modified by Pb exposure, probably as the result of elevated brain and blood CAT activity. Likewise, the motivational effects of EtOH were enhanced when brain ACD metabolism was prevented by ALDH2 inhibition, even in the Pb animals that evidenced reduced brain ALDH2 activity after chronic EtOH intake. Overall, these results suggest that brain EtOH metabolizing enzymes are modulated by Pb exposure with resultant central ACD accumulation and a prevalence of the reinforcing effects of the metabolite in brain against the aversive peripheral ACD accumulation. They also support the idea that early exposure to an environmental contaminant, even at low doses, predisposes at a later age to differential reactivity to challenging events, increasing, in this case, vulnerability to acquiring addictive behaviors, including excessive EtOH intake.

Keywords: ethanol, acetaldehyde, lead-exposure, catalase, ALDH2

OPEN ACCESS

Edited by:
Merce Correa, Jaume I University, Spain

Reviewed by:
Marcelo Tadeu Marin, Universidade Estadual Paulista Júlio Mesquita Filho, Brazil
Eduardo Karahanian, Universidad Autonoma de Chile, Chile

****Correspondence:***
Miriam B. Virgolini
mvirgoli@fcq.unc.edu.ar

Received: *25 January 2017*
Accepted: *04 May 2017*
Published: *23 May 2017*

Citation:
Virgolini MB, Mattalloni MS, Albrecht PA, Deza-Ponzio R and Cancela LM (2017) Modulation of Ethanol-Metabolizing Enzymes by Developmental Lead Exposure: Effects in Voluntary Ethanol Consumption. Front. Behav. Neurosci. 11:95. doi: 10.3389/fnbeh.2017.00095

INTRODUCTION

"The Barker hypothesis" (Osmond and Barker, 2000) first popularized the concept that parameters related to fetal, infant and childhood growth may be predictors of disease in later life. The original hypothesis has been extended to a range of components of the developmental environment such as the mother's nutrition, stress levels, lifestyle and exposure to chemicals, all factors that may play a powerful role in influencing later susceptibility to challenging events. Based on these considerations, this review article provides behavioral and biochemical evidence that aims to support the idea that early-life exposure to lead (Pb), an environmental neurotoxicant, produces an "imprint" in CNS functionality. We propose that this experience has health consequences over the life span, increasing vulnerability to addictive behaviors, in this case to the motivational responses to ethanol (EtOH), with brain acetaldehyde (ACD) and EtOH metabolizing enzymes playing a crucial role.

LEAD, ETHANOL AND THE TWO FACES OF REINFORCEMENT

Although a non-essential metal and with widely restricted industrial uses, Pb is present in the environment and in living organisms. Alarmingly, early-life Pb exposure even in trace amounts induces neurobehavioral manifestations that may not be evident until later in life (Vorvolakos et al., 2016). They include hyperactivity, cognitive deficits and altered responses to drugs of abuse including EtOH.

From the clinical perspective, a relationship between Pb and EtOH has been described (Cezard et al., 1992). Animals chronically exposed to high Pb levels during adulthood, showed higher, although less efficient, lever pressing for EtOH in a self-administration test, associated with increased EtOH intake (Nation et al., 1987). More recently, it was shown EtOH-induced hyperlocomotion after acute Pb administration (Correa et al., 2001). Similarly, perinatal low-level Pb exposure enhanced EtOH intake in a daily 2-h EtOH/water free-choice sessions. Moreover, Pb-exposed animals also showed elevated response rates in a FR-2 schedule of behavior associated with a higher breaking point compared to controls, evidencing their motivation to self-administer EtOH (Mattalloni et al., 2013).

Thus, to find a mechanism that explains these differential effects, the concepts of positive and negative reinforcing must be introduced. It is known that drug addiction is a process that progresses from an early condition of positive reinforcement, evidenced by the euphorizing and stimulant effects of the drug (compulsive desire for pleasure), to a later state of negative reinforcement, evidenced as dysphoria and anxiety as a result of drug removal (compulsive desire for relief). Thus, the two main sources of reinforcement play key roles in the allostatic processes that lead to drug abuse (Wise and Koob, 2014). Both aspects will be mentioned in this review article, with a focus on the positive reinforcement perspective, particularly related to EtOH and its bioproducts, with developmental Pb exposure as a determinant factor in the vulnerability of these animals to the motivational effects of EtOH.

A putative explanation for the Pb/EtOH interaction supported by the *negative reinforcement perspective* is based on the tension-reduction hypothesis (Pohorecky, 1990). This proposes that the anxiolytic properties of EtOH are the main factors that lead some individuals to consume excessive amounts of the drug to relieve negative emotionality. This mechanism involves both the hypothalamic-pituitary adrenal axis with corticosterone secretion as the final output (Fahlke et al., 1994), as well as the extrahypothalamic systems including the extended amygdala (Koob, 2008). The increased susceptibility to EtOH-anxiolytic effects and the enhanced EtOH intake reported in perinatally Pb-exposed animals was associated with elevated basal corticosterone levels (Virgolini et al., 1999). Thus, it is proposed that Pb-treated animals would ingest EtOH to diminish their basal anxiety in an attempt to cope with stressful situations. This line of research was not further investigated and deserves future endeavors.

The *positive reinforcement view*, on the other hand, is related to the motivational and stimulant effects of EtOH, mediated through the reported ability of EtOH, ACD and salsolinol (a tetrahydroisoquinoline product of dopamine (DA) and ACD condensation) to facilitate DA neurotransmission in the mesolimbic circuit. Moreover, central administration of EtOH (or its bioproducts) induces hyperlocomotion, conditioned place preference and promotes EtOH intake (reviewed in: Quertemont et al., 2005; Correa et al., 2012; Hipólito et al., 2012; Deehan et al., 2013; Israel et al., 2015; Peana et al., 2016). Therefore, the present review will particularly emphasize the modulation that the environmental neurotoxicant Pb exerts on the enzymes involved in central EtOH metabolism, given the positive reinforcing properties of EtOH, ACD and salsolinol.

With the reported low ADH activity in the brain, the CAT-H_2O_2 system in addition to being a peroxisomal redox regulator is the key enzyme involved in H_2O_2-dependent brain EtOH oxidation to ACD (Vetrano et al., 2005). It should be mentioned that blood catalase (CAT) activity is positively correlated with EtOH consumption in both rats (Amit and Aragon, 1988) and humans (Koechling and Amit, 1992). Several reports indicate that CAT activity is decreased after chronic adult Pb exposure in the brain (Jindal and Gill, 1999), liver (Flora et al., 2012a) and blood (Sajitha et al., 2010). Interestingly, developmental exposure to high Pb doses is able to increase CAT activity (brain: Valenzuela et al., 1989; brain, liver and heart: Somashekaraiah et al., 1992). Moreover, cumulative evidence demonstrated that acute (but not chronic) Pb administration raises brain CAT levels and increases the locomotor response to EtOH in mice (Correa et al., 1999, 2001). Similarly, we have reported that developmental Pb exposure increased basal blood CAT activity in periadolescent rats, an effect that persisted throughout their lifetime and was potentiated by EtOH intake. Although no differences between groups were observed in whole brain CAT activity, there was a region-specific increase in the Pb-exposed hippocampus and cerebellum, indicating that CAT-mediated EtOH oxidation is not homogeneous throughout the brain (Mattalloni et al., 2013, 2017).

On the other hand, ACD removal is mediated by ALDH2, a mitochondrial enzyme that belongs to the ALDH superfamily and catalyzes both brain and liver ACD oxidation to acetic acid (Crabb et al., 2004). The two evidences of an interaction between Pb and ALDH2 have shown that adult Pb exposure reduced liver ALDH2 (Flora and Tandon, 1987) whereas developmental Pb-exposure reduced brain ALDH2 activity (Mattalloni et al., 2017) after chronic EtOH consumption.

Thus, based on the premise that early-life Pb exposure will interfere with EtOH metabolism, brain ACD may be noted as the common site of action of the two neurotoxicants. Pharmacological manipulations of EtOH-metabolizing enzymes attempting to modulate brain ACD accumulation will therefore be described below, with the resultant changes evidenced at both behavioral and biochemical levels.

PHARMACOLOGICAL INTERFERENCE OF ETHANOL METABOLISM

The next section follows the two-dimensional model of alcohol consumption hypothesized over 30 years ago, supporting the idea

that "both brain CAT and ALDH may represent a biological marker system underlying the affinity of the animals to consume ethanol" (Aragon and Amit, 1985). Evidence is provided that Pb induces dynamic changes in the two main enzymes involved in brain EtOH metabolism, which may account for differential EtOH intake in response to pharmacological manipulations in these animals (**Figure 1**).

Brain Acetaldehyde Formation

One of the most commonly used CAT blockers employed to modulate stimulant responses to EtOH is 3-amino 1,2,4-triazole (AT), a fungicide that produces irreversible inhibition of the CAT-H_2O_2 site, thereby preventing *in vivo* brain EtOH oxidation to ACD (Aragon et al., 1989). Interestingly, the only report of an interaction among Pb, CAT, AT and EtOH showed that AT was able to reverse the increase in EtOH-induced hyperlocomotion and brain CAT activity observed after acute Pb administration (Correa et al., 2001). Similarly, we have reported that AT pretreatment prevented both elevated EtOH intake and blood and brain (hippocampus and cerebellum) CAT activity in developmentally Pb-exposed animals (Mattalloni et al., 2013). The absence of these effects in the control group suggests that the enzyme inhibition requires either high H_2O_2 (and ROS) levels that are increased as a result of Pb exposure (Flora et al., 2012b) or the excessive EtOH intake evidenced in Pb-exposed animals.

On the other hand, CAT overactivation can be achieved by the administration of 3-nitropropionic acid (3NPA), a mycotoxin that produces an irreversible inhibition of the succinate dehydrogenase (SDH) enzyme, along with ROS elevation and increased CAT activity, with resultant EtOH-induced hyperlocomotion (Manrique et al., 2006). Thus, 3NPA induced-CAT elevation was able to increase EtOH consumption in both, the Pb-exposed and the control animals, accompanied by higher blood and brain (striatal) CAT activity in the Pb group (Mattalloni et al., 2013).

Brain Acetaldehyde Removal

Cyanamide (CY) is a drug prescribed in some countries as a deterrent for alcoholics due to its ability to increase peripheral (aversive) ACD as a result of ALDH inhibition

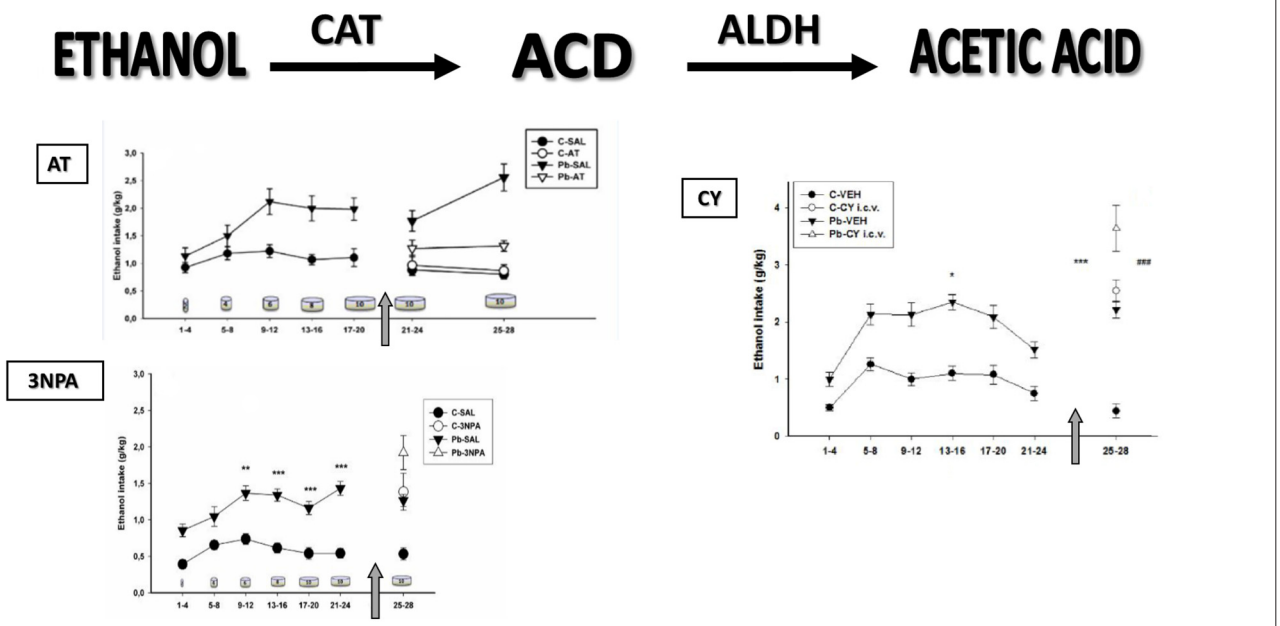

FIGURE 1 | Voluntary ethanol (EtOH) consumption measured in Wistar rats. Data (mean expressed as grams of EtOH per kilogram of body weight ± SE) grouped in 4-day blocks along the horizontal axis (in days) that correspond to EtOH intake in response to increasing EtOH concentrations symbolized as cylinders (days 1–4: 2%; days 5–8: 4%; days 9–12: 6%; days 13–16: 8%; and days 17–28: 10%). C, control; Pb, lead; CAT, catalase; SAL, saline; VEH, vehicle. Top, left: EtOH intake in response to 3-amino 1,2,4-triazole (AT) administration. The arrow signifies the start of SAL or AT administration (days 21–24 and 25–28; 250 mg/kg i.p.). C-SAL = 10; C-AT = 11; Pb-SAL = 11; Pb-AT = 9 animals per group (Mattalloni et al., 2013). Bottom, left: EtOH intake in response to 3-nitropropionic acid (3NPA) administration. The arrow signifies the start of SAL or 3NPA administration (days 25–28; 20 mg/kg s.c.). Baseline: *denotes statistical difference compared to controls at $^{**}p < 0.01$ and $^{***}p < 0.001$. C-SAL = 8; C-3NPA = 11; Pb-SAL = 9; Pb-3NPA = 9 animals per group (Mattalloni et al., 2013). Right: EtOH intake in response to intracerebroventricular cyanamide (CY) administration. The arrow signifies the start of VEH or CY administration (days 25–28; 0.3 mg i.c.v.). Baseline: *denotes differences compared to controls at $^{*}p < 0.05$. CY administration: *denotes differences between the C and Pb-exposed animals injected with VEH at $^{***}p < 0.001$; #denotes differences between the VEH and corresponding CY groups for both C and Pb-exposed animals at $^{\#\#\#}p < 0.001$. C-VEH = 11; C-CY i.c.v.= 14; Pb-VEH = 8; Pb-CY i.c.v.= 8 animals per group (Mattalloni et al., 2017).

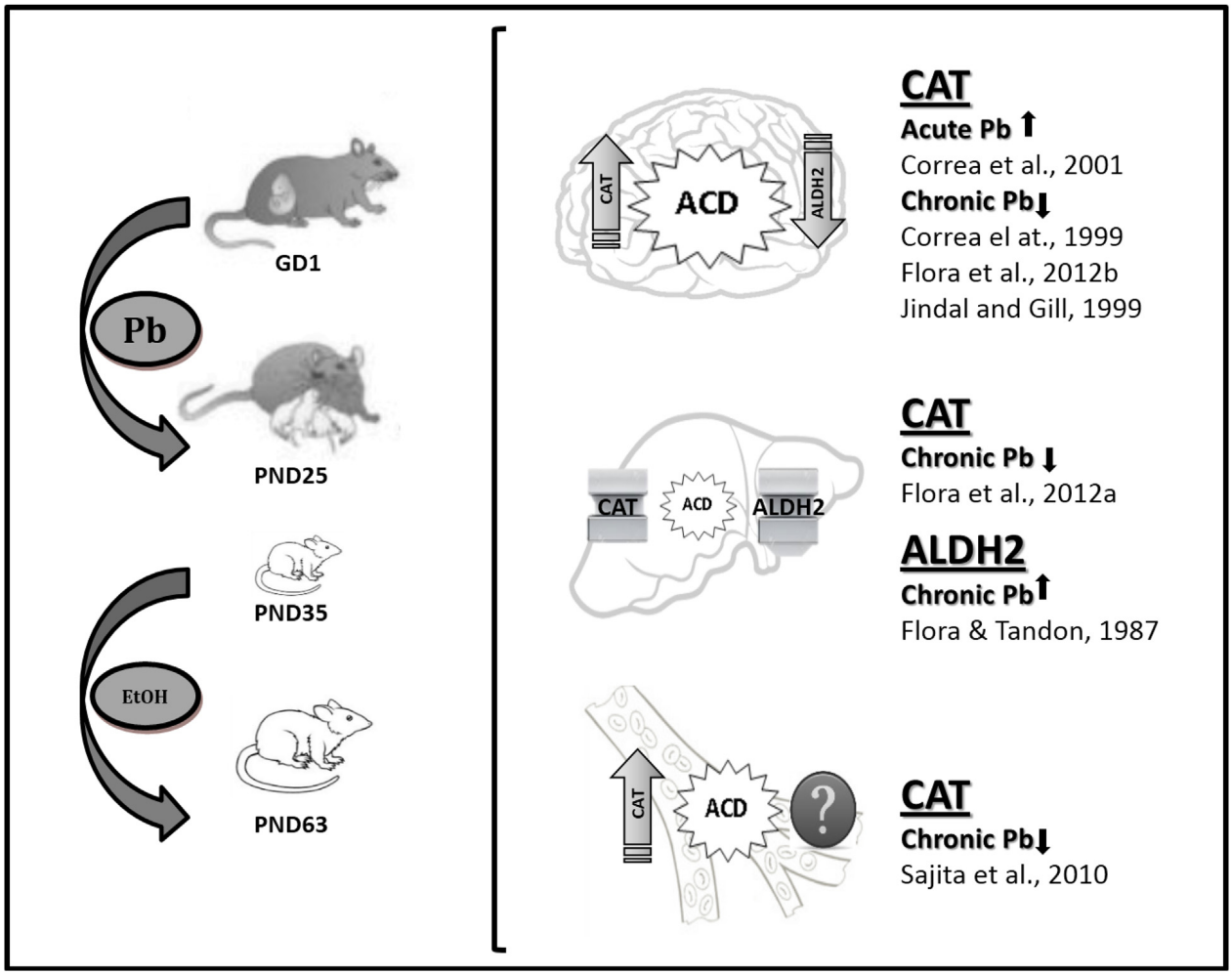

FIGURE 2 | Lead (Pb) exposure and EtOH intake with emphasis in EtOH metabolizing enzymes status. The square bracket comprises pictures for brain, liver and blood CAT and ALDH2 status and putative acetaldehyde (ACD) accumulation in the experimental model described in Mattalloni et al. (2013, 2017; as shown on the left). The references point-out CAT and ALDH2 data reported elsewhere as result of adult acute or chronic Pb exposure in animals with chronic EtOH intake. GD, gestational day; PND, postnatal day.

(Koppaka et al., 2012). Central CY administration enhanced EtOH intake in rats that had never consumed EtOH, an effect highly dependent on the CY dose (Critcher and Myers, 1987). We have demonstrated that i.c.v. CY administration inhibited brain ALDH2 and increased EtOH intake in control animals, whereas the Pb-exposed group also showed elevated EtOH intake although in the absence of brain ALDH2 inhibition (Mattalloni et al., 2017). This finding may be related to the reduced basal brain ALDH2 activity present in the Pb-exposed group, or to the fact that CY is a prodrug that, to convert itself to the active metabolite requires CAT and H_2O_2, a system that is modified by Pb-exposure.

CONCLUSION

This review article provides evidence of Pb modulation on the enzymes involved in either the production or the removal of brain ACD, i.e., CAT and ALDH2, the activities of which have been proposed as trait biomarkers of excessive EtOH intake (Aragon and Amit, 1985). The data demonstrate differential CAT and ALDH2 functionality in the developmentally Pb-exposed animals, with high blood and brain CAT activity and low brain ALDH2 activity, thereby promoting central ACD accumulation (**Figure 2**). This effect would directly influence EtOH self-administration, with Pb exposure representing a crucial variable in the behavioral and biochemical outputs described here. It can thus be postulated that one of the shared mechanisms between Pb and EtOH could be the result of differential EtOH metabolism in brain areas related to reward. Possibly, an imbalance towards a prevalence of the reinforcing effects of brain ACD vs. aversive peripheral ACD accumulation may play a key role in the differential motivational response to EtOH evidenced in Pb-exposed animals. Moreover, immunohistochemical studies have demonstrated that ALDH2 is widely expressed in the brain,

with low activity in the aminergic neurons, which coincidentally are the richest in CAT expression (Zimatkin, 1991; Zimatkin and Lindros, 1996), a fact that promotes brain ACD accumulation in the mesolimbic circuit, site of the reinforcing properties of addictive drugs. Immunostaining experiments are desirable for brain CAT and ALDH2 expression in the Pb-exposed animals.

Interestingly, there are differences in EtOH metabolism over the lifetime. CAT-H_2O_2 system activity is higher in pups than in adults (Hamby-Mason et al., 1997), thus promoting central ACD accumulation. Brain ALDH2 activity also increases gradually, reaching the activity specific for mature animals by periadolescence (Zamatkin and Lis, 1990). Hence, Pb exposure during development may have affected the functionality of these enzymes at a time of high ACD accumulation. This assumption has important clinical implications provided that the neurobehavioral outcomes showed no evidence of a safe threshold for Pb exposure in immature organisms and the ubiquity of this environmental neurotoxicant. Thus, these results indicate the existence of prenatal programming as a consequence of early Pb exposure, an experience that would leave an imprint that later in life may be responsible for differential responsiveness to events that generate a conflict in the individual, such as the initiation in addictive behaviors.

AUTHOR CONTRIBUTIONS

LMC and MBV conceived and designed the experiments. MSM, RD-P and PAA performed the experiments and analyzed the data. MBV wrote and LMC contributed to the writing of the manuscript.

ACKNOWLEDGMENTS

This work was supported by grants to MBV by the Consejo Nacional de Investigaciones Científicas y Técnicas (CONICET); Secretaría de Ciencia y Tecnología (SeCyT-UNC); Ministerio de Ciencia y Tecnología de la Provincia de Córdoba (MinCyT), and Fondo para la Investigación Científica y Tecnológica (FONCyT) from Argentina.

REFERENCES

Amit, Z., and Aragon, C. M. G. (1988). Catalase activity measured in rats naive to ethanol correlates with later voluntary ethanol consumption: possible evidence for a biological marker system of ethanol intake. *Psychopharmacology* 95, 512–515. doi: 10.1007/bf00172965

Aragon, C. M. G., and Amit, Z. (1985). A two-dimensional model of alcohol consumption: possible interaction of brain catalase and aldehyde dehydrogenase. *Alcohol* 2, 357–360. doi: 10.1016/0741-8329(85)90075-8

Aragon, C. M. G., Spivak, K., and Amit, Z. (1989). Effects of 3-amino-1,2,4-triazole on ethanol-induced open field activity: evidence for brain catalase mediation of ethanol's effects. *Alcohol. Clin. Exp. Res.* 13, 104–108. doi: 10.1111/j.1530-0277.1989.tb00293.x

Cezard, C., Demarquilly, C., Boniface, M., and Haguenoer, J. M. (1992). Influence of the degree of exposure to lead on relations between alcohol consumption and the biological indices of lead exposure: epidemiological study in a lead acid battery factory. *Br. J. Ind. Med.* 49, 645–647. doi: 10.1136/oem.49.9.645

Correa, M., Miquel, M., Sanchis-Segura, C., and Aragon, C. M. G. (1999). Effects of chronic lead administration on ethanol-induced locomotor and brain catalase activity. *Alcohol* 19, 43–49. doi: 10.1016/s0741-8329(99)00023-3

Correa, M., Salamone, J. D., Segovia, K. N., Pardo, M., Longoni, R., Spina, L., et al. (2012). Piecing together the puzzle of acetaldehyde as a neuroactive agent. *Neurosci. Biobehav. Rev.* 36, 404–430. doi: 10.1016/j.neubiorev.2011.07.009

Correa, M., Sanchis-Segura, C., and Aragon, C. M. G. (2001). Brain catalase activity is highly correlated with ethanol-induced locomotor activity in mice. *Physiol. Behav.* 73, 641–647. doi: 10.1016/s0031-9384(01)00511-x

Crabb, D. W., Matsumoto, M., Chang, D., and You, M. (2004). Overview of the role of alcohol dehydrogenase and aldehyde dehydrogenase and their variants in the genesis of alcohol-related pathology. *Proc. Nutr. Soc.* 63, 49–63. doi: 10.1079/pns2003327

Critcher, E. C., and Myers, R. D. (1987). Cyanamide given ICV or systemically to the rat alters subsequent alcohol drinking. *Alcohol* 4, 347–353. doi: 10.1016/0741-8329(87)90066-8

Deehan, G. A., Brodie, M. S., and Rodd, Z. A. (2013). What is in that drink: the biological actions of ethanol, acetaldehyde, and salsolinol. *Curr. Top. Behav. Neurosci.* 13, 163–184. doi: 10.1007/7854_2011_198

Fahlke, C., Engel, J. A., Eriksson, C. J. P., Hrd, E., and Söderpalm, B. (1994). Involvement of corticosterone in the modulation of ethanol consumption in the rat. *Alcohol* 11, 195–202. doi: 10.1016/0741-8329(94)90031-0

Flora, S. J., Gautam, P., and Dwivedi, N. (2012a). Dose-dependent effects of ethanol on lead-induced oxidative stress in rats. *J. Environ. Pathol. Toxicol. Oncol.* 31, 61–73. doi: 10.1615/jenvironpatholtoxicoloncol.v31.i1.70

Flora, S. J., Gautam, P., and Kushwaha, P. (2012b). Lead and ethanol co-exposure lead to blood oxidative stress and subsequent neuronal apoptosis in rats. *Alcohol Alcohol.* 47, 92–101. doi: 10.1093/alcalc/agr152

Flora, S. J. S., and Tandon, S. K. (1987). Effect of combined exposure to lead and ethanol on some biochemical indices in the rat. *Biochem. Pharmacol.* 36, 537–541. doi: 10.1016/0006-2952(87)90363-7

Hamby-Mason, R., Chen, J. J., Schenker, S., Perez, A., and Henderson, G. I. (1997). Catalase mediates acetaldehyde formation from ethanol in fetal and neonatal rat brain. *Alcohol. Clin. Exp. Res.* 21, 1063–1072. doi: 10.1097/00000374-199709000-00020

Hipólito, L., Sánchez-Catalán, M. J., Martí-Prats, L., Granero, L., and Polache, A. (2012). Revisiting the controversial role of salsolinol in the neurobiological effects of ethanol: old and new vistas. *Neurosci. Biobehav. Rev.* 36, 362–378. doi: 10.1016/j.neubiorev.2011.07.007

Israel, Y., Quintanilla, M. E., Karahanian, E., Rivera-Meza, M., and Herrera-Marschitz, M. (2015). The "first hit" toward alcohol reinforcement: role of ethanol metabolites. *Alcohol. Clin. Exp. Res.* 39, 776–786. doi: 10.1111/acer.12709

Jindal, V., and Gill, K. D. (1999). Ethanol potentiates lead-induced inhibition of rat brain antioxidant defense systems. *Pharmacol. Toxicol.* 85, 16–21. doi: 10.1111/j.1600-0773.1999.tb01057.x

Koechling, U. M., and Amit, Z. (1992). Relationship between blood catalase activity and drinking history in a human population, a possible biological marker of the affinity to consume alcohol. *Alcohol Alcohol.* 27, 181–188. doi: 10.1093/oxfordjournals.alcalc.a045218

Koob, G. F. (2008). A role for brain stress systems in addiction. *Neuron* 59, 11–34. doi: 10.1016/j.neuron.2008.06.012

Koppaka, V., Thompson, D. C., Chen, Y., Ellermann, M., Nicolaou, K. C., Juvonen, R. O., et al. (2012). Aldehyde dehydrogenase inhibitors: a comprehensive review of the pharmacology, mechanism of action, substrate specificity, and clinical application. *Pharmacol. Rev.* 64, 520–539. doi: 10.1124/pr.111.005538

Manrique, H. M., Miquel, M., and Aragon, C. M. G. (2006). Acute administration of 3-nitropropionic acid, a reactive oxygen species generator, boosts ethanol-

induced locomotor stimulation. New support for the role of brain catalase in the behavioural effects of ethanol. *Neuropharmacology* 51, 1137–1145. doi: 10.1016/j.neuropharm.2006.07.022

Mattalloni, M. S., De Giovanni, L. N., Molina, J. C., Cancela, L. M., and Virgolini, M. B. (2013). Participation of catalase in voluntary ethanol consumption in perinatally low-level lead-exposed rats. *Alcohol. Clin. Exp. Res.* 37, 1632–1642. doi: 10.1111/acer.12150

Mattalloni, M. S., Deza-Ponzio, R., Albrecht, P. A., Cancela, L. M., and Virgolini, M. B. (2017). Developmental lead exposure induces opposite effects on ethanol intake and locomotion in response to central vs. systemic cyanamide administration. *Alcohol* 58, 1–11. doi: 10.1016/j.alcohol.2016.11.002

Nation, J. R., Baker, D. M., Fantasia, M. A., Ruscher, A. E., and Clark, D. E. (1987). Ethanol consumption and free operant avoidance performance following exposure to dietary lead. *Neurotoxicology* 8, 561–568.

Osmond, C., and Barker, D. J. P. (2000). Fetal, infant, and childhood growth are predictors of coronary heart disease, diabetes and hypertension in adult men and women. *Environ. Health Perspect.* 108, 545–553. doi: 10.2307/3454545

Peana, A. T., Rosas, M., Porru, S., and Acquas, E. (2016). From ethanol to salsolinol: role of ethanol metabolites in the effects of ethanol. *J. Exp. Neurosci.* 10, 137–146. doi: 10.4137/jen.s25099

Pohorecky, L. A. (1990). Interaction of ethanol and stress: research with experimental animals—an update. *Alcohol Alcohol.* 25, 263–276. doi: 10.1093/oxfordjournals.alcalc.a045000

Quertemont, E., Tambour, S., and Tirelli, E. (2005). The role of acetaldehyde in the neurobehavioral effects of ethanol: a comprehensive review of animal studies. *Prog. Neurobiol.* 75, 247–274. doi: 10.1016/j.pneurobio.2005.03.003

Sajitha, G. R., Jose, R., Andrews, A., Ajantha, K. G., Augustine, P., and Augusti, K. T. (2010). Garlic oil and vitamin E prevent the adverse effects of lead acetate and ethanol separately as well as in combination in the drinking water of rats. *Indian J. Clin. Biochem.* 25, 280–288. doi: 10.1007/s12291-010-0042-x

Somashekaraiah, B. V., Padmaja, K., and Prasad, A. R. (1992). Lead-induced lipid peroxidation and antioxidant defense components of developing chick embryos. *Free Radic. Biol. Med.* 13, 107–114. doi: 10.1016/0891-5849(92)90071-n

Valenzuela, A., Lefauconnier, J. M., Chaudiere, J., and Bourre, J. M. (1989). Effects of lead acetate on cerebral glutathione peroxidase and catalase in the suckling rat. *Neurotoxicology* 10, 63–69.

Vetrano, A. M., Heck, D. E., Mariano, T. M., Mishin, V., Laskin, D. L., and Laskin, J. D. (2005). Characterization of the oxidase activity in mammalian catalase. *J. Biol. Chem.* 280, 35372–35381. doi: 10.1074/jbc.m503991200

Virgolini, M. B., Cancela, L. M., and Fulginiti, S. (1999). Behavioral responses to ethanol in rats perinatally exposed to low lead levels. *Neurotoxicol. Teratol.* 21, 551–557. doi: 10.1016/s0892-0362(99)00020-3

Vorvolakos, T., Arseniou, S., and Samakouri, M. (2016). There is no safe threshold for lead exposure: a literature review. *Psychiatriki* 27, 204–214.

Wise, R. A., and Koob, G. F. (2014). The development and maintenance of drug addiction. *Neuropsychopharmacology* 39, 254–262. doi: 10.1038/npp.2013.261

Zamatkin, S. M., and Lis, R. E. (1990). Aldehyde dehydrogenase activity in the rat brain during ontogenesis. *Arkh. Anat. Gistol. Embriol.* 98, 27–33.

Zimatkin, S. M. (1991). Histochemical study of aldehyde dehydrogenase in the rat CNS. *J. Neurochem.* 56, 1–11. doi: 10.1111/j.1471-4159.1991.tb02555.x

Zimatkin, S. M., and Lindros, K. O. (1996). Distribution of catalase in rat brain: aminergic neurons as possible targets for ethanol effects. *Alcohol Alcohol.* 31, 167–174. doi: 10.1093/oxfordjournals.alcalc.a008128

Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

frontiers in Behavioral Neuroscience

MINI REVIEW
published: 07 March 2017
doi: 10.3389/fnbeh.2017.00037

Pre-Clinical Studies with D-Penicillamine as a Novel Pharmacological Strategy to Treat Alcoholism: Updated Evidences

Alejandro Orrico[1†], Lucía Martí-Prats[2†], María J. Cano-Cebrián[3], Luis Granero[3], Ana Polache[3] and Teodoro Zornoza[3*]*

[1]Área de Investigación en Vacunas, Fundación para el Fomento de la Investigación Sanitaria y Biomédica de la Comunidad Valenciana (FISABIO), Valencia, Spain, [2]Department of Psychology, University of Cambridge, Cambridge, UK, [3]Department of Pharmacy and Pharmacy Technology and Parasitology, University of Valencia, Valencia, Spain

Ethanol, as other drugs of abuse, is able to activate the ventral tegmental area dopamine (VTA-DA) neurons leading to positively motivational alcohol-seeking behavior and use, and, ultimately to ethanol addiction. In the last decades, the involvement of brain-derived acetaldehyde (ACD) in the ethanol actions in the mesolimbic pathway has been widely demonstrated. Consistent published results have provided a mechanistic support to the use of ACD inactivating agents to block the motivational and reinforcing properties of ethanol. Hence, in the last years, several pre-clinical studies have been performed in order to analyze the effects of the sequestering ACD agents in the prevention of ethanol relapse-like drinking behavior as well as in chronic alcohol consumption. In this sense, one of the most explored interventions has been the administration of D-Penicillamine (DP). These pre-clinical studies, that we critically summarize in this article, are considered a critical step for the potential development of a novel pharmacotherapeutic strategy for alcohol addiction treatment that could improve the outcomes of current ones. Thus, on one hand, several experimental findings provide the rationale for using DP as a novel therapeutic intervention alone and/or in combination to prevent relapse into alcohol seeking and consumption. On the other hand, its effectiveness in reducing voluntary ethanol consumption in long-term experienced animals still remains unclear. Finally, this drug offers the additional advantage that has already been approved for use in humans, hence it could be easily implemented as a new therapeutic intervention for relapse prevention in alcoholism.

Keywords: D-penicillamine, pre-clinical studies, acetaldehyde sequestering agent, ethanol relapse prevention, voluntary alcohol consumption

OPEN ACCESS

Edited by:
Merce Correa,
Jaume I University, Spain

Reviewed by:
Yedy Israel,
University of Chile, Chile
Laura Font,
Jaume I University, Spain

***Correspondence:**
Ana Polache
ana.polache@uv.es
Teodoro Zornoza
teodoro.zornoza@uv.es

[†]These authors have contributed equally to this work.

Received: *27 December 2016*
Accepted: *21 February 2017*
Published: *07 March 2017*

Citation:
Orrico A, Martí-Prats L, Cano-Cebrián MJ, Granero L, Polache A and Zornoza T (2017) Pre-Clinical Studies with D-Penicillamine as a Novel Pharmacological Strategy to Treat Alcoholism: Updated Evidences. Front. Behav. Neurosci. 11:37. doi: 10.3389/fnbeh.2017.00037

INTRODUCTION

In the last years, numerous studies have supported the idea that, at least in part, motivational and neuropharmacological effects of ethanol are mediated by its first brain-derived metabolite, acetaldehyde (ACD) and/or its bioderivates (for extensive review, see Deehan et al., 2013; Peana et al., 2016). The most widely employed strategy to demonstrate the

involvement of ACD in the motivational and reinforcing effects of ethanol has been, for years, the pharmacological manipulation of the enzyme system activity implicated in the brain metabolism of this drug. For instance, modulating catalase (Correa et al., 1999, 2004; Sanchis-Segura et al., 1999a,b; Arizzi-LaFrance et al., 2006; Font et al., 2008), cytocrome P-4502E1 (Hipolito et al., 2009; Ledesma et al., 2014) or alcohol dehydrogenase (Escarabajal and Aragon, 2002) brain activity.

Furthermore, another strategy has allowed disentangling the role of ACD in different behavioral effects induced by ethanol: the use of ACD sequestering agents. Fortunately, ACD is a highly reactive molecule and, therefore, capable of being "sequestered" by thiol amino acids such as L-cysteine (L-cys) and D-penicillamine (DP), which react non-enzymatically with ACD to form stable non-toxic adducts. This fact has been evidenced not only *in vitro* (Nagasawa et al., 1980; Kera et al., 1985) but also *in vivo* (Serrano et al., 2007) experimental conditions. Interestingly, these compounds, besides being used in the aforementioned research strategy, could have other advantages, particularly from a clinical point of view. In this sense, these ACD-scavenging compounds would not alter neurotransmitter systems, thus avoiding the manifestation of unexpected side effects displayed by the most promising candidates which have been evaluated in pre-clinical studies (Salaspuro et al., 2006; Leggio et al., 2010). They act removing/blocking both hepatic and brain-derived ACD, thus potentially preventing the reinforcing and motivational properties of ethanol-derived ACD on specific regions and pathways of the brain. Behavioral studies have demonstrated that DP is able to: (i) dose-dependently prevent the ethanol- and ACD-induced conditioned place preference (CPP) in rodents (Font et al., 2006a,b; Peana et al., 2008, 2009); (ii) attenuate either behavioral depression caused by ACD or behavioral locomotion induced by ethanol in mice (Font et al., 2005); and (iii) prevent, in a dose-dependent manner, the motor activation induced by intra-ventral tegmental area (VTA) ethanol administration (Martí-Prats et al., 2010, 2013). Additionally, neurochemical studies have evidenced that this drug suppresses both ethanol- and ACD-induced stimulation of dopamine (DA) release in the nucleus accumbens shell as well as the ethanol-evoked excitation of VTA-DA neuron activity (Enrico et al., 2009). Moreover, in most of these articles, the specificity of DP effects has been addressed using other drugs of abuse such as cocaine, caffeine or morphine.

Considering these promising results, several groups have explored the pre-clinical validity of ACD inactivation with DP as an alternative strategy for the development of new pharmacological approaches for treatment of alcoholism. Hence, the effect of DP in the prevention of ethanol relapse-like drinking behavior as well as in voluntary alcohol consumption have been repeatedly demonstrated (Font et al., 2006b; Orrico et al., 2013; Martí-Prats et al., 2015). In addition, the fact that DP is currently approved by the Food and Drug Administration (FDA) and European Medicines Agency (EMA) for other indications, offers the additional advantage to its immediate potential clinical utility.

In this review article, readers will find a compilation of the most remarkable publications in which the potential use of DP in the prevention of ethanol relapse-like drinking behavior as well as in voluntary alcohol consumption has been investigated in a pre-clinical environment.

Effect of DP in Relapse-Like Drinking Behavior

Among the few animal models of relapse presently available, the Alcohol Deprivation Effect (ADE—a marked increase in ethanol consumption that follows periods of deprivation) has become a widely used model to examine the efficacy of potential medication providing excellent face and predictive validity (Rodd et al., 2004; Spanagel and Kiefer, 2008; Spanagel, 2009; Bell et al., 2012). For instance, the three medications currently used in the clinical setting—Acamprosate, Naltrexone (NTX) and Nalmefene—, although they have different pharmacological mechanisms of action, have all been proven to effectively reduce the ADE in rodents (Spanagel and Zieglgänsberger, 1997; Orrico et al., 2014; Spanagel et al., 2014; Vengeliene et al., 2014). Nowadays, Acamprosate's primary mechanism of action still remains unclear, although it is believed to normalize the balance between excitatory and inhibitory pathways throughout the glutamatercic system (De Witte et al., 2005). On the other hand, NTX as well as Nalmefene work as opioid antagonists at μ and δ receptors and as agonists at κ receptors (Wackernah et al., 2014).

The ADE phenomenon can be triggered under both operant (Hölter et al., 1997; Echeverry-Alzate et al., 2012) and home-cage free choice drinking, non-operant, conditions (Spanagel and Hölter, 1999; Vengeliene et al., 2014). In this sense, along the last years, our group has extensively employed this latter model to explore, for the first time, the potential role of DP in ethanol relapse prevention.

Non-Operant Procedures

Our recent work has successfully shown that DP is able to prevent the ADE in Wistar rats using a home-cage four-bottle free choice (water, ethanol 5%, 10% and 20%) paradigm (Orrico et al., 2013, 2014). Concretely, at the end of the fifth deprivation period and 48 h before the reintroduction of ethanol bottles, rats were subcutaneously (SC) implanted with a mini-osmotic pump delivering at a constant rate either vehicle or DP (1 or 0.25 mg/h), during 1 week. The results obtained demonstrated that DP dose-dependently prevented the ADE in long-term ethanol-experienced rats. In fact, the constant-rate SC infusion of DP at a dose of 1 mg/h, but not 0.25 mg/h, completely prevented the ADE phenomenon (**Figure 1A**), while the vehicle-treated group increased the ethanol intake along the four post-abstinence days compared to baseline. Hence, our data clearly indicate that systemic administration of DP is able to prevent the expression of the ADE without affecting total fluid consumption and body weight (Orrico et al., 2013).

In spite of these results, one important concern should be considered. As can be seen in detail in the following

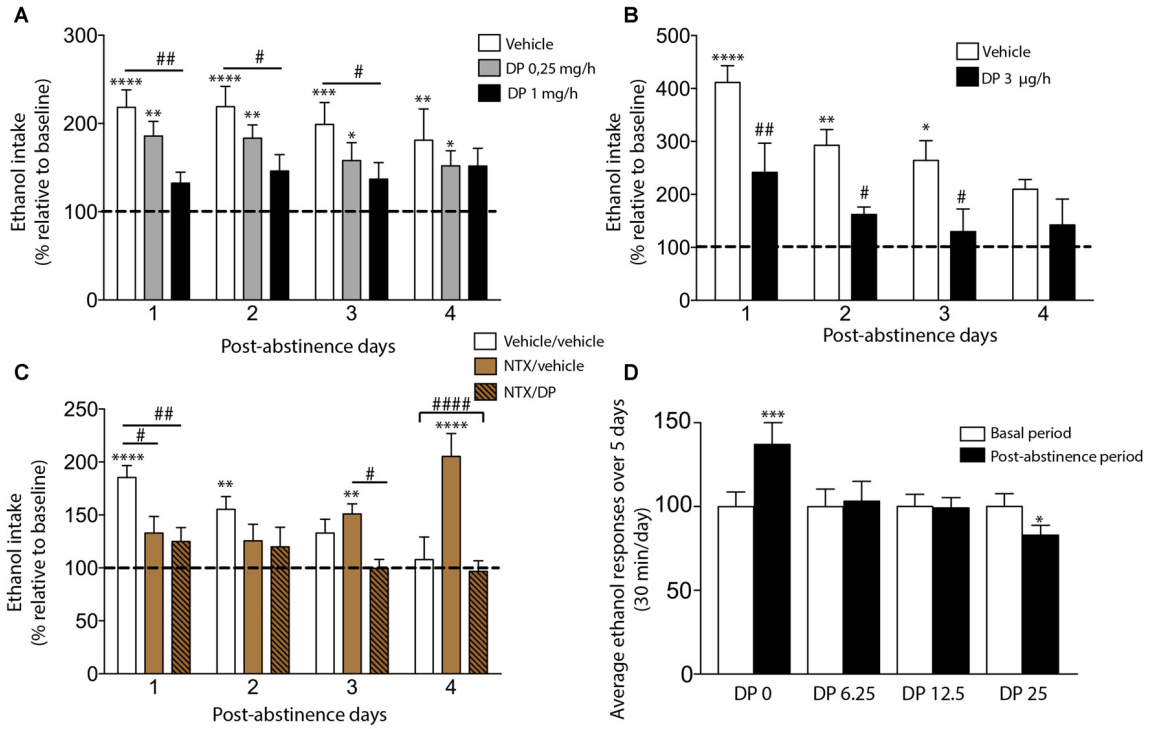

FIGURE 1 | Effect of D-penicillamine (DP) on alcohol deprivation effect (ADE) measurements under both non-operant (A–C) and operant paradigms **(D)**. **(A)** Subcutaneous DP treatment dose-dependently suppresses the ADE manifestation. Effect of subcutaneous infusion of vehicle or DP (0.25 or 1 mg/h) on ethanol intake. **(B)** Intra-ventral tegmental area (VTA) infusion of DP blocked the ADE expression. Effect of the intra-VTA infusion of vehicle or DP 3.0 µg/h on ethanol intake. **(C)** DP prevents the "delayed ADE" induced by the continuous blockade of the opioid receptor with Naltrexone (NTX). Effect of vehicle, NTX or NTX/DP administration on ethanol intake. In panels **(A–C)**, animals had 9–11 months of ethanol experience. The percentage of each rat's total daily ethanol intake during post-abstinence drinking days was calculated with respect to baseline drinking just before deprivation (dashed line). The basal alcohol intake was about 0.6–1 g/kg/day. The deprivation period lasted 14 days. The assayed treatments began 48 h before the reintroduction of ethanol bottles (available 24 h along the post-abstinence period). **(D)** DP is able to prevent the ADE under an operant paradigm. Average ethanol responses in animals intraperitoneally (i.p.) administrated with saline (DP 0) or DP (6.25, 12.5 and 25 mg/kg), 30 min before each self-administration session in the post-abstinence period. Asterisk denotes statistically significant differences relative to the respective baseline period ($^{*}p < 0.05$; $^{**}p < 0.01$; $^{***}p < 0.001$; $^{****}p < 0.0001$). *Post hoc* test showed differences between groups ($^{\#}p < 0.05$, $^{\#\#}p < 0.01$, $^{\#\#\#\#}p < 0.0001$; Adapted from Orrico et al., 2013, 2014; Martí-Prats et al., 2015).

section, some studies have demonstrated that systemic, but not intracerebroventricular (ICV), administration of DP is able to produce changes in the taste of fluid and food (Font et al., 2006a). Hence, the possibility that, at least, part of the preventive effects of DP on the ADE could be produced by an alteration in taste perception cannot be fully ruled out. Nonetheless, and in agreement with these results, our group also demonstrated that intra-posterior VTA (pVTA) administration of DP was able to suppress the ADE (**Figure 1B**), suggesting that the preventive effects of DP on ADE could, at least in part, be caused specifically by a mechanism independent of taste alteration (Orrico et al., 2013).

Most drugs of abuse, including ethanol, stimulate the release of DA in several limbic regions (Di Chiara, 2002). In recent years, it has been shown that ACD is a crucial component of the overall effects of ethanol on DA neurons of the VTA (Rodd-Henricks et al., 2002; Rodd et al., 2005, 2008; Melis et al., 2007; Deehan et al., 2013). Therefore, our results also showed, for the first time, that pVTA is a critical region for DP action in relapse-like drinking behavior and emphasize the role displayed by this brain area in the relapse phenomenon.

The abovementioned results encouraged us to further study the potential use of DP alone or in combination with other marketed drugs (such as NTX) as a promising strategy to increase the efficacy of current anti-relapse therapies, based on the neurochemical studies that have confirmed that the mechanism through which ethanol, or more probably ACD, excite DA neurons is dependent on the Mu-Opioid Receptors (MORs; Sánchez-Catalán et al., 2009; Peana et al., 2011). Accordingly, our group next conjectured that the NTX/DP combination, due to its distinct but complementary mechanisms of action to impede MORs activation could be more efficacious in ADE prevention (Orrico et al., 2014). Specifically, we explored whether the combination of DP with NTX could suppress the delayed ADE, i.e., the rebound in alcohol consumption detected in animal laboratory models after continuous blockade of the opioid receptor with antagonists such as NTX (Heyser et al., 2003) or naloxone (Hölter and Spanagel, 1999). In this sense, in several strains of rats using free choice

paradigms it has been demonstrated that NTX decreases ethanol consumption (Overstreet et al., 2006), however, tolerance to this effect was demonstrated after repeated drug administration, leading to an increase in alcohol consumption (Cowen et al., 1999; Juárez and Eliana, 2007). This fact is probably due to the MORs up-regulation associated with its continuous blockade (Hyytiä et al., 1999; Overstreet et al., 1999; Orrico et al., 2014). Thus, as can be seen in **Figure 1C**, in our experimental conditions, NTX powerfully blocked the ADE on post-abstinence days 1 and 2 in agreement with previously published data, but an increase in alcohol consumption, with respect to basal values, was detected on post-abstinence days 3 and 4 (the manifestation of the so-called "delayed ADE"). The results obtained supported the efficacy of the NTX/DP combination preventing not only the ADE expression, but also the delayed ADE. In fact, the combination of DP (0.25 mg/h; a non-effective dose in our previous article) and NTX (2 × 5 mg/kg SC per day) showed an adequate anti-relapse pre-clinical efficacy along the four post-abstinence days.

In summary, the reported data demonstrate that this therapeutic strategy, of combining two drugs with complementary actions—opioid receptor blockade (by NTX) and chemical ACD inactivation (by DP), shows adequate alcohol anti-relapse-like drinking efficacy in long-term ethanol-experienced rats. Moreover, it overcomes some therapeutic limitations of either drug alone, since this combination is able to block not only the delayed increase in ethanol consumption, typically occurring after chronic opioid antagonist administration, but it also allows the administration of sub-threshold DP doses. All in all, these findings suggest that sequestering agents of ACD, in general, and DP, in particular, may represent a valuable therapy in the management of relapse in alcohol-dependent patients.

Operant-Procedures

There is no doubt among researchers that in order to maximize the translational power of pre-clinical research, it is important to gather evidence for as many paradigms and different animal models as possible (Bell et al., 2012). Hence, the use of different paradigms to test the same observations would assure the reproducibility of pre-clinical data, which is a challenge for neuroscience (Steckler, 2015).

In this context, we were able to validate our previous work on the pre-clinical efficacy of DP using a different laboratory paradigm: an operant procedure. Several authors have used this operant paradigm to demonstrate the capacity of a number of drugs to reduce the expression of an operant ADE (Schroeder et al., 2005; Rodd et al., 2006; Dhaher et al., 2010). The results of our study showed that all DP doses tested (6.25, 12.5, or 25 mg/kg), intraperitoneally (i.p.) administered, were able to prevent the ADE in Wistar rats using an operant fixed ratio (FR) 1 procedure (**Figure 1D**). Contrarily to the saline group (named "DP 0"), DP blocked the increase in ethanol response following the imposed period of abstinence. Interestingly, animals treated with the higher DP dose (25 mg/kg) even reduced their response to ethanol significantly, by 20% below baseline levels. Moreover, DP did not modify the spontaneous motor activity of the rats indicating that the effectiveness of DP in preventing ADE cannot be due to a reduced locomotor performance of the animals (Martí-Prats et al., 2015). These results added reproducibility and robustness to previously reported data. Hence, to sum up, we were able to replicate our previous outcomes in a different laboratory (Laboratory of Psychobiology, Complutense University of Madrid) and using a different paradigm, (inter-lab reliability) leading to more robust conclusions on the use of DP as a potential new pharmacotherapy in the treatment of alcoholism.

Effect of DP on Voluntary Ethanol Consumption Behavior

As illustrated above, findings of different research groups working in the field agree with the efficacy of DP as a valid strategy to prevent alcohol relapse-like drinking behavior, denoting the relevant role of ACD on the relapse expression. Conversely, concerning voluntary ethanol intake, the few published studies reveal contradictory results on the efficacy of DP (Font et al., 2006b; Campos-Jurado et al., 2015; Gosalbez et al., 2015).

Font et al. (2006b) were the first group to evaluate the effect of DP on voluntary ethanol consumption. In their study, male Long-Evans rats had daily access to a 10% ethanol solution in their home-cages for a 15-min period. Under their experimental conditions, the systemic (50 and 75 mg/kg), as well as ICV (75 μg) administration of DP was able to decrease the ethanol intake during the 5-day treatment. Interestingly, after discontinuation of the treatment, animals recovered their previous consumption rates. These results represent the initial evidence of ACD sequestration usefulness as a possible valid strategy to prevent ethanol drinking. However, in the same study, the authors also showed that systemic, but not ICV, DP treatment modified sucrose intake. According to the taste reactivity test performed, authors attributed this effect to a modification of ethanol palatability due to DP administration. Thus, all these results suggest that, although part of the DP effect on modulating ethanol consumption could be ascribed to a taste modification, an effect on the central levels of ACD has also been demonstrated (Font et al., 2006b).

Since the abovementioned study, it was not until nearly 10 years later that new research evaluating the utility of DP on the voluntary ethanol intake was performed. Concretely, Peana et al. (2015) studied the validity of DP on the acquisition and maintenance of oral operant ethanol self-administration. For acquisition analysis, ethanol-naïve Wistar rats were i.p. administered with DP (50 mg/kg) concomitant with the access to ethanol solution under an FR-1 schedule of reinforcement. The ethanol concentration was gradually increased from 5% in the first three sessions to 10% in the eighth last session. In this phase, systemic DP treatment significantly reduced the number of ethanol nose pokes, consistent with an ethanol intake decrease from the second ethanol session until the end of the study. Nevertheless, when rats, after an acquisition period, self-administered 10% ethanol for at least 10 days, the same

DP treatment (50 m/kg) failed to reduce active nose pokes for ethanol. Indeed, the double dose of DP (100 mg/kg) was also unable to diminish ethanol self-administration. Furthermore, DP 50 mg/kg did not diminish the ethanol intake when the solution was changed from 10% to 5%, neither alone nor in combination with the catalase inhibitor amino-1,2,4-triazole (1 g/kg). Curiously, this catalase inhibitor, that has shown to be effective in reducing ethanol consumption during the acquisition phase, was ineffective along the maintenance phase (Peana et al., 2015). Hence, these results confirm the DP efficacy in impairing the acquisition of ethanol self-administration in naïve animals, but not in reducing active responses in ethanol-experienced animals. Indeed, ethanol-experienced rats increased their nose pokes when the ethanol concentration was reduced from 10% to 5% in order to obtain the same ethanol intake. According to these authors, a possible explanation for the failure of DP activity could be that ACD, paradoxically contributes to the perpetuation of ethanol self-administration, concretely the reduction of ACD levels due to the administered treatment might motivate rats to further seek and take ethanol to compensate for that decrease. Moreover, according to the abovementioned authors, additional explanations could be plausible since in these experimental conditions, due to its mechanism of action, DP would allow the increase of the non-metabolized fraction of ethanol in relation to the metabolized fraction (ACD and its derivatives). Under this condition, some studies have reported that ethanol, through the $GABA_A$ receptor, might lead to different behavioral responses such as decreasing the rat's locomotor activity (Martí-Prats et al., 2015) or increasing the responses in the paradigm of alcohol self-administration (Kumar et al., 2009; Kaminski et al., 2013).

Finally, two additional pre-clinical studies have recently evaluated the ability of DP to prevent voluntary ethanol consumption, in both ethanol-naïve and ethanol-experienced Wistar rats, in their home-cages and under non-operant procedures (Campos-Jurado et al., 2015; Gosalbez et al., 2015). In the former, animals were SC implanted with a mini-osmotic pump delivering either vehicle or DP 1 mg/h along a 1-week period. One day after surgery, every animal was exposed in its home-cage, for the first time, to a four-bottle alcohol self-administration model. Along the next 6 days of treatment, although DP did not modify the voluntary ethanol intake, a significant reduction in the preference for ethanol, with respect to total volume of consumed liquid, was detected in the animals receiving DP. No statistical differences were detected in the next 6 days after treatment (post-treatment phase) between both experimental groups (**Figures 2A,B**).

With regard to the ethanol-experienced rats, animals were exposed to ethanol, under a non-operant paradigm during a 14-week period. Next, ethanol intake was registered on a daily basis along 3 weeks, each week corresponding to a different experimental period: (i) Pre-treatment: baseline intake was established; (ii) Treatment: a mini-osmotic pump was implanted in animals delivering DP 1 mg/h along 1 week; (iii) Post-treatment: the mini-osmotic pump was removed. Thereafter, rats continued to drink freely for 2 weeks before repeating the same experimental procedure, although pumps delivered sterilized water (vehicle) instead of DP. In this study, no differences in ethanol consumption nor ethanol preference were reported when animals received 1 mg/h DP treatment (**Figures 2C,D**). Nevertheless, after DP was administered (post-treatment phase), rats increased their ethanol preference in relation to pre-treatment and treatment phases, however, ethanol intake remained unmodified. Yet, these data suggest that, at the tested DP dose, a change in the consumption pattern takes place, without altering the total ethanol consumption.

Although different studies denote the existence of a positive correlation between brain ACD levels and alcohol intake (Correa et al., 2012; Muggironi et al., 2013; Israel et al., 2015), some aspects still remain unclear. In this sense, the inhibition of the brain ACD formation has been related to a reduction in the voluntary ethanol intake in mice and rats (Aragon and Amit, 1985, 1992; Koechling and Amit, 1994; Karahanian et al., 2011; Quintanilla et al., 2012; Ledesma et al., 2014). Moreover, the increase of the ACD metabolism (Karahanian et al., 2015) or the reduction of ACD disposition (Font et al., 2006b; Peana et al., 2010) has also been associated with a significant inhibition of voluntary ethanol consumption. Conversely, and consistent with some of the results exposed herein, different groups have reported that several strategies aimed at reducing the ACD levels in the brain, inhibition of its formation (Quintanilla et al., 2012; Tampier et al., 2013; Karahanian et al., 2015; Peana et al., 2015) or, as shown before, ACD inactivation (Campos-Jurado et al., 2015; Gosalbez et al., 2015), have not been able to impair the ethanol intake when experimental animals have moderate to long ethanol-experience. Recently, some studies have suggested a differential influence of ACD on the ethanol intake. It has been proposed that the key role of ACD in the ethanol reinforcing properties could be limited to the initial ethanol experience (called *first hit*), while after this first phase, ethanol consumption may not depend on ACD levels (Israel et al., 2015; Quintanilla et al., 2016). However, alternative explanations in relation with divergent results have also been taken into account. On the one hand, some of the studies supporting the preceding theory (Quintanilla et al., 2012; Karahanian et al., 2015) have focused on the manipulation of ACD levels only in the VTA, leaving open the possibility that other brain areas could be involved in the maintenance of alcohol intake (Karahanian et al., 2015). On the other hand, ethanol-related cues could also support the perpetuation of ethanol drinking behavior independent of its reinforcing properties (Greeley et al., 1993; Miller and Gold, 1994; O'Brien et al., 1998; Tiffany and Carter, 1998; Everitt et al., 2001; Littleton et al., 2001; See, 2002; Ingjaldsson et al., 2003a,b; van de Laar et al., 2004; Weiss, 2010; Karahanian et al., 2015; Peana et al., 2015).

Taking all the data into consideration, several of the hypotheses exposed could be due to the different data obtained. However, the diversity of protocols, experimental animals, rodent strains and duration of ethanol exposition make it difficult to realistically compare the results obtained in the published studies hitherto. Hence, further studies are required to obtain a

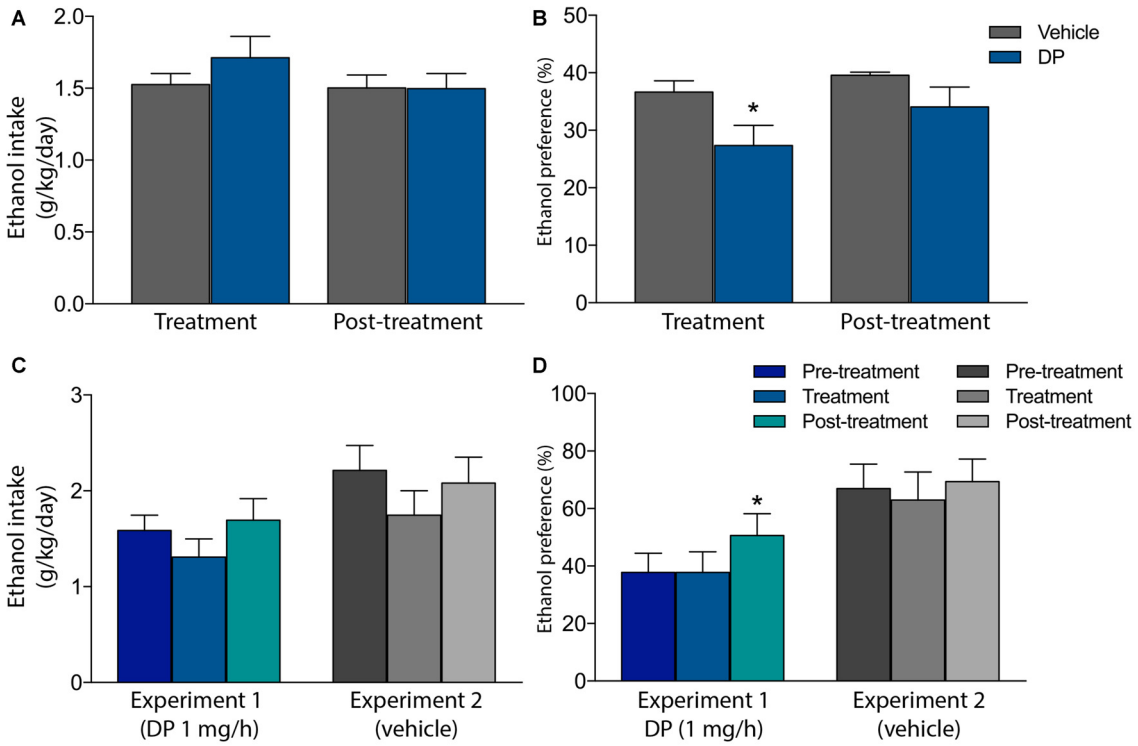

FIGURE 2 | Effect of systemic administration of DP on voluntary ethanol intake and alcohol preference in ethanol-naïve (A,B) or in long-term ethanol-experienced rats **(C,D)**. **(A)** DP does not modify voluntary ethanol intake in ethanol-naïve rats **(B)** DP reduced ethanol preference in ethanol-naïve rats. In panels **(A,B)**, animals received DP (1 mg/h) or vehicle, from a mini-osmotic pump subcutaneously (SC) implanted, along the treatment phase. Along the post-treatment phase no substance was administered. Asterisk denotes statistically significant differences with regard to the vehicle group ($p < 0.05$). **(C)** DP does not modify voluntary ethanol intake in long-term ethanol experienced rats. **(D)** DP does not modify ethanol preference in long-term ethanol experienced rats. A change in ethanol preference was detected after DP treatment only. In panels **(C,D)**, animals received DP (1 mg/h) or vehicle, from a mini-osmotic pump SC implanted, only along the treatment phase. Along the pre-treatment and post-treatment phase no substance was administered. Asterisk denotes statistically significant differences relative to the pre-treatment and treatment phases (*$p < 0.05$; Adapted from Campos-Jurado et al., 2015; Gosalbez et al., 2015).

firmer conclusion about DP efficacy in relation with voluntary ethanol intake.

CONCLUSIVE REMARKS AND FUTURE DIRECTIONS

To sum up, all of these experimental findings provide the rationale for using ACD sequestering agents, concretely DP, as a novel therapeutic intervention alone and or in combination to prevent relapse into alcohol seeking and consumption. In fact, there is a consensus among researchers that this potential therapeutic avenue deserves more attention and investigation (Melis et al., 2007; Sanchis and Aragón, 2007; Enrico et al., 2009; Peana et al., 2010; Orrico et al., 2013, 2014). This is also based on the fact that this drug acts as an ACD-scavenging compound (Nagasawa et al., 1980) without altering any neurotransmitter systems (Salaspuro et al., 2006). Hence, we hypothesized that DP could be a promising drug for preventing alcohol relapse. On the other hand, its effectiveness in reducing voluntary ethanol consumption in long-term experienced patients still remains unclear. In this sense, in our opinion, one possibility that could be clinically explored is its administration "as needed" in agreement with the latest trends in therapy such as with Nalmefene. Additionally, another point in favor of this strategy is the fact that DP has already been approved for its use in humans, hence, this fact would lead to a faster and easier way of inclusion in the present limited therapeutic arsenal. In conclusion, there is a vast amount of pre-clinical research that demonstrates the potential use of DP for treating alcoholism. At this point, it is time for clinical researchers to try to cross the so-called "Valley of Death" for alcohol drug development, i.e., the gap between the demonstrated efficacy in pre-clinical animal models and clinical testing. This gap has impeded several promising novel compounds from moving forward along the drug development pipeline (Litten et al., 2012).

AUTHOR CONTRIBUTIONS

AO and LM-P wrote the review. MJC-C and LG made substantial contributions to the design of the work and interpretation of data for the work. AP and TZ conceptualized and drafted the work critically for important intellectual content. All authors revised and approved the final version.

FUNDING

The research performed to elaborate this work was funded by grants from Conselleria de Educación (Generalitat Valenciana GVAICO2016-096), Universitat de València (UV-INV-AE11-42811) and (UV-INV-AE15-336743) (Spain) and Ministerio de Sanidad y Política Social (PND2009-021).

REFERENCES

Aragon, C. M., and Amit, Z. (1985). A two dimensional model of alcohol consumption: possible interaction of brain catalase and aldehyde dehydrogenase. *Alcohol* 2, 357–360. doi: 10.1016/0741-8329(85)90075-8

Aragon, C. M., and Amit, Z. (1992). The effect of 3-amino-1,2,4-triazole on voluntary ethanol consumption: evidence for brain catalase involvement in the mechanism of action. *Neuropharmacology* 31, 709–712. doi: 10.1016/0028-3908(92)90150-n

Arizzi-LaFrance, M. N., Correa, M., Aragon, C. M., and Salamone, J. D. (2006). Motor stimulant effects of ethanol injected into the substantia nigra pars reticulata: importance of catalase-mediated metabolism and the role of acetaldehyde. *Neuropsychopharmacology* 31, 997–1008. doi: 10.1038/sj.npp.1300849

Bell, R. L., Sable, H. J., Colombo, G., Hyytia, P., Rodd, Z. A., and Lumeng, L. (2012). Animal models for medications development targeting alcohol abuse using selectively bred rat lines: neurobiological and pharmacological validity. *Pharmacol. Biochem. Behav.* 103, 119–155. doi: 10.1016/j.pbb.2012.07.007

Campos-Jurado, Y., Peñuela, O., Orrico, A., Martí-Prats, L., Cano-Cebrián, M. J., and Zornoza, T. (2015). P-76 D-penicillamine, an acetaldehyde sequestering agent, reduces ethanol preference in alcohol-naïve rats. *Alcohol Alcohol.* 50:i64. doi: 10.1093/alcalc/agv080.76

Correa, M., Miquel, M., Sanchis-Segura, C., and Aragon, C. M. (1999). Acute lead acetate administration potentiates ethanol-induced locomotor activity in mice: the role of brain catalase. *Alcohol. Clin. Exp. Res.* 23, 799–805. doi: 10.1097/00000374-199905000-00006

Correa, M., Salamone, J. D., Segovia, K. N., Pardo, M., Longoni, R., Spina, L., et al. (2012). Piecing together the puzzle of acetaldehyde as a neuroactive agent. *Neurosci. Biobehav. Rev.* 36, 404–430. doi: 10.1016/j.neubiorev.2011.07.009

Correa, M., Sanchis-Segura, C., Pastor, R., and Aragon, C. M. (2004). Ethanol intake and motor sensitization: the role of brain catalase activity in mice with different genotypes. *Physiol. Behav.* 82, 231–240. doi: 10.1016/j.physbeh.2004.03.033

Cowen, M. S., Rezvani, A. H., Jarrott, B., and Lawrence, A. J. (1999). Ethanol consumption by Fawn-Hooded rats following abstinence: effect of naltrexone and changes in mu-opioid receptor density. *Alcohol. Clin. Exp. Res.* 23, 1008–1014. doi: 10.1111/j.1530-0277.1999.tb04218.x

De Witte, P., Littleton, J., Parot, P., and Koob, G. (2005). Neuroprotective and abstinence-promoting effects of acamprosate: elucidating the mechanism of action. *CNS Drugs* 19, 517–537. doi: 10.2165/00023210-200519060-00004

Deehan, G. A. Jr., Hauser, S. R., Wilden, J. A., Truitt, W. A., and Rodd, Z. A. (2013). Elucidating the biological basis for the reinforcing actions of alcohol in the mesolimbic dopamine system: the role of active metabolites of alcohol. *Front. Behav. Neurosci.* 7:104. doi: 10.3389/fnbeh.2013.00104

Dhaher, R., Hauser, S. R., Getachew, B., Bell, R. L., McBride, W. J., McKinzie, D. L., et al. (2010). The orexin-1 receptor antagonist SB-334867 reduces alcohol relapse drinking, but not alcohol-seeking, in alcohol-preferring (P) rats. *J. Addict. Med.* 4, 153–159. doi: 10.1097/ADM.0b013e3181bd893f

Di Chiara, G. (2002). Nucleus accumbens shell and core dopamine: differential role in behavior and addiction. *Behav. Brain Res.* 137, 75–114. doi: 10.1016/s0166-4328(02)00286-3

Echeverry-Alzate, V., Tuda-Arízcun, M., Bühler, K. M., Santos, Á., Giné, E., Olmos, P., et al. (2012). Cocaine reverses the naltrexone-induced reduction in operant ethanol self-administration: the effects on immediate-early gene expression in the rat prefrontal cortex. *Neuropharmacology* 63, 927–935. doi: 10.1016/j.neuropharm.2012.06.010

Enrico, P., Sirca, D., Mereu, M., Peana, A. T., Lintas, A., Golosio, A., et al. (2009). Acetaldehyde sequestering prevents ethanol-induced stimulation of mesolimbic dopamine transmission. *Drug Alcohol Depend.* 100, 265–271. doi: 10.1016/j.drugalcdep.2008.10.010

Escarabajal, M. D., and Aragon, C. M. (2002). Concurrent administration of diethyldithiocarbamate and 4-methylpyrazole enhances ethanol-induced locomotor activity: the role of brain ALDH. *Psychopharmacology* 160, 339–343. doi: 10.1007/s00213-001-0991-0

Everitt, B. J., Dickinson, A., and Robbins, T. W. (2001). The neuropsychological basis of addictive behaviour. *Brain Res Rev.* 36, 129–138. doi: 10.1016/s0165-0173(01)00088-1

Font, L., Aragon, C. M., and Miquel, M. (2006a). Ethanol-induced conditioned place preference, but not aversion, is blocked by treatment with D - penicillamine, an inactivation agent for acetaldehyde. *Psychopharmacology* 184, 56–64. doi: 10.1007/s00213-005-0224-z

Font, L., Aragon, C. M., and Miquel, M. (2006b). Voluntary ethanol consumption decreases after the inactivation of central acetaldehyde by d-penicillamine. *Behav. Brain Res.* 171, 78–86. doi: 10.1016/j.bbr.2006.03.020

Font, L., Miquel, M., and Aragon, C. M. (2005). Prevention of ethanol-induced behavioral stimulation by D-penicillamine: a sequestration agent for acetaldehyde. *Alcohol. Clin. Exp. Res.* 29, 1156–1164. doi: 10.1097/01.alc.0000171945.30494.af

Font, L., Miquel, M., and Aragon, C. M. (2008). Involvement of brain catalase activity in the acquisition of ethanol-induced conditioned place preference. *Physiol. Behav.* 93, 733–741. doi: 10.1016/j.physbeh.2007.11.026

Gosalbez, F. B., Cabañero, B., Orrico, A., Marti-Prats, L., Cano-Cebrián, M. J., and Zornoza, T. (2015). P-77 D-penicillamine, a potential ethanol anti-relapse drug, does not reduce the voluntary ethanol intake in long-term experienced rats. *Alcohol Alcohol.* 50, i64–i65. doi: 10.1093/alcalc/agv080.77

Greeley, J. D., Swift, W., Prescott, J., and Heather, N. (1993). Reactivity to alcohol-related cues in heavy and light drinkers. *J. Stud. Alcohol* 54, 359–368. doi: 10.15288/jsa.1993.54.359

Heyser, C. J., Moc, K., and Koob, G. F. (2003). Effects of naltrexone alone and in combination with acamprosate on the alcohol deprivation effect in rats. *Neuropsychopharmacology* 28, 1463–1471. doi: 10.1038/sj.npp.1300175

Hipolito, L., Sánchez-Catalán, M. J., Polache, A., and Granero, L. (2009). Induction of brain CYP2E1 changes the effects of ethanol on dopamine release in nucleus accumbens shell. *Drug Alcohol Depend.* 100, 83–90. doi: 10.1016/j.drugalcdep.2008.09.004

Hölter, S. M., Landgraf, R., Zieglgänsberger, W., and Spanagel, R. (1997). Time course of acamprosate action on operant ethanol self-administration after ethanol deprivation. *Alcohol. Clin. Exp. Res.* 21, 862–868. doi: 10.1097/00000374-199708000-00014

Hölter, S. M., and Spanagel, R. (1999). Effects of opiate antagonist treatment on the alcohol deprivation effect in long-term ethanol-experienced rats. *Psychopharmacology* 145, 360–369. doi: 10.1007/s002130051069

Hyytiä, P., Ingman, K., Soini, S. L., Laitinen, J. T., and Korpi, E. R. (1999). Effects of continuous opioid receptor blockade on alcohol intake and up-regulation of opioid receptor subtype signalling in a genetic model of high alcohol drinking. *Naunyn Schmiedebergs Arch. Pharmacol.* 360, 391–401. doi: 10.1007/s002109900070

Ingjaldsson, J. T., Thayer, J. F., and Laberg, J. C. (2003a). Craving for alcohol and pre-attentive processing of alcohol stimuli. *Int. J. Psychophysiol.* 49, 29–39. doi: 10.1016/s0167-8760(03)00075-8

Ingjaldsson, J. T., Thayer, J. F., and Laberg, J. C. (2003b). Preattentive processing of alcohol stimuli. *Scand. J. Psychol.* 44, 161–165. doi: 10.1111/1467-9450.00334

Israel, Y., Quintanilla, M. E., Karahanian, E., Rivera-Meza, M., and Herrera-Marschitz, M. (2015). The "first hit" toward alcohol reinforcement: role of ethanol metabolites. *Alcohol. Clin. Exp. Res.* 39, 776–786. doi: 10.1111/acer.12709

Juárez, J., and Eliana, B. T. (2007). Alcohol consumption is enhanced after naltrexone treatment. *Alcohol. Clin. Exp. Res.* 31, 260–264. doi: 10.1111/j.1530-0277.2006.00313.x

Kaminski, B. J., Van Linn, M. L., Cook, J. M., Yin, W., and Weerts, E. M. (2013). Effects of the benzodiazepine $GABA_A$ α1-preferring ligand, 3-propoxy-

β-carboline hydrochloride (3-PBC), on alcohol seeking and selfadministration in baboons. *Psychopharmacology* 227, 127–136. doi: 10.1007/s00213-012-2946-z

Karahanian, E., Quintanilla, M. E., Tampier, L., Rivera-Meza, M., Bustamante, D., Gonzalez-Lira, V., et al. (2011). Ethanol as a prodrug: brain metabolism of ethanol mediates its reinforcing effects. *Alcohol. Clin. Exp. Res.* 35, 606–612. doi: 10.1111/j.1530-0277.2011.01439.x

Karahanian, E., Rivera-Meza, M., Tampier, L., Quintanilla, M. E., Herrera-Marschitz, M., and Israel, Y. (2015). Long-term inhibition of ethanol intake by the administration of an aldehyde dehydrogenase-2 (ALDH2)-coding lentiviral vector into the ventral tegmental area of rats. *Addict. Biol.* 20, 336–344. doi: 10.1111/adb.12130

Kera, Y., Kiriyama, T., and Komura, S. (1985). Conjugation of acetaldehyde with cysteinylglycine, the first metabolite in glutathione breakdown by γ-glutamyltranspeptidase. *Agents Actions* 17, 48–52. doi: 10.1007/bf01966681

Koechling, U. M., and Amit, Z. (1994). Effects of 3-amino-1,2,4-triazole on brain catalase in the mediation of ethanol consumption in mice. *Alcohol* 11, 235–239. doi: 10.1016/0741-8329(94)90036-1

Kumar, S., Porcu, P., Werner, D. F., Matthews, D. B., Diaz-Granados, J. L., Helfand, R. S., et al. (2009). The role of $GABA_A$ receptors in the acute and chronic effects of ethanol: a decade of progress. *Psychopharmacology* 205, 529–564. doi: 10.1007/s00213-009-1562-z

Ledesma, J. C., Miquel, M., Pascual, M., Guerri, C., and Aragon, C. M. (2014). Induction of brain cytochrome P450 2E1 boosts the locomotor-stimulating effects of ethanol in mice. *Neuropharmacol.* 85, 36–44. doi: 10.1016/j.neuropharm.2014.05.018

Leggio, L., Cardone, S., Ferrulli, A., Kenna, G. A., Diana, M., Swift, R. M., et al. (2010). Turning the clock ahead: potential preclinical and clinical neuropharmacological targets for alcohol dependence. *Curr. Pharm. Des.* 16, 2159–2181. doi: 10.2174/138161210791516369

Litten, R. Z., Egli, M., Heilig, M., Cui, C., Fertig, J. B., Ryan, M. L., et al. (2012). Medications development to treat alcohol dependence: a vision for the next decade. *Addict. Biol.* 17, 513–527. doi: 10.1111/j.1369-1600.2012.00454.x

Littleton, J. M., Lovinger, D., Liljequist, S., Ticku, R., Matsumoto, I., and Barron, S. (2001). Role of polyamines and NMDA receptors in ethanol dependence and withdrawal. *Alcohol. Clin. Exp. Res.* 25, 132s–136s. doi: 10.1097/00000374-200105051-00023

Martí-Prats, L., Sánchez-Catalán, M. J., Hipólito, L., Orrico, A., Zornoza, T., Polache, A., et al. (2010). Systemic administration of D-penicillamine prevents the locomotor activation after intra-VTA ethanol administration in rats. *Neurosci. Lett.* 483, 143–147. doi: 10.1016/j.neulet.2010.07.081

Martí-Prats, L., Sánchez-Catalán, M. J., Orrico, A., Zornoza, T., Polache, A., and Granero, L. (2013). Opposite motor responses elicited by ethanol in the posterior VTA: the role of acetaldehyde and the non-metabolized fraction of ethanol. *Neuropharmacology* 72, 204–214. doi: 10.1016/j.neuropharm.2013.04.047

Martí-Prats, L., Zornoza, T., López-Moreno, J. A., Granero, L., and Polache, A. (2015). Acetaldehyde sequestration by D-penicillamine prevents ethanol relapse-like drinking in rats: evidence from an operant self-administration paradigm. *Psychopharmacology* 232, 3597–3606. doi: 10.1007/s00213-015-4011-1

Melis, M., Enrico, P., Peana, A. T., and Diana, M. (2007). Acetaldehyde mediates alcohol activation of the mesolimbic dopamine system. *Eur. J. Neurosci.* 26, 2824–2833. doi: 10.1111/j.1460-9568.2007.05887.x

Miller, N. S., and Gold, M. S. (1994). Dissociation of "conscious desire" (craving) from and relapse in alcohol and cocaine dependence. *Ann. Clin. Psychiatry* 6, 99–106. doi: 10.3109/10401239409148988

Muggironi, G., Fois, G. R., and Diana, M. (2013). Ethanol-derived acetaldehyde: pleasure and pain of alcohol mechanism of action. *Front. Behav. Neurosci.* 7:87. doi: 10.3389/fnbeh.2013.00087

Nagasawa, H. T., Elberling, J. A., and DeMaster, E. G. (1980). Structural requirements for the sequestration of metabolically generated acetaldehyde. *J. Med. Chem.* 23, 140–143. doi: 10.1002/chin.198026137

O'Brien, C. P., Childress, A. R., Ehrman, R., and Robbins, S. J. (1998). Conditioning factors in drug abuse: can they explain compulsion? *J. Psychopharmacol.* 12, 15–22. doi: 10.1177/026988119801200103

Orrico, A., Hipólito, L., Sánchez-Catalán, M. J., Martí-Prats, L., Zornoza, T., Granero, L., et al. (2013). Efficacy of D-penicillamine, a sequestering acetaldehyde agent, in the prevention of alcohol relapse-like drinking in rats. *Psychopharmacology* 228, 563–575. doi: 10.1007/s00213-013-3065-1

Orrico, A., Martí-Prats, L., Cano-Cebrián, M. J., Granero, L., Polache, A., and Zornoza, T. (2014). Improved effect of the combination naltrexone/D-penicillamine in the prevention of alcohol relapse-like drinking in rats. *J. Psychopharmacol.* 28, 76–81. doi: 10.1177/0269881113515063

Overstreet, D. H., Kampov-Polevoy, A. B., Rezvani, A. H., Braun, C., Bartus, R. T., and Crews, F. T. (1999). Suppression of alcohol intake by chronic naloxone treatment in P rats: tolerance development and elevation of opiate receptor binding. *Alcohol. Clin. Exp. Res.* 23, 1761–1771. doi: 10.1097/00000374-199911000-00008

Overstreet, D. H., Rezvani, A. H., Cowen, M., Chen, F., and Lawrence, A. J. (2006). Modulation of high alcohol drinking in the inbred Fawn-Hooded (FH/Wjd) rat strain: implications for treatment. *Addict. Biol.* 11, 356–373. doi: 10.1111/j.1369-1600.2006.00033.x

Peana, A. T., Assaretti, A. R., Muggironi, G., Enrico, P., and Diana, M. (2009). Reduction of ethanol-derived acetaldehyde induced motivational properties by L-cysteine. *Alcohol. Clin. Exp. Res.* 33, 43–48. doi: 10.1111/j.1530-0277.2008.00809.x

Peana, A. T., Enrico, P., Assaretti, A. R., Pulighe, E., Muggironi, G., Nieddu, M., et al. (2008). Key role of ethanol-derived acetaldehyde in the motivational properties induced by intragastric ethanol: a conditioned place preference study in the rat. *Alcohol. Clin. Exp. Res.* 32, 249–258. doi: 10.1111/j.1530-0277.2007.00574.x

Peana, A. T., Muggironi, G., Calvisi, G., Enrico, P., Mereu, M., Nieddu, M., et al. (2010). L-Cysteine reduces oral ethanol self-administration and reinstatement of ethanol-drinking behavior in rats. *Pharmacol. Biochem. Behav.* 94, 431–437. doi: 10.1016/j.pbb.2009.10.005

Peana, A. T., Muggironi, G., Fois, G. R., Zinellu, M., Vinci, S., and Acquas, E. (2011). Effect of opioid receptor blockade on acetaldehyde self-administration and ERK phosphorylation in the rat nucleus accumbens. *Alcohol* 45, 773–783. doi: 10.1016/j.alcohol.2011.06.003

Peana, A. T., Porcheddu, V., Bennardini, F., Carta, A., Rosas, M., and Acquas, E. (2015). Role of ethanol-derived acetaldehyde in operant oral self-administration of ethanol in rats. *Psychopharmacology* 232, 4269–4276. doi: 10.1007/s00213-015-4049-0

Peana, A. T., Rosas, M., Porru, S., and Acquas, E. (2016). From ethanol to salsolinol: role of ethanol metabolites in the effects of ethanol. *J. Exp. Neurosci.* 10, 137–146. doi: 10.4137/jen.s25099

Quintanilla, M. E., Rivera-Meza, M., Berrios-Cárcamo, P., Salinas-Luypaert, C., Herrera-Marschitz, M., and Israel, Y. (2016). Beyond the first hit: marked inhibition by N-acetyl cysteine of chronic ethanol intake but not of early ethanol intake. Parallel effects on ethanol-induced saccharin motivation. *Alcohol. Clin. Exp. Res.* 40, 1044–1051. doi: 10.1111/acer.13031

Quintanilla, M. E., Tampier, L., Karahanian, E., Rivera-Meza, M., Herrera-Marschitz, M., and Israel, Y. (2012). Reward and relapse: complete gene-induced dissociation in an animal model of alcohol dependence. *Alcohol. Clin. Exp. Res.* 36, 517–522. doi: 10.1111/j.1530-0277.2011.01606.x

Rodd, Z. A., Bell, R. L., McQueen, V. K., Davids, M. R., Hsu, C. C., Murphy, J. M., et al. (2005). Prolonged increase in the sensitivity of the posterior ventral tegmental area to the reinforcing effects of ethanol following repeated exposure to cycles of ethanol access and deprivation. *J. Pharmacol. Exp. Ther.* 315, 648–657. doi: 10.1124/jpet.105.084350

Rodd, Z. A., Bell, R. L., Sable, H. J., Murphy, J. M., and McBride, W. J. (2004). Recent advances in animal models of alcohol craving and relapse. *Pharmacol. Biochem. Behav.* 79, 439–450. doi: 10.1016/j.pbb.2004.08.018

Rodd, Z. A., McKinzie, D. L., Bell, R. L., McQueen, V. K., Murphy, J. M., Schoepp, D. D., et al. (2006). The metabotropic glutamate 2/3 receptor agonist LY404039 reduces alcohol-seeking but not alcohol self-administration in alcohol-preferring (P) rats. *Behav. Brain Res.* 171, 207–215. doi: 10.1016/j.bbr.2006.03.032

Rodd, Z. A., Oster, S. M., Ding, Z. M., Toalston, J. E., Deehan, G., Bell, R. L., et al. (2008). The reinforcing properties of salsolinol in the ventral tegmental area: evidence for regional heterogeneity and the involvement of serotonin and dopamine. *Alcohol. Clin. Exp. Res.* 32, 230–239. doi: 10.1111/j.1530-0277.2007.00572.x

Rodd-Henricks, Z. A., Melendez, R. I., Zaffaroni, A., Goldstein, A., McBride, W. J., and Li, T. K. (2002). The reinforcing effects of acetaldehyde in the posterior

ventral tegmental area of alcohol-preferring rats. *Pharmacol. Biochem. Behav.* 72, 55–64. doi: 10.1016/s0091-3057(01)00733-x

Salaspuro, V. J., Hietala, J. M., Marvola, M. L., and Salaspuro, M. P. (2006). Eliminating carcinogenic acetaldehyde by cysteine from saliva during smoking. *Cancer Epidemiol. Biomarkers Prev.* 15, 146–149. doi: 10.1158/1055-9965.epi-05-0248

Sánchez-Catalán, M. J., Hipólito, L., Zornoza, T., Polache, A., and Granero, L. (2009). Motor stimulant effects of ethanol and acetaldehyde injected into the posterior ventral tegmental area of rats: role of opioid receptors. *Psychopharmacology (Berl)* 204, 641–653. doi: 10.1007/s00213-009-1495-6

Sanchis, C., and Aragón, C. M. (2007). What we drink when we drink? The role of the acetaldehyde in the alcohol consumption. *Adicciones* 19, 5–11. doi: 10.1037/e527342012-358

Sanchis-Segura, C., Miquel, M., Correa, M., and Aragon, C. M. (1999a). The catalase inhibitor sodium azide reduces ethanol-induced locomotor activity. *Alcohol* 19, 37–42. doi: 10.1016/s0741-8329(99)00016-6

Sanchis-Segura, C., Miquel, M., Correa, M., and Aragon, C. M. (1999b). Cyanamide reduces brain catalase and ethanol-induced locomotor activity: is there a functional link? *Psychopharmacology* 144, 83–89. doi: 10.1007/s002130050980

Schroeder, J. P., Overstreet, D. H., and Hodge, C. W. (2005). The mGluR5 antagonist MPEP decreases operant ethanol self-administration during maintenance and after repeated alcohol deprivations in alcohol-preferring (P) rats. *Psychopharmacology* 179, 262–270. doi: 10.1007/s00213-005-2175-9

Serrano, E., Pozo, O. J., Beltrán, J., Hernández, F., Font, L., Miquel, M., et al. (2007). Liquid chromatography/tandem mass spectrometry determination of (4S,2RS)-2,5,5-trimethylthiazolidine-4-carboxylic acid, a stable adduct formed between D-(-)-penicillamine and acetaldehyde (main biological metabolite of ethanol), in plasma, liver and brain rat tissues. *Rapid Commun. Mass Spectrom.* 21, 1221–1229. doi: 10.1002/rcm.2951

See, R. E. (2002). Neural substrates of conditioned-cued relapse to drug-seeking behavior. *Pharmacol. Biochem. Behav.* 71, 517–529. doi: 10.1016/s0091-3057(01)00682-7

Spanagel, R. (2009). Alcoholism: a systems approach from molecular physiology to addictive behavior. *Physiol. Rev.* 89, 649–705. doi: 10.1152/physrev.00013.2008

Spanagel, R., and Hölter, S. M. (1999). Long-term alcohol self-administration with repeated alcohol deprivation phases: an animal model of alcoholism? *Alcohol Alcohol.* 34, 231–243. doi: 10.1093/alcalc/34.2.231

Spanagel, R., and Kiefer, F. (2008). Drugs for relapse prevention of alcoholism: ten years of progress. *Trends Pharmacol. Sci.* 29, 109–115. doi: 10.1016/j.tips.2007.12.005

Spanagel, R., Vengeliene, V., Jandeleit, B., Fischer, W. N., Grindstaff, K., Zhang, X., et al. (2014). Acamprosate produces its anti-relapse effects via calcium. *Neuropsychopharmacology* 39, 783–791. doi: 10.1038/npp.2013.264

Spanagel, R., and Zieglgänsberger, W. (1997). Anti-craving compounds for ethanol: new pharmacological tools to study addictive processes. *Trends Pharmacol. Sci.* 18, 54–59. doi: 10.1016/s0165-6147(97)89800-8

Steckler, T. (2015). Preclinical data reproducibility for RandD–the challenge for neuroscience. *Psychopharmacology* 232, 317–320. doi: 10.1007/s00213-014-3836-3

Tampier, L., Quintanilla, M. E., Karahanian, E., Rivera-Meza, M., Herrera-Marschitz, M., and Israel, Y. (2013). The alcohol deprivation effect: marked inhibition by anticatalase gene administration into the ventral tegmental area in rats. *Alcohol. Clin. Exp. Res.* 37, 1278–1285. doi: 10.1111/acer.12101

Tiffany, S. T., and Carter, B. L. (1998). Is craving the source of compulsive drug use? *J. Psychopharmacol.* 12, 23–30. doi: 10.1177/026988119801200104

van de Laar, M. C., Licht, R., Franken, I. H., and Hendriks, V. M. (2004). Event-related potentials indicate motivational relevance of cocaine cues in abstinent cocaine addicts. *Psychopharmacology (Berl)* 177, 121–129. doi: 10.1007/s00213-004-1928-1

Vengeliene, V., Bilbao, A., and Spanagel, R. (2014). The alcohol deprivation effect model for studying relapse behavior: a comparison between rats and mice. *Alcohol* 48, 313–320. doi: 10.1016/j.alcohol.2014.03.002

Wackernah, R. C., Minnick, M. J., and Clapp, P. (2014). Alcohol use disorder: pathophysiology, effects, and pharmacologic options for treatment. *Subst. Abuse Rehabil.* 23, 1–12. doi: 10.2147/SAR.S37907

Weiss, F. (2010). "Advances in animal models of relapse for addiction research," in *Advances in the Neuroscience of Addiction*, eds C. M. Kuhn and G. F. Koob (Boca Raton, FL: CRC Press/Taylor and Francis Llc), 1–26.

Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

The reviewer LF and handling Editor declared their shared affiliation, and the handling Editor states that the process nevertheless met the standards of a fair and objective review.

frontiers in Behavioral Neuroscience

ORIGINAL RESEARCH
published: 14 July 2017
doi: 10.3389/fnbeh.2017.00133

Fenofibrate Administration Reduces Alcohol and Saccharin Intake in Rats: Possible Effects at Peripheral and Central Levels

*Mario Rivera-Meza[1], Daniel Muñoz[2], Erik Jerez[1], María E. Quintanilla[3], Catalina Salinas-Luypaert[1], Katia Fernandez[4] and Eduardo Karahanian[2,5]**

[1]*Department of Pharmacological and Toxicological Chemistry, Faculty of Chemical and Pharmaceutical Sciences, University of Chile, Santiago, Chile,* [2]*Center for Biomedical Research, Faculty of Health Sciences, Universidad Autónoma de Chile, Santiago, Chile,* [3]*Program of Molecular and Clinical Pharmacology, Faculty of Medicine, Institute of Biomedical Sciences, University of Chile, Santiago, Chile,* [4]*CIB, Faculty of Health and Dentistry, Universidad Diego Portales, Santiago, Chile,* [5]*Research Center for the Study of Alcohol Drinking Behavior in Adolescents, Universidad Autónoma de Chile, Santiago, Chile*

We have previously shown that the administration of fenofibrate to high-drinker UChB rats markedly reduces voluntary ethanol intake. Fenofibrate is a peroxisome proliferator-activated receptor alpha (PPARα) agonist, which induces the proliferation of peroxisomes in the liver, leading to increases in catalase levels that result in acetaldehyde accumulation at aversive levels in the blood when animals consume ethanol. In these new studies, we aimed to investigate if the effect of fenofibrate on ethanol intake is produced exclusively in the liver (increasing catalase and systemic levels of acetaldehyde) or there might be additional effects at central level. High drinker rats (UChB) were allowed to voluntary drink 10% ethanol for 2 months. Afterward, a daily dose of fenofibrate (25, 50 or 100 mg/kg/day) or vehicle (as control) was administered orally for 14 days. Voluntary ethanol intake was recorded daily. After that time, animals were deprived of ethanol access for 24 h and administered with an oral dose of ethanol (1 g/kg) for acetaldehyde determination in blood. Fenofibrate reduced ethanol voluntary intake by 60%, in chronically drinking rats, at the three doses tested. Acetaldehyde in the blood rose up to between 80 μM and 100 μM. Considering the reduction of ethanol consumption, blood acetaldehyde levels and body weight evolution, the better results were obtained at a dose of 50 mg fenofibrate/kg/day. This dose of fenofibrate also reduced the voluntary intake of 0.2% saccharin by 35% and increased catalase levels 2.5-fold in the liver but showed no effects on catalase levels in the brain. To further study if fenofibrate administration changes the motivational properties of ethanol, a conditioned-place preference experiment was carried out. Animals treated with fenofibrate (50 mg/kg/day) did not develop ethanol-conditioned place preference (CPP).In an additional experiment, chronically ethanol-drinking rats underwent two cycles of ethanol deprivation/re-access, and fenofibrate (50 mg/kg/day) was given only in deprivation periods; under this paradigm, fenofibrate was also able to generate a prolonged (30 days) decreasing of ethanol consumption, suggesting some effect beyond the acetaldehyde-generated aversion. In summary, reduction of ethanol intake by fenofibrate appears

OPEN ACCESS

Edited by:
Merce Correa,
Jaume I University, Spain

Reviewed by:
Li Jing,
National Institutes of Health, United States
Salim Yalcin Inan,
University of Konya-NE, Turkey

****Correspondence:***
Eduardo Karahanian
eduardo.karahanian@uautonoma.cl

Received: *22 March 2017*
Accepted: *03 July 2017*
Published: *14 July 2017*

Citation:
Rivera-Meza M, Muñoz D, Jerez E, Quintanilla ME, Salinas-Luypaert C, Fernandez K and Karahanian E (2017) Fenofibrate Administration Reduces Alcohol and Saccharin Intake in Rats: Possible Effects at Peripheral and Central Levels. Front. Behav. Neurosci. 11:133. doi: 10.3389/fnbeh.2017.00133

to be a consequence of a combination of catalase induction in the liver and central pharmacological effects.

Keywords: fibrates, catalase, alcoholism, treatment, peroxisome proliferator-activated receptor alpha

INTRODUCTION

Alcohol dehydrogenase (ADH) and mitochondrial aldehyde dehydrogenase (ALDH2) are the main enzymes involved in ethanol metabolism in the liver. However, catalase also plays an important role in the conversion of ethanol to acetaldehyde (Handler and Thurman, 1988a). Catalase is localized mainly in peroxisomes and oxygen peroxide required for its activity is provided via the peroxisomal oxidation of fatty acids (Handler and Thurman, 1988b). It was reported that oral administration of fenofibrate—a peroxisome proliferator-activated receptor alpha (PPARα) specific agonist—to rats increases catalase activity in the liver (Henninger et al., 1987; Steinberg et al., 1988; Clouet et al., 1990; Arnaiz et al., 1995; Karahanian et al., 2014). Furthermore, Karahanian et al. (2014) found that fenofibrate reduced daily ethanol intake by 70% in high-alcohol drinking UChB rats and Blednov et al. (2015) reported similar findings in fenofibrate-treated mice. It is known that the increase of systemic acetaldehyde concentration generates an aversion to ethanol consumption (Mizoi et al., 1983), and this was the basis for the treatment of alcohol-dependent individuals with disulfiram. Karahanian et al. (2014) demonstrated that the administration of fenofibrate to alcohol-drinking rats up-regulates catalase activity in the liver, leading to a higher acetaldehyde concentration in blood which ultimately produces the aversion to voluntary ethanol intake. Accordingly, they found that an oral dose of 1 g ethanol/kg produced a marked increase in blood acetaldehyde in fenofibrate-treated animals.

An alternative hypothesis also emerged to explain the effect of PPARα agonists on alcohol intake: these drugs would act in the brain where they change the expression of genes related to reward response, ultimately leading to a reduced ethanol drinking (Blednov et al., 2015). Accordingly, upregulation of several neuropeptide-coding genes in GABAergic neurons located in the amygdala (an area involved in memory consolidation and conditioning) and the prefrontal cortex (involved in the executive decision) was reported (Ferguson et al., 2014). Interestingly, upregulation of genes involved in dopaminergic transmission and downregulation of genes involved in glutamate signaling were also found (Ferguson et al., 2014), both of which important pathways related to alcohol consumption. There is strong evidence that PPARα agonists reduce the consumption of another drug of abuse, such as nicotine, decreasing its reinforcing properties in the brain (Melis et al., 2008; Mascia et al., 2011; Panlilio et al., 2012); therefore, it is plausible that a similar mechanism is also involved in decreasing ethanol intake.

In this work, we aimed at contributing to the clarification of the likely mechanisms of action of PPARα agonists on ethanol intake. Our aim was to study the peripheral (liver) and potential central effects of fenofibrate that determine its capacity to reduce ethanol consumption. Generally accepted paradigms to determine whether a drug can reduce central rewarding properties are to evaluate: (i) the prevention of conditioned place preference (CPP); and (ii) its effects on the consumption of sweet substances such as saccharin (Hajnal et al., 2004). Selectively bred alcohol-preferring UChB rats were found to consume significantly larger quantities of saccharin solution than their alcohol-avoiding counterparts (Tampier and Quintanilla, 2005). Although a common mechanism for the association between consumption of sweet solutions and ethanol intake has not been identified, this mechanism is likely involved in mediating the rewarding properties of both sweet solutions and ethanol. It has been shown that various drugs of abuse and sweet foods share the ability to increase the extracellular concentration of dopamine in the nucleus accumbens (Di Chiara, 1998; Hajnal et al., 2004) suggesting that alcohol and sweet taste may share a common dopaminergic mechanism in mediating their hedonic effects.

In this study, we administered fenofibrate to alcohol- or saccharin-drinking UChB rats, in order to establish whether the effect of this drug on the reduction of alcohol consumption is due to effects at the central level or to an increase of the aversive properties mediated by acetaldehyde generated in the liver after ethanol consumption. With this aim, we evaluated the effects of fenofibrate administration on: (i) voluntary ethanol intake; (ii) saccharin intake; and (iii) ethanol-CPP in alcohol-preferring UChB rats.

MATERIALS AND METHODS

Animals

High-drinker UChB rats derived from the Wistar strain and bred selectively for their high alcohol intake (Mardones and Segovia-Riquelme, 1983; Quintanilla et al., 2006) were used. Two-month-old male rats (240 g ± 17 g) were housed in individual cages in temperature-controlled rooms under a regular 12-h light/12-h dark cycle. For 60 days, rats were offered a choice between 10% (v/v) ethanol solution and water, or 0.2% w/v saccharin and water. Food (Mardones rat formula, Alimentos Cisternas, Santiago, Chile) was provided *ad libitum* and the volume of water, ethanol and saccharin solutions consumed was recorded daily. After this time, 10% ethanol solution consumption stabilized at ∼70 ml/kg/day, and saccharin solution mean intake was 108 ml/kg/day. All procedures used in this study were revised by and in compliance with the Bioethics Committee on Animal Research, Faculty of Medicine, Universidad de Chile (Protocol CBA0767FMUCH).

Fenofibrate Treatment

After 60 days of continuous free choice between 10% (v/v) ethanol solution and water (or 0.2% w/v saccharin and water), rats were divided into six groups (4 for ethanol drinking and 2 for saccharin drinking, n = 7 animals per group). Ethanol-

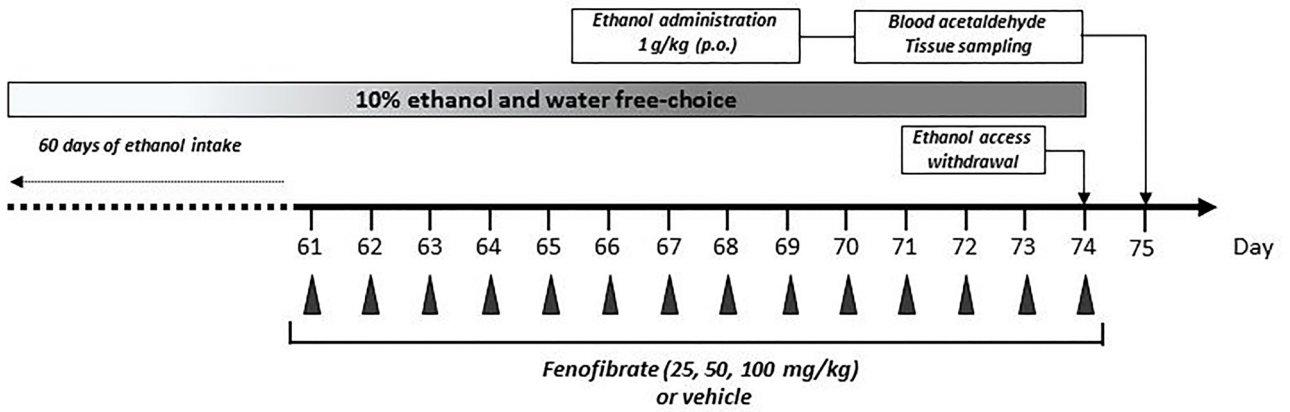

FIGURE 1 | Graphical time schedule for the ethanol-drinking experiments. Twenty-eight UChB male rats were given 24 h free choice between 10% v/v ethanol and water for 60 days. Starting at day 61, 25, 50 or 100 mg/kg/day of fenofibrate or vehicle were given orally for 14 days. At the end of fenofibrate treatment, animals were deprived for ethanol access for 24 h to allow complete elimination of ethanol and acetaldehyde from the blood. At day 75, one ethanol dose (1 g/kg) was given orally, and acetaldehyde in arterial blood was measured. In the end, blood, liver and brain tissues were collected for biochemical measurements.

drinking groups were treated with micronized fenofibrate (Fibronil®, Royal Pharma, Chile) administered orally as an aqueous suspension at a doses of 25, 50 or 100 mg/kg/day, respectively, or a similar volume of vehicle (water) as control for 14 consecutive days (**Figure 1**). Saccharin-drinking groups were treated with fenofibrate at a dose of 50 mg/kg/day or a similar volume of vehicle as control for 14 consecutive days.

In another experiment, 13 UChB rats were exposed for 59 days to a free choice between 10% (v/v) ethanol and water, animals were deprived of ethanol access on day 60 and fenofibrate (50 mg/kg, oral, $n = 7$) or vehicle ($n = 6$) was administered in a daily basis during 14 days (61–74). On day 78, a second period of free choice between 10% (v/v) ethanol and water was re-instated for 24 days (78–101). On day 102, ethanol access was suppressed again and fenofibrate or vehicle administration was repeated for 14 days (103–116). After that, a third period of free choice between 10% (v/v) ethanol and water was re-instated on day 117, which lasted for 38 days (117–154).

Determination of Acetaldehyde in Blood

Acetaldehyde levels in arterial blood were determined as described previously (Karahanian et al., 2014). Briefly, rats under chronic ethanol consumption that were treated with fenofibrate (25, 50, 100 mg/kg/day) or vehicle for 14 days were deprived for ethanol access for 24 h to allow complete elimination of ethanol and acetaldehyde from the blood. At day 75 ethanol (1 g/kg) was given orally (as a 20% solution in saline; **Figure 1**). Acetaldehyde in arterial blood was measured by head-space gas chromatography at 5, 10, 15, 30 and 60 min post-ethanol administration. After blood acetaldehyde determination, animals were sacrificed by decapitation and tissues (blood, liver and brain) were sampled. Blood was collected in EDTA-tubes and centrifuged at 1500× *g* at 4°C for 10 min to obtain plasma. Liver and brain were quickly excised, weighed and stored at −80°C for posterior analysis.

Quantification of Catalase Levels on Liver and Brain by Western Blot

Liver and brain tissues were homogenized in a pestle with 1% Triton X-100 in phosphate buffer [50 mM (pH 7.4)] containing a complete EDTA-free protease inhibitor cocktail (Pierce, Rockford, IL, USA). Cell debris was removed by centrifugation and protein content was determined with the Micro BCA protein assay kit (Pierce, Rockford, IL, USA). Samples were analyzed by western blot with an anti-catalase primary antibody (Pierce PA523246) and beta-actin (Pierce PA1183) plus a goat anti-rabbit secondary antibody conjugated to HRP (Pierce 31460). Total protein loaded per lane was adjusted to 100 μg. Blotting membranes were revealed for chemiluminiscence with Pierce ECL Western Blotting Substrate. As catalase is far more abundant in liver than in the brain, the exposure of the autoradiography films was adjusted separately to obtain clear signals in both samples.

Conditioned Place Preference

Adult male UChB rats had free access to two bottles containing 10% ethanol and water for 60 days at the same conditions described above. Ethanol access was then restricted for 14 days, and animals were divided into two groups: one group was treated with fenofibrate 50 mg/kg p.o. for 14 days, and the other group was given the vehicle as the control ($n = 7$ animals per group). At the start of fenofibrate treatment, alcohol-induced CPP was assessed according to the methodology described by Quintanilla and Tampier (2011). Briefly, animals were placed in boxes with three compartments separated by removable guillotine doors. One compartment is painted black, the other is white and the central compartment is painted gray. The procedure had three phases: preconditioning, conditioning and post-conditioning. In the preconditioning phase, three sessions were conducted 24 h apart to determine the initially non-preferred

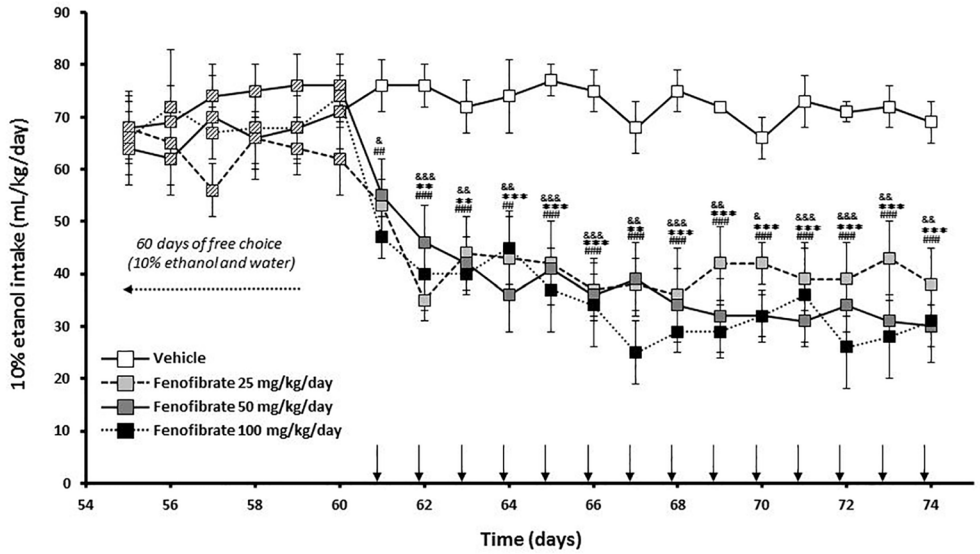

FIGURE 2 | Ethanol voluntary intake of fenofibrate-treated animals. UChB rats were given 24 h free choice between 10% v/v ethanol and water for 60 days. Starting at day 61, 25, 50 or 100 mg/kg/day of fenofibrate (n = 7) or vehicle (n = 7) were given orally for 14 days (arrow). Ethanol intake was recorded daily, every 24 h. Deviations shown are SEM. two-way ANOVA test indicates that alcohol intake in the fenofibrate-treated groups was significantly lower than in the control group ($^{\&}p < 0.05$, $^{\&\&}p < 0.01$, $^{\&\&\&}p < 0.001$ for fenofibrate 25 mg/kg/day vs. vehicle; $^{**}p < 0.01$, $^{***}p < 0.001$ for fenofibrate 50 mg/kg/day vs. vehicle; $^{\#\#}p < 0.01$, $^{\#\#\#}p < 0.001$ for fenofibrate 100 mg/kg/day vs. vehicle).

side of the apparatus for each animal. In each session, rats were placed in the passageway leading to the conditioning chambers for 15 min. The time each rat spent in each chamber was recorded. During the conditioning phase (days 4–14), guillotine doors separated the compartments so the rats were confined to one side of the conditioning apparatus. Rats were administered ethanol orally (1 g/kg), placed immediately in their less preferred compartment and left there for 15 min. On alternate days, the animals were administered saline and placed in their preferred compartment. As a result of this conditioning schedule, ethanol has been paired five times with the less preferred compartment. The post-conditioning phase began 24 h after the last conditioning trial: a 15 min choice test was performed with no administrations in which the rats could move freely between the two chambers, and the time spent by each rat in the drug-paired compartment was recorded. Data are expressed as percentage of total time spent in the ethanol-paired compartment.

Determination of Serum Transaminases

After treatment with fenofibrate or vehicle for 14 days, blood was extracted to determine the levels of alanine transferase (ALAT) and aspartate transaminase (ASAT) to assess liver damage (ALAT/GPT and ASAT/GOT Kits, Valtek Diagnostics, Chile). The activity was calculated as International Units/liter (IU/L), according to the indications of the supplier.

Statistical Analyses

Data are expressed as means ± SEM. Statistical differences are analyzed by Student's t-test or two-way analysis of variance (ANOVA) followed by Bonferroni's multiple comparison *post hoc* test. A level of $p < 0.05$ was considered for statistical significance.

RESULTS

Effect of Fenofibrate Administration on Voluntary Intake of Ethanol and Blood Acetaldehyde Levels

Twenty-eight male UChB rats were allowed to voluntarily drink ethanol 10% (v/v) or water for 60 days until consumption was stabilized at ∼70 ml ethanol (10%)/kg/day. The mean daily ethanol consumption of the animals between 1 day and 60 days was 68.9 ± 1.4 mL of 10% ethanol/kg/day. Starting at day 61, animals were divided into four groups (n = 7 animals per group) given fenofibrate at a daily oral dose (25, 50 or 100 mg/kg/day) or vehicle for 14 days. The effect of repeated administration of fenofibrate on ethanol intake was evaluated using a 24 h continuous-access paradigm. As shown in **Figure 2**, ethanol intake quickly decreases after fenofibrate administration, attaining 60% reduction after 2 days for all doses of fenofibrate ($F_{(3,336)}$ = 127.0, $p < 0.001$ for drug effect). This decrease remained constant throughout the remaining days of fenofibrate treatment. There were no statistically significant differences between fenofibrate groups ($F_{(2,252)}$ = 3.755, $p > 0.05$ for drug effect).

Figure 3 shows that 5 min after the administration of an oral ethanol dose (1 g/kg) to fenofibrate-treated UChB rats, a marked 5-fold elevation of acetaldehyde levels in arterial blood was verified for the three fenofibrate doses tested in comparison to vehicle treated rats (from 15 μM up to 80–100 μM,

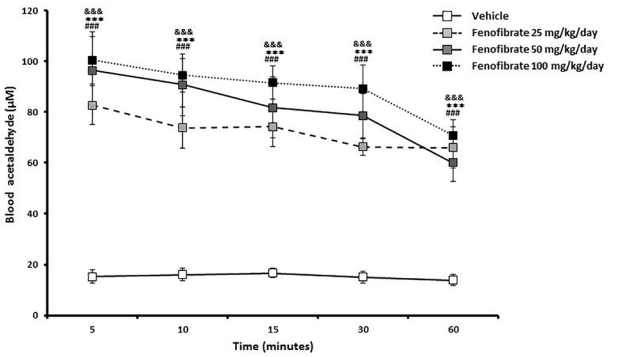

FIGURE 3 | Blood acetaldehyde levels in fenofibrate-treated animals after a single oral dose of ethanol. UChB rats were allowed to voluntarily drink 10% v/v alcohol and water for 60 days on a 24-h basis. On the subsequent 14 days, 25, 50 or 100 mg/kg/day fenofibrate was given orally to three groups (n = 7) while the control group (n = 7) was given vehicle. Thereafter, animals were deprived from ethanol access for 24 h and ethanol (1 g/kg) was administered orally to both fenofibrate-treated and control (vehicle) animals. Blood samples for acetaldehyde measurement were drawn from the carotid artery at different times. Deviations shown are SEM.; two-way ANOVA test indicates that blood acetaldehyde levels in the fenofibrate-treated group were significantly higher than in the control group ($^{\&\&\&}p < 0.001$ for fenofibrate 25 mg/kg/day vs. vehicle; $^{***}p < 0.001$ for fenofibrate 50 mg/kg/day vs. vehicle; $^{\#\#\#}p < 0.001$ for fenofibrate 100 mg/kg/day vs. vehicle).

$F_{(3,120)} = 90.87$, $p < 0.001$ for drug effect). A two-way ANOVA analysis of time-course acetaldehyde levels for fenofibrate groups showed that they were statistically different ($F_{(2,90)} = 4.330$, $p < 0.05$ for drug effect), but a posthoc test of individual time-points showed no significant differences between them. Blood acetaldehyde levels remained significantly elevated in fenofibrate-treated rats beyond 1 h after ethanol administration.

Figure 4 shows the evolution of body weight of the same group of UChB rats used to study the effects of fenofibrate on voluntary ethanol intake. Results show that fenofibrate administration at doses of 25 and 50 mg/kg/day did not induce changes in animal body weight compared to the vehicle group ($F_{(2,36)} = 0.7778$, $p > 0.05$ for drug effect). By contrast, administration of 100 mg/kg/day of fenofibrate reduced body weight compared to vehicle treated animals ($F_{(1,24)} = 16.44$, $p < 0.001$ for drug effect). Animals showed no signs of sickness after fenofibrate treatment. Fenofibrate-treated animals compensate their decreased consumption of ethanol solution by increasing water intake (data not shown).

Effect of Fenofibrate Administration on Voluntary Intake of Saccharin

Taking into account that the fenofibrate dose of 25 mg/kg/day achieved the lower reduction of ethanol consumption (**Figure 2**) and lower blood acetaldehyde levels (**Figure 3**) and that fenofibrate dosing of 100 mg/kg/day produced alterations in the body weights of the animals (**Figure 4**), we decided to continue the following experiments with a dose of 50 mg/kg/day. In the case of saccharin consumption, animals were offered a free choice between 0.2% w/v saccharin solution and water, from two

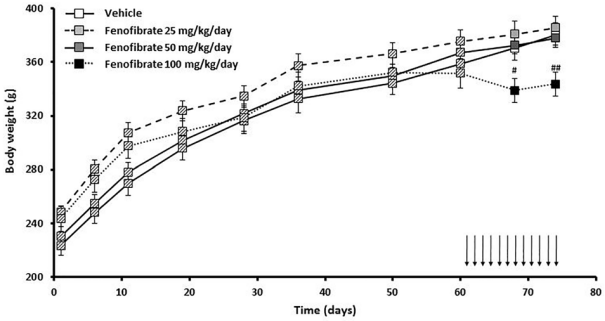

FIGURE 4 | Body weight evolution of fenofibrate-treated animals. Body weight was measured weekly in the group of UChB rats used to study the effects of fenofibrate on voluntary ethanol intake. Arrows indicate the daily administration of fenofibrate (25, 50 or 100 mg/kg/day) or vehicle to the animals. Deviations shown are SEM ($^{\#}p < 0.05$, $^{\#\#}p < 0.01$ for fenofibrate 100 mg/kg/day vs. vehicle).

graduated bottles. After 60 days, 0.2% saccharin consumption averaged 108 ml/kg/day. The daily saccharin consumption prior to fenofibrate administration (days 1–60) was not different between the two groups ($F_{(1,720)} = 0.48$, $p = 0.48$; **Figure 5**). At that time, fenofibrate (50 mg/kg/day, p.o.) or vehicle were administered for 14 days, as described in Materials and Methods. As it can be seen in **Figure 5**, saccharin consumption was reduced in the first 3 days of fenofibrate or vehicle administration in both groups. After those first 3 days, saccharin intake returned to pre-treatment levels in the control group (the apparent difference between pre- and post-vehicle administration is not statistically significant ($F_{(1,280)} = 2.6$, $p > 0.05$). However, in the fenofibrate-treated group, saccharin consumption raised only up to 65% of pre-treatment levels ($F_{(1,140)} = 36.25$, $p < 0.0001$). Fenofibrate-treated animals compensate their decreased consumption of saccharin solution by increasing water intake (data not shown).

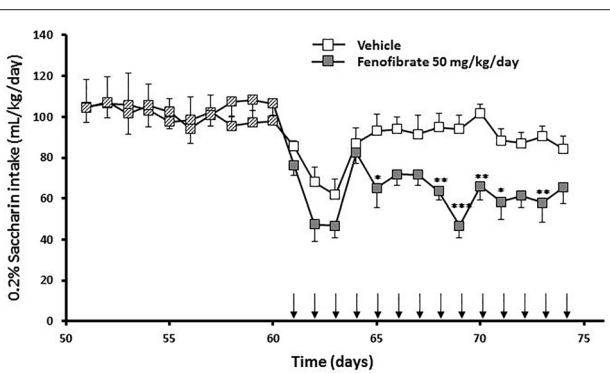

FIGURE 5 | Saccharin consumption of fenofibrate-treated animals. UChB rats were allowed to voluntary drink 0.2% w/v saccharin and water for 60 days on a 24-h basis. After this period, 50 mg/kg/day of fenofibrate (n = 7) or vehicle (n = 7) was given orally for 14 days (arrows). Saccharin intake was recorded daily, every 24 h. Deviations shown are SEM.; two-way ANOVA test indicates that saccharin intake in the fenofibrate-treated group was significantly lower than in the control group from day 65 onwards ($^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$).

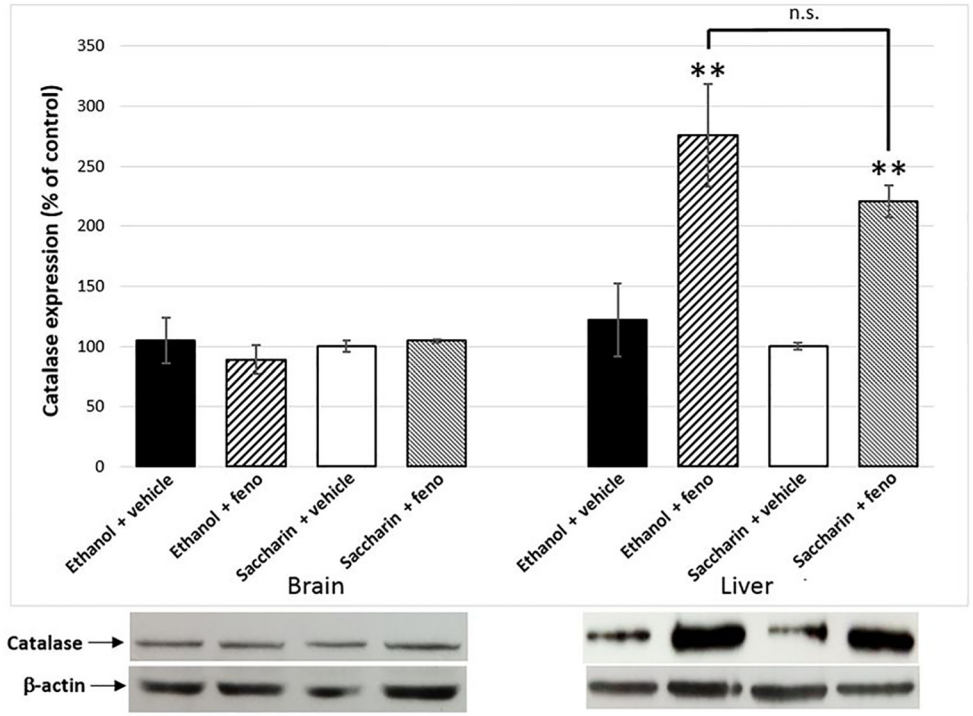

FIGURE 6 | Catalase expression in liver and the brain of fenofibrate-treated animals. Catalase levels were determined by western blot in brain and liver of ethanol or saccharin drinking rats (treated with fenofibrate or vehicle; $n = 7$ for each group). Catalase levels were normalized against β-actin. Bars represent the normalized densitometric quantification of the bands. Catalase expression of the saccharin group without fenofibrate administration was used as control (100%). $^{**}p < 0.01$ with respect to control; n.s. = difference statistically non-significant.

Effect of Fenofibrate on Catalase Levels in Liver and Brain

One of the effects of the administration of fenofibrate is the increase of catalase activity in the liver. In previous studies, we observed a 2.5-fold increase in liver catalase activity in fenofibrate-treated animals (Karahanian et al., 2014). As catalase-generated acetaldehyde in the brain is rewarding rather than aversive (Karahanian et al., 2011; Israel et al., 2015), it was also important to test if fenofibrate increases catalase levels in this organ. Western blot analysis showed ∼2.5-fold increase of catalase in the liver of fenofibrate-treated animals (**Figure 6**). Conversely, catalase levels were unaltered in the brain (**Figure 6**). Chronic ethanol or saccharin consumption by UChB rats did not induce significant changes in catalase levels in neither the liver nor brain.

Effect of Fenofibrate on Ethanol-Induced Conditioned Place Preference

It has been reported that after 60 days of voluntary alcohol drinking, UChB rats develop ethanol-induced CPP when administered daily doses of 1 g/kg ethanol (Quintanilla and Tampier, 2011). As acetaldehyde generated by the upregulation of catalase would produce an ethanol-avoiding effect, we expected that fenofibrate would affect ethanol-induced CPP in UChB rats. As shown in **Figure 7**, animals treated with fenofibrate or vehicle did not present any significant difference in the time spent in the less preferred side before conditioning (15.1 ± 2.3 vs. 15.9 ± 3.9% of time; *t*-test: $t = 0.18$, $df = 12$, $p = 0.85$, white bars). On alternate days, animal received five conditioning sessions on which ethanol (1 g/kg, p.o.) was administered and placed for 15 min in the less preferred side. The postconditioning session revealed that rats treated with vehicle developed a marked CPP, expressed as a 2.4-fold increase in the time spent in the ethanol-paired compartment compared to the preconditioning value (15.9 ± 3.9 vs. 38.7 ± 12.1% of time; *t*-test: $t = 1.8$, $df = 12$, $p < 0.05$). The postconditioning time spent in the ethanol-paired compartment by fenofibrate-treated rats showed that fenofibrate prevented the development of ethanol-induced CPP (14.7 ± 2.6 vs. 18.8 ± 6.3% of time; *t*-test: $t = 0.6$, $df = 12$, $p = 0.27$).

Effect of Fenofibrate Administration during Periods of Alcohol Withdrawal

We were also interested in investigating whether fenofibrate treatment would have any medium or long-term action on ethanol consumption after its administration. To do this, UChB rats were exposed for 60 days to a free-choice between 10% ethanol and water, after that animals were withdrawn from ethanol access (18 days) and at that time the administration of fenofibrate was started at a daily dose of 50 mg/kg/day for

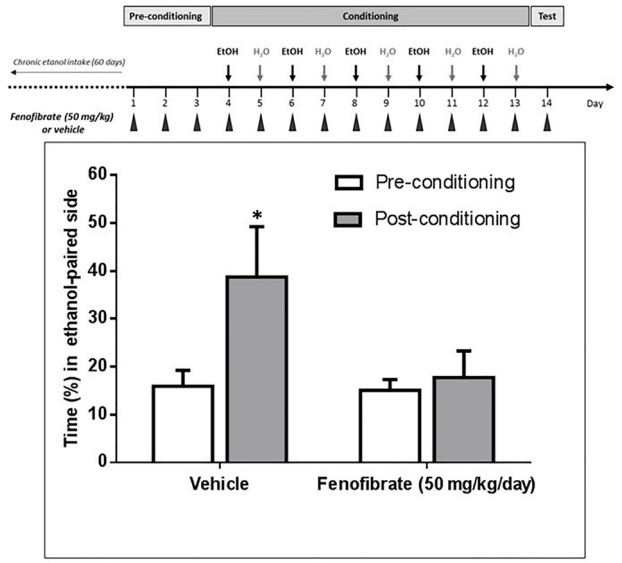

FIGURE 7 | Fenofibrate administration prevents ethanol-induced conditioned place preference (CPP) in UChB rats. CPP was not generated by ethanol administration (1 g/kg oral) in UChB rats treated with fenofibrate. In the control group, CPP was clearly developed. Values represent mean time (±SEM) spent in the ethanol-paired side during the pre-conditioning and post-conditioning phases (*$p < 0.05$). $n = 7$ per group.

14 days. Subsequently, the access to ethanol was restored for 24 days. **Figure 8** shows that after re-access, animals treated with fenofibrate markedly reduced its mean voluntary ethanol intake by 42% compared to animals treated with vehicle (3.7 ± 0.2 vs. 6.3 ± 0.1 g ethanol/kg/day; two-way ANOVA, $F_{(1,264)} = 288.2$, $p < 0.0001$). A Bonferroni's *post hoc* test shows that reduction of ethanol intake was statistically significant during the first 12 days of ethanol re-access.

At that time, a second cycle of deprivation/reaccess was performed, depriving alcohol and administering fenofibrate for 14 days, then withdrawing fenofibrate and allowing free consumption of ethanol for 38 days. As can be seen in **Figure 8**, after this second re-access, animals treated with fenofibrate reduced its mean voluntary ethanol intake by 41% compared to animals treated with vehicle (3.7 ± 0.1 vs. 6.2 ± 0.1 g ethanol/kg/day; two-way ANOVA, $F_{(1,418)} = 311, 5$, $p < 0.0001$). A Bonferroni's *post hoc* test shows that reduction of ethanol intake in this second re-access was statistically significant during the first 15 days.

Effect of Fenofibrate Treatment on Serum Transaminases

The effect of fenofibrate administration in conjunction with voluntary chronic alcohol consumption on liver damage had not been studied. It was also important to determine if co-administration of ethanol and fenofibrate produce toxic effects in the liver that could affect the behavior of animals. To assess this effect, we evaluated as markers of liver damage the activity of the enzymes ASAT and ALAT. As shown in **Figure 9**, there are no differences in ASAT and ALAT activity between ethanol, ethanol plus fenofibrate, saccharin or saccharin plus fenofibrate treatments, indicating that no damage to the liver occurs when both ethanol and fenofibrate are administered at the same time. The apparent differences in ALAT activity between ethanol and saccharin groups are not statistically significant.

DISCUSSION

In a previous report, we showed that fenofibrate is able to reduce voluntary alcohol consumption by 70% in high alcohol-drinker UChB rats (Karahanian et al., 2014). We also showed that fenofibrate treatment produced a 2.5-fold increase in catalase activity in the liver and a 10-fold increase (70–95 μM) in blood acetaldehyde levels after animals were administered an oral dose of 1 g/kg ethanol. This excess of systemic acetaldehyde then would produce an aversive effect towards ethanol consumption.

However, we cannot rule out the possibility that fenofibrate exerts part of its effect at the CNS level (e.g., by altering the preference and/or the hedonistic effect of ethanol). This latter hypothesis was proposed by Ferguson et al. (2014), as they found that fenofibrate produced alterations of neuropeptide and dopaminergic gene expression in the amygdala. Indeed, fenofibrate is able to reach the brain (Weil et al., 1988; Blednov et al., 2015). With these antecedents, we had proposed that the reduction of alcohol intake seen after the administration of PPARα agonists could be a dual phenomenon: (i) PPARα activation is important in the liver, where an increase in catalase activity leads to blood acetaldehyde accumulation whose aversive effects produce a decrease in alcohol intake; and (ii) PPARα could have central effects altering neuronal circuits that are important for the ethanol drinking behavior (Karahanian et al., 2015). In order to determine the contribution of hepatic or/and CNS effects in the reduction of ethanol intake elicited by fenofibrate, we measured alcohol and saccharin intake in UChB rats after administration of fenofibrate (50 mg/kg/day). As it can be seen in **Figure 2**, UChB rats voluntary drink ∼70 ml of 10% ethanol solution/kg/day. The next after starting the administration of a daily oral doses of 25, 50 or 100 mg/kg fenofibrate, alcohol intake decreased by 60%. This reduced alcohol consumption remained basically constant throughout the 14 days of fenofibrate treatment. This is in total agreement with data we reported in a previous work (Karahanian et al., 2014). There were no differences between the three doses of fenofibrate tested. Accordingly, the three doses of fenofibrate tested produced a similar increase in blood acetaldehyde levels after the administration of a dose of alcohol (1 g/kg) to the animals (80–100 μM vs. 15 μM in controls; **Figure 3**).

Interestingly, while the 25 and 50 mg/kg/day doses did not produce effects on the body weight of the animals, the dose of 100 mg/kg/day produced a decrease in this parameter (**Figure 4**). It is possible that this higher dose has caused discomfort, or some type of damage or simply a lower consumption of food in the animals. In any case, it has been reported that in humans

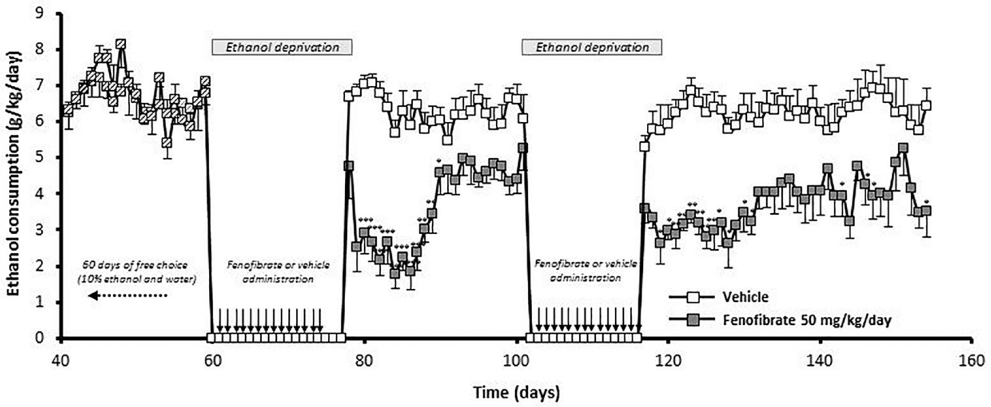

FIGURE 8 | The administration of fenofibrate in periods of alcohol deprivation elicit a persistent reduction of post-deprivation ethanol consumption. Rats that consumed alcohol chronically for 60 days, were subjected to two cycles of ethanol withdrawal plus fenofibrate administration followed by re-access to ethanol without fenofibrate administration. Fenofibrate-treated animals reduced significantly its voluntary ethanol intake compared to vehicle-treated animals (two-way ANOVA test; $p < 0.0001$). Black arrows indicate the administration of fenofibrate (50 mg/kg/day) or vehicle to the animals. Deviations shown are SEM (*$p < 0.05$; **$p < 0.01$; ***$p < 0.001$).

only 5% of patients suffer from gastrointestinal discomfort resulting from treatment with fenofibrate (Mahley and Bersot, 2001).

In the case of saccharin-drinking rats, the administration of fenofibrate lowered saccharin consumption by 35% (**Figure 5**). Therefore, we suggest that a significant part of the effect of fenofibrate on the reduction in ethanol intake could take place at the central level. There is evidence that ethanol and palatable foods (e.g., sweet taste) ingestion share common mechanisms involving μ-opioid receptors and dopaminergic transmission in the brain reward system (Di Chiara, 1998; Kelley et al., 2002; Hajnal et al., 2004). Tampier and Quintanilla (2005) showed that UChB rats (bred for their high ethanol intake) have a higher preference for saccharin intake than UChA rats (low alcohol drinking). Furthermore, a long-term exposure to a 10% alcohol solution containing 0.2% saccharin induced a significant increase in alcohol consumption in UChB rats once saccharin was faded out, whereas alcohol consumption in UChA rats returned to the previous low value (Tampier and Quintanilla, 2005). In the same line, when UChB rats exposed for 3 months to a free choice between 10% ethanol and water were offered with a third bottle containing 0.2% saccharin, they maintained their levels of ethanol consumption but showing a 2.3-fold increase in their consumption of saccharin solution (Tampier and Quintanilla, 2009). These results suggest the existence of common neuronal mechanisms determining the rewarding properties of ethanol and saccharin.

While there is a direct relationship between the activation of PPARα mediated by fenofibrate with an increased catalase activity in the liver and subsequent aversion to alcohol intake due to increased levels of ethanol-derived acetaldehyde in blood, the connection between PPARα activation and the decrease in the rewarding properties of ethanol has not a straightforward explanation. PPARα is expressed throughout the central nervous system, including the midbrain (Cullingford et al., 1998) and the nucleus accumbens core and shell (Moreno et al., 2004); this last area is essential for the rewarding properties of many drugs of abuse. Ferguson et al. (2014) reported that fenofibrate produces several changes in genes related to synaptic transmission in brain regions relevant to addictive behaviors (amygdala and prefrontal cortex). However, a mechanistic relation between PPARα activation in the brain and changes in the expression patterns of those genes is still lacking. Dopamine neurons in the ventral tegmental area (VTA) express nicotinic acetylcholine receptors (Clarke et al., 1985), whose activation lead to an increased dopaminergic activity (Pidoplichko et al., 1997). It has been reported that activation of PPARα induces a yet-unidentified tyrosine kinase(s) which phosphorilates and negatively regulates β2-nicotinic acetylcholine receptors, thus decreasing the dopaminergic activity of VTA neurons (Melis et al., 2008, 2010). According to this, one possibility is that fenofibrate-mediated activation of PPARα would diminish dopamine release in the mesolimbic system, thus decreasing reward. The effect of fenofibrate at the central level would not be surprising, since it has been described that PPARα activation leads to a lower consumption of nicotine, due to a decrease of its rewarding properties (Mascia et al., 2011; Panlilio et al., 2012). In these studies, PPAR-α agonists dose-dependently decreased nicotine-induced excitation of dopamine neurons in the VTA and nicotine-induced elevations of dopamine levels in the nucleus accumbens shell of rats.

Disulfiram inhibits ALDH2 in the liver, leading to a buildup of acetaldehyde in the periphery when the individual consumes alcohol. This excess of acetaldehyde finally produces aversion to alcoholic beverages. This effect is clearly observed in UChB rats when they initiate their alcohol intake (Tampier et al., 2008); however, when these rats have ingested ethanol chronically, disulfiram although having an identical effect in elevating blood

acetaldehyde levels is completely ineffective in reducing ethanol intake (Tampier et al., 2008). It is noteworthy that in these studies, acetaldehyde accumulated at even higher concentrations (150 μM) than those achieved in the studies presented here (80–100 μM). Similarly, in humans, the success rate of treatment with disulfiram is quite low because many patients do not show aversion to ethanol ingestion and further develop tolerance to disulfiram. Recent placebo-controlled clinical work and meta-analyses also show that disulfiram—as a drug (in blind studies)—is not different from placebo in reducing ethanol relapse in alcoholics (Skinner et al., 2014; Yoshimura et al., 2014). These studies might be taken as an indication that following chronic ethanol intake a systemic elevation of acetaldehyde does not inhibit ethanol consumption. However, increases in systemic acetaldehyde following the administration of an adenoviral vector (which does not enter the brain) coding for an antisense-RNA that inhibits ALDH2 synthesis or another adenoviral vector that also overexpresses ADH, markedly inhibited (50%–65%) voluntary ethanol intake of rats that had ingested ethanol chronically for 60–75 days (Ocaranza et al., 2008; Rivera-Meza et al., 2012). Rather, the lack of disulfiram effect on ethanol intake in animals fed alcohol chronically (and in alcoholics) may stem from the fact that disulfiram crosses the blood-brain barrier and also inhibits ALDH2 in the brain (Hellström and Tottmar, 1982), increasing acetaldehyde levels in this organ. A number of studies have shown that oppositely to its peripherally aversive actions, acetaldehyde possesses reinforcing and stimulating effects in the brain (Rodd et al., 2005). Thus, in ethanol-fed rats brain disulfiram might contribute an added hedonistic effect of ethanol to counter the aversive effects of acetaldehyde in the periphery. Therefore, in order to find a drug that is more effective than disulfiram in reducing alcohol consumption in patients, this drug should ideally stimulate the production of acetaldehyde in the periphery, and not in the brain. To clarify whether treatment with fenofibrate increases levels of catalase in the brain, we quantified catalase levels by western blot. Clearly, no increase in catalase is observed in the brain after the treatment with fenofibrate. In contrast, levels of catalase in the liver undergo a marked increase (**Figure 6**). As fenofibrate induced a reduction on voluntary ethanol intake in chronically drinker rats, we hypothesized that it can also interfere with the development of CPP. In fact, the administration of fenofibrate fully blocked CPP (**Figure 7**). It is interesting that fenofibrate was also able to reduce alcohol consumption when it was administered not simultaneously with ethanol (**Figure 8**). In animals that have been consuming alcohol for 60 days, when alcohol was withdrawn and fenofibrate was given for 14 days, a significant decrease in consumption was observed on the following days of ethanol re-access. A second cycle of deprivation/administration of fenofibrate and subsequent re-access to ethanol also showed a decrease in consumption, and this effect lasted longer than in the first cycle (12 days vs. 15 days). These observations have two possible explanations: (i) the levels of catalase in the liver may remain elevated several days after withdrawal of fenofibrate, so that the aversive reaction to peripheral acetaldehyde would continue to occur; or (ii) fenofibrate produced effects at the central level, decreasing the preference for ethanol when it was offered back to the animals. Further experiments are needed to clarify this point.

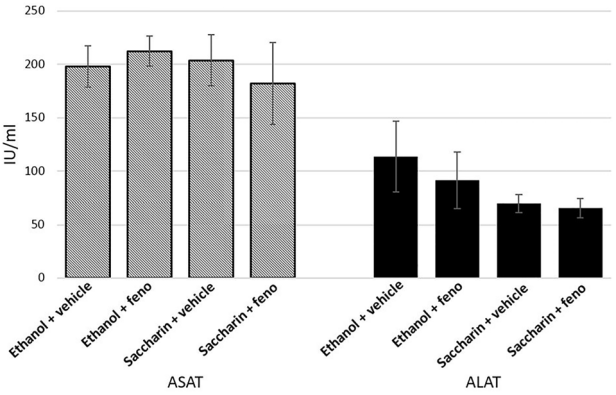

FIGURE 9 | Enzyme markers of liver damage when fenofibrate was administered alone or in conjunction with ethanol. Aspartate transaminase (ASAT) and alanine transferase (ALAT) levels were determined in serum from ethanol or saccharin drinking rats (treated or non-treated with fenofibrate; $n = 7$ for each group).

One of our concerns was that the co-administration of fenofibrate in conjunction with voluntary alcohol consumption could somehow produce liver damage. Under our experimental conditions, no changes in ASAT and ALAT were detected in the serum of ethanol-drinking fenofibrate-treated animals (**Figure 9**). Tsutsumi and Takase (2001) studied the effect of fenofibrate administration to rats subjected to forced consumption of alcohol (ethanol-containing liquid diet); they observed not only that fenofibrate administered together with ethanol did not produce liver damage, but that some indicators improved (ALAT decreased in serum). In addition, treatment with fenofibrate reverted the hepatic steatosis induced by the consumption of the ethanol-containing liquid diet.

In summary, fenofibrate produces a 60% decrease in voluntary ethanol intake in high-drinker rats and 35% reduction in saccharin intake. These results suggest that fenofibrate reduces ethanol appetence by a combination of peripherally (aversive) and central effects. Thus, fenofibrate administration can be further explored as a new pharmacological strategy for the treatment of alcoholism.

AUTHOR CONTRIBUTIONS

All authors contributed to the experiments. EK and MR-M wrote the article.

ACKNOWLEDGMENTS

This work was supported by Fondo Nacional de Desarrollo Científico y Tecnológico (FONDECYT; #1150850 to EK and #11130241 to MR-M) and Comisión Nacional de Investigación Científica y Tecnológica (CONICYT) ACT1411 to EK. The authors greatly appreciate Dr. Yedy Israel for the critical revision of the manuscript and to Mr. Juan Santibañez for the technical support.

REFERENCES

Arnaiz, S. L., Travacio, M., Llesuy, S., and Boveris, A. (1995). Hydrogen peroxide metabolism during peroxisome proliferation by fenofibrate. *Biochim. Biophys. Acta* 1272, 175–180. doi: 10.1016/0925-4439(95)00084-4

Blednov, Y. A., Benavidez, J. M., Black, M., Ferguson, L. B., Schoenhard, G. L., Goate, A. M., et al. (2015). Peroxisome proliferator-activated receptors α and γ are linked with alcohol consumption in mice and withdrawal and dependence in humans. *Alcohol. Clin. Exp. Res.* 39, 136–145. doi: 10.1111/acer.12610

Clarke, P. B., Schwartz, R. D., Paul, S. M., Pert, C. B., and Pert, A. (1985). Nicotinic binding in rat brain: autoradiographic comparison of [3H]acetylcholine, [3H]nicotine and [125I]-alpha-bungarotoxin. *J. Neurosci.* 5, 1307–1315.

Clouet, P., Henninger, C., Niot, I., Boichot, J., and Bezard, J. (1990). Short term treatment by fenofibrate enhances oxidative activities towards long-chain fatty acids in the liver of lean Zucker rats. *Biochem. Pharmacol.* 40, 2137–2143. doi: 10.1016/0006-2952(90)90246-h

Cullingford, T. E., Bhakoo, K., Peuchen, S., Dolphin, C. T., Patel, R., and Clark, J. B. (1998). Distribution of mRNAs encoding the peroxisome proliferator-activated receptor α, β and γ and the retinoid X receptor α, β and γ in rat central nervous system. *J. Neurochem.* 70, 1366–1375. doi: 10.1046/j.1471-4159.1998.70041366.x

Di Chiara, G. (1998). A motivational learning hypothesis of the role of mesolimbic dopamine in compulsive drug use. *J. Psychopharmacol.* 12, 54–67. doi: 10.1177/026988119801200108

Ferguson, L. B., Most, D., Blednov, Y. A., and Harris, R. A. (2014). PPAR agonists regulate brain gene expression: relationship to their effects on ethanol consumption. *Neuropharmacology* 86, 397–407. doi: 10.1016/j.neuropharm.2014.06.024

Hajnal, A., Smith, G. P., and Norgren, R. (2004). Oral sucrose stimulation increases accumbens dopamine in the rat. *Am. J. Physiol. Regul. Integr. Comp. Physiol.* 286, R31–R37. doi: 10.1152/ajpregu.00282.2003

Handler, J. A., and Thurman, R. G. (1988a). Hepatic ethanol metabolism is mediated predominantly by catalase-H_2O_2 in the fasted state. *FEBS Lett.* 238, 139–141. doi: 10.1016/0014-5793(88)80243-6

Handler, J. A., and Thurman, R. G. (1988b). Catalase-dependent ethanol oxidation in perfused rat liver. requirement for fatty-acid-stimulated H_2O_2 production by peroxisomes. *Eur. J. Biochem.* 176, 477–484. doi: 10.1111/j.1432-1033.1988.tb14305.x

Hellström, E., and Tottmar, O. (1982). Effects of aldehyde dehydrogenase inhibitors on enzymes involved in the metabolism of biogenic aldehydes in rat liver and brain. *Biochem. Pharmacol.* 31, 3899–3905. doi: 10.1016/0006-2952(82)90308-2

Henninger, C., Clouet, P., Cao Danh, H., Pascal, M., and Bezard, J. (1987). Effects of fenofibrate treatment on fatty acid oxidation in liver mitochondria of obese zucker rats. *Biochem. Pharmacol.* 36, 3231–3236. doi: 10.1016/0006-2952(87)90638-1

Israel, Y., Quintanilla, M. E., Karahanian, E., Rivera-Meza, M., and Herrera-Marschitz, M. (2015). The "first hit" toward alcohol reinforcement: role of ethanol metabolites. *Alcohol. Clin. Exp. Res.* 39, 776–786. doi: 10.1111/acer.12709

Karahanian, E., Quintanilla, M. E., Tampier, L., Rivera-Meza, M., Bustamante, D., Gonzalez-Lira, V., et al. (2011). Ethanol as a prodrug: brain metabolism of ethanol mediates its reinforcing effects. *Alcohol. Clin. Exp. Res.* 35, 606–612. doi: 10.1111/j.1530-0277.2011.01439.x

Karahanian, E., Quintanilla, M. E., Fernandez, K., and Israel, Y. (2014). Fenofibrate—a lipid-lowering drug—reduces voluntary alcohol drinking in rats. *Alcohol* 48, 665–670. doi: 10.1016/j.alcohol.2014.08.004

Karahanian, E., Rivera-Meza, M., Quintanilla, M. E., Muñoz, D., Fernández, K., and Israel, Y. (2015). PPARα agonists reduce alcohol drinking: do they act in the brain or in the liver? *Alcohol Alcohol.* 50, 717–718. doi: 10.1093/alcalc/agv060

Kelley, A. E., Bakshi, V. P., Haber, S. N., Steininger, T. L., Will, M. J., and Zhang, M. (2002). Opioid modulation of taste hedonics within the ventral striatum. *Physiol. Behav.* 76, 365–377. doi: 10.1016/s0031-9384(02)00751-5

Mahley, R. W., and Bersot, T. P. (2001). "Drug Therapy for Hypercholesterolemia and Dyslipidemia," in *Goodman and Gilman's the Pharmacological Basis of Therapeutics*, eds J. G. Hardman, L. E. Limbird (New York, NY: McGraw-Hill), 971–1002.

Mardones, J., and Segovia-Riquelme, N. (1983). Thirty-two years of selection of rats by ethanol preference: UChA and UChB strains. *Neurobehav. Toxicol. Teratol.* 5, 171–178.

Mascia, P., Pistis, M., Justinova, Z., Panlilio, L. V., Luchicchi, A., Lecca, S., et al. (2011). Blockade of nicotine reward and reinstatement by activation of alpha-type peroxisome proliferator-activated receptors. *Biol. Psychiatry* 69, 633–641. doi: 10.1016/j.biopsych.2010.07.009

Melis, M., Pillolla, G., Luchicchi, A., Muntoni, A. L., Yasar, S., Goldberg, S. R., et al. (2008). Endogenous fatty acid ethanolamides suppress nicotine-induced activation of mesolimbic dopamine neurons through nuclear receptors. *J. Neurosci.* 28, 13985–13994. doi: 10.1523/JNEUROSCI.3221-08.2008

Melis, M., Carta, S., Fattore, L., Tolu, S., Yasar, S., Goldberg, S. R., et al. (2010). Peroxisome proliferator-activated receptors-alpha modulate dopamine cell activity through nicotinic receptors. *Biol. Psychiatry* 68, 256–264. doi: 10.1016/j.biopsych.2010.04.016

Mizoi, Y., Tatsuno, Y., Adachi, J., Kogame, M., Fukunaga, T., Fujiwara, S., et al. (1983). Alcohol sensitivity related to polymorphism of alcohol-metabolizing enzymes in Japanese. *Pharmacol. Biochem. Behav.* 18, 127–133. doi: 10.1016/0091-3057(83)90159-4

Moreno, S., Farioli-Vecchioli, S., and Cerù, M. P. (2004). Immunolocalization of peroxisome proliferator-activated receptors and retinoid X receptors in the adult rat CNS. *Neuroscience* 123, 131–145. doi: 10.1016/j.neuroscience.2003.08.064

Ocaranza, P., Quintanilla, M. E., Tampier, L., Karahanian, E., Sapag, A., and Israel, Y. (2008). Gene therapy reduces ethanol intake in an animal model of alcohol dependence. *Alcohol. Clin. Exp. Res.* 32, 52–57. doi: 10.1111/j.1530-0277.2007.00553.x

Panlilio, L. V., Justinova, Z., Mascia, P., Pistis, M., Luchicchi, A., Lecca, S., et al. (2012). Novel use of a lipid lowering fibrate medication to prevent nicotine reward and relapse: preclinical findings. *Neuropsychopharmacology* 37, 1838–1847. doi: 10.1038/npp.2012.31

Pidoplichko, V. I., DeBiasi, M., Williams, J., and Dani, J. A. (1997). Nicotine activates and desensitizes midbrain dopamine neurons. *Nature* 390, 401–404. doi: 10.1038/37120

Quintanilla, M. E., Israel, Y., Sapag, A., and Tampier, L. (2006). The UChA and UChB rat lines: metabolic and genetic differences influencing ethanol intake. *Addict. Biol.* 11, 310–323. doi: 10.1111/j.1369-1600.2006.00030.x

Quintanilla, M. E., and Tampier, L. (2011). Place conditioning with ethanol in rats bred for high (UChB) and low (UChA) voluntary alcohol drinking. *Alcohol* 45, 751–762. doi: 10.1016/j.alcohol.2011.06.002

Rivera-Meza, M., Quintanilla, M. E., and Tampier, L. (2012). Reduction of ethanol consumption in alcohol-preferring rats by dual expression gene transfer. *Alcohol Alcohol.* 47, 102–108. doi: 10.1093/alcalc/agr161

Rodd, Z. A., Bell, R. L., Zhang, Y., Murphy, J. M., Goldstein, A., Zaffaroni, A., et al. (2005). Regional heterogeneity for the intracranial self-administration of ethanol and acetaldehyde within the ventral tegmental area of alcohol-preferring (P) rats: involvement of dopamine and serotonin. *Neuropsychopharmacology* 30, 330–338. doi: 10.1038/sj.npp.1300561

Skinner, M. D., Lahmek, P., Pham, H., and Aubin, H. J. (2014). Disulfiram efficacy in the treatment of alcohol dependence: a meta-analysis. *PLoS One* 9:e87366. doi: 10.1371/journal.pone.0087366

Steinberg, P., Schladt, L., Dienes, H. P., Timms, C., and Oesch, F. (1988). Microsomal and cytosolic epoxide hydrolases, the peroxisomal fatty acid beta-oxidation system and catalase. activities, distribution and induction in rat liver parenchymal and non-parenchymal cells. *Eur. J. Biochem.* 176, 39–45. doi: 10.1111/j.1432-1033.1988.tb14248.x

Tampier, L., and Quintanilla, M. E. (2005). Saccharin consumption and the effect of a long-term exposure to a sweetened alcoholic solution in high-(UChB) and low-(UChA) alcohol-drinking rats. *Alcohol* 37, 47–52. doi: 10.1016/j.alcohol.2005.10.002

Tampier, L., Quintanilla, M. E., and Israel, Y. (2008). Tolerance to disulfiram induced by chronic alcohol intake in the rat. *Alcohol. Clin. Exp. Res.* 32, 937–941. doi: 10.1111/j.1530-0277.2008.00658.x

Tampier, L., and Quintanilla, M. E. (2009). Effect of concurrent saccharin intake on ethanol consumption by high-alcohol-drinking (UChB) rats. *Addict. Biol.* 14, 276–282. doi: 10.1111/j.1369-1600.2009.00147.x

Tsutsumi, M., and Takase, S. (2001). Effect of fenofibrate on fatty liver in rats treated with alcohol. *Alcohol. Clin. Exp. Res.* 25, 75S–79S. doi: 10.1111/j.1530-0277.2001.tb02423.x

Weil, A., Caldwell, J., and Strolin-Benedetti, M. (1988). The metabolism and disposition of fenofibrate in rat, guinea pig and dog. *Drug Metab. Dispos.* 16, 302–309.

Yoshimura, A., Kimura, M., Nakayama, H., Matsui, T., Okudaira, F., Akazawa, S., et al. (2014). Efficacy of disulfiram for the treatment of alcohol dependence assessed with a multicenter randomized controlled trial. *Alcohol. Clin. Exp. Res.* 38, 572–578. doi: 10.1111/acer.12278

Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

frontiers in Behavioral Neuroscience

ORIGINAL RESEARCH
published: 01 May 2017
doi: 10.3389/fnbeh.2017.00071

Changes in Dopamine Transmission in the Nucleus Accumbens Shell and Core during Ethanol and Sucrose Self-Administration

Valentina Bassareo[1,2], Flavia Cucca[1], Roberto Frau[1] and Gaetano Di Chiara[1,2,3]**

[1]*Department of Biomedical Sciences, University of Cagliari, Cagliari, Italy,* [2]*Institute of Neuroscience, Cagliari Section, University of Cagliari, Cagliari, Italy,* [3]*National Institute of Neuroscience CNR, University of Cagliari, Cagliari, Italy*

Ethanol, like other substances of abuse, preferentially increases dopamine (DA) transmission in the rat nucleus accumbens (NAc) following passive administration. It remains unclear, however, whether ethanol also increases NAc DA transmission following operant oral self-administration (SA). The NAc is made-up of a ventro-medial compartment, the shell and a dorso-lateral one, the core, where DA transmission responds differentially following exposure to drugs of abuse. Previous studies from our laboratory investigated changes in dialysate DA in the NAc shell and core of rats responding for sucrose pellets and for drugs of abuse. As a follow up to these studies, we recently investigated the changes in NAc shell and core DA transmission associated to oral SA of a 10% ethanol solution. For the purpose of comparison with literature studies utilizing sucrose + ethanol solutions, we also investigated the changes in dialysate DA associated to SA of 20% sucrose and 10% ethanol + 20% sucrose solutions. Rats were trained to acquire oral SA of the solutions under a Fixed Ratio 1 (FR1) schedule of nose-poking. After training, rats were monitored by microdialysis on three consecutive days under response contingent (active), reward omission (extinction trial) and response non-contingent (passive) presentation of ethanol, sucrose or ethanol + sucrose solutions. Active and passive ethanol administration produced a similar increase in dialysate DA in the two NAc subdivisions, while under extinction trial DA increased preferentially in the shell compared to the core. Conversely, under sucrose SA and extinction DA increased exclusively in the shell. These observations provide unequivocal evidence that oral SA of 10% ethanol increases dialysate DA in the NAc, and also suggest that stimuli conditioned to ethanol exposure contribute to the increase of dialysate DA observed in the NAc following ethanol SA. Comparison between the pattern of DA changes detected in the NAc subdivisions under sucrose and ethanol SA likewise suggests that the NAc shell and core DA play different roles in sucrose as compared to ethanol reinforcement.

Keywords: mesolimbic system, self-administration, sucrose, ethanol, dopamine, shell, core

OPEN ACCESS

Edited by:
John D. Salamone, University of Connecticut, USA

Reviewed by:
Simona Cabib, Sapienza University of Rome, Italy
Akiko Shimamoto, Meharry Medical College, USA

****Correspondence:***
Valentina Bassareo
bassareo@unica.it
Gaetano Di Chiara
dichiara@unica.it

Received: *20 January 2017*
Accepted: *07 April 2017*
Published: *01 May 2017*

Citation:
Bassareo V, Cucca F, Frau R and Di Chiara G (2017) Changes in Dopamine Transmission in the Nucleus Accumbens Shell and Core during Ethanol and Sucrose Self-Administration. Front. Behav. Neurosci. 11:71. doi: 10.3389/fnbeh.2017.00071

INTRODUCTION

Drinking of alcohol plays an important social role in numerous cultures, with alcoholic beverages being legally available throughout the majority of countries worldwide. However, alcohol consumption may lead to dependance; indeed, the high prevalence of alcoholism in the general population represents a major social and health issue, and considerable

effort has been devoted on clarifying the neurobiological bases of this condition.

A large number of studies have focused on the ability of ethanol to stimulate *in vivo* dopamine (DA) transmission in the nucleus accumbens (NAc) of rats and mice (Imperato and Di Chiara, 1986; Weiss et al., 1993; Gonzales and Weiss, 1998; Melendez et al., 2002; Doyon et al., 2003, 2005; Tang et al., 2003; for review see Gonzales et al., 2004). The NAc, however, is not a homogeneous structure, being made up of a ventro-medial subdivision, the shell and a dorso-lateral one, the core, possessing different input-out connections and functions. Drugs of abuse such as cocaine, amphetamine, morphine, heroin, THC, MDMA and nicotine preferentially activate shell DA transmission following response non-contingent (passive) as well as response-contingent exposure (Pontieri et al., 1995, 1996; Tanda et al., 1997; Lecca et al., 2006a,b, 2007a,b; Aragona et al., 2008).

While the ability of response non-contingent ethanol to increase dialysate DA in the NAc, and in particular the shell, is well established (Di Chiara and Imperato, 1985; Imperato and Di Chiara, 1986; Di Chiara et al., 1996; Bassareo et al., 2003; Howard et al., 2008), it remains unclear whether the same also applies to response-contingent oral administration (self-administration, SA). Previous microdialysis studies in rats self-administering oral ethanol solutions have estimated the changes in DA transmission in the NAc by taking as basal (100%) values the levels of dialysate DA in samples collected in the rat's home cage (Weiss et al., 1993; Gonzales and Weiss, 1998; Doyon et al., 2003, 2005; Howard et al., 2009). However, transfer to the Skinner box equipped for microdialysis monitoring was found to increase *per se* dialysate DA in the NAc (Weiss et al., 1993; Gonzales and Weiss, 1998; Doyon et al., 2003, 2005; Howard et al., 2009). Accordingly, as highlighted by Gonzales and Weiss (1998), it is unclear to what extent the increase in dialysate DA observed in the NAc under ethanol SA is affected by the DA-activating influence of transfer from the home cage to the Skinner box (Weiss et al., 1993; Gonzales and Weiss, 1998; Doyon et al., 2003, 2005; Howard et al., 2009). On the other hand, in studies from the same group that distinguished the NAc shell from core, no increase of DA was observed, except for microdialysis probe placements at the border between the shell and core, an area that the same authors indicate as "shore" but devoid of anatomical and physiological identity (Howard et al., 2008). A further point of uncertainty in the paradigm adopted in the above studies arises from the failure of sucrose SA to increase dialysate DA in any subdivision of the NAc (Howard et al., 2009). This observation is in contrast with a large number of studies showing that sucrose SA increases dialysate DA, depending on the experimental conditions, specifically in the NAc shell, or in both the shell and core (Bassareo and Di Chiara, 1997, 1999; Brown et al., 2011; Cacciapaglia et al., 2012; Bassareo et al., 2015a,b,c).

This premise shows that it is still unclear if indeed oral ethanol SA increases DA transmission in the NAc, as estimated by microdialysis. In an attempt to shed light on this issue we compared changes in dialysate DA in the shell and core of rats trained to respond for oral ethanol (10% solution), sucrose (20% solution) and 10% ethanol in 20% sucrose solution. Rats were tested on three consecutive days under operant (active), extinction trial and passive ethanol presentation. This procedure has previously been used by our group in studies of sucrose pellet reinforcement (Bassareo et al., 2015a,b,c).

MATERIALS AND METHODS

Animals

Male Sprague-Dawley rats (Harlan Italy, Udine, Italy) weighing 250–275 g were housed in group of six per cage (h:20 cm × w:38 cm × l:59 cm) with standard chow (Stefano Morini, S. Polo D'Enza, RE, Italy) and water available *ad libitum*, for at least 1 week in the central animal room under constant temperature (23°C), humidity (60%) and a 12 h light/dark cycle (light on from 8 a.m. to 8 p.m.).

This study was carried out in accordance with the guidelines for care and use of experimental animals of the European Communities Council (2010/63/UE L 276 20/10/2010) and with Italian law (DL: 04.03.2014, N°26), and approved by the Organism for animal care of University of Cagliari (OPBA). Every effort was made to minimize suffering and reduce the numbers of animals used.

Surgery

Rats were anesthetized as previously reported by our group (Bassareo et al., 2015a). A guide cannula (Plasticone, Roanoke, VA, USA; Ø: 0,022 mm) was stereotaxically and unilaterally implanted, randomly in the left or in the right hemisphere according to the following coordinates: NAc shell (A: 2.0 mm; L: 1 mm from bregma, V: −3.6 mm from dura), NAc core (A: 1.6 mm; L: 1.9 mm from bregma, V: −3.4 mm from dura) according to the atlas of Paxinos and Watson (1998). Guide cannulae were plugged with a dummy cannula.

After surgery, rats were housed in individual cages (45 × 21 × 24 cm) under the same conditions mentioned above. Rats were left to recover for 10 days, and Gentamicin sulfate (40 mg/Kg s.c.) was administered over the first 5 days. Rats were handled once daily for 5 min throughout the training period to habituate them to contact with the operator and all procedures.

After recovery rats were fed daily with 20 g standard chow (Stefano Morini, S. Polo D' Enza, RE, Italy) and their weight maintained at approx. 95% their *ad libitum* weight. Water was available *ad libitum* throughout the duration of experiments.

Microdialysis

Probe Preparation

Microdialysis probes were prepared according to the method of Lecca et al. (2006a,b) and reported by us (Bassareo et al., 2015c), using AN69 membrane (Hospal Dasco, Italy). The length of the dialyzing portion of the probe was 1.5 mm. A new probe was used for each experimental session.

Microdialysis Experiments

At the beginning of each microdialysis session, microdialysis probes were connected to an infusion pump and perfused with Ringer's solution (147 mM NaCl, 4 mM KCl, 2.2 mM $CaCl_2$; see Lecca et al., 2006a on the use of 2.2 mM Ca^{2+} in the Ringer) at a constant rate of 1 μl/min. The dummy cannula was removed and the microdialysis probe inserted through the guide cannula. Dialysate samples (10 μl) were taken every 10 min and injected without purification into either a high-performance liquid chromatograph (HPLC) or an ultra HPLC (UHPLC; ALEXYS Neurotransmitter analyzer, Antec).

The HPLC was equipped with a reverse phase column (LC-18 DB, 15 cm, 5 μm particle size, Supelco) and a coulometric detector (ESA, Coulochem II, Bedford, MA, USA) to quantify DA. The first electrode of the detector was set at +125 mV (oxidation) and the second at −175 mV (reduction). The composition of the mobile phase was: 50 mM NaH_2PO_4, 0.1 mM Na_2-EDTA, 0.5 mM n-octyl sodium sulfate, 15% (v/v) methanol, pH 5.5 (obtained adding Na_2HPO_4). Under these conditions, sensitivity of the assay for DA was 5 fmol/sample. The UHPLC was equipped with a NeuroSep (C18 110, 1.0 × 100 mm, 1.7 μm) column and an electrochemical amperometric detector (DECADE II SCC). Composition of the mobile phase was: 100 mM phosphoric acid, 100 mM citric acid, 0.1 mM EDTA, acetonitrile 8% v/v, 3 mM. Using these conditions sensitivity of the assay for DA was 5 fmol/sample. At the end of each microdialysis session the probe was removed and the guide cannula was once again plugged with a sterilized dummy cannula.

Sucrose

The sucrose solution (20%, w/v) was obtained using granulated sugar (SADAM S.p.A., Villasor, Cagliari, Italy) and tap water.

Ethanol

The 10% ethanol solution (w/v) was obtained using 95° ethanol (Farmitalia Carlo Erba, Milan, Italy) and tap water or 20% sucrose solution.

Training

Ten days after guide cannula implant, rats were trained every day for 3 weeks, with the exception of weekends. Sessions lasted 1 h and took place between 9.00 am and 2.00 pm in acoustically isolated and ventilated operant cages (Coulbourn Instruments, Allentown, NJ, USA). Two nose-poke holes were placed on one wall, 2 cm from the cage floor. The active nose-poke was illuminated by a green-yellow light, and the inactive one by a red light. The liquid solenoid valve for solution delivery was inserted between the nose-pokes holes and a light placed above it. A loudspeaker emitting a tone of 4500 Hz was located on the same wall.

Rats were trained to respond for 20% sucrose solution, for 10% ethanol in 20% sucrose solution, or for 10% ethanol solution under a Fixed Ratio 1 (FR1) schedule. Each active nose poke = 0.28 ml of each solution. The number of nose-pokes performed and rewards earned were recorded by Graphic State 2 software, Coulbourn Instruments, Whitehall, PA, USA.

Each 1-h session was made up of a cyclic alternation of three phases:

- Phase 1, lasting 15 s, during which the house light and nose poke lights were turned on and a tone was activated to signal reward availability. Failure to respond correctly for more than 15 s resulted in switch off of visual and auditory cues and start of phase 3, bypassing phase 2.
- Phase 2: 0.28 ml of 20% sucrose solution, or 10% ethanol in 20% sucrose solution, or 10% ethanol solution were delivered into the valve and the light above the valve was switched on. After 5 s phase 3 was initiated.
- Phase 3 (time out): all cues were turned off and reward was not available for 7 s.

A significant difference ($p < 0.05$) between active and inactive nose pokes for at least five consecutive sessions (1 session × day) was taken as criterion for full training.

Microdialysis after Training

Following completion of training rats were tested in three daily microdialysis sessions performed on three consecutive days. Sessions were started as soon as DA basal dialysate levels had stabilized (i.e., after approximately 1 h).

First day: FR1 responding for 20% sucrose solution, or for 10% ethanol in 20% sucrose solution, or for 10% ethanol solution.

Second day: responding under extinction i.e., by substituting tap water for ethanol or sucrose solutions, but in the presence of all the stimuli preceding and following each response.

Third day: non-contingent presentation of 20% sucrose, 10% ethanol in 20% sucrose or 10% ethanol solutions at the same mean rate of operant responding in the absence of discriminative cues signaling reward availability.

Histology

At the end of all experimental procedures, rats were anesthetized as previously reported (Bassareo et al., 2015b), and the brains removed and postfixed for 5 days. Brains were cut in 100-μm-thick serial coronal slices on a Vibratome (Technical Products International, Saint Louis, MO, USA) to establish the location of dialysis probes. Probe location was reconstructed and referred to the atlas of Paxinos and Watson (1998; **Figure 1**).

Statistics

Statistical analysis was carried out using Statistica for Windows. Basal dialysate DA was calculated as the mean of the last three consecutive samples differing by no more than 10%, collected during the 60-min period preceding each experimental session. Inter-group comparison of basal dialysate DA values, expressed as femtomoles per 10-μl dialysate, was performed using one or two-way ANOVA. Changes in dialysate DA were expressed as percentage of respective baseline values and were analyzed using two-way ANOVA for repeated measures. Cumulative nose-pokes registered during training and during experiments were analyzed by three-way ANOVA. The number of nose-pokes registered during each 10 min sampling were compared by

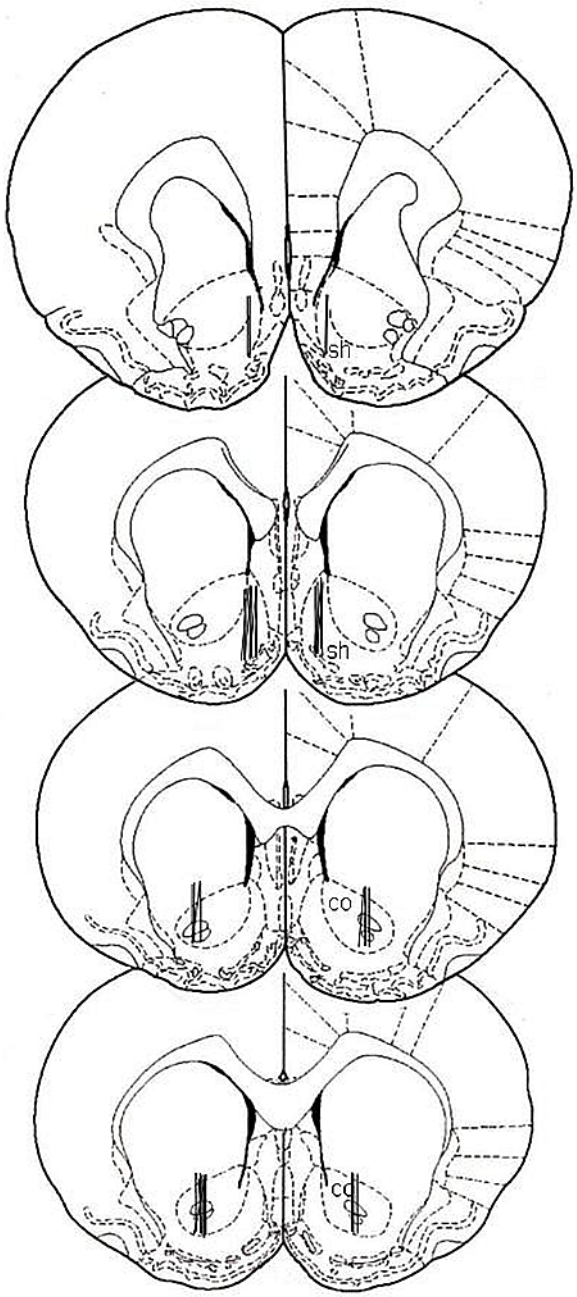

FIGURE 1 | Localization of dialysis probes (dialysis portion) within the nucleus accumbens (NAc) shell and core according to Paxinos and Watson (1998). sh, shell; co, core.

two-way ANOVA between rats whose probes were implanted either in the shell or the core and combined in the absence of statistical differences. The amount of solutions (in ml) and ethanol intake (in g/Kg) were analyzed using two-way ANOVA for repeated measures.

The results of treatments showing significant overall changes were subjected to *post hoc* Tukey's test, with statistical significance set at $p < 0.05$.

RESULTS

Basal values of dialysate DA (fmoles/sample, means ± SEM) corresponded to 46 ± 3 (N = 43) in the shell and 48 ± 3 (N = 48) in the core, with no differences between the two areas ($F_{(1,89)} = 3.17$; $p = 0.08$). Values obtained were consistent across all groups. Accordingly, two-way ANOVA of basal dialysate DA obtained from the different groups revealed no significant differences between reward utilized or microdialysis sessions either in the shell ($F_{\text{reward } 2,34} = 0.02$; $p = 0.98$; $F_{\text{sessions } 2,34} = 0.60$; $p = 0.55$; $F_{\text{reward} \times \text{sessions } 4,34} = 0.34$; $p = 0.85$) or the core ($F_{\text{reward } 2,39} = 0.001$; $p = 1$; $F_{\text{sessions } 2,39} = 0.29$; $p = 0.75$; $F_{\text{reward} \times \text{sessions } 4,39} = 0.12$; $p = 0.97$). The lack of statistically significant differences in absolute basal levels between groups justifies the subsequent analysis of changes in dialysate DA as % of baseline.

Responding for 20% Sucrose

Training

Figure 2 shows the number of cumulative active and inactive nose pokes performed during training for FR1 responding for sucrose, and on the first and second experiment days. Nose-poking increased selectively on the active hole.

Three-way ANOVA showed a main effect of nose poke ($F_{(1,12)} = 529.3$; $p < 0.01$), days ($F_{(16,192)} = 53.7$; $p < 0.01$) and a nose poke × days interaction ($F_{(16,192)} = 47.3$; $p < 0.01$). Tukey's test revealed no differences in the temporal profile of acquisition between the shell- and core-implanted groups.

Two-way ANOVA of the amount of solution (ml) consumed by rats showed a main effect of days ($F_{(16,80)} = 28.4$; $p < 0.01$). *Post hoc* test revealed that the amount of 20% sucrose solution (ml) consumed by rats increased selectively during the training period, but did not reveal any difference between shell- and core-implanted rats (**Figure 3**).

Monitoring dialysate DA in FR1 trained rats.

Operant Session

Figures 4A,D shows the time-course of dialysate DA in the NAc shell and core and the number of active nose-pokes performed during FR1 responding for 20% sucrose solution.

Responding for 20% sucrose solution increased dialysate DA in the shell alone. Core DA remained unchanged throughout the session. A peak in shell DA was displayed at the first sample, and returned to basal levels at the 3rd sample. Responding remained high at four additional samples taken subsequent to the increase of DA in the shell.

Two-way ANOVA showed an effect of area ($F_{(1,6)} = 32.1$; $p < 0.01$), time ($F_{(9,54)} = 44.5$; $p < 0.01$) and an area × time interaction ($F_{(9,54)} = 32.4$; $p <= 0.01$). *Post hoc* test revealed an increase of dialysate DA in the NAc shell but not in the core.

Extinction Session

Figures 4B,E shows the time-course of dialysate DA in the NAc shell and core and of active nose-pokes under extinction in the presence of cues signaling sucrose availability and associated to sucrose delivery.

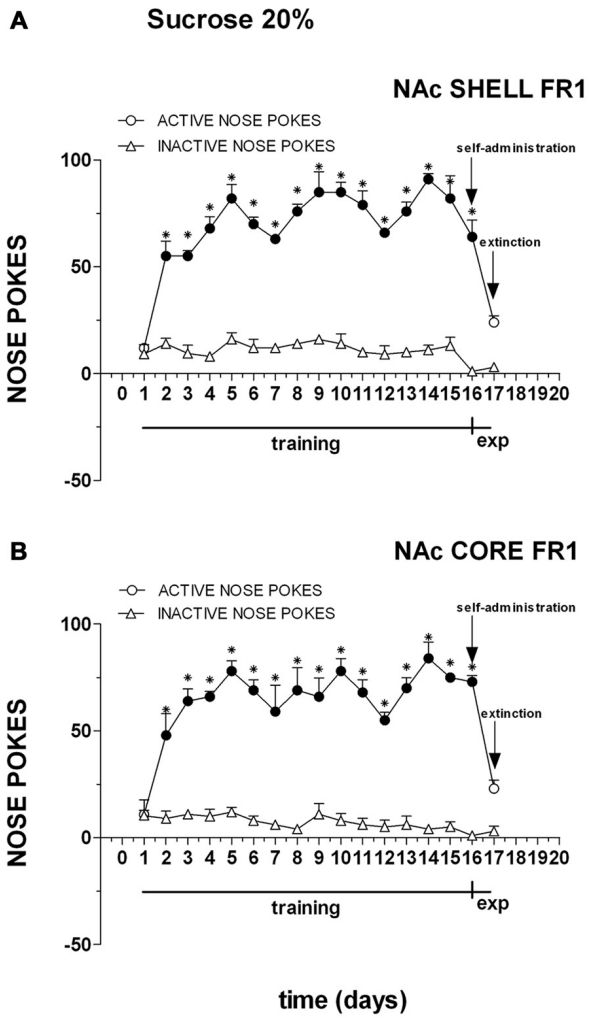

FIGURE 2 | Cumulative active (circles) and inactive (triangles) nose-pokes during training to respond for 20% sucrose under an fixed ratio 1 (FR1) schedule and during dialysis experiments. Data are means ± SEM of the results obtained in rats implanted with guide cannulas in the NAc shell **(A)** (N = 4) and rats in the NAc core **(B)** (N = 4). Filled symbols, $p < 0.05$ vs. 1st day; *$p < 0.05$ vs. inactive nose pokes.

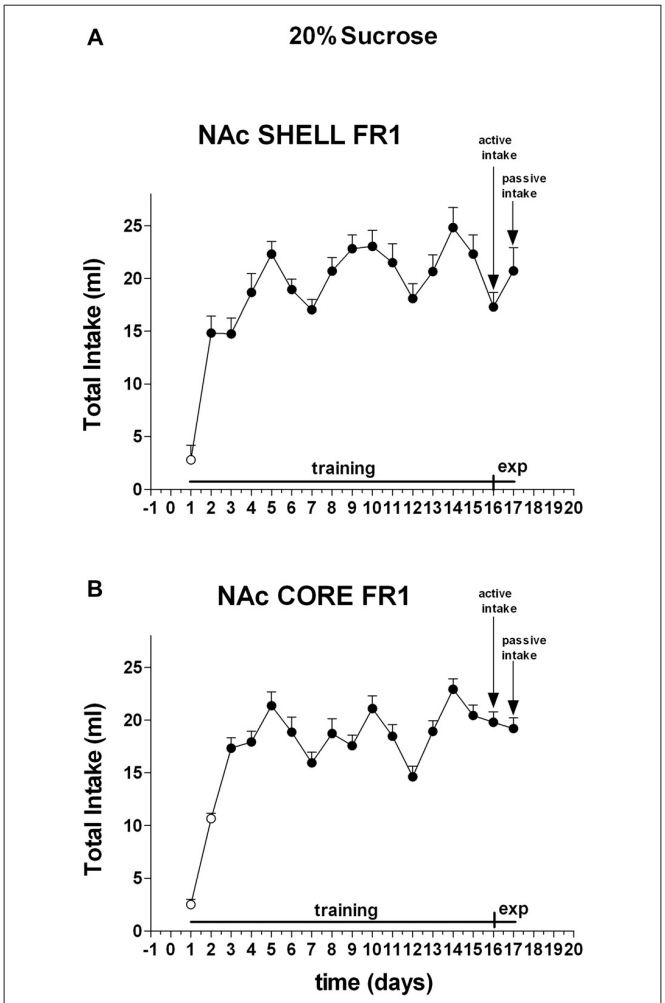

FIGURE 3 | Total intake expressed in ml of 20% sucrose solution. Data are means ± SEM of the results obtained in rats implanted with guide cannulas in the NAc shell **(A)** (N = 4) and rats in the NAc core **(B)** (N = 4). Filled symbols, $p < 0.05$ vs. 1st day.

Responding under extinction was associated to a selective increase of DA in the shell. NAc core DA remained unchanged throughout the entire session. A peak in shell DA was displayed at the first sample, and had returned to baseline levels by the 4th sample.

Responding was high for the three samples in the presence of increased DA in the shell.

Two-way ANOVA showed an effect of area ($F_{(1,6)}$ = 11.5; p = 0.014), time ($F_{(9,54)}$ = 30.7; $p < 0.01$) and an interaction area × time ($F_{(9,54)}$ = 15.4; $p < 0.01$). *Post hoc* test showed an increase of DA in the NAc shell but not in the core.

Non-Contingent 20% Sucrose Solution

Figure 4C shows the time-course of DA in the NAc shell and core following non-contingent 20% sucrose solution presentation.

In contrast to responding for sucrose and to extinction, non-contingent sucrose presentation was associated with an increase of dialysate DA in both the NAc shell and core.

Shell DA peaked at the first sample and had returned to basal values by the 5th sample; Core DA peaked at the 2nd sample and had returned to baseline by the 4th sample.

Two-way ANOVA showed an effect of time ($F_{(9,45)}$ = 48.9; $p < 0.01$) and an interaction area × time ($F_{(9,45)}$ = 11.8; $p < 0.01$). *Post hoc* test showed an earlier increase in DA in the NAc shell than in the core.

10% Ethanol in 20% Sucrose

Training

Figure 5 shows the number of cumulative active and inactive nose pokes performed during training for FR1 responding for ethanol and sucrose solution, and on the first and the second

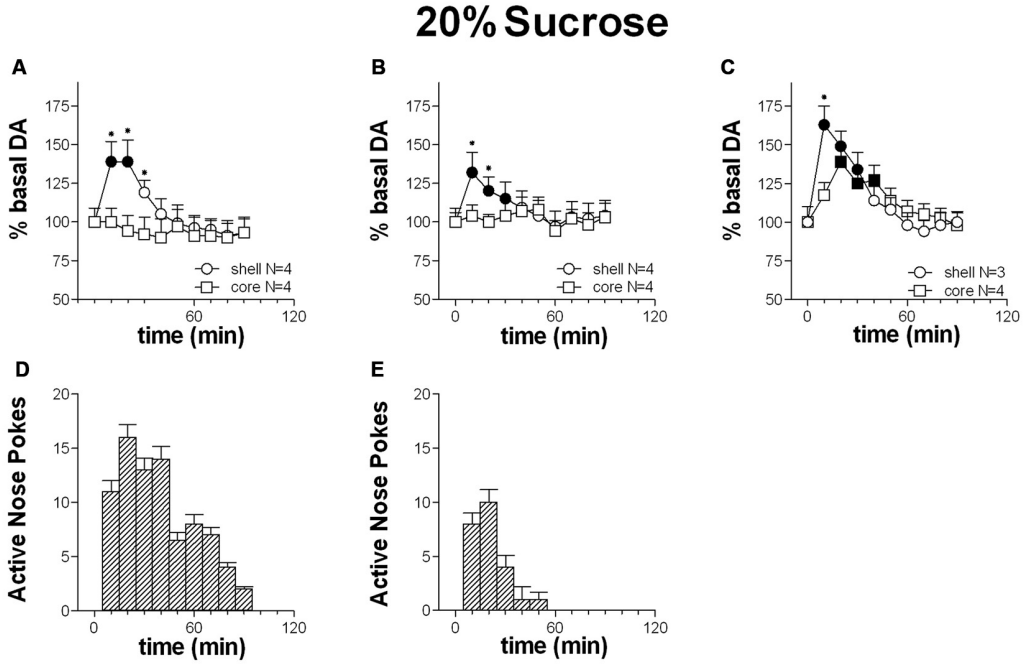

FIGURE 4 | Time-course of dialysate dopamine (DA) in the NAc shell (circles) and core (squares) and active nose pokes (Bars) every 10 min under FR1 responding for 20% sucrose solution (A,D), extinction **(B,E)** and non-contingent sucrose presentation **(C)**. Data are means ± SEM of the results, expressed as % of basal values, obtained in the number (*N*) of rats indicated in the figure. Filled symbols: $p < 0.05$ vs. basal values; *$p < 0.05$ vs. values obtained in the core.

experiment days. Nose-poking increased selectively on the active hole.

Three-way ANOVA showed a main effect of nose poke ($F_{(1,18)} = 152.9$; $p < 0.01$), days ($F_{(16,288)} = 7.6$; $p < 0.01$) and a nose poke × days interaction ($F_{(16,288)} = 10.0$; $p < 0.01$). Tukey's test revealed no differences in the temporal profile of acquisition between the shell- and core-implanted groups.

Two-way ANOVA of the amount of solution (ml) consumed by rats showed a main effect of days ($F_{(16,128)} = 9.76$; $p < 0.01$). *Post hoc* test revealed that the amount of 10% ethanol in 20% sucrose solution (ml) consumed by rats increased selectively during the training period but did not reveal any difference between shell- and core-implanted rats (**Figure 6**).

Two-way ANOVA of the amount of ethanol (g/Kg) consumed by rats showed a main effect of days ($F_{(16,128)} = 9.72$; $p < 0.01$). *Post hoc* test revealed that the amount of ethanol (g/Kg) consumed by rats increased selectively during the training period but did not reveal any difference between shell- and core-implanted rats (**Figure 6**).

Operant Session

Figures 7A,D shows the time-course of dialysate DA in the NAc shell and core and the number of active nose-pokes performed during FR1 responding for 10% ethanol in 20% sucrose solution.

Responding for 10% ethanol in 20% sucrose solution increased dialysate DA in both the shell and the core. Responding remained high throughout the session and in the presence of increased DA in the two areas.

Two-way ANOVA showed an effect of area ($F_{(1,8)} = 5.7$; $p = 0.04$), time ($F_{(9,72)} = 26.8$; $p < 0.01$) and an area × time interaction ($F_{(9,72)} = 5.3$; $p < 0.01$). *Post hoc* test revealed a higher increase of DA in the NAc core than in the shell.

Extinction Session

Figures 7B,E shows the time-course of dialysate DA in the NAc shell and core and of active nose-pokes under extinction in the presence of cues signaling reward availability and associated to reward delivery. Responding under extinction was associated to an increase of DA in both the shell and the core. Responding was high over the first 30 min.

Two-way ANOVA showed an effect of area ($F_{(1,10)} = 12.6$; $p < 0.01$), time ($F_{(9,90)} = 30.3$; $p < 0.01$) and an interaction area × time ($F_{(9,90)} = 4.48$; $p < 0.01$). *Post hoc* test revealed a higher increase in DA in the NAc shell than in the core.

Non-Contingent 10% Ethanol in 20% Sucrose

Figure 7C shows the time-course of DA in the NAc shell and core following the non-contingent presentation of 10% ethanol in 20% sucrose solution.

Non-contingent ethanol-sucrose presentation was associated with an increase in dialysate DA both in the NAc shell and core.

Two-way ANOVA showed an effect of area ($F_{(1,8)} = 10.9$; $p = 0.01$), time ($F_{(9,72)} = 32.6$; $p < 0.01$) and an interaction area × time ($F_{(9,72)} = 12.5$; $p < 0.01$). *Post hoc* test revealed a prolonged increase of DA in the NAc shell compared with the core.

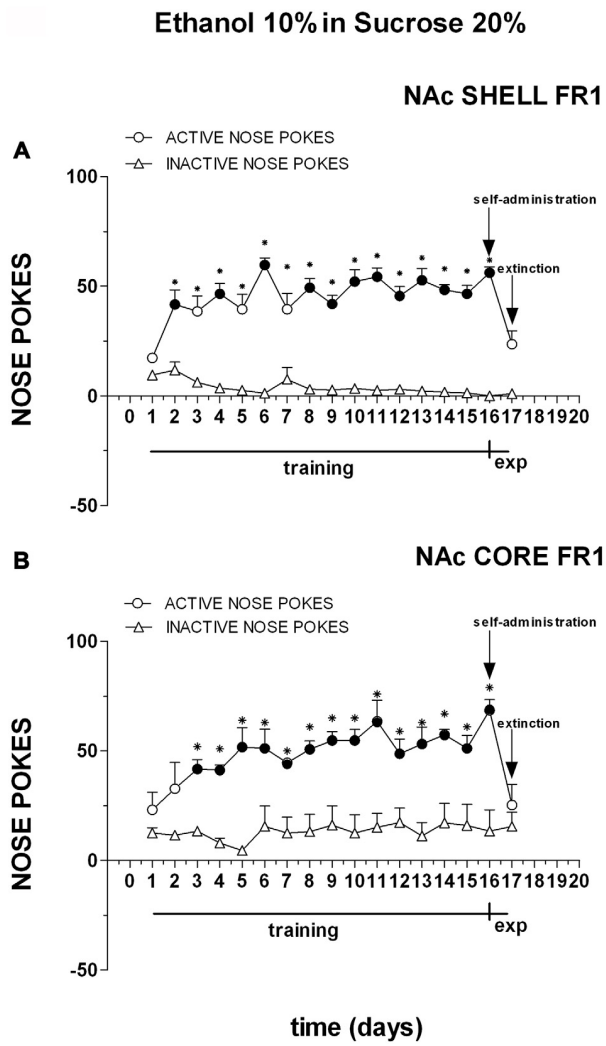

FIGURE 5 | Cumulative active (circles) and inactive (triangles) nose-pokes during training for FR1 responding for 10% ethanol in 20% sucrose solution and during dialysis experiments. Data are means ± SEM of the results obtained in rats implanted with guide cannulas in the NAc shell **(A)** ($N = 6$) and rats in the NAc core **(B)** ($N = 6$). Filled symbols, $p < 0.05$ vs. 1st day; $^*p < 0.05$ vs. inactive nose pokes.

10% Ethanol

Training

Figure 8 shows the number of cumulative active and inactive nose pokes performed during training for FR1 responding for 10% ethanol, and on the first and the second experiment days. Nose-poking increased selectively on the active hole.

Three-way ANOVA showed a main effect of nose poke ($F_{(1,22)} = 54.9$; $p < 0.01$), days ($F_{(16,352)} = 3.5$; $p < 0.01$) and a nose poke × days interaction ($F_{(16,352)} = 47.3$; $p < 0.01$). Tukey's test revealed no differences in the temporal profile of acquisition between the shell- and core-implanted groups.

Two-way ANOVA of the amount of solution (ml) consumed by rats showed a main effect of days ($F_{(16,160)} = 4.9$; $p < 0.01$). *Post hoc* test revealed that the amount of 10% ethanol solution (ml) consumed by rats increased selectively during the training period but did not reveal any difference between shell- and core-implanted rats (**Figure 9**).

Two-way ANOVA of the amount of ethanol (g/Kg) consumed by rats showed a main effect of days ($F_{(16,160)} = 4.2$; $p < 0.01$). *Post hoc* test revealed that the amount of ethanol (g/Kg) consumed by rats increased selectively during the training period but did not reveal any difference between shell- and core-implanted rats (**Figure 9**).

Operant Session

Figures 10A,D shows the time-course of dialysate DA in the NAc shell and core and the number of active nose-pokes performed during FR1 responding for 10% ethanol solution.

Responding for 10% ethanol solution increased dialysate DA to a similar extent in both the shell and the core. Responding remained high for the first 30 min.

Two-way ANOVA showed an effect of area ($F_{(1,12)} = 7.9$; $p = 0.02$), and time ($F_{(9,108)} = 14.9$; $p < 0.01$). *Post hoc* Tukey's test revealed no differences between the two groups of animals.

Extinction Session

Figures 10B,E shows the time-course of dialysate DA in the NAc shell and core and of active nose-pokes under extinction in the presence of cues signaling ethanol availability and associated to ethanol delivery.

Responding under extinction was associated to an increase of DA in both the shell and the core. Responding was high over the first 20 min.

Two-way ANOVA showed an effect of area ($F_{(1,12)} = 5.4$; $p = 0.04$), time ($F_{(9,108)} = 39.2$; $p < 0.01$) and an interaction area × time ($F_{(9,108)} = 7.7$; $p < 0.01$). *Post hoc* test revealed a higher increase in DA in the NAc shell than in the core.

Non-Contingent Presentation

Figure 10C shows the time-course of DA in the NAc shell and core following non-contingent presentation of 10% ethanol solution.

Non-contingent ethanol presentation was associated with an increase of dialysate DA both in the NAc shell and core.

Two-way ANOVA showed an effect of area ($F_{(1,10)} = 6.5$; $p = 0.03$) and of time ($F_{(9,90)} = 10.1$; $p < 0.01$). *Post hoc* test did not reveal any differences between the two areas.

DISCUSSION

This study investigated by microdialysis the responsiveness of NAc shell and core DA transmission to 10% ethanol solution compared to 10% ethanol + 20% sucrose and 20% sucrose solutions; each solution was administered under three different conditions (response-contingent, under extinction trial and response non-contingent) in rats previously trained to respond on a continuous reinforcement (FR1) schedule. An important difference between the present study and previous studies (Weiss et al., 1993; Gonzales and Weiss, 1998;

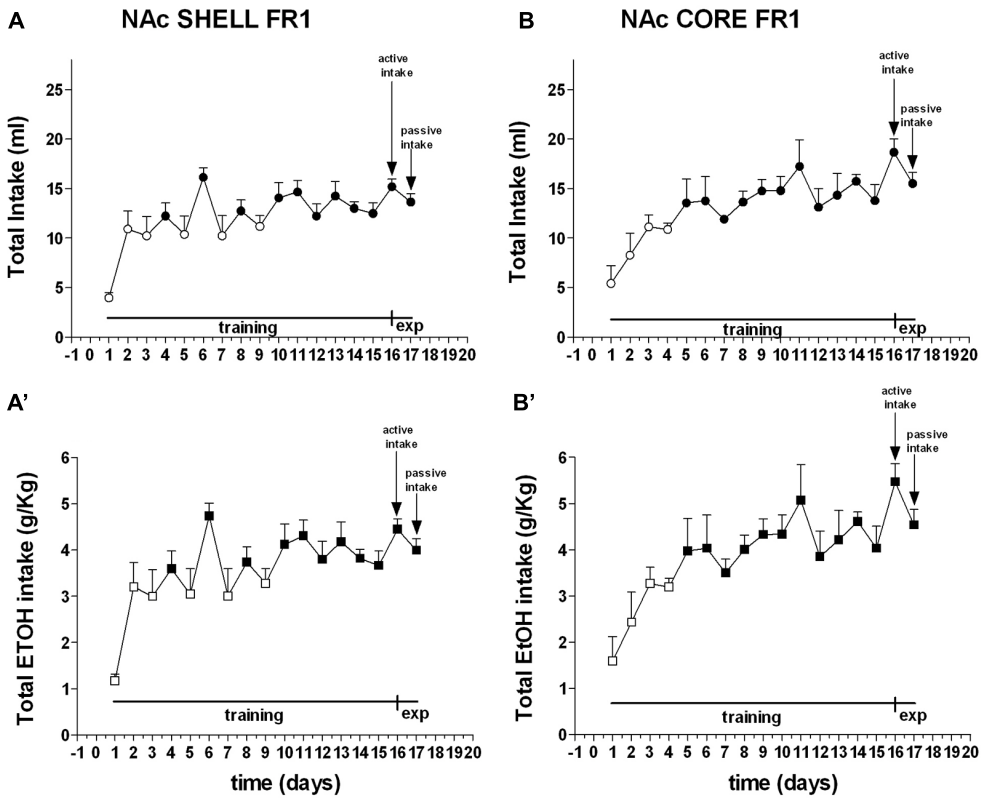

FIGURE 6 | Total intake expressed as ml of 10% ethanol in 20% sucrose solution and g/Kg of ethanol. Data are means ± SEM of the results obtained in rats implanted with guide cannulas in the NAc shell (**A**:ml; **A'**:g/Kg; *N* = 6) and rats in the NAc core (**B**:ml; **B'**:g/Kg; *N* = 6). Filled symbols, $p < 0.05$ vs. 1st day.

Doyon et al., 2003, 2005; Howard et al., 2009) is that basal levels of dialysate DA were obtained in the Skinner box in which microdialysis was performed. This condition enabled us to avoid the potential artifact of transfer from the home cage to the Skinner box, as in the case of previous studies in which basal samples had been collected in the home cage. Under these conditions, responding for 20% sucrose solution was associated with a selective increase of DA in the shell, while responding for ethanol and ethanol + sucrose solutions was associated with increased dialysate DA both in the shell and core. The same qualitative pattern of changes in DA transmission was obtained in extinction trials. Conversely, response non-contingent presentation of sucrose and ethanol solutions was associated with an increase of DA both in the NAc shell and core. These results provide the first unequivocal evidence that oral ethanol SA increases DA transmission in the rat NAc.

20% Sucrose

In rats fully trained to respond for 20% sucrose solutions under a continuous schedule of reinforcement (FR1), dialysate DA increased maximally in the NAc shell over the first 20 min and then decreased, returning to baseline levels by the 4th sample (40 min). In contrast, responding remained high throughout the entire session (60 min), in agreement with our previous observations in rats responding for sucrose pellets (Bassareo et al., 2015c). Thus, in both studies, after an initial surge, the increase in dialysate DA observed in the NAc shell subsequently returned to basal values despite sustained responding, thus becoming dissociated from instrumental action and from the actual intake of sucrose. No change in dialysate DA was observed in the NAc core, again in agreement with our previous observations with sucrose pellets (Bassareo et al., 2015c).

This study also extends to sucrose solutions our previous finding that in instrumentally trained rats, non-contingent presentation and feeding of sucrose pellets (Bassareo et al., 2015c) increases DA in the shell in spite of repeated exposure to sucrose. This finding has been taken to indicate that training to respond for sucrose induces a loss of the habituation of NAc shell DA responsiveness observed in naive rats following response non-contingent sucrose presentation and feeding (Bassareo et al., 2015c). We argued, however, that the loss of habituation of DA responsiveness to sucrose feeding in rats trained for sucrose responding is only apparent, being due to the fact that in these rats NAc shell DA transmission is

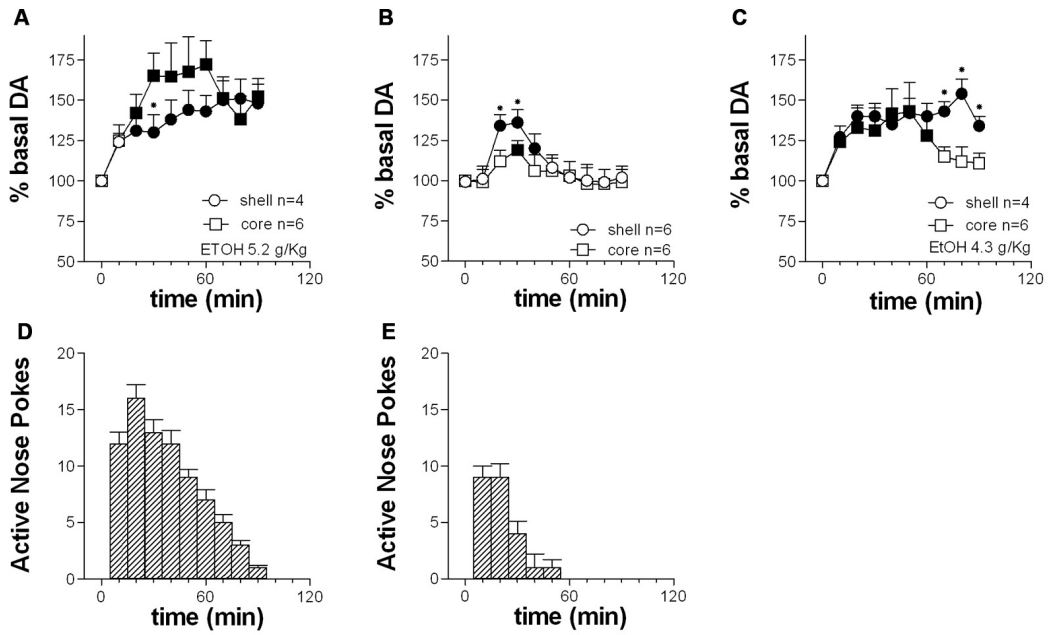

FIGURE 7 | Time-course of dialysate DA in the NAc shell (circles) and core (squares) and active nose pokes (Bars) every 10 min under FR1 responding for 10% ethanol in 20% sucrose solution (A,D), extinction **(B,E)** and non-contingent 10% ethanol in 20% sucrose solution presentation **(C)**. Data are means ± SEM of the results, expressed as % of basal values, obtained in the number (*N*) of rats indicated in the figure. Filled symbols: $p < 0.05$ vs. basal values; *$p < 0.05$ vs. values obtained in the core.

driven by sucrose discriminative/conditioned stimuli (DS/CS) rather than by sucrose unconditioned stimulus (US; Bassareo et al., 2015c). This hypothesis is based on the assumption that in fully trained rats, the sucrose US is fully predicted by DS/US stimuli, and therefore its DA-stimulant property is lost and transferred to the DS/US stimuli (Schultz et al., 1997). This interpretation, in turn, is consistent with the observation that a similar, although shorter lasting, increase of DA is observed in the NAc shell under an extinction trial, a condition in which sucrose US is absent but DS/CS cues are still present (Bassareo et al., 2015a,c and present study). These observations indicate that the pattern of DA responsiveness in the NAc shell and core compartments to sucrose is strongly dependent on response contingency; specifically, in rats responding by nose-poking, the response of NAc core DA, while allowed under non-contingent sucrose, seems actively suppressed under response-contingent sucrose presentation and feeding.

On the other hand, adaptive modulation of NAc core DA responsiveness depends on the nature of the motor response itself. Indeed, in rats trained to respond for sucrose pellets by lever pressing instead of nose poking, we recently observed that instrumental responding, as well as extinction, was associated with an increase in dialysate DA in both the NAc shell and core. As nose-poking is a rodent-specific, innate motor response, while lever pressing is an unnatural response acquired as a result of training (skill), we suggested that NAc core DA is required to achieve lever pressing but not nose-poking (Bassareo et al., 2015b). Accordingly, whilst NAc core DA may be useful in responding by lever pressing, in the case of nose-poking it is not required, and may even prove counterproductive, potentially inducing "vicious" behavior (stereotypies) that interferes with goal-directed action (Bassareo et al., 2015c), and is therefore suppressed. This hypothesis is consistent with evidence that the NAc shell DA is involved in the inhibition of inappropriate responses in goal-directed action (Ambroggi et al., 2011). Thus, in a context that signals alcohol unavailability, inactivation of NAc shell, rather than inhibiting responding, actually increases it (Chaudhri et al., 2008).

Responding for 10% Ethanol + 20% Sucrose and for 10% Ethanol

Responding for ethanol, either alone or in association with sucrose, was efficiently acquired, as indicated by the low rate of responding on the inactive compared to the active hole, and by the rapid extinction of responding when ethanol solutions were substituted with water. The rate of responding for 10% ethanol in 20% sucrose was higher than that observed for 10% ethanol alone, but lower than for 20% sucrose. Responding for ethanol alone was maximal for the first two 10 min fractions and then progressively decreased, reaching very low levels in the last 10 min fraction of the session.

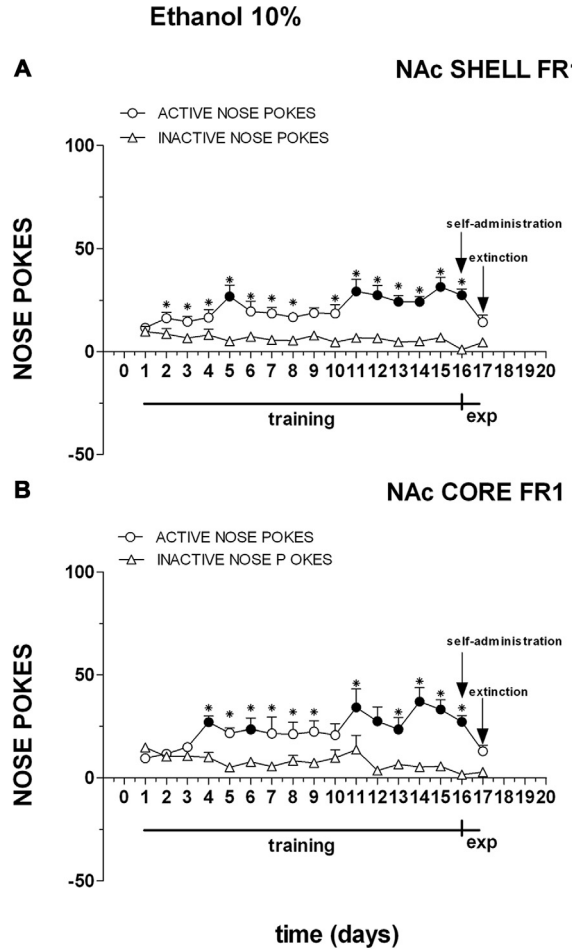

FIGURE 8 | Cumulative active (circles) and inactive (triangles) nose-pokes during training for FR1 responding for 10% ethanol solution and during dialysis experiments. Data are means ± SEM of the results obtained in rats implanted with guide cannulas in the NAc shell **(A)** ($N = 7$) and rats in the NAc core **(B)** ($N = 7$). Filled symbols, $p < 0.05$ vs. 1st day; $^*p < 0.05$ vs. inactive nose pokes.

With regard to the changes in dialysate NAc DA, a series of differences were observed in rats self-administering ethanol solutions compared to rats responding for sucrose. In rats responding for ethanol solutions, dialysate DA increased in both the shell and core, while in rats responding for sucrose the increase was selective in the shell. On the other hand, similarly to sucrose, non-contingent ethanol solutions increased DA in both the shell and core.

Moreover, responding for sucrose and ethanol solutions further differed in that, in the case of sucrose, the increase of DA in the NAc returned to basal values during the session, when responding was still high, whilst with ethanol solutions DA increased up to a plateau that was maintained beyond the session, in spite of a within session progressive decrease of responding, as occurred with 10% ethanol. We suggest that this intra-session reduction of responding for ethanol is due to the attainment of a plateau of ethanol concentrations in the brain that reduces the need for further ethanol intake. In the case of ethanol, its direct intracerebral action interacts with indirect, cue-related influences on DA to potentially overcome or amplify these. An extensive presence of these interactions is expected during SA of ethanol + sucrose solutions, owing to the fact that the influence of sucrose on DA transmission is peripheral and largely cue-related. Consistent with this suggestion, following ethanol + sucrose SA the pattern of DA increase in the shell vs. core is actually reversed compared to sucrose alone as, rather than being selective to the shell, the increase of DA is actually higher in the core.

During the extinction trial the time-course of DA paralleled that of responding; thus, DA maximally increased in the shell and core at the first sample and then progressively returned to basal values during the session. Moreover, in the extinction trial, DA response was higher in the shell than in the core, in contrast with observations made during operant responding for ethanol, when DA increased to a similar extent in the NAc shell and core.

Although previous studies investigating the changes in NAc DA transmission under operant responding for ethanol did not distinguish between effects produced in the shell and the core (Weiss et al., 1993; Gonzales and Weiss, 1998; Doyon et al., 2003, 2005), the findings of the present study, reporting a similar increase of dialysate DA in the shell and core allow the comparison between our observations and those of studies making no distinction between the shell and core subdivisions of the NAc.

Our observation of an increase in DA in the shell and core under operant responding for sucrose solutions seems in agreement with the conclusions reached by Weiss et al. (1993); Gonzales and Weiss (1998); Doyon et al. (2003, 2005), on the basis of microdialysis studies that made no distinction between shell and core. However, in the above studies, attribution of the increase in NAc DA to ethanol SA is uncertain. Indeed, as previously mentioned in the Introduction, in the studies quoted, basal levels of dialysate DA were obtained from samples collected in the home cage, while dialysate samples of the operant session were collected in the Skinner boxes after a waiting period of 15–20 min. Transfer of rats trained to respond for ethanol solutions from their home cage to the SA cage, or even simple transfer of naive rats from their home cage to a new cage, by itself increases dialysate DA in the NAc (Weiss et al., 1993; Gonzales and Weiss, 1998; Doyon et al., 2003, 2005; Howard et al., 2009). As discussed by Gonzales and Weiss (1998) and by Gonzales et al. (2004) this transfer-induced increase of NAc DA might be due, depending on the conditions, to the acquisition of reinforcer-predictive properties by the SA cage or to the unconditioned incentive properties of novelty. Whatever the mechanism, the transfer-induced rise of DA is a potential confound of any increase in DA during ethanol SA. Admittedly, as pointed out by Gonzales and Weiss (1998), the presence of a similar "artifact" makes it difficult to establish whether, and to what extent, the increase of DA above basal levels is related to ethanol or ethanol + sucrose SA or rather to the combined effect of cage transfer and ethanol SA. In the studies published by Weiss et al. (1993) and by Gonzales and Weiss (1998), the increase in dialysate DA in the NAc is maximal in the first sample after transfer and progressively fades as the rat becomes acclimatized to the

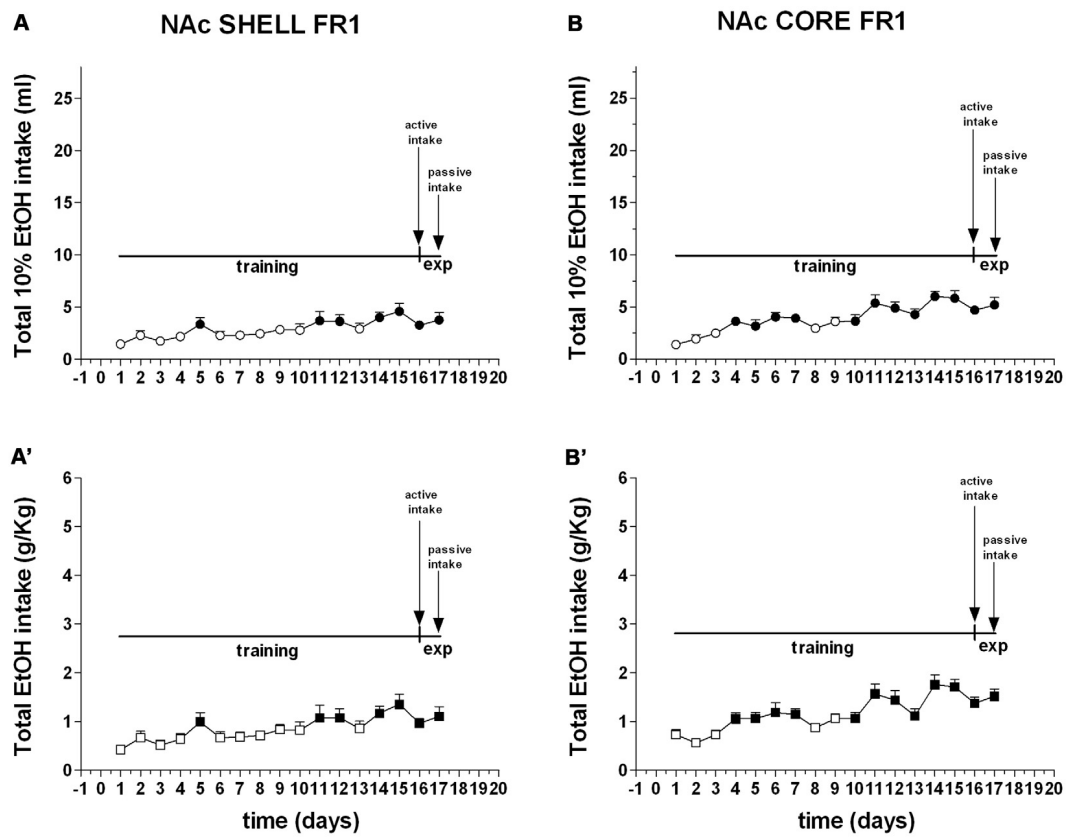

FIGURE 9 | Total intake expressed as ml of 10% ethanol solution and g/Kg of ethanol. Data are means ± SEM of the results obtained in rats implanted with guide cannulas in the NAc shell (**A**:ml; **A'**:g/Kg; *N* = 7) and rats in the NAc core (**B**:ml; **B'**:g/Kg; *N* = 7). Filled symbols, $p < 0.05$ vs. 1st day.

test cage. However, the habituation time (wait) before starting ethanol SA after transfer (15–20 min.) might be insufficient to allow DA levels to return to baseline. For example, according to Howard et al. (2009), cage transfer increases DA by 20%–30% in the shell and core, with the increase lasting for at least 15 min. This might explain the failure to observe an increase in DA in the NAc shell and core in rats responding for ethanol + sucrose (Howard et al., 2009).

In the present study, dialysate samples were taken directly in the Skinner box in which operant sessions were performed, and mean levels of DA obtained from three consecutive samples, and differing by no more than 10%, were taken as basal values. These conditions obviated the influence of cage transfer and allowed for full stabilization of DA, thus providing appropriate basal reference levels of dialysate DA.

In the article, Howard et al. (2009) reported that ethanol SA increases dialysate DA in an area of the NAc that they call the "shore" (see "Introduction" Section), but not in the shell nor in the core. The "shore", however, is a virtual area, comprised of the shell and core tissue located along their adjoining border. Accordingly, microdialysis probes placed in this area would recover DA, depending on the precise location, from the shell and, respectively, from the core. In view of this, a more parsimonious interpretation of the results by Howard et al is that ethanol increases dialysate DA both in the shell and in the core, in agreement with our observations.

Our observations might also be discussed in terms of the nature of changes in dialysate DA observed following exposure to ethanol. Doyon et al. (2003, 2005) suggested that changes in dialysate DA in rats responding for ethanol are not the result of a direct central effect of ethanol since their peak precedes that of ethanol in the blood. This suggestion is consistent with our previous observations that in rats given ethanol through intraoral cannulas, the increase of DA in the NAc shell is biphasic, with an initial peak related to the taste of ethanol and a second peak coincident with the rise of ethanol in dialysates (Bassareo et al., 2003). These observations, however, were made in rats naive to the taste of ethanol, in which habituation to the taste-induced activation of DA is observed. In an operant paradigm ethanol taste might undergo habituation as a primary US and be converted into a CS as a result of instrumental conditioning. As long as it is reinforced, taste CS-induced increase of DA in the NAc shell should not habituate, thus explaining the apparent loss of habituation of DA responsiveness in rats given

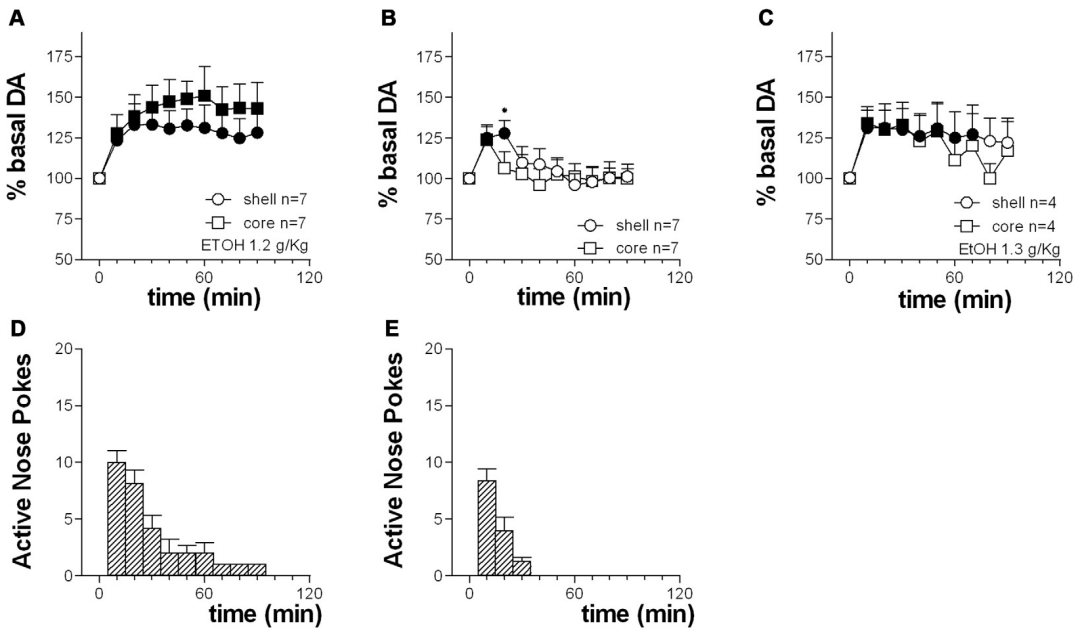

FIGURE 10 | Time-course of dialysate DA in the NAc shell (circles) and core (squares) and active nose pokes (Bars) every 10 min under FR1 responding for 10% ethanol solution (A,D), extinction **(B,E)** and non-contingent 10% ethanol solution presentation **(C)**. Data are means ± SEM of the results, expressed as % of basal values, obtained in the number (*N*) of rats indicated in the figure. Filled symbols: $p < 0.05$ vs. basal values; $^{*}p < 0.05$ vs. values obtained in the core.

ethanol solutions non-contingently. Consistent with a role of non-taste ethanol-conditioned CSs, dialysate DA increases in both the shell and core in extinction trials, when ethanol is substituted by water. Similar observations have been made under extinction from sucrose pellets, in which case, however, they were selective to the shell (Bassareo et al., 2015c and present study).

Due to the limited temporal resolution of microdialysis techniques, the relative contribution of non-taste DS/CS preceding taste US/CS to the increase in dialysate DA in rats responding for ethanol solutions cannot be ascertained. It is however clear that with both ethanol and sucrose, the changes in dialysate DA obtained during the extinction trial follow the same shell/core pattern as during operant sessions; this finding suggests that in instrumentally trained rats discriminative CS are *per se* sufficient to account for the changes in dialysate DA that occur during operant sessions.

From this point of view ethanol differs from other drugs of abuse. Thus, while DA remains flat during extinction trials from heroin, cocaine, nicotine and Win 55,212-2 SA both in the shell and core (Lecca et al., 2006a,b, 2007a,b), it increases in both shell and core under responding for ethanol. In our opinion, these major differences are linked to the fact that ethanol is administered orally and to the acquisition of taste as a highly salient secondary reinforcer. Thus, once the predictive association between the taste of ethanol and its systemic effects has been made, rats work for ethanol taste, a mechanism that likely applies also to sucrose reinforcement. With training, instrumental conditioning also generalizes to non-taste cues, thus explaining the increase of dialysate DA under extinction trials, in the absence of taste cues. A notable difference between sucrose and ethanol reinforcement is related to the pattern of DA responsiveness in the shell and core under operant responding and extinction. Indeed, on exposure to sucrose a similar pattern of response is observed in the two conditions, since DA increases selectively in the shell; conversely, in the presence of ethanol + sucrose, DA increases preferentially in the core during reinforced, and in the shell during non-reinforced, sessions. Once again, this might be due to the action of ethanol, which activates DA neurons in reinforced sessions and thus overcomes the influence of other cues on DA transmission.

These observations seem to suggest that the differences observed between ethanol and sucrose are partly due to the fact that ethanol similarly activates NAc DA in the shell and in the core; this in turn distinguishes ethanol from other drugs of abuse that preferentially activate DA in the shell after i.v. SA (Lecca et al., 2006a,b, 2007a,b). It is however unclear whether the differences observed are due, and to what extent, to the use of diverse routes of administration for ethanol (oral) and for the other drugs (i.v.).

The ability of ethanol SA to activate DA in the NAc core, and the ability of sucrose to activate DA selectively in the shell, may underlie the differential roles played by NAc shell and core DA in paradigms of pavlovian to instrumental transfer (PIT), recently

reported by Corbit et al. (2016). PIT consists in the ability of a conditioned stimulus (CS) previously paired to a given US, to invigorate responding for the same (outcome-selective PIT) or for a different US (general PIT; Corbit et al., 2007). The two forms of PIT are differentially dependent on the integrity of the two subdivisions of the NAc. While general PIT depends on the NAc core, outcome-selective PIT depends on the shell (Corbit et al., 2007). Similarly, while ethanol-paired stimuli increase responding both for ethanol on the ethanol lever and for sucrose on the sucrose lever (general PIT), sucrose paired stimuli increase responding on the sucrose but not on the ethanol lever (outcome-selective PIT), with these effects being selectively impaired by inactivation of the core and, respectively, of the shell (Corbit et al., 2016). We suggest that these differences are related to the differential pattern of activation of NAc shell and core DA during instrumental performance, that differentially interacts with the incentive properties of pavlovian stimuli conditioned to ethanol and sucrose, respectively.

CONCLUSIONS

In conclusion, the present study, provides clear evidence unconfounded by the influence of transfer from the home cage to the SA box, that oral SA of a 10% ethanol solution is associated with a rapid activation of DA transmission in the NAc shell and core that reaches a plateau and is maintained throughout the session despite a progressive decrease in responding following the initial surge. Comparison of the pattern of activation of DA transmission following the administration of sucrose, sucrose + ethanol and ethanol solutions highlights the existence of important differences between sucrose and ethanol rewards, suggesting that the different patterns obtained are the result of a complex interaction between the direct intracerebral action of ethanol and the action of peripheral stimuli arising from sucrose and ethanol USs and from their related DS/CSs. A key aspect in the pattern of DA transmission following exposure to an ethanol solution is the activation of NAc core DA, which contrasts with the selective activation of NAc shell DA by sucrose SA; this in turn is at variance with the combined activation of shell and core DA following passive sucrose presentation and consumption. These observations suggest that major adaptive mechanisms, such as active inhibition of NAc core DA transmission, are activated during instrumental responding for sucrose. These mechanisms may be related to a basic function of NAc shell DA, that of preventing the expression of changes (e.g., increase of NAc core DA) inappropriate for correct goal-directed action. Conversely, following exposure to ethanol, the direct action of ethanol and the resulting increase in NAc core DA may be amplified by its stimulus properties or by DS/CS cues. This in turn would result in a general motivational arousal, overcoming fine adaptive mechanisms and resulting in a peculiar behavioral abnormality whereby pavlovian stimuli unrelated to ethanol are nonetheless capable of invigorating ethanol seeking and consumption, much like the general transfer effect displayed in experimental PIT paradigms. Such generalized activation might contribute to the abnormal features of ethanol reward as compared to a conventional one like sucrose.

AUTHOR CONTRIBUTIONS

VB: study design; acquisition, analysis and interpretation of data; draft of the article. FC: acquisition and analysis of data. RF: acquisition and analysis of data. GDC: study design, theoretical elaboration of the data, draft and final revision of the article.

FUNDING

This work was supported by grants from Fondazione Banco di Sardegna (2011.1047. Prot n.U1140.2013/AI.1059.MGB).

ACKNOWLEDGMENTS

Authors would like to thank Mr Valerio Lecca for animal care.

REFERENCES

Ambroggi, F., Ghazizadeh, A., Nicola, S. M., and Fields, H. L. (2011). Roles of nucleus accumbens core and shell in incentive-cue responding and behavioral inhibition. *J. Neurosci.* 31, 6820–6830. doi: 10.1523/JNEUROSCI.6491-10.2011

Aragona, B. J., Cleaveland, N. A., Stuber, G. D., Day, J. J., Carelli, R. M., and Wightman, R. M. (2008). Preferential enhancement of dopamine transmission within the nucleus accumbens shell by cocaine is attributable to a direct increase in phasic dopamine release events. *J. Neurosci.* 28, 8821–8831. doi: 10.1523/JNEUROSCI.2225-08.2008

Bassareo, V., Cucca, F., Frau, R., and Di Chiara, G. (2015a). Monitoring dopamine transmission in the rat nucleus accumbens shell and core during acquisition of nose-poking for sucrose. *Behav. Brain Res.* 287, 200–206. doi: 10.1016/j.bbr.2015.03.056

Bassareo, V., Cucca, F., Frau, R., and Di Chiara, G. (2015b). Differential activation of accumbens shell and core dopamine by sucrose reinforcement with nose poking and with lever pressing. *Behav. Brain Res.* 294, 215–223. doi: 10.1016/j.bbr.2015.08.006

Bassareo, V., Cucca, F., Musio, P., Lecca, D., Frau, R., and Di Chiara, G. (2015c). Nucleus accumbens shell and core dopamine responsiveness to sucrose in rats: role of response contingency and discriminative/conditioned cues. *Eur. J. Neurosci.* 41, 802–809. doi: 10.1111/ejn.12839

Bassareo, V., and Di Chiara, G. (1997). Differential influence of associative and nonassociative learning mechanisms on the responsiveness of prefrontal and accumbal dopamine transmission to food stimuli in rats fed *ad libitum*. *J. Neurosci.* 17, 851–861.

Bassareo, V., and Di Chiara, G. (1999). Differential responsiveness of dopamine transmission to food-stimuli in nucleus accumbens shell/core compartments. *Neuroscience* 89, 637–641. doi: 10.1016/S0306-4522(98)00583-1

Bassareo, V., De Luca, M. A., Aresu, M., Aste, A., Ariu, T., and Di Chiara, G. (2003). Differential adaptive properties of accumbens shell dopamine responses to ethanol as a drug and as a motivational stimulus. *Eur. J. Neurosci.* 17, 1465–1472. doi: 10.1046/j.1460-9568.2003.02556.x

Brown, H. D., McCutcheon, J. E., Cone, J. J., Ragozzino, M. E., and Roitman, M. F. (2011). Primary food reward and reward-predictive stimuli evoke different patterns of phasic dopamine signaling throughout the striatum. *Eur. J. Neurosci.* 34, 1997–2006. doi: 10.1111/j.1460-9568.2011.07914.x

Cacciapaglia, F., Saddoris, M. P., Wightman, R. M., and Carelli, R. M. (2012). Differential dopamine release dynamics in the nucleus accumbens core and shell track distinct aspects of goal-directed behavior for sucrose. *Neuropharmacology* 62, 2050–2056. doi: 10.1016/j.neuropharm.2011.12.027

Chaudhri, N., Sahuque, L. L., Cone, J. J., and Janak, P. H. (2008). Reinstated ethanol-seeking in rats is modulated by environmental context and requires the nucleus accumbens core. *Eur. J. Neurosci.* 28, 2288–2298. doi: 10.1111/j.1460-9568.2008.06517.x

Corbit, L. H., Fischbach, S. C., and Janak, P. H. (2016). Nucleus accumbens core and shell are differentially involved in general and outcome-specific forms of Pavlovian-instrumental transfer with alcohol and sucrose rewards. *Eur. J. Neurosci.* 43, 1229–1236. doi: 10.1111/ejn.13235

Corbit, L. H., Janak, P. H., and Balleine, B. W. (2007). General and outcome-specific forms of Pavlovian-instrumental transfer: the effect of shifts in motivational state and inactivation of the ventral tegmental area. *Eur. J. Neurosci.* 26, 3141–3149. doi: 10.1111/j.1460-9568.2007.05934.x

Di Chiara, G., Acquas, E., and Tanda, G. (1996). Ethanol as a neurochemical surrogate of conventional reinforcers: the dopamine-opioid link. *Alcohol* 13, 13–17. doi: 10.1016/0741-8329(95)02034-9

Di Chiara, G., and Imperato, A. (1985). Ethanol preferentially stimulates dopamine release in the nucleus accumbens of freely moving rats. *Eur. J. Pharmacol.* 115, 131–132. doi: 10.1016/0014-2999(85)90598-9

Doyon, W. M., Anders, S. K., Ramachandra, V. S., Czachowski, C. L., and Gonzales, R. A. (2005). Effect of operant self-administration of 10% ethanol plus 10% sucrose on dopamine and ethanol concentrations in the nucleus accumbens. *J. Neurochem.* 93, 1469–1481. doi: 10.1111/j.1471-4159.2005.03137.x

Doyon, W. M., York, J. L., Diaz, L. M., Samson, H. H., Czachowski, C. L., and Gonzales, R. A. (2003). Dopamine activity in the nucleus accumbens during consummatory phases of oral ethanol self-administration. *Alcohol. Clin. Exp. Res.* 27, 1573–1582. doi: 10.1097/01.ALC.0000089959.66222.B8

Gonzales, R. A., Job, M. O., and Doyon, W. M. (2004). The role of mesolimbic dopamine in the development and maintenance of ethanol reinforcement. *Pharmacol. Ther.* 103, 121–146. doi: 10.1016/j.pharmthera.2004.06.002

Gonzales, R. A., and Weiss, F. (1998). Suppression of ethanol-reinforced behavior by naltrexone is associated with attenuation of the ethanol-induced increase in dialysate dopamine levels in the nucleus accumbens. *J. Neurosci.* 18, 10663–10671.

Howard, E. C., Schier, C. J., Wetzel, J. S., Duvauchelle, C. L., and Gonzales, R. A. (2008). The shell of the nucleus accumbens has a higher dopamine response compared with the core after non-contingent intravenous ethanol administration. *Neuroscience* 154, 1042–1053. doi: 10.1016/j.neuroscience.2008.04.014

Howard, E. C., Schier, C. J., Wetzel, J. S., and Gonzales, R. A. (2009). The dopamine response in the nucleus accumbens core-shell border differs from that in the core and shell during operant ethanol self-administration. *Alcohol. Clin. Exp. Res.* 33, 1355–1365. doi: 10.1111/j.1530-0277.2009.00965.x

Imperato, A., and Di Chiara, G. (1986). Preferential stimulation of dopamine release in the nucleus accumbens of freely moving rats by ethanol. *J. Pharmacol. Exp. Ther.* 239, 219–228.

Lecca, D., Cacciapaglia, F., Valentini, V., and Di Chiara, G. (2006a). Monitoring extracellular dopamine in the rat nucleus accumbens shell and core during acquisition and maintenance of intravenous WIN 55,212–2 self-administration. *Psychopharmacology* 188, 63–74. doi: 10.1007/s00213-006-0475-3

Lecca, D., Cacciapaglia, F., Valentini, V., Gronli, J., Spiga, S., and Di Chiara, G. (2006b). Preferential increase of extracellular dopamine in the rat nucleus accumbens shell as compared to that in the core during acquisition and maintenance of intravenous nicotine self-administration. *Psychopharmacology* 184, 435–446. doi: 10.1007/s00213-005-0280-4

Lecca, D., Cacciapaglia, F., Valentini, V., Acquas, E., and Di Chiara, G. (2007a). Differential neurochemical and behavioral adaptation to cocaine after response contingent and noncontingent exposure in the rat. *Psychopharmacology* 191, 653–667. doi: 10.1007/s00213-006-0496-y

Lecca, D., Valentini, V., Cacciapaglia, F., Acquas, E., and Di Chiara, G. (2007b). Reciprocal effects of response contingent and noncontingent intravenous heroin on *in vivo* nucleus accumbens shell versus core dopamine in the rat: a repeated sampling microdialysis study. *Psychopharmacology* 194, 103–116. doi: 10.1007/s00213-007-0815-y

Melendez, R. I., Rodd-Henricks, Z. A., Engleman, E. A., Li, T. K., McBride, W. J., and Murphy, J. M. (2002). Microdialysis of dopamine in the nucleus accumbens of alcohol-preferring (P) rats during anticipation of operant sel-administration of ethanol. *Alcohol. Clin. Exp. Res.* 26, 318–325. doi: 10.1111/j.1530-0277.2002.tb02540.x

Paxinos, G., and Watson, C. (1998). *The Rat Brain in Stereotaxic Coordinates*. 4th Edn. SanDiego, CA: Academic Press.

Pontieri, F. E., Tanda, G., and Di Chiara, G. (1995). Intravenous cocaine, morphine, and amphetamine preferentially increase extracellular dopamine in the "shell" as compared with the "core" of the rat nucleus accumbens. *Proc. Natl. Acad. Sci. U S A* 92, 12304–12308. doi: 10.1073/pnas.92.26.12304

Pontieri, F. E., Tanda, G., Orzi, F., and Di Chiara, G. (1996). Effects of nicotine on the nucleus accumbens and similarity to those of addictive drugs. *Nature* 382, 255–257. doi: 10.1038/382255a0

Schultz, W., Dayan, P., and Montague, P. R. (1997). A neural substrate of prediction and reward. *Science* 275, 1593–1599. doi: 10.1126/science.275.5306.1593

Tanda, G., Pontieri, F. E., and Di Chiara, G. (1997). Cannabinoid and heroin activation of mesolimbic dopamine transmission by a common μ1 opioid receptor mechanism. *Science* 276, 2048–2050. doi: 10.1126/science.276.5321.2048

Tang, A., George, M. A., Randall, J. A., and Gonzales, R. A. (2003). Ethanol increases extracellular dopamine concentration in the ventral striatum in C57BL/6 mice. *Alcohol. Clin. Exp. Res.* 27, 1083–1089. doi: 10.1097/01.alc.0000075825.14331.65

Weiss, F., Lorang, M. T., Bloom, F. E., and Koob, G. F. (1993). Oral alcohol self-administration stimulates dopamine release in the rat nucleus accumbens: genetic and motivational determinants. *J. Pharmacol. Exp. Ther.* 267, 250–258.

Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

frontiers in Behavioral Neuroscience

MINI REVIEW
published: 09 February 2017
doi: 10.3389/fnbeh.2017.00023

Acetaldehyde, Motivation and Stress: Behavioral Evidence of an Addictive *ménage à trois*

*Anna Brancato, Gianluca Lavanco, Angela Cavallaro, Fulvio Plescia and Carla Cannizzaro **

Department of Sciences for Health Promotion and Mother and Child Care "G. D'Alessandro", University of Palermo, Palermo, Italy

Acetaldehyde (ACD) contributes to alcohol's psychoactive effects through its own rewarding properties. Recent studies shed light on the behavioral correlates of ACD administration and the possible interactions with key neurotransmitters for motivation, reward and stress-related response, such as dopamine and endocannabinoids. This mini review article critically examines ACD psychoactive properties, focusing on behavioral investigations able to unveil ACD motivational effects and their pharmacological modulation *in vivo*. Similarly to alcohol, rats spontaneously drink ACD, whose presence is detected in the brain following chronic self-administration paradigm. ACD motivational properties are demonstrated by operant paradigms tailored to model several drug-related behaviors, such as induction and maintenance of operant self-administration, extinction, relapse and punishment resistance. ACD-related addictive-like behaviors are sensitive to pharmacological manipulations of dopamine and endocannabinoid signaling. Interestingly, the ACD-dopamine-endocannabinoids relationship also contributes to neuroplastic alterations of the NPYergic system, a stress-related peptide critically involved in alcohol abuse. The understanding of the *ménage-a-trois* among ACD, reward- and stress-related circuits holds promising potential for the development of novel pharmacological approaches aimed at reducing alcohol abuse.

Keywords: acetaldehyde, operant behavior, dopamine, endocannabinoids, stress

OPEN ACCESS

Edited by:
Merce Correa, Jaume I University, Spain

Reviewed by:
Elio Acquas, University of Cagliari, Italy
Luis Granero, University of Valencia, Spain

***Correspondence:**
Carla Cannizzaro
carla.cannizzaro@unipa.it

Received: *25 November 2016*
Accepted: *27 January 2017*
Published: *09 February 2017*

Citation:
Brancato A, Lavanco G, Cavallaro A, Plescia F and Cannizzaro C (2017) Acetaldehyde, Motivation and Stress: Behavioral Evidence of an Addictive ménage à trois. Front. Behav. Neurosci. 11:23. doi: 10.3389/fnbeh.2017.00023

INTRODUCTION

It is a matter of fact that the efficacy of current medications for alcohol-related pathological traits remains modest, since the incomplete understanding of the neurobiological background beyond alcohol central effects hampers the development of successful pharmacological therapies (Franck and Jayaram-Lindström, 2013). Alcohol acts at multiple biological targets (Mascia et al., 2001; Martire et al., 2002; Martí-Prats et al., 2013; Zorumski et al., 2014) and its extended use profoundly dysregulates key neurochemical circuits that drive incentive-salience/reward (dopamine, endocannabinoids) and stress-related response (corticotropin-releasing hormone [CRH], Neuropeptide Y [NPY]) within the brain (Koob, 2013). Moreover, the products of alcohol biotransformation, primarily acetaldehyde (ACD), also contribute to its mechanism of action with their own behavioral and neuropharmacological effects (Arizzi et al., 2003; Correa et al., 2003; Pardo et al., 2013; Segovia et al., 2013).

ACD is produced in the human body after the consumption of alcohol in a tissue-specific fashion (Cohen et al., 1980; Ramchandani et al., 2001; Edenberg, 2007), and occurs naturally in alcoholic

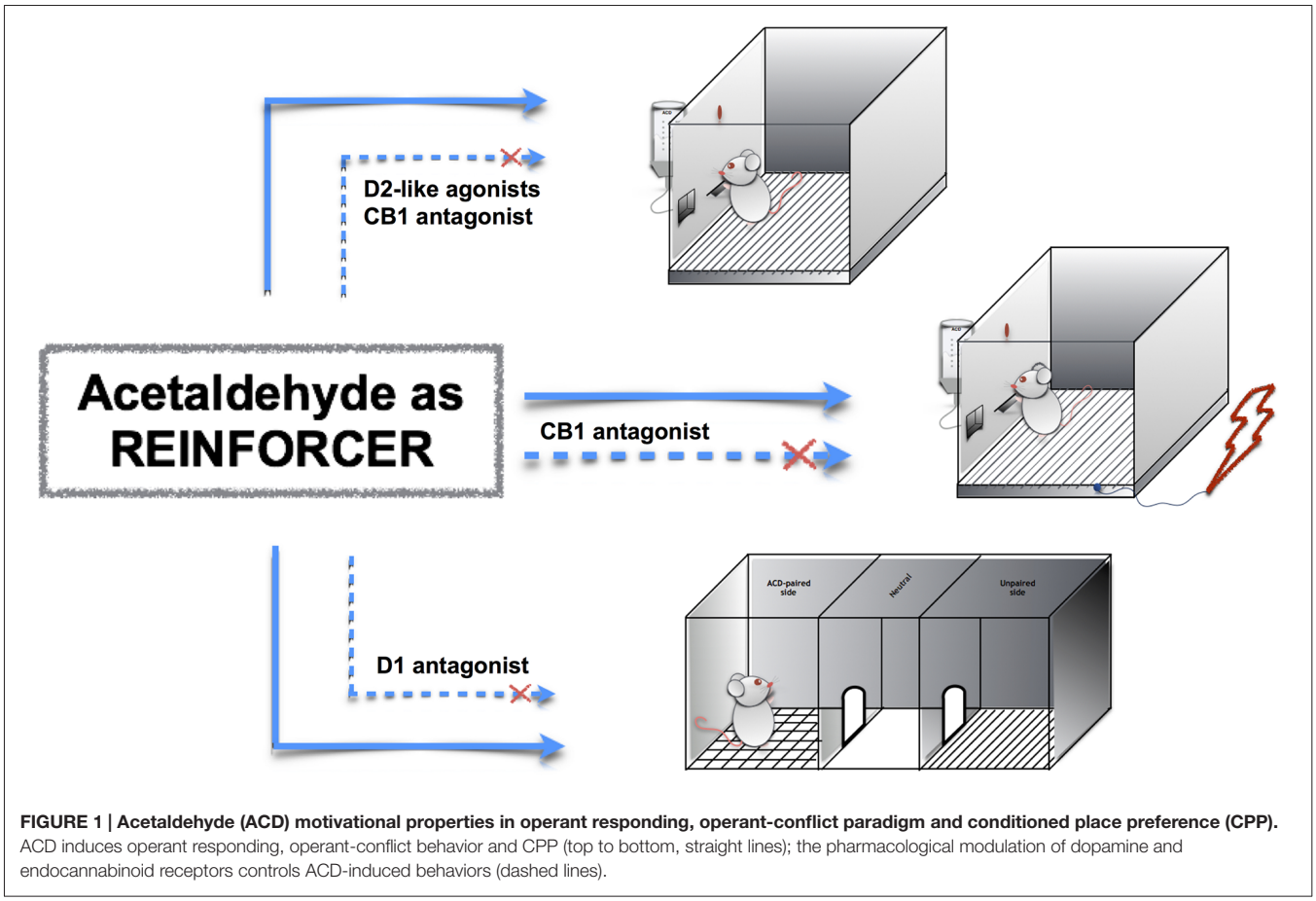

FIGURE 1 | Acetaldehyde (ACD) motivational properties in operant responding, operant-conflict paradigm and conditioned place preference (CPP). ACD induces operant responding, operant-conflict behavior and CPP (top to bottom, straight lines); the pharmacological modulation of dopamine and endocannabinoid receptors controls ACD-induced behaviors (dashed lines).

beverages. Indeed, high ACD concentrations were detected in a number of products, including apple wines and ciders from Germany, France and Scotland, fortified wines and spirits such as sugarcane spirits from Guatemal and Brazil (cuxa; cachaça) (Miranda et al., 2007; Oliveira et al., 2008; Kanteres et al., 2009), agave spirits from Mexico (Lachenmeier et al., 2006), certain spirits from China and calvados from Europe (Lachenmeier and Sohnius, 2008; Linderborg et al., 2008; Lachenmeier et al., 2015). Despite preclinical research has traditionally disregarded the role of taste and post-ingestive influences as independent regulators of motivation to drink alcohol, clinical studies on alcoholism have frequently recognized the significance of alcohol chemosensory stimuli in eliciting craving and associated drug-seeking responses in alcohol-experienced individuals (Stormark et al., 1995; Grüsser et al., 2000). Both alcohol and ACD possess complex chemosensory attributes detected via sensory receptors, which gain immediate access to the central nervous system. Importantly, these sensory pathways are linked to limbic forebrain and cortical areas involved in controlling ingestive motivation and feeding (Kareken et al., 2004; Yamamoto, 2006; Filbey et al., 2008).

Depending on alcohol doses and modalities of administration, peripheral ACD can cross the blood-brain barrier and potentially add to locally formed ACD, produced from alcohol via the brain specific catalase system. On the other hand, increasing evidence shows that ACD is detected in the brain after its oral introduction, when single high systemic concentrations are used, or when chronic exposure occurs (Tabakoff et al., 1976; Heap et al., 1995; Ward et al., 1997; Quertemont et al., 2004; Plescia et al., 2014, 2015a; Jamal et al., 2016).

Whatever its source, either as original substance or as alcohol metabolite, ACD has been largely involved in the mediation of alcohol effect, although its contribution to the development of alcohol abuse still needs to be elucidated.

The neuropharmacology of ACD is of particular interest, as ACD interacts both with reward- and stress-related circuits in the brain. For this reason, this mini-review article focuses on ACD motivational properties and examines its interplay with relevant neurotransmitters for motivation-and stress-related response, such as dopamine and endocannabinoids (**Figure 1**). Indeed, a deeper understanding of ACD neuropharmacology could provide further venue for the development of innovative medication for alcohol use disorders.

ACD MIRRORING ALCOHOL EFFECT IN THE BRAIN

Looking at the literature, ACD has been investigated primarily as a metabolite raising the idea that it could not only produce aversive reactions in the whole body, but rather may contribute

to alcohol mechanism of action in the brain (Plescia et al., 2015b; Cavallaro et al., 2016). Progressively the observation that direct administration of ACD in experimental animals resulted in behavioral effects that are comparable to those induced by alcohol strengthened this concept (Quertemont et al., 2005; Correa et al., 2012). For instance, systemic ACD administrations induced depression in locomotor activity (Myers et al., 1987), impairment of spatial memory (Abe et al., 1999), alcohol-like discrimination (Redila et al., 2002), sedative and hypnotic effects (Quertemont et al., 2004).

Notably, ACD has been implicated in alcohol stimulating effects on the reward pathway in the brain, i.e., ventral tegmental area (VTA) and nucleus accumbens (NAc), that lead to positive reinforcement and mediate alcohol consumption (Brown et al., 1979; Amit and Smith, 1985; Aragon and Amit, 1992; Tampier et al., 1995; Smith et al., 1997). Recently, specific gene-blocking techniques that allow inhibiting catalase in the VTA and thus, the production of ACD from alcohol, demonstrated that ACD mediates alcohol-reinforcing effect in self-administration paradigms. In these studies, microinjection of lentiviral vector encoding anticatalase shRNA into the VTA strongly decreased voluntary alcohol consumption in rats and abolished the increased dopamine release in NAc induced by acute administration of alcohol (Karahanian et al., 2011; Quintanilla et al., 2012). Moreover, VTA anticatalase shRNA injection reduced the marked increase in alcohol intake that follows a period of deprivation, an effect that was proposed to reflect increased reinforcing value of alcohol (Tampier et al., 2013).

If ACD formed from alcohol is responsible for the development of alcohol-related behaviors, then chronic administration of ACD alone should produce behavioral and neurochemical responses of an "addictive" -type.

ACD AS A REINFORCER

ACD's own reinforcing properties were first shown by conditioned place preference (CPP), behavioral paradigm widely used to explore rewarding effects of drugs. Laboratory rats receiving intracerebroventricular (icv) ACD infusions showed increased preference for environmental cues previously paired with the drug administration (Smith et al., 1984).

A strong preference for ACD-paired environment and stimuli was also observed when ACD administration was either intraperitoneal or oral (Quertemont and De Witte, 2001; Peana et al., 2008).

Place conditioning is suggestive of drug-associated reinforcement, although it may not be clear what exactly the procedure measures. Indeed, it focuses on automatic or implicit expressions of reward, rather than active demonstration of motivated behavior.

Thus the positive reinforcing properties of ACD were more specifically explored by the evaluation of acquisition and maintenance of ACD drinking behavior in self-administration paradigms in rats. Drug self-administration is directly under rat control, and the amount of drug consumed is widely used to infer drug hedonic properties: positively reinforcing drugs will be readily and avidly self-administrated. As alcohol, ACD is voluntary self-administered in two-bottle choice-drinking paradigm and its consummatory behavior was dose-dependent, in that ACD intake increased when higher solution strength was provided (Plescia et al., 2015a; Brancato et al., 2016b). The flavor and taste of ACD solution have been proposed to take part to the reinforcing properties and may actually serve as conditioned stimuli of post-ingestional effects (Cannizzaro et al., 2011).

Little is known on the molecular targets that account for ACD complex flavor. However, ACD directly activates the sensory neuronal TRP channels TRPA1 that are relevant for taste and chemesthesis (Bang et al., 2007; Roper, 2014). With chronic exposure, sensory and post-ingestive inputs become intimately integrated, such that these stimuli gain meaning for the addicted organism (Brasser et al., 2015). Natural ACD self-administration provides a framework for moving beyond the dissociation between the sensory and post-absorptive effects of ACD to the understanding of their neurobiological integration and significance for sensory processing of alcoholic beverages and alcohol addiction.

The suggestion that ACD may be endowed with positive reinforcing properties was further investigated by using a variety of operant self-administration paradigms.

The operant self-administration is a commonly used model in which animals are trained to emit a specific response (lever press or nose poke) for gaining the reinforcement (Samson et al., 1988). Operant behavior for ACD was readily acquired by rats, both through icv and intravenous routes of administration (Brown et al., 1980; Myers et al., 1984). In details, Rodd et al. (2003, 2005) demonstrated that rats selectively bred as alcohol drinkers self-administered both alcohol and ACD directly into the VTA, where ACD showed reinforcing effects at concentrations 1000 lower than those required for alcohol. Unselected animals also perform lever pressing for obtaining ACD through the natural oral route. Indeed ACD was reported to induce and maintain operant drinking behavior according to fixed and progressive ratios of reinforcement (Peana et al., 2011; Cacace et al., 2012). Apart from drug taking, the operant conditioning paradigm serves as an invaluable tool in addiction research, since it enables researchers to explore discrete features of addictive behavior, as reported for humans in the DSM-V (American Psychiatric Association, 2013). Indeed, different schedules of drug reinforcement critically model distinct aspects of incentive motivation for the drug, such as drug seeking and relapse following periods of abstinence, and maintained alcohol use despite adverse consequences that constitute central issues of the translational research on addiction. The employment of such tailored paradigms showed that ACD acts as positive reinforcement that elicits challenging behavior, such as craving and relapse, as shown for alcohol. Indeed, ACD-drinking rats displayed resistance to extinction i.e., the emission of high number of operant responses when reinforce delivery was withheld- and a powerful deprivation-effect when ACD availability was resumed after repeated cycles of deprivation (Peana et al., 2010; Cacace et al., 2012; Plescia et al., 2013; Brancato et al., 2014). The motivational properties of ACD have been further measured by the operant-conflict paradigm, where

an aversive stimulus is associated with rat operant response for ACD. Indeed, when a mild foot-shock was delivered following each lever press rewarded with ACD (punished response), ACD-drinking rats were not discouraged from lever pressing and emitted higher number of punished responses than control rats (Cacace et al., 2012). In the Geiller-Seifter procedure, anxiolytic drugs do not affect the unpunished component of operant responses, whereas drugs with non-specific motor effects decrease it. Actually, ACD was able to increase the unpunished responses, although to a lesser extent than the punished ones, suggesting a prevailing motivational effect, rather than anti-conflict properties (Cannizzaro et al., 2011; Cacace et al., 2012).

ACD AND STRESS RESPONSE

A large and consistent body of literature, on the other hand, shows that both acute and chronic ACD peripheral administrations were associated with anxiety-like behavior in the elevated plus maze (Correa et al., 2005; Plescia et al., 2015a) and with the recruitment of peripheral and central stress response. In particular, ACD was shown to mediate alcohol-induced activation of the hypothalamic-pituitary-adrenal (HPA) axis, since ACD increased plasma corticosterone levels (Kinoshita et al., 2001; Escrig et al., 2012) and induced the release of CRH in a dose-dependent manner (Cannizzaro et al., 2010). Notably, when the oxidation of alcohol into ACD by catalase was inhibited by 3-amino-1,2,4-triazole, CRH release in the presence of alcohol was prevented. Furthermore, the administration of D-penicillamine, an ACD-trapping agent, inhibited ACD-induced CRH release, demonstrating that ACD is the primary mediator of alcohol activity on the HPA axis (Cannizzaro et al., 2010).

The stress system contributes to various extents to the development of alcohol-related behaviors.

Indeed, alcohol- and ACD induce an activation of the stress system that can facilitate behavioral reactivity in aversive conditions (Cacace et al., 2011, 2012; Plescia et al., 2015a). On the other hand repeated cycles of alcohol and ACD intoxication deeply affect the homeostasis of brain stress- and anti-stress system. Indeed, both chronic alcohol and ACD excessive consumption decreased the expression of the anxiolytic peptide NPY in limbic brain regions, such as hippocampus and ventral striatum (Kinoshita et al., 2000; Olling et al., 2007; Plescia et al., 2014). Most importantly, the discontinuation of chronic and high doses of ACD induced a constellation of behavioral signs, such as general hyperactivity, irritability, tail tremors, tail stiffness, general tremor and spasticity, which recall alcohol withdrawal syndrome. ACD withdrawal signs exhibited minor severity, were observed at 12 h from the last ACD administration, started to decline at 16 h and disappeared at 36 h abstinence (Plescia et al., 2014). It is worth noting that during this time CRH expression increased and NPY expression levels decreased in limbic brain areas and in the hypothalamus, causing the occurrence of the aversive psychological state characteristic of withdrawal. These modifications are consistent with the so-called involvement of the "dark side", or stress systems, in the development of alcohol use problems and abuse vulnerability (Thiele et al., 1998; Koob, 2013; Barkley-Levenson et al., 2016). Individuals would consume alcohol in an attempt to return to homeostasis via a negative reinforcement process that maintains and promotes drug taking. Similarly to alcohol, ACD would contribute to engender an aversive (anxious, depressive) state by bidirectional effects on the two major and functionally opposite stress-related peptides, CRH and NPY, thus perpetuating excessive alcohol consumption.

PHARMACOLOGICAL CHARACTERIZATION OF ACD-RELATED BEHAVIOR

ACD and Dopamine

Although the mechanisms by which ACD elicits its effects are poorly explored, this compound activates the neuronal firing of dopaminergic neurons in the VTA, an effect that is mediated by salsolinol, the condensation product of ACD and dopamine (Melis et al., 2007, 2015). Importantly, ACD elicits dopamine release in the NAc shell (Foddai et al., 2004; Melis et al., 2007; Enrico et al., 2009) at the same doses used in CPP studies (Peana et al., 2008, 2009; Spina et al., 2010).

This is not surprising, since the acquisition of drug-induced CPP is critically controlled by dopamine transmission and D1 receptors in the NAc shell (Di Chiara et al., 2004; Tzschentke, 2007). Spina et al. (2010) demonstrated that this also applies to ACD. The blockade of D1 receptors during ACD conditioning, through the pre-treatment with SCH 39166, a D1 dopamine receptor antagonist, also prevented the acquisition of CPP for ACD. In this regard, interference not only on incentive learning processes but also on dopamine-mediated reward has been proposed (Di Chiara et al., 2004).

Besides, dopamine plays a fundamental role in the expression of operant behavior elicited by rewards and reward-related stimuli. Release of dopamine in the NAc shell by Pavlovian stimuli induces an appetitive state of incentive arousal (state—hedonia, euphoria) that facilitates the rate of current instrumental behavior, the acquisition and expression of secondary reinforcement, as well as the consolidation of mnemonic traces of salient stimuli associated with affective states.

It is proven that ACD stimulates dopamine release, and that dopamine increase in the limbic regions accounts for addictive behavioral traits in the rat. Thus, modulating dopamine release or deactivating dopamine signaling could represent a tool able to interrupt the addictive cycle. Indeed, the involvement of dopamine transmission in ACD-related operant behavior was explored by the administration of a D2 dopamine receptor agonist, quinpirole, which at low doses preferentially activates presynaptic D2 dopamine autoreceptors. Thus, by functionally reducing ACD-induced dopamine release, quinpirole decreased the number of lever presses for ACD, also during extinction and, after ACD deprivation, during relapse (Rodd et al., 2005; Brancato et al., 2014). Quinpirole was then able to restrain dopamine signal as supporter of the incentive and rewarding properties of ACD, which indeed was less demanded by

the rats. Interestingly, in accordance with chronic alcohol-induced down regulation of dopamine signaling in the limbic regions, chronic ACD could exert a profound disarrangement in dopamine output to the NAc during withdrawal (Rossetti et al., 1992). Indeed, when ropinirole was sub-chronically administered during ACD deprivation, a decrease in operant responses and ACD intake was observed during relapse (Brancato et al., 2014). This post-synaptic D2 dopamine receptor agonist, useful to restore dopaminergic tone in Parkinson's disease (Tel et al., 2002), likely produced a stimulation of dopamine D2 signaling able to turn off rats craving when ACD was available. This evidence contributes to the suggestion that ACD interaction with the dopamine system plays a role in the development of discrete features of addictive behavior that can be especially relevant to alcohol use disorders.

ACD and Cannabinoids

Along with the dopaminergic transmission, the endocannabinoid system plays an important role in value attribution processing and in modulation of drug-seeking behavior (Serrano and Parsons, 2011; Brancato et al., 2016a; Henderson-Redmond et al., 2016), in view of its role as fine modulator of incoming inputs within the limbic brain regions (D'Amico et al., 2004; Cannizzaro et al., 2006; Melis et al., 2012). Indeed, in rodents, treatment with the CB1 receptor inverse agonist SR141716A (Rimonabant), or CB1 genetic deletion, lead to a reduction in alcohol operant drinking and a decrease in stress-induced alcohol relapse, whereas cannabinoid antagonists mitigate alcohol withdrawal symptoms (Kleczkowska et al., 2016).

Consistently with this significant background, the systemic administration of the selective CB1 receptor antagonist AM281 was evaluated on the operant behavior for ACD. In details, CB1 receptor blockage decreased ACD-seeking behavior during extinction and decreased ACD lever pressing and intake following forced abstinence. Most importantly, the CB1 antagonist decreased the punishment resistance observed in ACD-drinking rats in the operant-conflict paradigm, when the foot-shock was associated with ACD delivery (Plescia et al., 2013). These data suggest that the reinforcing properties of ACD involve endocannabinoids production, which in turn, modulate dopamine mesocorticolimbic pathway and stress response through CB1 receptors. Indeed a recent research employing a binge-like drinking paradigm, pointed to the endocannabinoids as mediators of the detrimental effects exerted by ACD chronic consumption and withdrawal on neuropeptidergic homeostasis, and in particular on the expression of the anti-stress NPY (Plescia et al., 2014). In this study, the administration of the CB1 receptor antagonist was able to ameliorate the behavioral signs that followed withdrawal from chronic ACD; this effect was accompanied by a time- and region-dependent increase in the number of NPY-positive neurons both in the hippocampus and in the NAc. These data prompted us to speculate that ACD binge-like treatment might increase the production of endocannabinoids, thus resulting in downregulation of NPY expression in the hippocampus and in the NAc. Accordingly, during early and prolonged ACD withdrawal, endocannabinoids production may decrease while NPY expression progressively rises. This return to homeostasis can likely contribute to controlling neuronal hyperexcitability and the related behavioral signs (Plescia et al., 2014). Hence, the pharmacological inhibition of CB1 signaling represents a promising strategy for counteracting the neurochemical imbalance associated with ACD- and alcohol-withdrawal syndrome.

CONCLUSIONS

A deeper understanding of the "*ménage à trois*" between ACD, reward- and stress systems is crucial to untangle the etiology of alcohol-related behaviors. Increasing attention must be paid to alcohol, and indirectly to ACD, ingestion during gestation and lactation since the neuronal systems suffer from a severe vulnerability (Cannizzaro et al., 2002, 2005), and ACD has not been studied in the perinatal period yet.

The pharmacological targeting of the endocannabinoid system can exert profound influence on the positive and negative reinforcing effects of ACD, and might accelerate the development of more effective therapeutic interventions to reduce the incidence of alcohol abuse and alcoholism.

AUTHOR CONTRIBUTIONS

AB, GL, AC, FP and CC wrote major parts of the article. All authors critically reviewed and edited the article. The review article was written based on the expertise of the authors, who have sourced the article on PubMed and Google Scholar.

FUNDING

This study was supported by research grant 2012-ATE-0167 provided by the University of Palermo, Italy.

REFERENCES

Abe, K., Yamaguchi, S., Sugiura, M., and Saito, H. (1999). The ethanol metabolite acetaldehyde inhibits the induction of long-term potentiation in the rat dentate gyrus *in vivo*. *Br. J. Pharmacol.* 127, 1805–1810. doi: 10.1038/sj.bjp.0702738

American Psychiatric Association. (2013). *The Diagnostic and Statistical Manual of Mental Disorders: DSM 5.* Washington, DC: Bookpoint US.

D'Amico, M., Cannizzaro, C., Preziosi, P., and Martire, M. (2004). Inhibition by anandamide and synthetic cannabimimetics of the release of [^{3}H] D-aspartate and [^{3}H] GABA from synaptosomes isolated from the rat hippocampus. *Neurochem. Res.* 29, 1553–1561. doi: 10.1023/b:nere.0000029569.20266.3f

Amit, Z., and Smith, B. R. (1985). A multi-dimensional examination of the positive reinforcing properties of acetaldehyde. *Alcohol* 2, 367–370. doi: 10.1016/0741-8329(85)90077-1

Aragon, C. M., and Amit, Z. (1992). The effect of 3-amino-1,2,4-triazole on voluntary ethanol consumption: evidence for brain catalase involvement in the mechanism of action. *Neuropharmacology* 31, 709–712. doi: 10.1016/0028-3908(92)90150-n

Arizzi, M. N., Correa, M., Betz, A. J., Wisniecki, A., and Salamone, J. D. (2003). Behavioral effects of intraventricular injections of low doses of ethanol, acetaldehyde and acetate in rats: studies with low and high rate operant schedules. *Behav. Brain Res.* 147, 203–210. doi: 10.1016/s0166-4328(03)00158-x

Bang, S., Kim, K. Y., Yoo, S., Kim, Y. G., and Hwang, S. W. (2007). Transient receptor potential A1 mediates acetaldehyde-evoked pain sensation. *Eur. J. Neurosci.* 26, 2516–2523. doi: 10.1111/j.1460-9568.2007.05882.x

Barkley-Levenson, A. M., Ryabinin, A. E., and Crabbe, J. C. (2016). Neuropeptide Y response to alcohol is altered in nucleus accumbens of mice selectively bred for drinking to intoxication. *Behav. Brain Res.* 302, 160–170. doi: 10.1016/j.bbr.2016.01.015

Brancato, A., Lavanco, G., Cavallaro, A., Plescia, F., and Cannizzaro, C. (2016a). The use of the emotional-object recognition as an assay to assess learning and memory associated to an aversive stimulus in rodents. *J. Neurosci. Methods.* 274, 106–115. doi: 10.1016/j.jneumeth.2016.09.010

Brancato, A., Plescia, F., Lavanco, G., Cavallaro, A., and Cannizzaro, C. (2016b). Continuous and intermittent alcohol free-choice from pre-gestational time to lactation: focus on drinking trajectories and maternal behavior. *Front. Behav. Neurosci.* 10:31. doi: 10.3389/fnbeh.2016.00031

Brancato, A., Plescia, F., Marino, R. A., Maniaci, G., Navarra, M., and Cannizzaro, C. (2014). Involvement of dopamine D2 receptors in addictive-like behaviour for acetaldehyde. *PLoS One* 9:e99454. doi: 10.1371/journal.pone.0099454

Brasser, S. M., Castro, N., and Feretic, B. (2015). Alcohol sensory processing and its relevance for ingestion. *Physiol. Behav.* 148, 65–70. doi: 10.1016/j.physbeh.2014.09.004

Brown, Z. W., Amit, Z., and Rockman, G. E. (1979). Intraventricular self-administration of acetaldehyde, but not ethanol, in naive laboratory rats. *Psychopharmacology (Berl)* 64, 271–276. doi: 10.1007/bf00427509

Brown, Z. W., Amit, Z., and Smith, B. (1980). Intraventricular self-administration of acetaldehyde and voluntary consumption of ethanol in rats. *Behav. Neural Biol.* 28, 150–155. doi: 10.1016/s0163-1047(80)91487-9

Cacace, S., Plescia, F., Barberi, I., and Cannizzaro, C. (2012). Acetaldehyde oral self-administration: evidence from the operant-conflict paradigm. *Alcohol. Clin. Exp. Res.* 36, 1278–1287. doi: 10.1111/j.1530-0277.2011.01725.x

Cacace, S., Plescia, F., La Barbera, M., and Cannizzaro, C. (2011). Evaluation of chronic alcohol self-administration by a 3-bottle choice paradigm in adult male rats. Effects on behavioural reactivity, spatial learning and reference memory. *Behav. Brain Res.* 219, 213–220. doi: 10.1016/j.bbr.2011.01.004

Cannizzaro, C., D'Amico, M., Preziosi, P., and Martire, M. (2006). Presynaptic effects of anandamide and WIN55,212-2 on glutamatergic nerve endings isolated from rat hippocampus. *Neurochem. Int.* 48, 159–165. doi: 10.1016/j.neuint.2005.10.009

Cannizzaro, C., La Barbera, M., Plescia, F., Cacace, S., and Tringali, G. (2010). Ethanol modulates corticotropin releasing hormone release from the rat hypothalamus: does acetaldehyde play a role? *Alcohol. Clin. Exp. Res.* 34, 588–593. doi: 10.1111/j.1530-0277.2009.01127.x

Cannizzaro, E., Martire, M., Gagliano, M., Plescia, F., La Barbera, M., Mantia, G., et al. (2005). Reversal of prenatal diazepam-induced deficit in a spatial-object learning task by brief, periodic maternal separation in adult rats. *Behav. Brain Res.* 161, 320–330. doi: 10.1016/j.bbr.2005.02.022

Cannizzaro, C., Martire, M., Steardo, L., Cannizzaro, E., Gagliano, M., Mineo, A., et al. (2002). Prenatal exposure to diazepam and alprazolam, but not to zolpidem, affects behavioural stress reactivity in handling-naïve and handling-habituated adult male rat progeny. *Brain Res.* 953, 170–180. doi: 10.1016/s0006-8993(02)03282-1

Cannizzaro, C., Plescia, F., and Cacace, S. (2011). Role of acetaldehyde in alcohol addiction: current evidence and future perspectives. *Malta Med. J.* 23, 27–31.

Cavallaro, A., Lavanco, G., Cannizzaro, C., Brancato, A., Di Majo, D., Giammanco, M., et al. (2016). Acetaldehyde as the first hit of addictive behaviour. *J. Biol. Res.* 86:6206. doi: 10.4081/jbr.2016.6206

Di Chiara, G., Bassareo, V., Fenu, S., De Luca, M. A., Spina, L., Cadoni, C., et al. (2004). Dopamine and drug addiction: the nucleus accumbens shell connection. *Neuropharmacology* 47, 227–241. doi: 10.1016/j.neuropharm.2004.06.032

Cohen, G., Sinet, P. M., and Heikkila, R. (1980). Ethanol oxidation by rat brain *in vivo*. *Alcohol. Clin. Exp. Res.* 4, 366–370. doi: 10.1111/j.1530-0277.1980.tb04833.x

Correa, M., Arizzi, M. N., Betz, A., Mingote, S., and Salamone, J. D. (2003). Open field locomotor effects in rats after intraventricular injections of ethanol and the ethanol metabolites acetaldehyde and acetate. *Brain Res. Bull.* 62, 197–202. doi: 10.1016/j.brainresbull.2003.09.013

Correa, M., Pascual, M., Sanchis-Segura, C., Guerri, C., and Aragon, C. M. (2005). Lead-induced catalase activity differentially modulates behaviors induced by short-chain alcohols. *Pharmacol. Biochem. Behav.* 82, 443–452. doi: 10.1016/j.pbb.2005.09.016

Correa, M., Salamone, J. D., Segovia, K. N., Pardo, M., Longoni, R., Spina, L., et al. (2012). Piecing together the puzzle of acetaldehyde as a neuroactive agent. *Neurosci. Biobehav. Rev.* 36, 404–430. doi: 10.1016/j.neubiorev.2011.07.009

Edenberg, H. J. (2007). The genetics of alcohol metabolism: role of alcohol dehydrogenase and aldehyde dehydrogenase variants. *Alcohol Res. Health* 30, 5–13.

Enrico, P., Sirca, D., Mereu, M., Peana, A. T., Lintas, A., Golosio, A., et al. (2009). Acetaldehyde sequestering prevents ethanol-induced stimulation of mesolimbic dopamine transmission. *Drug Alcohol Depend.* 100, 265–271. doi: 10.1016/j.drugalcdep.2008.10.010

Escrig, M. A., Pardo, M., Aragon, C. M., and Correa, M. (2012). Anxiogenic and stress-inducing effects of peripherally administered acetaldehyde in mice: similarities with the disulfiram-ethanol reaction. *Pharmacol. Biochem. Behav.* 100, 404–412. doi: 10.1016/j.pbb.2011.10.002

Filbey, F. M., Claus, E., Audette, A. R., Niculescu, M., Banich, M. T., Tanabe, J., et al. (2008). Exposure to the taste of alcohol elicits activation of the mesocorticolimbic neurocircuitry. *Neuropsychopharmacology* 33, 1391–1401. doi: 10.1038/sj.npp.1301513

Foddai, M., Dosia, G., Spiga, S., and Diana, M. (2004). Acetaldehyde increases dopaminergic neuronal activity in the VTA. *Neuropsychopharmacology* 29, 530–536. doi: 10.1038/sj.npp.1300326

Franck, J., and Jayaram-Lindström, N. (2013). Pharmacotherapy for alcohol dependence: status of current treatments. *Curr. Opin. Neurobiol.* 23, 692–699. doi: 10.1016/j.conb.2013.05.005

Grüsser, S. M., Heinz, A., and Flor, H. (2000). Standardized stimuli to assess drug craving and drug memory in addicts. *J. Neural Transm.* 107, 715–720. doi: 10.1007/s007020070072

Heap, L., Ward, R. J., Abiaka, C., Dexter, D., Lawlor, M., Pratt, O., et al. (1995). The influence of brain acetaldehyde on oxidative status, dopamine metabolism and visual discrimination task. *Biochem. Pharmacol.* 50, 263–270. doi: 10.1016/0006-2952(94)00539-x

Henderson-Redmond, A. N., Guindon, J., and Morgan, D. J. (2016). Roles for the endocannabinoid system in ethanol-motivated behavior. *Prog. Neuropsychopharmacol. Biol. Psychiatry* 65, 330–339. doi: 10.1016/j.pnpbp.2015.06.011

Jamal, M., Ameno, K., Tanaka, N., Ito, A., Takakura, A., Kumihashi, M., et al. (2016). Ethanol and acetaldehyde after intraperitoneal administration to Aldh2-knockout mice-reflection in blood and brain levels. *Neurochem. Res.* 41, 1029–1034. doi: 10.1007/s11064-015-1788-6

Kanteres, F., Lachenmeier, D. W., and Rehm, J. (2009). Alcohol in mayan guatemala: consumption, distribution, production and composition of cuxa. *Addiction* 104, 752–759. doi: 10.1111/j.1360-0443.2009.02507.x

Karahanian, E., Quintanilla, M. E., Tampier, L., Rivera-Meza, M., Bustamante, D., Gonzalez-Lira, V., et al. (2011). Ethanol as a prodrug: brain metabolism of ethanol mediates its reinforcing effects. *Alcohol. Clin. Exp. Res.* 35, 606–612. doi: 10.1111/j.1530-0277.2011.01439.x

Kareken, D. A., Claus, E. D., Sabri, M., Dzemidzic, M., Kosobud, A. E., Radnovich, A. J., et al. (2004). Alcohol-related olfactory cues activate the nucleus accumbens and ventral tegmental area in high-risk drinkers: preliminary findings. *Alcohol. Clin. Exp. Res.* 28, 550–557. doi: 10.1097/01.alc.0000122764.60626.af

Kinoshita, H., Jessop, D. S., Finn, D. P., Coventry, T. L., Roberts, D. J., Ameno, K., et al. (2000). Acute ethanol decreases NPY mRNA but not POMC mRNA in the arcuate nucleus. *Neuroreport* 11, 3517–3519. doi: 10.1097/00001756-200011090-00023

Kinoshita, H., Jessop, D. S., Finn, D. P., Coventry, T. L., Roberts, D. J., Ameno, K., et al. (2001). Acetaldehyde, a metabolite of ethanol, activates the hypothalamic-pituitary-adrenal axis in the rat. *Alcohol Alcohol.* 36, 59–64. doi: 10.1093/alcalc/36.1.59

Kleczkowska, P., Smaga, I., Filip, M., and Bujalska-Zadrozny, M. (2016). Cannabinoid ligands and alcohol addiction: a promising therapeutic tool

or a humbug? *Neurotox. Res.* 29, 173–196. doi: 10.1007/s12640-015-9555-7

Koob, G. F. (2013). Theoretical frameworks and mechanistic aspects of alcohol addiction: alcohol addiction as a reward deficit disorder. *Curr. Top. Behav. Neurosci.* 13, 3–30. doi: 10.1007/7854_2011_129

Lachenmeier, D. W., Gill, J. S., Chick, J., and Rehm, J. (2015). The total margin of exposure of ethanol and acetaldehyde for heavy drinkers consuming cider or vodka. *Food Chem. Toxicol.* 83, 210–214. doi: 10.1016/j.fct.2015.05.006

Lachenmeier, D. W., and Sohnius, E. M. (2008). The role of acetaldehyde outside ethanol metabolism in the carcinogenicity of alcoholic beverages: evidence from a large chemical survey. *Food Chem. Toxicol.* 46, 2903–2911. doi: 10.1016/j.fct.2008.05.034

Lachenmeier, D. W., Sohnius, E. M., Attig, R., and López, M. G. (2006). Quantification of selected volatile constituents and anions in Mexican agave spirits (Tequila, Mezcal, Sotol, Bacanora). *J. Agric. Food Chem.* 54, 3911–3915. doi: 10.1021/jf060094h

Linderborg, K., Joly, J. P., Visapää, J. P., and Salaspuro, M. (2008). Potential mechanism for Calvados-related oesophageal cancer. *Food Chem. Toxicol.* 46, 476–479. doi: 10.1016/j.fct.2007.08.019

Martí-Prats, L., Sánchez-Catalán, M. J., Orrico, A., Zornoza, T., Polache, A., and Granero, L. (2013). Opposite motor responses elicited by ethanol in the posterior VTA: the role of acetaldehyde and the non-metabolized fraction of ethanol. *Neuropharmacology* 72, 204–214. doi: 10.1016/j.neuropharm.2013.04.047

Martire, M., Altobelli, D., Cannizzaro, C., Maurizi, S., and Preziosi, P. (2002). Prenatal diazepam exposure functionally alters the $GABA_A$ receptor that modulates [^{3}H] noradrenaline release from rat hippocampal synaptosomes. *Dev. Neurosci.* 24, 71–78. doi: 10.1159/000064947

Mascia, M. P., Maiya, R., Borghese, C. M., Lobo, I. A., Hara, K., Yamakura, T., et al. (2001). Does acetaldehyde mediate ethanol action in the central nervous system? *Alcohol. Clin. Exp. Res.* 25, 1570–1575. doi: 10.1111/j.1530-0277.2001.tb02163.x

Melis, M., Carboni, E., Caboni, P., and Acquas, E. (2015). Key role of salsolinol in ethanol actions on dopamine neuronal activity of the posterior ventral tegmental area. *Addict. Biol.* 20, 182–193. doi: 10.1111/adb.12097

Melis, M., Enrico, P., Peana, A. T., and Diana, M. (2007). Acetaldehyde mediates alcohol activation of the mesolimbic dopamine system. *Eur. J. Neurosci.* 26, 2824–2833. doi: 10.1111/j.1460-9568.2007.05887.x

Melis, M., Muntoni, A. L., and Pistis, M. (2012). Endocannabinoids and the processing of value-related signals. *Front. Pharmacol.* 3:7. doi: 10.3389/fphar.2012.00007

Miranda, M. B., Martins, N. G. S., Belluco, A. E. S., Horii, J., and Alcarde, A. R. (2007). Chemical quality of Brazilian sugarcane spirits. *Cienc. Tecnol. Aliment.* 27, 897–901. doi: 10.1590/S0101-20612007000400034

Myers, W. D., Gibson, S., Ng, K. T., and Singer, G. (1987). Sex differences in acetaldehyde on body temperature and open-field performance in the rat. *Drug Alcohol Depend.* 19, 1–6. doi: 10.1016/0376-8716(87)90081-0

Myers, W. D., Ng, K. T., Marzuki, S., Myers, R. D., and Singer, G. (1984). Alteration of alcohol drinking in the rat by peripherally self-administered acetaldehyde. *Alcohol* 1, 229–236. doi: 10.1016/0741-8329(84)90103-4

Oliveira, V. A., Vicente, M. A., Fietto, L. G., Castro, I. M., Coutrim, M. X., Schu-ller, D., et al. (2008). Biochemical and molecular characterization of *Saccharomyces cerevisiae* strains obtained from sugar-cane juice fermentations and their impact in cacha-a production. *Appl. Environ. Microbiol.* 74, 693–701. doi: 10.1128/aem.01729-07

Olling, J. D., Ulrichsen, J., Haugbøl, S., Glenthøj, B., Hemmingsen, R., and Woldbye, D. P. (2007). Decreased gene expression of neuropeptide Y and its receptors in hippocampal regions during ethanol withdrawal in rats. *Neurosci. Lett.* 424, 160–164. doi: 10.1016/j.neulet.2007.07.050

Pardo, M., Betz, A. J., San Miguel, N., López-Cruz, L., Salamone, J. D., and Correa, M. (2013). Acetate as an active metabolite of ethanol: studies of locomotion, loss of righting reflex and anxiety in rodents. *Front. Behav. Neurosci.* 7:81. doi: 10.3389/fnbeh.2013.00081

Peana, A. T., Assaretti, A. R., Muggironi, G., Enrico, P., and Diana, M. (2009). Reduction of ethanol-derived acetaldehyde induced motivational properties by L-cysteine. *Alcohol. Clin. Exp. Res.* 33, 43–48. doi: 10.1111/j.1530-0277.2008.00809.x

Peana, A. T., Enrico, P., Assaretti, A. R., Pulighe, E., Muggironi, G., Nieddu, M., et al. (2008). Key role of ethanol-derived acetaldehyde in the motivational properties induced by intragastric ethanol: a conditioned place preference study in the rat. *Alcohol. Clin. Exp. Res.* 32, 249–258. doi: 10.1111/j.1530-0277.2007.00574.x

Peana, A. T., Muggironi, G., and Diana, M. (2010). Acetaldehyde-reinforcing effects: a study on oral self-administration behavior. *Front. Psychiatry* 1:23. doi: 10.3389/fpsyt.2010.00023

Peana, A. T., Muggironi, G., Fois, G. R., Zinellu, M., Vinci, S., and Acquas, E. (2011). Effect of opioid receptor blockade on acetaldehyde self-administration and ERK phosphorylation in the rat nucleus accumbens. *Alcohol* 45, 773–783. doi: 10.1016/j.alcohol.2011.06.003

Plescia, F., Brancato, A., Marino, R. A., and Cannizzaro, C. (2013). Acetaldehyde as a drug of abuse: insight into AM281 administration on operant-conflict paradigm in rats. *Front. Behav. Neurosci.* 7:64. doi: 10.3389/fnbeh.2013.00064

Plescia, F., Brancato, A., Marino, R. A., Vita, C., Navarra, M., and Cannizzaro, C. (2014). Effect of acetaldehyde intoxication and withdrawal on NPY expression: focus on endocannabinoidergic system involvement. *Front. Psychiatry* 5:138. doi: 10.3389/fpsyt.2014.00138

Plescia, F., Brancato, A., Venniro, M., Maniaci, G., Cannizzaro, E., Sutera, F. M., et al. (2015a). Acetaldehyde self-administration by a two-bottle choice paradigm: consequences on emotional reactivity, spatial learning and memory. *Alcohol* 49, 139–148. doi: 10.1016/j.alcohol.2015.01.002

Plescia, F., Cannizzaro, E., Brancato, A., Martines, F., Di Naro, A., Mucia, M., et al. (2015b). Acetaldehyde effects in the brain. *Acta Med. Mediterr.* 31, 813–817.

Quertemont, E., and De Witte, P. (2001). Conditioned stimulus preference after acetaldehyde but not ethanol injections. *Pharmacol. Biochem. Behav.* 68, 449–454. doi: 10.1016/s0091-3057(00)00486-x

Quertemont, E., Grant, K. A., Correa, M., Arizzi, M. N., Salamone, J. D., Tambour, S., et al. (2005). The role of acetaldehyde in the central effects of ethanol. *Alcohol. Clin. Exp. Res.* 29, 221–234. doi: 10.1097/01.ALC.0000156185.39073.D2

Quertemont, E., Tambour, S., Bernaerts, P., Zimatkin, S. M., and Tirelli, E. (2004). Behavioral characterization of acetaldehyde in C57BL/6J mice: locomotor, hypnotic, anxiolytic and amnesic effects. *Psychopharmacology (Berl)* 177, 84–92. doi: 10.1007/s00213-004-1911-x

Quintanilla, M. E., Tampier, L., Karahanian, E., Rivera-Meza, M., Herrera-Marschitz, M., and Israel, Y. (2012). Reward and relapse: complete gene-induced dissociation in an animal model of alcohol dependence. *Alcohol. Clin. Exp. Res.* 36, 517–522. doi: 10.1111/j.1530-0277.2011.01606.x

Ramchandani, V. A., Bosron, W. F., and Li, T. K. (2001). Research advances in ethanol metabolism. *Pathol. Biol. (Paris)* 49, 676–682. doi: 10.1016/s0369-8114(01)00232-2

Redila, V. A., Aliatas, E., Smith, B. R., and Amit, Z. (2002). Effects of ethanol on an acetaldehyde drug discrimination with a conditioned taste aversion procedure. *Alcohol* 28, 103–109. doi: 10.1016/s0741-8329(02)00270-7

Rodd, Z. A., Bell, R. L., Kuc, K. A., Murphy, J. M., Lumeng, L., Li, T. K., et al. (2003). Effects of repeated alcohol deprivations on operant ethanol self-administration by alcohol-preferring (P) rats. *Neuropsychopharmacology* 28, 1614–1621. doi: 10.1038/sj.npp.1300214

Rodd, Z. A., Bell, R. L., Zhang, Y., Murphy, J. M., Goldstein, A., Zaffaroni, A., et al. (2005). Regional heterogeneity for the intracranial self-administration of ethanol and acetaldehyde within the ventral tegmental area of alcohol-preferring (P) rats: involvement of dopamine and serotonin. *Neuropsychopharmacology* 30, 330–338. doi: 10.1038/sj.npp.1300561

Roper, S. D. (2014). TRPs in taste and chemesthesis. *Handb. Exp. Pharmacol.* 223, 827–871. doi: 10.1007/978-3-319-05161-1_5

Rossetti, Z. L., Melis, F., Carboni, S., Diana, M., and Gessa, G. L. (1992). Alcohol withdrawal in rats is associated with a marked fall in extraneuronal dopamine. *Alcohol. Clin. Exp. Res.* 16, 529–532. doi: 10.1111/j.1530-0277.1992.tb01411.x

Samson, H. H., Pfeffer, A. O., and Tolliver, G. A. (1988). Oral ethanol self-administration in rats: models of alcohol-seeking behavior. *Alcohol. Clin. Exp. Res.* 12, 591–598. doi: 10.1111/j.1530-0277.1988.tb00248.x

Segovia, K. N., Vontell, R., López-Cruz, L., Salamone, J. D., and Correa, M. (2013). c-Fos immunoreactivity in prefrontal, basal ganglia and limbic areas of the rat brain after central and peripheral administration of ethanol and its metabolite acetaldehyde. *Front. Behav. Neurosci.* 7:48. doi: 10.3389/fnbeh.2013.00048

Serrano, A., and Parsons, L. H. (2011). Endocannabinoid influence in drug reinforcement, dependence and addiction-related behaviors. *Pharmacol. Ther.* 132, 215–241. doi: 10.1016/j.pharmthera.2011.06.005

Smith, B. R., Amit, Z., and Splawinsky, J. (1984). Conditioned place preference induced by intraventricular infusions of acetaldehyde. *Alcohol* 1, 193–195. doi: 10.1016/0741-8329(84)90097-1

Smith, B. R., Aragon, C. M., and Amit, Z. (1997). Catalase and the production of brain acetaldehyde: a possible mediator of the psychopharmacological effects of ethanol. *Addict. Biol.* 2, 277–290. doi: 10.1080/13556219772570

Spina, L., Longoni, R., Vinci, S., Ibba, F., Peana, A. T., Muggironi, G., et al. (2010). Role of dopamine D1 receptors and extracellular signal regulated kinase in the motivational properties of acetaldehyde as assessed by place preference conditioning. *Alcohol. Clin. Exp. Res.* 34, 607–616. doi: 10.1111/j.1530-0277.2009.01129.x

Stormark, K. M., Laberg, J. C., Bjerland, T., Nordby, H., and Hugdahl, K. (1995). Autonomic cued reactivity in alcoholics: the effect of olfactory stimuli. *Addict. Behav.* 20, 571–584. doi: 10.1016/0306-4603(95)00017-7

Tabakoff, B., Anderson, R. A., and Ritzmann, R. F. (1976). Brain acetaldehyde after ethanol administration. *Biochem. Pharmacol.* 25, 1305–1309. doi: 10.1016/0006-2952(76)90094-0

Tampier, L., Quintanilla, M. E., Karahanian, E., Rivera-Meza, M., Herrera-Marschitz, M., and Israel, Y. (2013). The alcohol deprivation effect: marked inhibition by anticatalase gene administration into the ventral tegmental area in rats. *Alcohol. Clin. Exp. Res.* 37, 1278–1285. doi: 10.1111/acer.12101

Tampier, L., Quintanilla, M. E., and Mardones, J. (1995). Effects of aminotriazole on ethanol, water and food intake and on brain catalase in UChA and UChB rats. *Alcohol* 12, 341–344. doi: 10.1016/0741-8329(95)00014-i

Tel, B. C., Zeng, B. Y., Cannizzaro, C., Pearce, R. K., Rose, S., and Jenner, P. (2002). Alterations in striatal neuropeptide mRNA produced by repeated administration of L-DOPA, ropinirole or bromocriptine correlate with dyskinesia induction in MPTP-treated common marmosets. *Neuroscience* 115, 1047–1058. doi: 10.1016/s0306-4522(02)00535-3

Thiele, T. E., Marsh, D. J., Ste Marie, L., Bernstein, I. L., and Palmiter, R. D. (1998). Ethanol consumption and resistance are inversely related to neuropeptide Y levels. *Nature* 396, 366–369. doi: 10.1038/24614

Tzschentke, T. M. (2007). Measuring reward with the conditioned place preference (CPP) paradigm: update of the last decade. *Addict. Biol.* 12, 227–462. doi: 10.1111/j.1369-1600.2007.00070.x

Ward, R. J., Colantuoni, C., Dahchour, A., Quertemont, E., and De Witte, P. (1997). Acetaldehyde-induced changes in monoamine and amino acid extracellular microdialysate content of the nucleus accumbens. *Neuropharmacology* 36, 225–232. doi: 10.1016/s0028-3908(97)00007-5

Yamamoto, T. (2006). Neural substrates for the processing of cognitive and affective aspects of taste in the brain. *Arch. Histol. Cytol.* 69, 243–255. doi: 10.1679/aohc.69.243

Zorumski, C. F., Mennerick, S., and Izumi, Y. (2014). Acute and chronic effects of ethanol on learning-related synaptic plasticity. *Alcohol* 48, 1–17. doi: 10.1016/j.alcohol.2013.09.045

Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

frontiers in Behavioral Neuroscience

ORIGINAL RESEARCH
published: 02 November 2016
doi: 10.3389/fnbeh.2016.00206

Ethanol and Caffeine Effects on Social Interaction and Recognition in Mice: Involvement of Adenosine A_{2A} and A_1 Receptors

*Laura López-Cruz[1], Noemí San-Miguel[1], Pilar Bayarri[1], Younis Baqi[2], Christa E. Müller[2], John D. Salamone[3] and Mercé Correa[1,3]**

[1] Àrea de Psicobiologia, Campus de Riu Sec, Universitat Jaume I, Castelló, Spain, [2] Pharma-Zentrum Bonn, Pharmazeutisches Institut, Pharmazeutische Chemie, Universität Bonn, Bonn, Germany, [3] Department of Psychological Sciences, University of Connecticut, Storrs, CT, USA

Ethanol and caffeine are frequently consumed in combination and have opposite effects on the adenosine system: ethanol metabolism leads to an increase in adenosine levels, while caffeine is a non-selective adenosine A_1/A_{2A} receptor antagonist. These receptors are highly expressed in striatum and olfactory tubercle, brain areas involved in exploration and social interaction in rodents. Ethanol modulates social interaction processes, but the role of adenosine in social behavior is still poorly understood. The present work was undertaken to study the impact of ethanol, caffeine and their combination on social behavior, and to explore the involvement of A_1 and A_{2A} receptors on those actions. Male CD1 mice were evaluated in a social interaction three-chamber paradigm, for preference of conspecific vs. object, and also for long-term recognition memory of familiar vs. novel conspecific. Ethanol showed a biphasic effect, with low doses (0.25 g/kg) increasing social contact and higher doses (1.0–1.5 g/kg) reducing social interaction. However, no dose changed social preference; mice always spent more time sniffing the conspecific than the object, independently of the ethanol dose. Ethanol, even at doses that did not change social exploration, produced amnestic effects on social recognition the following day. Caffeine reduced social contact (15.0–60.0 mg/kg), and even blocked social preference at higher doses (30.0–60.0 mg/kg). The A_1 antagonist Cyclopentyltheophylline (CPT; 3–9 mg/kg) did not modify social contact or preference on its own, and the A_{2A} antagonist MSX-3 (1.5–6 mg/kg) increased social interaction at all doses. Ethanol at intermediate doses (0.5–1.0 g/kg) was able to reverse the reduction in social exploration induced by caffeine (15.0–30.0 mg/kg). Although there was no interaction between ethanol and CPT or MSX-3 on social exploration in the first day, MSX-3 blocked the amnestic effects of ethanol observed on the following day. Thus, ethanol impairs the formation of social memories, and A_{2A} adenosine antagonists can prevent the amnestic effects of ethanol, so that animals can recognize familiar conspecifics. On the other hand, ethanol can counteract the social withdrawal induced by caffeine, a non-selective adenosine A_1/A_{2A} receptor antagonist. These results show the complex set of interactions between ethanol and caffeine, some of which could be the result of the opposing effects they have in modulating the adenosine system.

Keywords: ethanol, adenosine, social exploration, social memory, anxiety, caffeine

OPEN ACCESS

Edited by:
Donald A. Wilson, New York University School of Medicine, USA

Reviewed by:
Susan A. Masino, Trinity College, USA
Charlis Raineki, University of British Columbia, Canada

****Correspondence:***
Mercé Correa
correa@psb.uji.es

Received: *05 August 2016*
Accepted: *10 October 2016*
Published: *02 November 2016*

Citation:
López-Cruz L, San-Miguel N, Bayarri P, Baqi Y, Müller CE, Salamone JD and Correa M (2016) Ethanol and Caffeine Effects on Social Interaction and Recognition in Mice: Involvement of Adenosine A_{2A} and A_1 Receptors. Front. Behav. Neurosci. 10:206. doi: 10.3389/fnbeh.2016.00206

INTRODUCTION

Alcohol and caffeine are the most consumed psychoactive drugs worldwide. In recent times, it has become common to consume high doses of caffeine in combination with ethanol in order to reduce the intoxicating effects of the alcohol (Ferré and O'Brien, 2011; López-Cruz et al., 2013; Correa et al., 2014). Caffeine and ethanol act on the adenosine system in distinct ways that can result in opposite physiological and behavioral effects. Caffeine is a non-selective adenosine antagonist that acts mainly on A_1 and A_{2A} receptors (Fredholm et al., 1999), whereas ethanol has been demonstrated to increase the adenosinergic tone by inhibiting the endonucleotid transporter type-1, thus, blocking adenosine uptake (Nagy et al., 1990; Krauss et al., 1993), and also by increasing the synthesis of adenosine during ethanol metabolism (Carmichael et al., 1991; López-Cruz et al., 2013).

Adenosine is a neuromodulator in the central nervous system (CNS) that plays an important role in the regulation of synaptic transmission and neuronal excitability (Cunha, 2001; Sebastião and Ribeiro, 2009). Several subtypes of adenosine receptors are expressed in the brain, with A_1 and A_{2A} being the most abundant. A_{2A} receptors are expressed at high levels, mostly in the striatum and olfactory bulbs and tubercle (Schiffmann et al., 1991; Fredholm et al., 2001), regions that are involved in the regulation of motivated (Salamone and Correa, 2002, 2012; Hauber and Sommer, 2009), and social behaviors (Sano et al., 2008; Pena et al., 2014). However, A_1 receptors have a widespread distribution in the brain, with a somewhat higher concentration in hippocampus (Schwarzschild et al., 2006).

It is well known that ethanol consumption facilitates interactions with peers and alleviates anxiety (Varlinskaya and Spear, 2002; Kirchner et al., 2006). In rodent models of social interaction, acute ethanol administration at low doses produces social facilitation (Nadal et al., 1993; Varlinskaya and Spear, 2009), but dose-related decrements in social interaction after high doses also have been observed in mice (Lister and Hilakivi, 1988; Hilakivi et al., 1989). Caffeine was shown to decrease social interaction in mice and rats (Baldwin and File, 1989; Baldwin et al., 1989; Hilakivi et al., 1989), effects that have been suggested to be related to its anxiogenic actions (Baldwin et al., 1989; Hilakivi et al., 1989; Prediger et al., 2004). However, very little is known about the interaction of both substances on social exploration and social memory (Hilakivi et al., 1989; Spinetta et al., 2008). The amnestic effect of ethanol is well known. Although ethanol at low doses can act as a short-term social memory enhancer in mice (Manrique et al., 2005), high doses of ethanol can cause amnesia, or impaired retrieval of memory, after the drug wears off (Goodwin, 1995; Hartzler and Fromme, 2003). This effect of ethanol could be explained by the fact that adenosine and adenosine receptor agonists have been demonstrated to impair short-term social recognition memory in rats (Prediger and Takahashi, 2005). On the other hand, selective A_1 and A_{2A} receptor antagonists can improve short-term social memory (Prediger and Takahashi, 2005).

The present work evaluated the effect of a broad range of doses of caffeine, in combination with ethanol, on social motivation as measured by preference towards a conspecific vs. a neutral object. Our procedure minimized anxiety induced by aggression, avoiding whole-body contact. In a second phase of the behavioral test, long-term social recognition memory was studied 24 h after the drug was administered and the preference test had taken place. In addition, the role of A_1 and A_{2A} receptors on social motivation and memory were also evaluated using selective adenosine antagonists alone or in combination with ethanol.

MATERIALS AND METHODS

Subjects

Adult male CD1 mice (30–45 g) were purchased from Janvier (France). Mice were housed in groups of three per cage, with standard laboratory rodent chow and tap water available *ad libitum*. They were maintained in the colony at 22 ± 1°C with lights on from 8:00 to 20:00 h. All experimental procedures were approved by "Comité de bienestar animal, UJI" and complied with the European Community Council directive (86/609/ECC) for the use of laboratory animal subjects and with the "Guidelines for the Care and Use of Mammals in Neuroscience and Behavioral Research" (National Research Council 2003).

Drugs

Caffeine (Sigma-Aldrich, Spain) and MSX3 ((*E*)-phosphoric acid mono-[3-[8-[2-(3-methoxphenyl)vinyl]-7-methyl-2,6-dioxo-1-prop-2-ynyl-1,2,6,7-tetrahydropurin-3-yl] propyl] ester disodium salt; synthesized at the laboratory of Dr. Christa E. Müller at the Pharmazeutisches Institut, Universität Bonn, Germany) were dissolved in 0.9% w/v saline. 8-cyclopentyltheophylline (CPT; purchased from Sigma-Aldrich, Spain) was dissolved in distilled water (pH = 8.0). All these drugs were administered intraperitoneally (IP) 30 min before testing. Ethanol (Panreac Quimica S.A., Spain) was diluted to 20% (v/v) in physiological saline (0.9% w/v) and administered IP 10 min before testing. Saline solution was used as vehicle. These doses and time leads were selected based on previous studies done in our laboratory with the same strain of mice (Correa et al., 2008; Pardo et al., 2013; López-Cruz et al., 2014). For the interaction studies we selected doses that did not impair locomotion, but showed some effect in the social procedures. The dose of CPT was selected because it was the one closest to reaching a significant effect in the social interaction test.

Behavioral Apparatus and Testing Procedures

Social Preference and Social Recognition Tests

The effects of adenosine antagonists on social preference were measured in a three-chambered social box (originally developed by Crawley, 2004). The general procedure was adapted from Chévere-Torres et al. (2012). Every mouse had two consecutive habituation sessions in the chambers: in the first one, they freely explored the empty social arena during

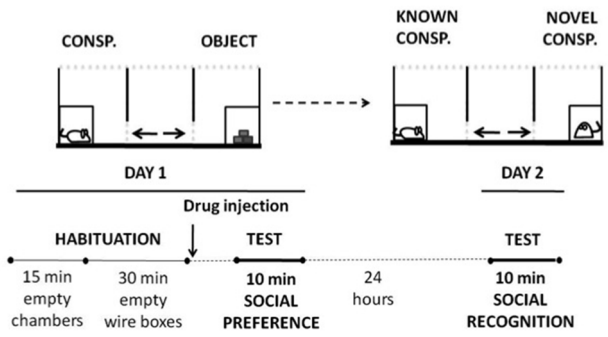

FIGURE 1 | Schematic representation of social preference and social recognition tests settings and timeline.

15 min, and immediately there was a second exploration session, that lasted 30 min, in the presence of two wire cages, one in each of the side-compartments. After the 45-min habituation period, different groups of animals received their corresponding treatment and were placed in an individual cage during 10 or 30 min (depending on the drug). After this time, mice were placed in the center chamber of the social interaction apparatus and test started. During the test session (10 min), the three-chambered arena contained a caged conspecific on one side, and on the other side there was a small wire cage containing an object. The center compartment was empty (see **Figure 1** for a schematic on the procedure). The placement of the conspecific or the object was counterbalanced between animals. A trained experimenter who was unaware of the experimental conditions, registered manually time spent sniffing each target (conspecific vs. object) as a measure of social preference. Vertical and horizontal locomotion were also registered. Twenty-four hours after the social preference test, mice were placed back in the central chamber and were subjected to a 10 min social recognition test (Moy et al., 2004). No drugs were administered before this second test. During the recognition test a novel mouse replaced the object, and the experimental mice were given the choice to interact with the familiar conspecific (same conspecific used in the social preference test the day before) vs. a novel conspecific. Time spent sniffing each conspecific was registered.

Statistics

One-way analysis of variance (ANOVA) was used to analyze the effect of drug administration on the different dependent variables; time spent sniffing conspecific, object, familiar and novel conspecific, and vertical and horizontal locomotion. Two-way factorial ANOVA was used for the interaction studies. When the overall ANOVA was significant, non-orthogonal planned comparisons using the overall error term were used to compare each treatment with the control group (Keppel, 1991). For these comparisons, α level was kept at 0.05 because the number of comparisons was restricted to the number of treatments minus one. Student's *t*-test for dependent samples was used to analyze "preference" (e.g., conspecific vs. object, or familiar vs. novel conspecifics). A probability level of 0.05 or smaller was used to indicate statistical significance. Statistics were done using STATISTICA 7 software.

RESULTS

Experiment 1: Effect of Ethanol on Social Preference and Locomotion: Impact on Long-Term Social Recognition Memory

In this experiment, mice (N = 45) received saline or ethanol (0.25, 0.5, 1.0 or 1.5 g/kg) 10 min before been evaluated in the social preference test. The following day, the same animals were tested for social recognition memory in the absence of drug. Ethanol treatment, as shown by the one-way ANOVA, had a significant effect on time spent sniffing the conspecific ($F_{(4,40)}$ = 20.12, $p < 0.01$), and planned comparisons revealed that ethanol at the lowest dose (0.25 g/kg) increased conspecific exploration ($p < 0.01$) in comparison with vehicle treatment, while higher doses decreased time with conspecific (1.0 and 1.5 g/kg, $p < 0.05$ and $p < 0.01$ respectively). The one-way ANOVA for time spent sniffing the object ($F_{(4,40)}$ = 4.45, $p < 0.01$) was also significant. However, only the highest dose of ethanol (1.5 g/kg) significantly reduced ($p < 0.01$) time spent sniffing the object compared to the vehicle treated group (**Figure 2A**). When comparing time exploring both stimuli in the same animals, Student *t*-test for dependent samples showed that in the vehicle group there was a significant difference in time spent sniffing the conspecific vs. the object ($t = -8.28$, $p < 0.01$), a pattern that was repeated at all doses of ethanol (0.25 g/kg, $t = -5.49$, $p < 0.01$; 0.5 g/kg, $t = -5.75$, $p < 0.01$; 1.0 g/kg, $t = 2.61$, $p < 0.05$; 1.5 g/kg $t = -2.76$, $p < 0.01$; **Figure 2A**). Thus, independently of the ethanol dose used, all groups explored the conspecific more than the object, showing a clear preference for social interaction.

There was no significant effect of ethanol treatment on total crosses ($F_{(4,40)}$ = 0.59, n.s.; **Figure 2C**) and on vertical locomotion ($F_{(4,40)}$ = 2.25, n.s.; **Figure 2D**).

One day after the social interaction test took place, social recognition was evaluated, and the results of the one-way ANOVA showed an overall effect of previous exposure to ethanol on time spent sniffing the familiar conspecific ($F_{(4,40)}$ = 2.08, $p < 0.05$). Ethanol at doses of 0.25 and 1.5 g/kg increased time spent at sniffing the familiar conspecific ($p < 0.05$ and $p < 0.01$ respectively) compared to the group previously treated with vehicle. A significant effect of ethanol administered the previous day was also observed on time spent sniffing the novel conspecific ($F_{(4,40)}$ = 5.78, $p < 0.01$). Only animals that had received the lowest dose of ethanol (0.25 g/kg) increased time spent sniffing the novel conspecific in comparison with the vehicle group ($p < 0.01$; **Figure 2B**). Student's *t*-test for dependent samples showed that vehicle animals spent more time sniffing the novel than the familiar conspecific (t = 5.32, $p < 0.01$), a pattern that was only observed in the group that had received the lower dose of ethanol (0.25 g/kg, t = 2.46, $p < 0.05$), suggesting that ethanol, even at doses that had no effect

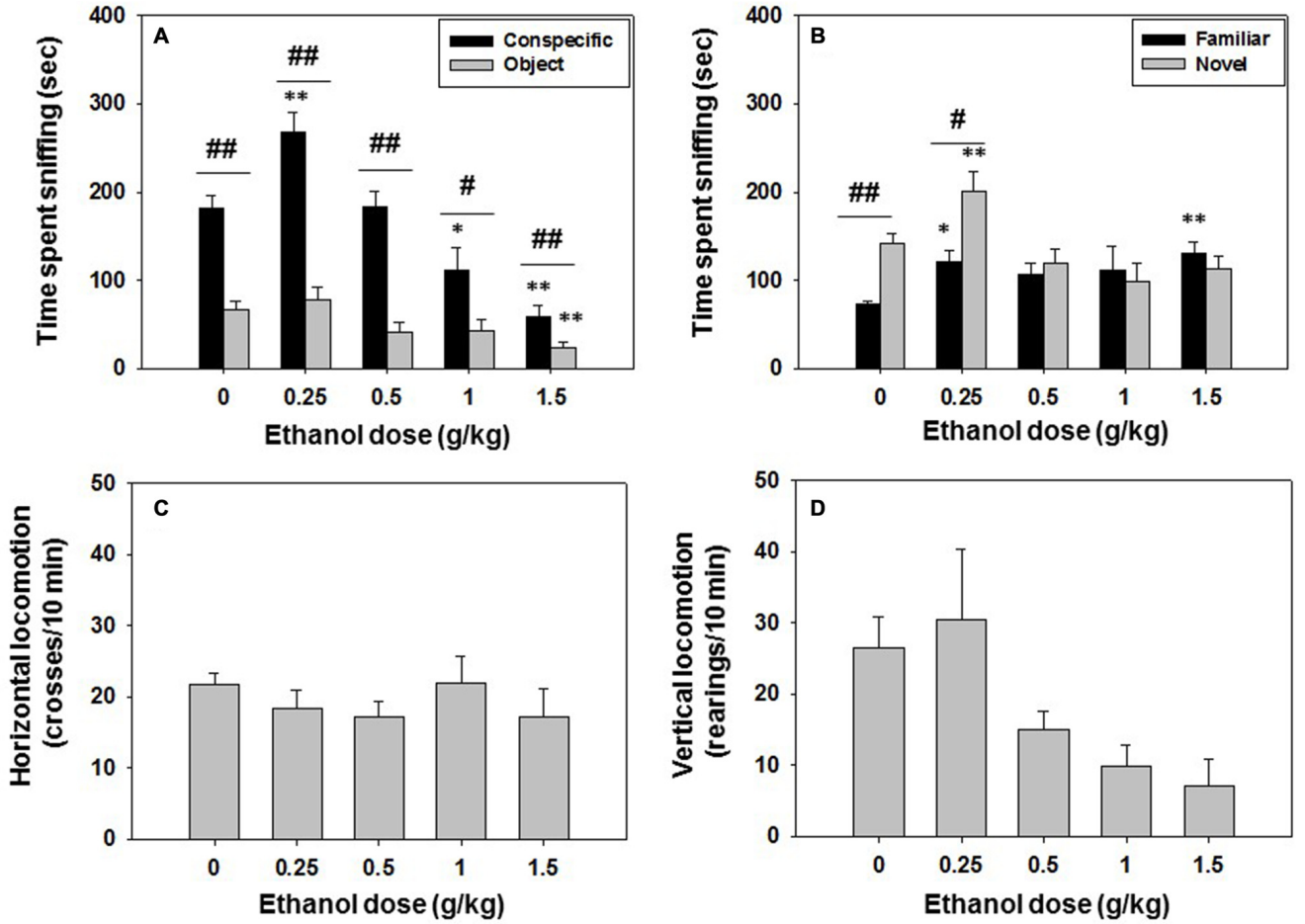

FIGURE 2 | Effect of ethanol in social preference and recognition tests. Data are expressed as mean (±SEM) of time spent sniffing **(A)** conspecific and object in the social preference test, **(B)** familiar and novel conspecifics in the social recognition test, and **(C)** horizontal and **(D)** vertical locomotion during the social preference test. $^{*}p < 0.05$, $^{**}p < 0.01$ significant differences from a vehicle for the same target. $^{\#}p < 0.05$, $^{\#\#}p < 0.01$ significant differences between time spent sniffing both targets for the same dose of ethanol.

on social exploration the day before (0.5 g/kg), can impair social recognition 24 h after been administered.

Experiment 2: Effect of The Non-Selective Adenosine A_1/A_{2A} Antagonist Caffeine on Social Preference and Locomotion: Impact on Long-Term Social Recognition Memory

Mice (N = 44) were injected with saline or caffeine (7.5, 15.0, 30.0 or 60.0 mg/kg) 30 min before the social interaction test started. The following day (24 h later) no drugs were administered and social recognition was evaluated as described before. The one-way ANOVA revealed an overall effect of caffeine on time spent sniffing the conspecific ($F_{(4,39)}$ = 21.12, $p < 0.01$). Planned comparison analysis showed a significant decrement in time spent sniffing the conspecific after caffeine administration at doses of 15.0, 30.0 and 60.0 mg/kg ($p < 0.01$). The one-way ANOVA for the effect of caffeine on time spent sniffing the object ($F_{(3.39)}$ = 4.03, $p < 0.01$) was also significant, and the planned comparisons revealed that the same doses of caffeine (15.0, 30.0 and 60.0 mg/kg) decreased time spent sniffing the object compared to vehicle ($p < 0.05$, $p < 0.01$ and $p < 0.01$, respectively). The Student's t-test for dependent samples was used to compare time spent sniffing the conspecific with time spent sniffing the object. The vehicle treated group spent more time exploring the conspecific than the object ($t = 5.24$, $p < 0.01$), and this pattern of behavior was also preserved after the administration of moderate doses of caffeine (7.5 and 15.0 mg/kg; $t = 6.28$, $p < 0.01$, $t = 3.84$, $p < 0.01$ respectively) but not after the highest doses (30.0 and 60.0 mg/kg; **Figure 3A**), indicating a lack of preference for the conspecific after mice received the higher doses of caffeine.

The one-way ANOVA revealed an overall effect of caffeine on horizontal locomotion ($F_{(4,39)}$ = 7.90 $p < 0.01$). Caffeine significantly increased horizontal locomotion at low to intermediate doses (7.5 and 15.0 mg/kg; $p < 0.01$) compared to vehicle, but did not have a significant effect at

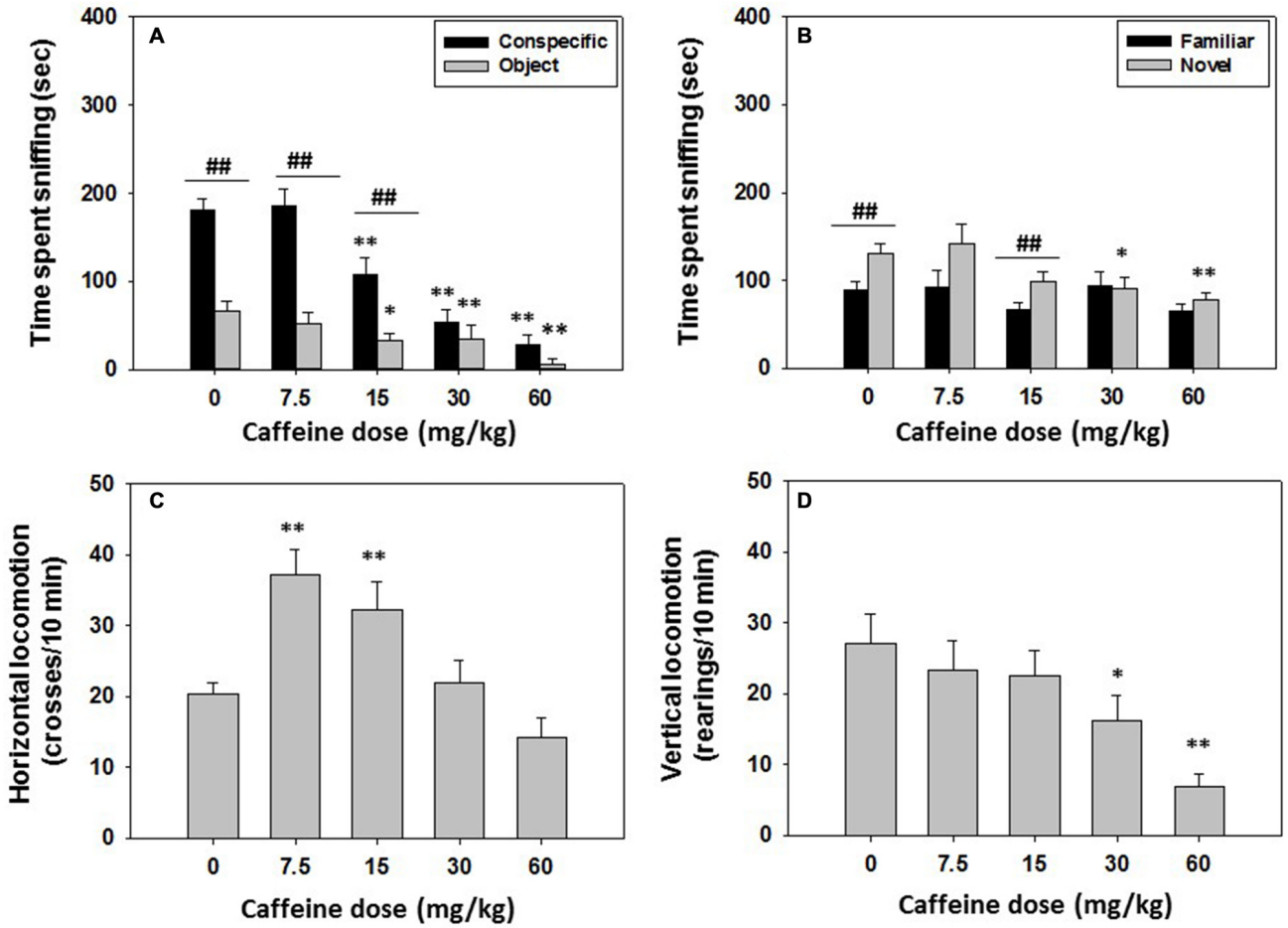

FIGURE 3 | Effect of caffeine on social preference and recognition tests. Data are expressed as mean (±SEM) of time spent sniffing **(A)** conspecific and object in the social preference test, **(B)** familiar and novel conspecifics in the social recognition test, and **(C)** horizontal and **(D)** vertical locomotion during the social preference test. $^{*}p < 0.05$, $^{**}p < 0.01$ significant differences from a vehicle for the same target. $^{\#\#}p < 0.01$ significant differences between time spent sniffing both targets for the same dose of caffeine.

higher doses. The one-way ANOVA for vertical locomotion ($F_{(4,39)} = 4.60$ $p < 0.01$) was also significant, but for this dependent variable, planned comparisons revealed that the higher doses (30.0 and 60.0 mg/kg), significantly decreased vertical locomotion in comparison with the vehicle treated group ($p < 0.05$ and $p < 0.01$, respectively; **Figures 3C,D**). This decrease in locomotion could be influencing the reduction in time dedicated to targeted exploration, more importantly, to conspecific exploration.

For the social recognition results, the one-way ANOVA revealed no significant effect of the previous treatment with caffeine on time spent sniffing the familiar conspecific ($F_{(4,39)} = 1.37$, n.s.). However, there was an overall effect of previous caffeine treatment on time spent sniffing the novel conspecific ($F_{(4,39)} = 3.83$, $p < 0.01$). Planned comparisons revealed that compared with vehicle the highest doses of caffeine (30.0 and 60.0 mg/kg) significantly decreased time spent sniffing the novel conspecific ($p < 0.05$ and $p < 0.01$, respectively; **Figure 3B**). Student's t-test for dependent samples showed that the vehicle group spent more time sniffing the novel conspecific than sniffing the familiar one ($t = -3.40$, $p < 0.01$), and this was also observed in the group that received 15.0 mg/kg of caffeine ($t = -3.31$, $p < 0.01$), but not the rest of the doses (**Figure 3B**).

Experiment 3: Effect of Caffeine-Ethanol Co-Administration on Social Preference and Locomotion: Impact on Long-Term Social Recognition Memory

For experiment 3, mice ($N = 74$) received an injection of vehicle or caffeine (15.0 or 30.0 mg/kg; 30 min before being tested) plus vehicle or ethanol (0.5 or 1.0 g/kg; 10 min before test), and were evaluated for social preference and locomotion. The following day, the same animals were tested in the social recognition test. Factorial ANOVA (Caffeine × Ethanol) on time

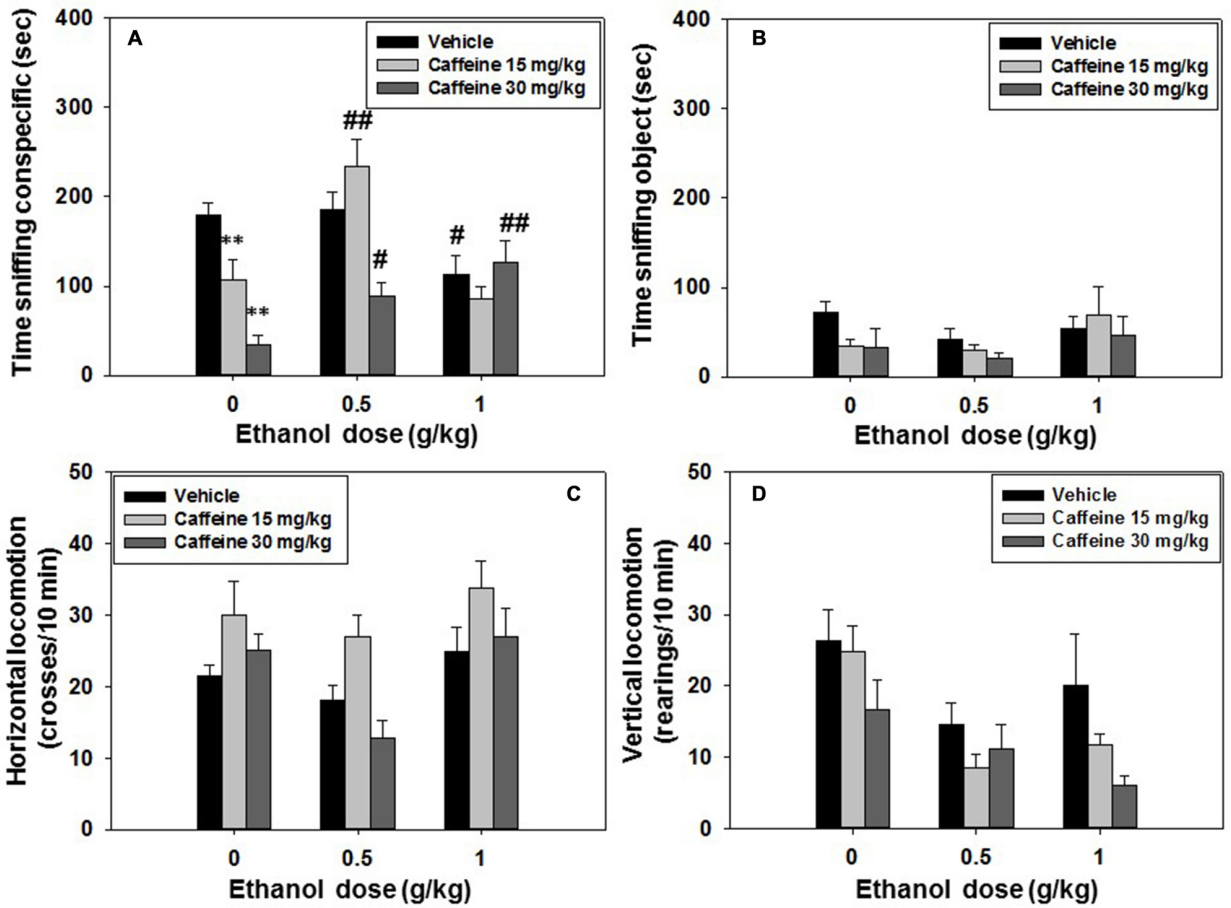

FIGURE 4 | Effect of caffeine plus ethanol interaction in the social preference test. Data are expressed as mean (±SEM) of time spent sniffing **(A)** conspecific, **(B)** object, **(C)** horizontal and **(D)** vertical locomotion during the social preference test. $^{*}p < 0.05$, $^{**}p < 0.01$ significantly different from the vehicle group in the same dose of ethanol. $^{\#}p < 0.05$, $^{\#\#}p < 0.01$ significantly different from the group that received the same dose of caffeine plus ethanol 0.0 g/kg.

spent sniffing the conspecific showed overall effects of caffeine ($F_{(2,65)} = 13.33$, $p < 0.01$), and ethanol ($F_{(2,65)} = 9.97$, $p < 0.01$) and also a significant interaction ($F_{(4,65)} = 8.99$, $p < 0.05$). Planned comparisons confirmed that when compared with the vehicle-vehicle group only the highest dose of ethanol used in the present study (1.0 g/kg) reduced conspecific exploration ($p < 0.05$), and that the two doses of caffeine (15.0 and 30.0 mg/kg) selected for this experiment also reduced social exploration ($p < 0.01$). In terms of the interactions, the group that received the lowest dose of caffeine (15.0 mg/kg) in combination with the lowest dose of ethanol (0.5 g/kg) was significantly different ($p < 0.01$) from the group that had received that dose of caffeine but no ethanol, pointing to a reversal effect of ethanol on the caffeine-induced impairment. However, the effect of this dose of caffeine was not reversed when given in combination with the highest dose of ethanol (1.0 g/kg). As for the impairing effect on conspecific exploration observed in the group that had received the highest dose of caffeine (30.0 mg/kg) plus vehicle, this effect was partially reversed by the two doses of ethanol ($p < 0.05$ and $p < 0.01$ respectively; **Figure 4A**). The factorial ANOVA (Caffeine × Ethanol) for the dependent variable time spent sniffing, the conspecific did not show a significant effect of caffeine ($F_{(2,65)} = 1.31$, n.s.), of ethanol ($F_{(2,65)} = 1.69$, n.s.) or the interaction ($F_{(4,65)} = 0.71$, n.s.), (**Figure 4B**).

Factorial ANOVA (Caffeine × Ethanol) for total crosses as a measure of horizontal locomotion revealed an overall effect of caffeine ($F_{(2,65)} = 7.22$, $p < 0.01$), and ethanol ($F_{(2,65)} = 6.27$, $p < 0.01$), but no significant interaction ($F_{(4,65)} = 0.77$, n.s.), (**Figure 4C**). A separate factorial ANOVA for vertical locomotion showed the same pattern of results. It revealed an effect of caffeine ($F_{(2,65)} = 4.23$, $p < 0.05$), and of ethanol ($F_{(2,65)} = 7.74$, $p < 0.01$), but no significant caffeine-ethanol interaction ($F_{(4,65)} = 0.81$, n.s.; **Figure 4D**).

The results for the impact of these pharmacological manipulations on social recognition memory evaluated the day after the drug injection, and the preference test, are shown in **Table 1**. The factorial ANOVA (Caffeine × Ethanol) showed an overall effect of caffeine ($F_{(2,65)} = 3.72$, $p < 0.05$), and of ethanol ($F_{(2,65)} = 8.27$, $p < 0.01$) on time spent sniffing

TABLE 1 | Effect of caffeine-ethanol coadministration on social recognition memory.

Etoh (g/kg)	0.0		0.5		1.0	
Caffeine (mg/kg)	**Familiar**	**Novel**	**Familiar**	**Novel**	**Familiar**	**Novel**
0.0	87.5 ± 9.1	136.4 ± 12.1$^{\#\#}$	111.4 ± 14.5	124.1 ± 16.6	115.9 ± 21.7	106 ± 19.1
15.0	71.2 ± 7.1	100.1 ± 13.2$^{\#}$	120.6 ± 27.3	98.1 ± 12.5	72.1 ± 10.5	102.3 ± 21.7
30.0	33.0 ± 11.1	31.9 ± 21.1	103.6 ± 11.8	137.2 ± 21.9	83.3 ± 11.2	91.1 ± 12.7

Data are expressed as mean ± SEM of time (in seconds) spent sniffing the novel and the familiar conspecifics. $^{\#\#}p < 0.01$, $^{\#}p < 0.05$ significant differences between time with familiar vs. time with novel conspecific for the same dose of ethanol group.

the familiar conspecific. However, there was no significant caffeine × ethanol interaction ($F_{(4,65)} = 1.49$, n.s.). In terms of time spent sniffing the novel conspecific, the factorial ANOVA revealed no significant effect of ethanol ($F_{(2,65)} = 2.37$, n.s.), but a significant effect of caffeine ($F_{(2,65)} = 3.43$, $p < 0.05$), and a significant interaction ($F_{(2,65)} = 0.91$, $p < 0.01$). The Student's *t*-test for dependent samples comparing time spent sniffing familiar conspecific vs. novel conspecific revealed that the group that had received vehicle-vehicle injections the day before spent significantly more time sniffing the novel conspecific than the familiar conspecific ($t = 4.96$, $p < 0.01$), and the same was true for the animals treated with the low dose of caffeine (15.0 mg/kg) plus saline ($t = 2.85$, $p < 0.05$), indicating that mice recognized the familiar conspecific. However, the lower dose of caffeine (15.0 mg/kg) did not block the impairing effect on recognition produced by ethanol (0.5 or 1.0 g/kg).

Experiment 4: Effect of The Selective Adenosine A_1 Receptor Antagonist CPT on Social Preference and Locomotion: Impact on Long-Term Social Recognition Memory

Mice ($N = 37$) were injected with vehicle or CPT at doses of 3.0, 6.0, or 9.0 mg/kg 30 min before being tested in the social preference task, and 24 h later the same animals were tested in the social recognition test. The effect of CPT on time spent sniffing the conspecific analyzed by a one-way ANOVA revealed no significant effect ($F_{(3,33)} = 2.13$, n.s.). However, the one-way ANOVA on the effect of CPT on time spent sniffing the non-social target was significant ($F_{(3,33)} = 5.21$, $p < 0.01$). Planned comparison revealed that CPT significantly decreased time spent exploring the object at all doses of CPT in comparison with the vehicle group ($p < 0.01$; **Figure 5A**), suggesting an increase in relative preference for the conspecific. Student's *t*-test for dependent samples showed significant differences in all the groups in time spent sniffing the conspecific vs. the object. Animals spent more time sniffing the conspecific after saline ($t = 5.37$, $p < 0.05$), CPT 3.0 mg/kg ($t = 11.25$, $p < 0.01$), CPT 6.0 mg/kg ($t = 6.38$, $p < 0.01$), and CPT 9.0 mg/kg ($t = 5.95$, $p < 0.01$).

These doses of CPT did not affect the horizontal ($F_{(3,33)} = 1.03$, n.s.) or vertical locomotion ($F_{(3,33)} = 1.42$, n.s.), as analyzed by one-way ANOVA's (**Figures 5C,D**).

For the social recognition test, the one-way ANOVA did not show a significant effect of CPT dose on time spent sniffing the familiar conspecific ($F_{(3,33)} = 0.14$, n.s.), or on time spent sniffing the novel conspecific ($F_{(3,33)} = 0.02$, n.s.). Student's *t*-test for dependent samples showed significant differences between time spent sniffing the novel vs. the familiar conspecific in the vehicle group ($t = -3.82$, $p < 0.01$), as expected when animals recognized the previously explored conspecific, and this effect was also observed in the animals that had received the highest dose of CPT 9.0 mg/kg the day before ($t = -3.25$, $p < 0.05$), but not the lower doses (**Figure 5B**).

Experiment 5: Effect of CPT–Ethanol Co-Administration on Social Preference and Locomotion: Impact on Long-Term Social Recognition Memory

Mice ($N = 60$) received an injection of vehicle or CPT 6.0 mg/kg 20 min before the test, and a second injection of vehicle or ethanol (0.5 or 1.0 g/kg) 10 min before the social preference test started. The following day, the same animals were tested in the social recognition test with no drug been administered. A factorial ANOVA (CPT × Ethanol) showed an overall effect of ethanol ($F_{(2,41)} = 5.33$, $p < 0.05$), but no significant effect of CPT ($F_{(1,41)} = 0.32$, n.s) or CPT-ethanol interaction ($F_{(2,41)} = 1.60$, n.s.) on time spent sniffing the conspecific (**Figure 6A**). The factorial ANOVA for time spent sniffing the object (**Figure 6B**) did not reveal a significant effect of CPT ($F_{(1,41)} = 0.43$, n.s.), of ethanol ($F_{(2,41)} = 1.46$, n.s.), or of the interaction ($F_{(2,41)} = 2.21$, n.s.) either.

The factorial ANOVA (CPT × Ethanol) on horizontal locomotion yield no significant effect of ethanol ($F_{(2,41)} = 0.55$, n.s.), CPT ($F_{(1,42)} = 2.36$, n.s) or CPT-ethanol interaction ($F_{(2,41)} = 2.86$, n.s.; **Figure 6C**). As for vertical locomotion, there was a significant effect of ethanol ($F_{(2,41)} = 6.59$, $p < 0.01$), but not a significant effect of CPT ($F_{(1,41)} = 0.03$, n.s.) or of CPT-ethanol interaction ($F_{(2,41)} = 1.82$, n.s.; **Figure 6D**).

For the social recognition test the factorial ANOVA (CPT × Ethanol) did not show a significant effect of CPT ($F_{(1,41)} = 1.06$, n.s.), of ethanol ($F_{(2,41)} = 0.97$, n.s.), or of the interaction ($F_{(2,41)} = 0.05$, n.s.) on time spent sniffing the familiar conspecific. The factorial ANOVA for the variable time spent sniffing the novel conspecific, did not show an overall effect of CPT ($F_{(1,41)} = 0.38$, n.s), ethanol ($F_{(2,41)} = 1.78$,

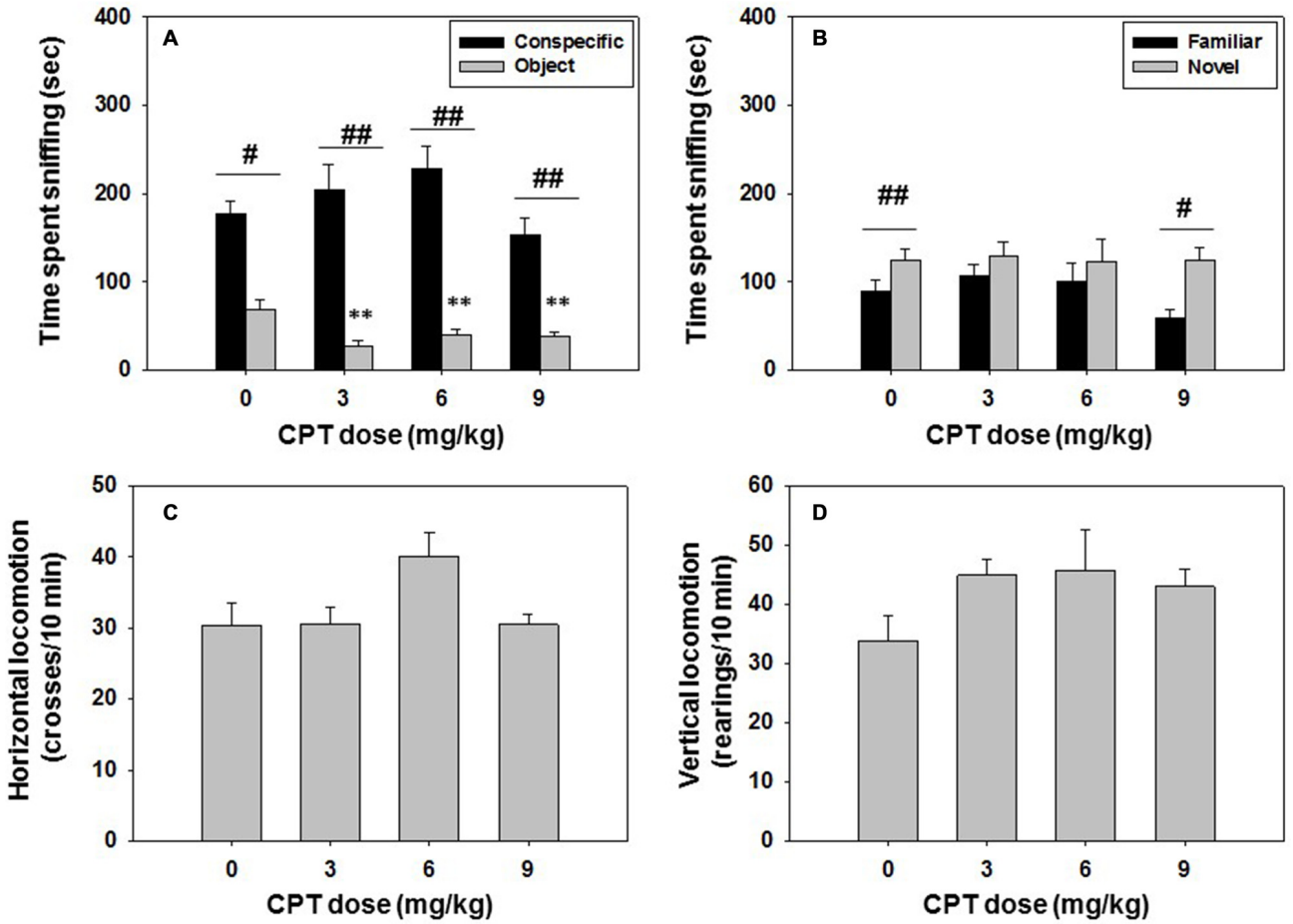

FIGURE 5 | Effect of Cyclopentyltheophylline (CPT) in the social preference and recognition tests. (A) Conspecific and object in the social preference test, **(B)** familiar and novel conspecifics in the social recognition test, and **(C)** horizontal and **(D)** vertical locomotion during the social preference test. $^{**}p < 0.01$ significant differences from vehicle for the same target. $^{\#}p < 0.05$, $^{\#\#}p < 0.01$ significant differences between time spent sniffing both targets for the same dose of CPT.

n.s.), or CPT-ethanol interaction ($F_{(2,41)}$ = 1.11, n.s.) either. Student's *t*-test for dependent samples showed significant differences between time spent at sniffing the novel vs. familiar conspecific only in the control group (t = 4.7, $p < 0.01$), confirming that ethanol as shown before impaired social recognition at all doses, and indicating that CPT (6 mg/kg) did not block the amnestic effects of ethanol (data shown in **Table 2**).

Experiment 6: Effect of The Selective Adenosine A_{2A} Receptor Antagonist MSX-3 on Social Preference and Locomotion: Impact on Long-Term Social Recognition Memory

Different groups of mice (N = 36) received an acute administration of vehicle or MSX-3 at doses of 1.5, 3.0, or 6.0 mg/kg, 30 min before the social interaction test. The same animals were tested 24 h later in the social recognition test. The one-way ANOVA revealed an overall effect of MSX-3 on time spent sniffing the conspecific ($F_{(3,32)}$ = 4.58, $p < 0.01$), and planned comparison showed that all doses increased significantly the time spent sniffing the social target (1.5 mg/kg, $p < 0.05$; 3.0 mg/kg and 6.0 mg/kg, $p < 0.01$) compared with the vehicle treated group. The one-way ANOVA for the dependent variable time spent exploring the object was also significant ($F_{(3,32)}$ = 3.63, $p < 0.05$). MSX-3 significantly decreased the time exploring the object at all doses (1.5 mg/kg, $p < 0.05$; 3.0 mg/kg and 6.0 mg/kg, $p < 0.01$) when compared with the vehicle group. Student *t*-test for dependent samples demonstrated that there were significant differences in time spent sniffing the conspecific vs. the object in the vehicle group (t = 12.96, $p < 0.01$), but also in all the MSX-3 treated groups (MSX-3 1.5 mg/kg, t = 7.96, $p < 0.01$; MSX-3 3.0 mg/kg, t = 10.33 $p < 0.01$, and MSX-3 6.0 mg/kg, t = 6.87 $p < 0.01$; **Figure 7A**).

The impact of MSX-3 on locomotion is shown in **Figures 7C,D**. The ANOVA for the effect of MSX-3 on horizontal locomotion was significant ($F_{(3,32)}$ = 3.66, $p < 0.05$), and planned comparisons showed a significant effect of all

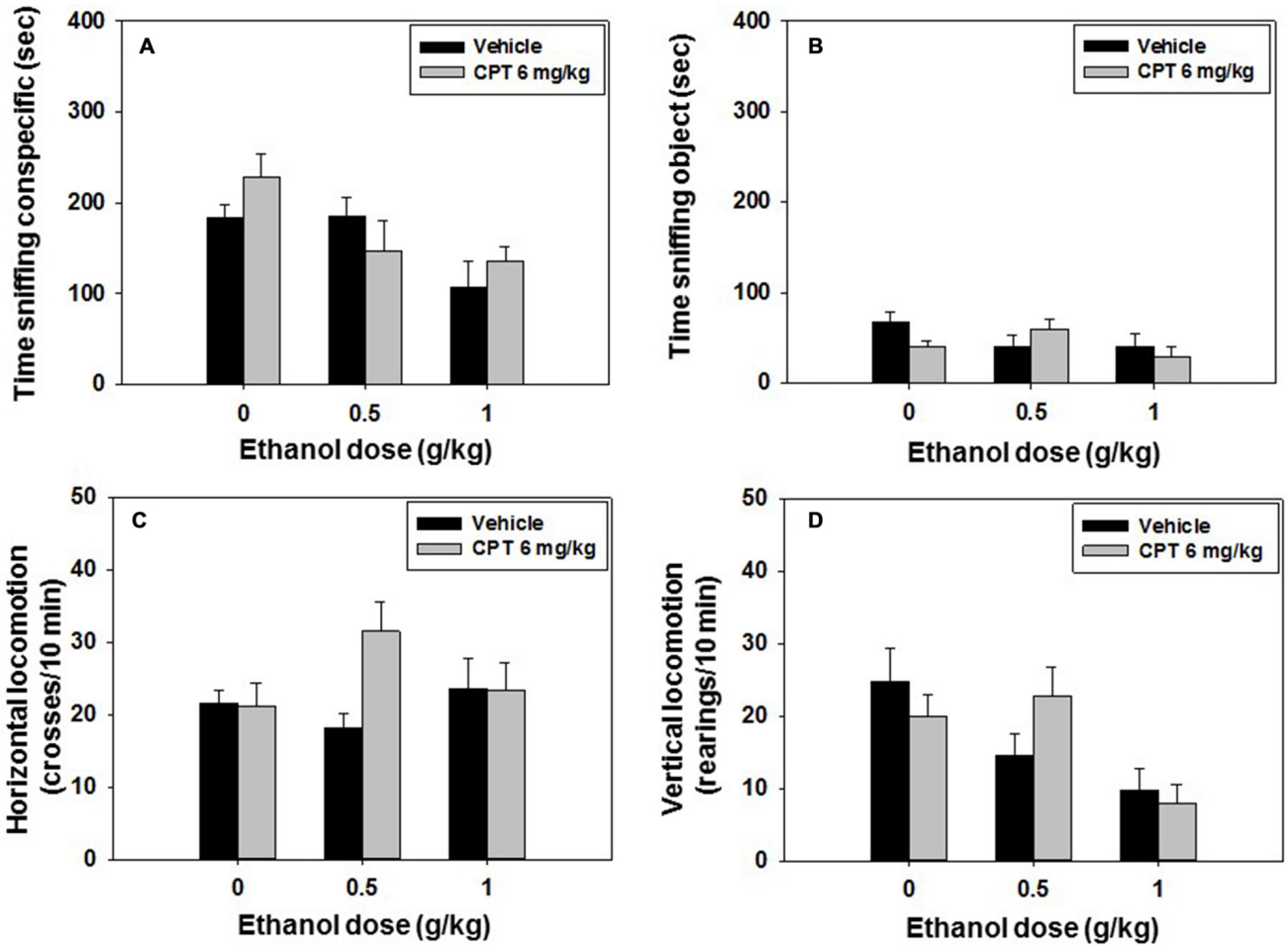

FIGURE 6 | Effect of CPT plus ethanol interaction on the social preference test. Data are expressed as mean (±SEM) of time spent sniffing **(A)** conspecific, **(B)** object, **(C)** horizontal and **(D)** vertical locomotion during the social preference test.

TABLE 2 | Effects of Cyclopentyltheophylline (CPT) ethanol combination on social recognition memory.

Etoh (g/kg)	0.0		0.5		1.0	
CPT (mg/kg)	**Familiar**	**Novel**	**Familiar**	**Novel**	**Familiar**	**Novel**
0.0	74.1 ± 4.5	139.4 ± 12.4$^{\#\#}$	122.6 ± 12.4	124.1 ± 16.6	99.0 ± 22.0	102.5 ± 12.4
6.0	100.1 ± 20.5	123.5 ± 25.3	111.0 ± 14.6	172.3 ± 27.8	119.6 ± 23.3	105.0 ± 27.8

Data are expressed as mean ± SEM of time in seconds spent sniffing novel and familiar conspecifics. $^{\#\#}p < 0.01$ significant differences between time in familiar vs. time in novel conspecific for the same dose of CPT and ethanol.

doses of MSX-3 on total crosses between compartments as a measure of horizontal locomotion (1.5 mg/kg and 3.0 mg/kg, $p < 0.05$; and 6.0 mg/kg, $p < 0.01$). However, the one-way ANOVA for vertical locomotion was not significant ($F_{(3,32)} = 1.83$, n.s.).

For the social recognition test, the one-way ANOVA revealed no significant effect of MSX-3 on time spent sniffing the familiar conspecific ($F_{(3,32)} = 1.83$, n.s.), and also no significant effect of this drug on novel conspecific exploration ($F_{(3,32)} = 0.61$, n.s.; **Figure 7B**). Student's *t*-test for dependent samples showed significant differences between time spent sniffing novel vs. familiar conspecific in the vehicle group ($t = -4.71$, $p < 0.01$), as expected, and this pattern was also observed in the MSX-3 1.5 mg/kg, ($t = -2.64$, $p < 0.05$) and the MSX-3 6.0 mg/kg groups ($t = -2.42$, $p < 0.05$). The intermediate dose of MSX-3 3.0 mg/kg almost reach significant levels ($t = -2.13$, $p = 0.06$). Thus, MSX-3 administered the day before did not affect social recognition memory.

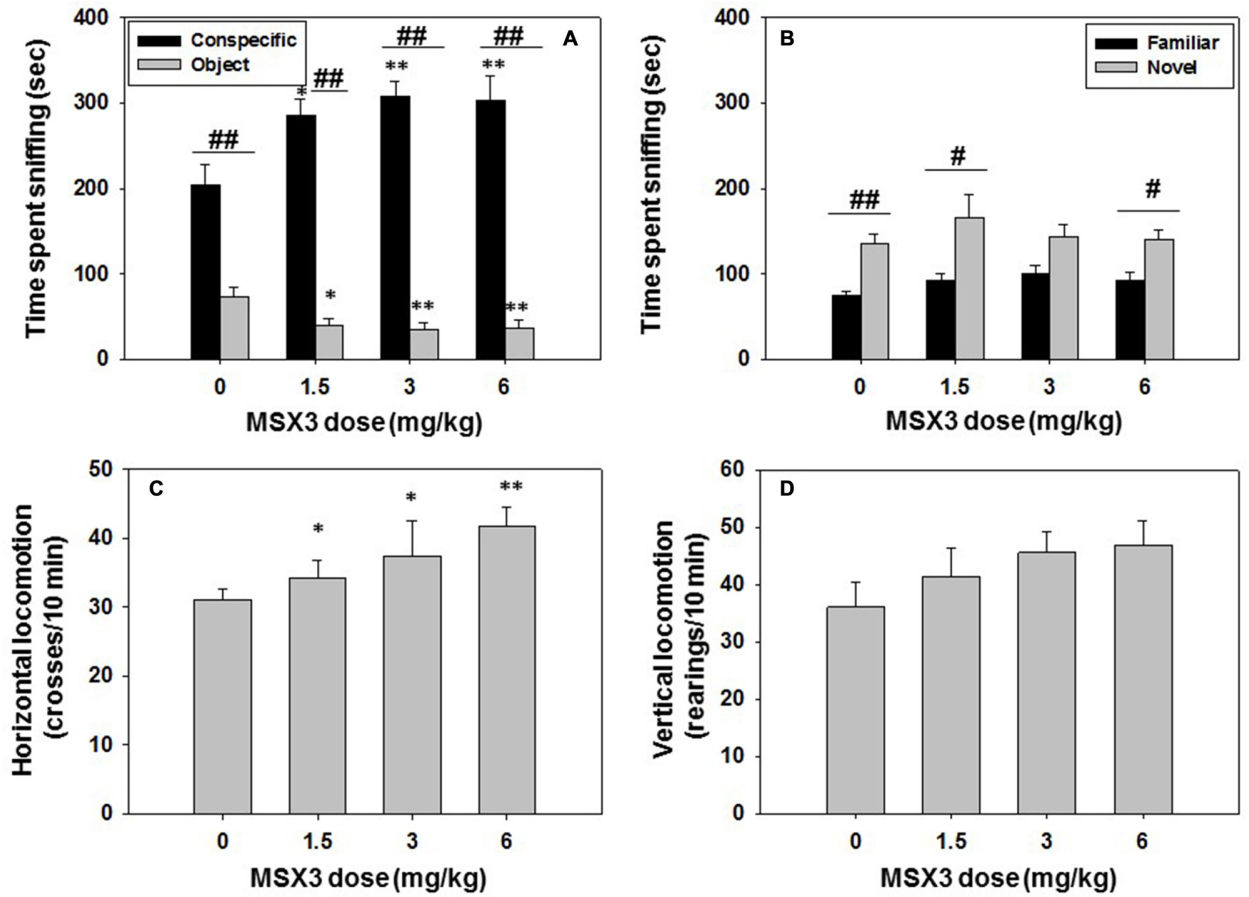

FIGURE 7 | Effect of MSX3 in social preference and recognition tests. Data are expressed as mean (±SEM) of time spent sniffing **(A)** conspecific and object in the social preference test, **(B)** familiar and novel conspecifics in the social recognition test, and **(C)** horizontal and **(D)** vertical locomotion during the social preference test. $^{*}p < 0.05$, $^{**}p < 0.01$ significant differences from vehicle for the same target. $^{\#}p < 0.05$, $^{\#\#}p < 0.01$ significant differences between time spent sniffing both targets for the same dose of MSX3.

Experiment 7: Effect of MSX3–Ethanol Co-Administration on Social Preference and Locomotion: Impact on Long-Term Social Recognition Memory

Mice ($N = 50$) received a dose of vehicle or of the lowest dose of MSX-3 (1.5 mg/kg) that was effective in experiment 6. MSX-3 was administered 20 min before test, and 10 min before the social preference test, a second injection of vehicle or ethanol (0.5 or 1.0 g/kg) was administered. The following day, the same animals were tested for social long-term memory. A factorial ANOVA (MSX-3 × Ethanol) revealed an overall effect of MSX-3 ($F_{(1,43)} = 40.65$, $p < 0.01$), and of ethanol ($F_{(2,43)} = 3.36$, $p < 0.05$) on time spent sniffing the conspecific. However, there was not a significant interaction ($F_{(2,43)} = 0.34$, n.s.; **Figure 8A**). The factorial ANOVA for time spent sniffing the object did not reveal a significant effect of MSX-3 ($F_{(1,43)} = 1.45$, n.s.), or ethanol ($F_{(2,43)} = 0.49$, n.s.), and no significant interaction ($F_{(2,43)} = 2.23$, n.s.) either (**Figure 8B**).

Total crosses between compartments as a measure of horizontal locomotion were overall affected by MSX-3 ($F_{(1,43)} = 21.18$, $p < 0.01$), but not by ethanol ($F_{(2,43)} = 2.42$, n.s.), and there was no significant interaction either ($F_{(2,43)} = 0.30$, n.s.). The one-way ANOVA for vertical locomotion revealed a significant effect of ethanol ($F_{(2,43)} = 3.99$, $p < 0.05$), but no effect of MSX3 ($F_{(1,43)} = 2.27$, n.s.), and no significant interaction ($F_{(2,43)} = 0.11$, n.s; See **Figures 8C,D**).

As for the impact of these drugs on recognition of the conspecific presented during the preference test, the factorial ANOVA (MSX-3 × Ethanol) for time spent sniffing the familiar conspecific showed a significant effect of ethanol ($F_{(2,43)} = 6.97$, $p < 0.01$), but did not show an effect of MSX-3 ($F_{(1,43)} = 0.02$, n.s.), and no MSX-3 × ethanol interaction on this variable ($F_{(2,43)} = 2.14$, n.s.; **Table 3**). Another factorial ANOVA for the variable time spent sniffing the novel conspecific, did not reveal an effect of MSX-3 ($F_{(1,43)} = 0.14$, n.s.), it did not show a significant effect of ethanol although it was close to significance ($F_{(2,43)} = 2.73$, $p = 0.08$), and the interaction was not

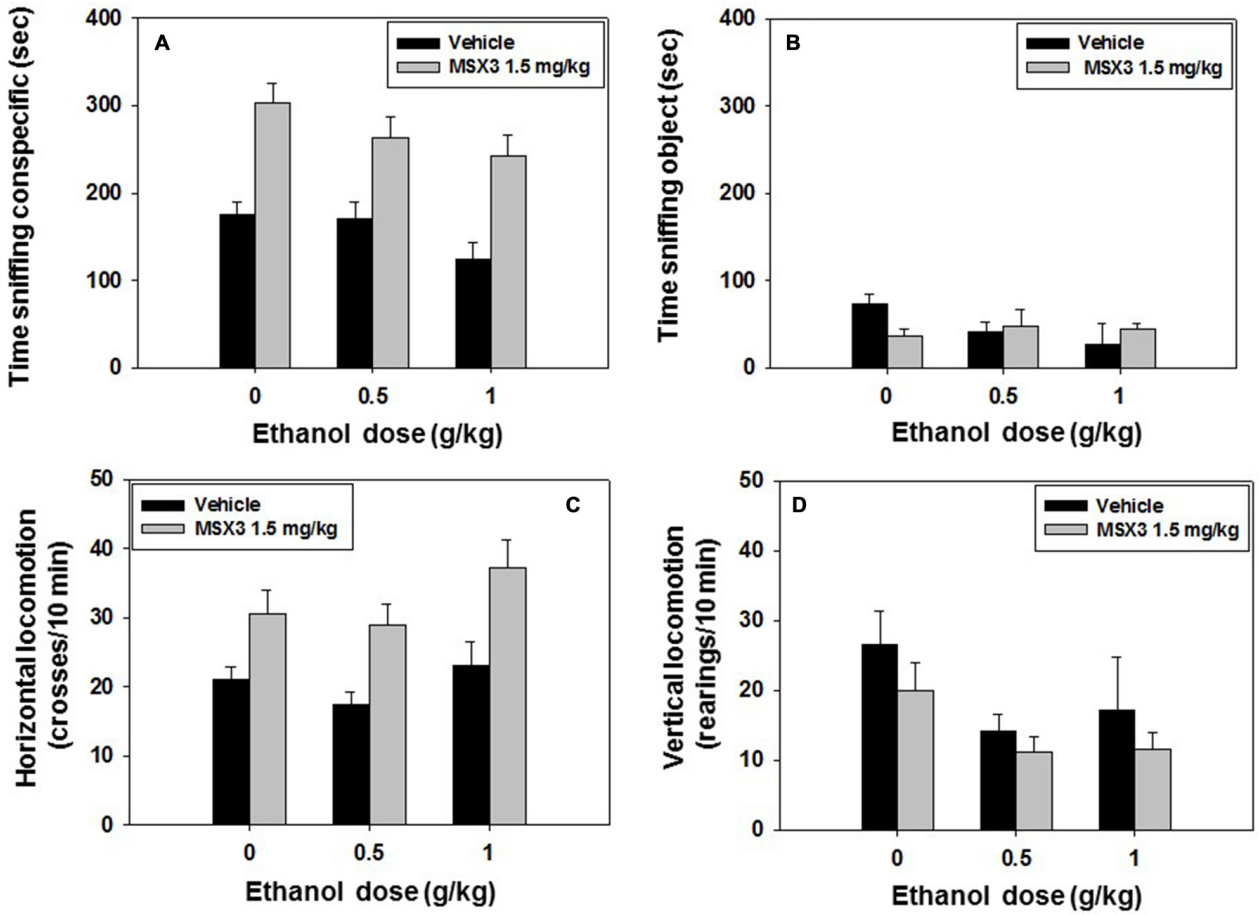

FIGURE 8 | Effect of MSX3 plus ethanol interaction in the social preference test. Data are expressed as mean (±SEM) of time spent sniffing **(A)** conspecific, **(B)** object, **(C)** horizontal and **(D)** vertical locomotion during the social preference test.

TABLE 3 | Effects of MSX3-ethanol combination on social recognition memory.

Etoh (g/kg)	0.0		0.5		1.0	
MSX3 (mg/kg)	**Familiar**	**Novel**	**Familiar**	**Novel**	**Familiar**	**Novel**
0.0	75.1 ± 4.9	138.3 ± 14.0$^{\#\#}$	105.6 ± 11.9	118.7 ± 13.3	120.0 ± 24.6	114.6 ± 19.8
1.5	84.1 ± 10.9	160.1 ± 24.8$^{\#}$	68.7 ± 7.8	109.3 ± 12.3$^{\#}$	142.6 ± 20.0	117.8 ± 12.2

Data are expressed as mean ± SEM of time in seconds spent sniffing novel or familiar conspecifics. $^{\#\#}p < 0.01$, $^{\#}p < 0.05$ significant differences between time in familiar vs. time in novel conspecific for the same dose of MSX3 and ethanol.

significant ($F_{(2,43)} = 0.43$, n.s.). When comparing the behavior of every group of animals in the exploration of the known and novel conspecific, the control group that had been treated with vehicle-vehicle the day before spent significantly more time sniffing the novel conspecific vs. the familiar conspecific as expected if the animal recognizes the known conspecific ($t = 4.71$, $p < 0.01$). This result was also observed in animals treated with MSX-3 1.5 mg/kg plus vehicle ($t = 2.64$, $p < 0.05$). As expected, animals treated with vehicle plus ethanol (at either dose) did not recognize the familiar animal and explored both conspecific equally. However, MSX-3 1.5 mg/kg blocked the effect of the lowest dose of ethanol 0.5 g/kg ($t = 2.52$, $p < 0.05$), although not the highest dose of ethanol. Thus, it seems that MSX-3 had a preventive effect only when the dose of ethanol was low.

DISCUSSION

In the present study, we characterize the impact of two of the most commonly consumed drugs of abuse, caffeine and alcohol, on motivation for social contact as manifested by social preference or avoidance, and also on consolidation of social

memories. We evaluated the possibility of a common mechanism of action for both drugs via the adenosine system. Thus, we hypothesized that low to intermediate doses of alcohol could lead to an increase in adenosine levels that would counteract the effect of caffeine, which acts as a non-selective A_1 and A_{2A} antagonist. In order to test that hypothesis, the effects of selective A_1 and A_{2A} receptor antagonists were also assessed alone or in combination with ethanol.

Our results show that the suppressing effects of high doses of caffeine on social approach and preference can be counteracted by low doses of ethanol, but this reversal effect reaches a ceiling when ethanol starts to mildly impair social approach and preference on its own. Social interaction has been mostly used to evaluate anxiety in rodents, because it was found that anxiolytics increase time spent in active social interaction while anxiogenic drugs decrease social contact independently of any change in activity (File and Hyde, 1978; Guy and Gardner, 1985). Thus, the reduction in social preference observed after caffeine administration could be explained by an increase in anxiety, since doses ranging from 25.0 to 100.0 mg/kg have been demonstrated to have a substantial anxiogenic effect in this strain of mice as seen in the elevated plus maze (López-Cruz et al., 2013). It is also possible that anxiolysis induced by ethanol could be playing a role in potentiating social interaction as suggested by previous researchers (Hilakivi et al., 1989; Nadal et al., 1993). However, it cannot be the only explanation for this effect since doses of ethanol that induced anxiolysis in this strain of mice (0.5 and 1.0 g/kg) in an elevated plus maze (Correa et al., 2008) were not able to reverse social preferences to normal levels. Moreover, in the present study we used a procedure developed to minimize anxiety in the experimental mouse by eliminating the possibility of physical aggression since the target mouse was enclosed in a wire cage (Crawley, 2004; Moy et al., 2004). Thus, in this paradigm it is possible to assess preference or avoidance for social interaction based on free choice. Furthermore, none of the pharmacological manipulations used in the present series of studies produced a significant avoidance for the compartment where the conspecific was located (data not shown). The effects of caffeine and ethanol alone or in combination on social behavior do not seem to be mediated by their effects on locomotion either, because the range of doses used do not clearly impair locomotion, and an increase in locomotion induced by the lowest doses of caffeine (7.5 and 15.0 mg/kg) seems to be unrelated to social exploration.

Although a strength of the present study was the use of a broad range of doses for all drugs, including the studies of drug interaction (most of the previous studies have used a single dose approach), it is not clear that the effect of high doses of caffeine were mediated by its actions on adenosine A_1 and A_{2A} receptors, since neither of the selective adenosine receptors reduced social interaction at the doses tested. Because in the present paradigm the experimental mouse has to explore a broad area that separates the two targets (conspecific and object), we selected doses of caffeine and selective adenosine antagonists based on results from previous work showing no impairing effects on ambulation and rearing in an open field (Pardo et al., 2013; López-Cruz et al., 2014), in order to avoid the possibility of mediating variables related to motor function. Thus, the A_1 antagonist CPT did not produce a significant change in social approach and preference, although mice spent more time in the conspecific compartment at the low doses (data not shown), and there was no interaction with ethanol on these parameters. It is possible, however, that higher doses of CPT could mimic the effects of caffeine on social preference, specially taking into account that previous studies have demonstrated that caffeine, at the same dose used in the present study (30.0 mg/kg), and the A_1 antagonist DPCPX produced an anxiogenic-like effect in mice, and reversed ethanol anxiolytic actions (Prediger et al., 2004). On the other hand, the A_{2A} receptor antagonist MSX-3 did have a significant effect, increasing preference for the social target and reducing it for the object. It is also worth noting that although general exploration (crossings between the three compartments) increased, MSX-3 did not disturb focused social exploration. Moreover, there was no significant interaction between MSX-3 and ethanol on any of these parameters; the improving effect of MSX-3 on preference was maintained at the same level independently of the dose of ethanol (0.5 or 1.0 g/kg) that the animals received. Consistently, high levels of social interaction have been observed in A_{2A} receptor KO mice, and these animals were not affected by a dose of ethanol (1.0 g/kg) that impaired social interaction (López-Cruz et al., in press). Interestingly, A_{2A}KO mice showed an anxiogenic profile, which again argues against a straight relationship between anxiety and social interaction (López-Cruz et al., in press).

A decrease in exploring a familiar conspecific when a new one is also present has been interpreted as an index of social recognition (Thor and Holloway, 1982; Crawley, 2004; Moy et al., 2004), which some authors consider to be also an index of preference for novelty seeking (Costa et al., 2014). Whatever the interpretation, it is required that the animal consolidates a memory for the familiar conspecific. Adenosine seems to modulate short-term social memory in rats by acting on both A_1 and A_{2A} receptors, with adenosine receptor agonists and antagonists respectively disrupting and enhancing social recognition memory (Prediger and Takahashi, 2005). Thus, the selective A_1 agonist CCPA and the A_{2A} agonist DPMA disrupted juvenile recognition in adult rats (Prediger and Takahashi, 2005). This impairment of short-term social memory induced by adenosine agonists was reversed by caffeine, the A_1 antagonist DPCPX, and the A_{2A} antagonist ZM24138 (Prediger and Takahashi, 2005). Moreover, acute administration of caffeine or selective A_{2A} antagonists reversed the disruption of social recognition memory in ageing rats (Prediger et al., 2005a), and also in spontaneously hypertensive rats (Prediger et al., 2005b) in which some alterations in adenosine neurotransmission have been reported (Davies et al., 1987; Matias et al., 1993; Lopes et al., 1999). However, all these studies evaluated short-term social memory and not long-term social memory. If the recognition test is carried 24 h after the first presentation it can be considered as a test of long-term memory processes. The development and consolidation of long-term potentiation seems to be also modulated by adenosine

receptor-dependent mechanisms in the hippocampus (Tanaka et al., 1990; de Mendonca and Ribeiro, 1994; Hauber and Bareiss, 2001). Data from the present study indicates that caffeine at high doses impaired recognition on the following day, especially at those doses (30.0 and 60.0 mg/kg) that had reduced relative preference for social interaction the day before. Thus, mice explored familiar and novel conspecifics equally, which could be explained by the fact that animals had explored the conspecific for much less time the day before than animals under control conditions. It is possible that the ability of caffeine to improve memory at low doses could be seen under different experimental conditions. In fact, theophylline (another non-selective A_1/A_{2A} antagonist) has been shown to facilitate long-term spatial reference memory in retention sessions, but not in working memory, both of which are tasks that are highly dependent on hippocampus (Hauber and Bareiss, 2001). Thus, when the nature of the task involves optimal performance during basal conditions, it is very difficult to improve performance.

It is well known that ethanol can produce amnestic effects and impair retrieval of memories after the drug wears off (Goodwin, 1995; Hartzler and Fromme, 2003; Gulick and Gould, 2007, 2009). Ethanol-induced memory impairments can be produced by disruption of attention, and also by affecting neural mechanisms involved in memory consolidation such as the adenosinergic system (Tanaka et al., 1990; Gulick and Gould, 2007, 2009). In experiment 1, ethanol, even at doses that did not impair social interaction (0.5 g/kg), impaired social recognition 24 h later. Although this situation was characterized by low performance, caffeine (15.0 or 30.0 mg/kg) co-administration was not able to block the amnestic effects of ethanol. A previous study in rats explored the effect of caffeine-ethanol interaction on long-term memory using social odors (Spinetta et al., 2008). In that study ethanol was administered immediately after exposure to the social odor, and a recognition test was performed 24 h later (Spinetta et al., 2008). Caffeine, at a low dose that did not have an effect on its own (5.0 mg/kg), was able to prevent the disruptive effects of ethanol (1.0 g/kg) on memory consolidation (Spinetta et al., 2008). It is possible that in our study lower doses of caffeine could have improved ethanol-induced deficits. The behavioral effects induced by methylxantines at low doses are likely to be mediated by nonselective adenosine A_1/A_{2A} receptor blockade, while higher doses might involve additional mechanisms such as inhibition of phosphodiesterases (Nehlig et al., 1992; Hauber and Bareiss, 2001).

As for the role of selective adenosine receptor antagonists, it appears that although CPT did not affect social interaction, it mildly impaired long-term social recognition at low doses, an effect that was not observed at high doses. CPT was not able to reverse the ethanol-induced impairment of recognition memory. In contrast, the selective A_{2A} antagonist MSX-3, which increased preference for the conspecific when administered alone, did not impair social recognition, and was able to block the amnestic effect of the lower dose of ethanol (0.5 g/kg). Thus, in our studies a selective A_{2A} antagonist was able to improve social memory under conditions of suboptimal performance (ethanol amnestic effects), but not under optimal performance (i.e., non-treated animals). This improvement in memory might be due to actions on processes involved in learning, such as attention and wakefulness, but may also be related to direct actions on memory systems. Alternatively, it is possible that MSX3 blocks ethanol's amnestic effects because it robustly increases active sniffing of the conspecific. It has been demonstrated that sensory impoverishment in rats (by whisker clipping) exacerbates ethanol-induced deficits in social interaction (Wellmann and Mooney, 2015). Thus, under different experimental conditions that promote sensory exploration (such as sniffing behavior), it could be possible that ethanol's amnestic effects would be diminished.

Although it is clear that normal social interaction is required for normal retrieval of social memories, the data from the present studies indicate a relative independence between social preference and social long-term memory processes. The results available at the present moment also suggest that A_1 receptors do not seem to regulate social motivation and social recognition, since blocking their tonic activity has very little effect. A_1 receptor antagonists appear to play only a modest role in the regulation of dopamine-dependent aspects of motivated behaviors (Pardo et al., 2012; Salamone and Correa, 2012). A_{2A} antagonists have similar motivational effects to dopamine uptake inhibitors (Yohn et al., 2016a,b), and since A_{2A} receptors are densely localized in dopamine rich areas such as the nucleus accumbens (Fredholm et al., 2001), it is possible that the modulation provided by A_{2A} antagonists on ethanol effects could be the result of a potentiation of the motivational functions regulated by this nucleus. Moreover, because selective A_1 and A_{2A} antagonists did not mimic the effects of caffeine, it is possible that blockade of both receptors is necessary for producing a caffeine-like action. Alternatively, it is possible that at high doses caffeine may not be acting solely as an adenosine antagonist. Thus, although an increase in adenosine levels could be mediating ethanol effects, the usefulness of highly caffeinated drinks in counteracting ethanol-induced impairments on these normal social processes is questionable.

AUTHOR CONTRIBUTIONS

LL-C conducted the experiments, analyzed the data and wrote the draft of the manuscript, NS-M and PB conducted part of the experiments and helped in the evaluation of the variables. YB and CEM provided information and synthesized the drug MSX3. JDS and MC were involved in the design of experiments, and writing of the manuscript.

ACKNOWLEDGMENTS

This work was supported by a grant to MC from Plan Nacional de Drogas (2010/024) Spain. LL-C was supported by a personal grant awarded by FPU (AP2010–3793) Ministerio de Educación, NS-M was supported by a personal grant from UJI (Predoc-UJI/2012/28) and PB was supported by Plan Nacional de Drogas (2010/024).

REFERENCES

Baldwin, H. A., and File, S. E. (1989). Caffeine-induced anxiogenesis: the role of adenosine, benzodiazepine and noradrenergic receptors. *Pharmacol. Biochem. Behav.* 32, 181–186. doi: 10.1016/0091-3057(89)90230-x

Baldwin, H. A., Johnston, A. L., and File, S. E. (1989). Antagonistic effects of caffeine and yohimbine in animal tests of anxiety. *Eur. J. Pharmacol.* 159, 211–215. doi: 10.1016/0014-2999(89)90709-7

Carmichael, F. J., Israel, Y., Crawford, M., Minhas, K., Saldivia, V., Sandrin, S., et al. (1991). Central nervous system effects of acetate: contribution to the central effects of ethanol. *J. Pharmacol. Exp. Ther.* 259, 403–408.

Chévere-Torres, I., Maki, J. M., Santini, E., and Klann, E. (2012). Impaired social interactions and motor learning skills in tuberous sclerosis complex model micenovelty seeking behavior during decision making. *Neurobiol. Dis.* 128, 556–566. doi: 10.1016/j.nbd.2011.07.018

Correa, M., Manrique, H. M., Font, L., Escrig, M. A., and Aragon, C. M. (2008). Reduction in the anxiolytic effects of ethanol by centrally formed acetaldehyde: the role of catalase inhibitors and acetaldehyde-sequestering agents. *Psychopharmacology (Berl)* 200, 455–464. doi: 10.1007/s00213-008-1219-3

Correa, M., San Miguel, N., López-Cruz, N., Bayarri, P., Monferrer, L., and Salamone, J. D. (2014). "Caffeine modulation of alcohol intake: impact on its psychomotor effects and withdrawal," in *Caffeine: Consumption, Side Effects and Impact on Performance and Mood*, ed. A. S. Tolley (New York, NY: NOVA Science Publishers, Inc.), 89–111.

Costa, V. D., Tran, V. L., Turchi, J., and Averbeck, B. B. (2014). Dopamine modulates novelty seeking behavior during decision making. *Behav. Neurosci.* 128, 556–566. doi: 10.1037/a0037128

Crawley, J. N. (2004). Designing mouse behavioral tasks relevant to autistic-like behaviors. *Ment. Retard. Dev. Disabil. Res. Rev.* 10, 248–258. doi: 10.1002/mrdd.20039

Cunha, R. A. (2001). Adenosine as a neuromodulator and as a homeostatic regulator in the nervous system: different role, different sources and different receptors. *Neurochem. Int.* 38, 107–125. doi: 10.1016/s0197-0186(00)00034-6

Davies, L. P., Hambley, J. W., and Johnston, G. A. R. (1987). Reduced adenosine deaminase activity in the CNS of spontaneously hypertensive rats. *Neurochem. Int.* 10, 533–536. doi: 10.1016/0197-0186(87)90081-7

de Mendonca, A., and Ribeiro, J. A. (1994). Endogenous adenosine modulates long-term potentiation in the hippocampus. *Neuroscience* 62, 385–390. doi: 10.1016/0306-4522(94)90373-5

Ferré, S., and O'Brien, M. C. (2011). Alcohol and caffeine: the perfect storm. *J. Caffeine Res.* 1, 153–162. doi: 10.1089/jcr.2011.0017

File, S. E., and Hyde, J. R. (1978). Can social interaction be used to measure anxiety? *Br. J. Pharmacol.* 62, 9–24. doi: 10.1111/j.1476-5381.1978.tb07001.x

Fredholm, B. B., Bättig, K., Holmén, J., Nehlig, A., and Zvartau, E. (1999). Actions of caffeine in the brain with special reference to factors that contribute to its widespread use. *Pharmacol. Rev.* 51, 83–133.

Fredholm, B. B., Ijzerman, A. P., Jacobson, K. A., Klotz, K. N., and Linden, J. (2001). International Union of Pharmacology. XXV. Nomenclature and classification of adenosine receptors. *Pharmacol. Rev.* 53, 527–552.

Goodwin, D. W. (1995). Alcohol amnesia. *Addiction* 90, 315–317. doi: 10.1080/09652149542292

Gulick, D., and Gould, T. J. (2007). Acute ethanol has biphasic effects on short- and long-term memory in both foreground and background contextual fear conditioning in C57BL/6 mice. *Alcohol. Clin. Exp. Res.* 31, 1528–1537. doi: 10.1111/j.1530-0277.2007.00458.x

Gulick, D., and Gould, T. J. (2009). Effects of ethanol and caffeine on behavior in C57BL/6 mice in the plus-maze discriminative avoidance task. *Behav. Neurosci.* 123, 1271–1278. doi: 10.1037/a0017610

Guy, A. P., and Gardner, C. R. (1985). Pharmacological characterisation of a modified social interaction model of anxiety in the rat. *Neuropsychobiology* 13, 194–200. doi: 10.1159/000118187

Hartzler, B., and Fromme, K. (2003). Fragmentary and en bloc blackouts: similarity and distinction among episodes of alcohol-induced memory loss. *J. Stud. Alcohol* 64, 547–550. doi: 10.15288/jsa.2003.64.547

Hauber, W., and Bareiss, A. (2001). Facilitative effects of an adenosine A_1/A_2 receptor blockade on spatial memory performance of rats: selective enhancement of reference memory retention during the light period. *Behav. Brain Res.* 118, 43–52. doi: 10.1016/s0166-4328(00)00307-7

Hauber, W., and Sommer, S. (2009). Prefrontostriatal circuitry regulates effort-related decision-making. *Cereb. Cortex* 19, 2240–2247. doi: 10.1093/cercor/bhn241

Hilakivi, L. A., Durcan, M. J., and Lister, R. G. (1989). Effects of caffeine on social behavior, exploration and locomotor activity: interactions with ethanol. *Life Sci.* 44, 543–553. doi: 10.1016/0024-3205(89)90616-4

Keppel, G. (1991). *Design and Analysis: A Researcher's Handbook*. Englewood Cliffs, NJ: Prentice-Hall.

Kirchner, T. R., Sayette, M. A., Cohn, J. F., Moreland, R. L., and Levine, J. M. (2006). Effects of alcohol on group formation among male social drinkers. *J. Stud. Alcohol* 67, 785–793. doi: 10.15288/jsa.2006.67.785

Krauss, S. W., Ghirnikar, R. B., Diamond, I., and Gordon, A. S. (1993). Inhibition of adenosine uptake by ethanol is specific for one class of nucleoside transporters. *Mol. Pharmacol.* 44, 1021–1026.

Lister, R. G., and Hilakivi, L. A. (1988). The effects of novelty, isolation, light and ethanol on the social behavior of mice. *Psychopharmacology (Berl)* 96, 181–187. doi: 10.1007/bf00177558

Lopes, L. V., Cunha, R. A., and Ribeiro, J. A. (1999). Increase in the number, G protein coupling and efficiency of facilitatory adenosine A_{2A} receptors in the limbic cortex, but not striatum, of aged rats. *J. Neurochem.* 73, 1733–1738. doi: 10.1046/j.1471-4159.1999.731733.x

López-Cruz, L., Carbó-Gas, M., Pardo, M., Bayarri, P., et al. (in press). Motivation for social interaction and impact of anxiety levels in A_{2A} receptor KO mice: involvement of anterior cingulate and amygdala. *Behav. Brain Res.*

López-Cruz, L., Pardo, M., Salamone, J. D., and Correa, M. (2014). Differences between the nonselective adenosine receptor antagonists caffeine and theophylline in motor and mood effects: studies using medium to high doses in animal models. *Behav. Brain Res.* 1270, 213–222. doi: 10.1016/j.bbr.2014.05.020

López-Cruz, L., Salamone, J. D., and Correa, M. (2013). The impact of caffeine on the behavioral effects of ethanol related to abuse and addiction: a review of animal studies. *J. Caffeine Res.* 3, 9–21. doi: 10.1089/jcr.2013.0003

Manrique, H. M., Miquel, M., and Aragon, C. M. (2005). Brain catalase mediates potentiation of social recognition memory produced by ethanol in mice. *Drug Alcohol Depend.* 79, 343–350. doi: 10.1016/j.drugalcdep.2005.02.007

Matias, A., Zimmer, F. J., Lorenzen, A., Keil, R., and Schwabe, U. (1993). Affinity of central adenosine A_1 receptors is decreased in spontaneously hypertensive rats. *Eur. J. Pharmacol.* 244, 223–230. doi: 10.1016/0922-4106(93)90147-2

Moy, S. S., Nadler, J. J., Perez, A., Barbaro, R. P., Johns, J. M., Magnuson, T. R., et al. (2004). Sociability and preference for social novelty in five inbred strains: an approach to assess autistic-like behavior in mice. *Genes Brain Behav.* 3, 287–302. doi: 10.1111/j.1601-1848.2004.00076.x

Nadal, R. A., Pallares, M. A., and Ferré, N. S. (1993). The effects of caffeine in the social interaction test and on exploration in rats: comparison with ethanol and clorazepate. *Behav. Pharmacol.* 4, 501–508. doi: 10.1097/00008877-199310000-00005

Nagy, L. E., Diamond, I., Casso, D. J., Franklin, C., and Gordon, A. S. (1990). Ethanol increases extracellular adenosine by inhibiting adenosine uptake via the nucleoside transporter. *J. Biol. Chem.* 265, 1946–1951.

Nehlig, A., Daval, J.-L., and Debry, G. (1992). Caffeine and the central nervous system: mechanisms of action, biochemical, metabolic and psychostimulant effects. *Brain Res. Brain Res. Rev.* 17, 139–170. doi: 10.1016/0165-0173(92)90012-b

Pardo, M., López-Cruz, L., Valverde, O., Ledent, C., Baqi, Y., Müller, C. E., et al. (2012). Adenosine A_{2A} receptor antagonism and genetic deletion attenuate the effects of dopamine D2 antagonism on effort-based decision making in mice. *Neuropharmacology* 62, 2068–2077. doi: 10.1016/j.neuropharm.2011.12.033

Pardo, M., López-Cruz, L., Valverde, O., Ledent, C., Baqi, Y., Müller, C. E., et al. (2013). Effect of subtype-selective adenosine receptor antagonists on basal or haloperidol-regulated striatal function: studies of exploratory locomotion and c-Fos immunoreactivity in outbred and A_{2A}R KO mice. *Behav. Brain Res.* 247, 217–226. doi: 10.1016/j.bbr.2013.03.035

Pena, R. R., Pereira-Caixeta, A. R., Moraes, M. F., and Pereira, G. S. (2014). Anisomycin administered in the olfactory bulb and dorsal hippocampus

impaired social recognition memory consolidation in different time-points. *Brain Res. Bull.* 109, 151–157. doi: 10.1016/j.brainresbull.2014.10.009

Prediger, R. D., Batista, L. C., and Takahashi, R. N. (2004). Adenosine A_1 receptors modulate the anxiolytic-like effect of ethanol in the elevated plus-maze in mice. *Eur. J. Pharmacol.* 499, 147–154. doi: 10.1016/j.ejphar.2004.07.106

Prediger, R. D., Batista, L. C., and Takahashi, R. N. (2005a). Caffeine reverses age-related deficits in olfactory discrimination and social recognition memory in rats. Involvement of adenosine A_1 and A_{2A} receptors. *Neurobiol. Aging* 26, 957–964. doi: 10.1016/j.neurobiolaging.2004.08.012

Prediger, R. D., Fernandes, D., and Takahashi, R. N. (2005b). Blockade of adenosine A_{2A} receptors reverses short-term social memory impairments in spontaneously hypertensive rats. *Behav. Brain Res.* 159, 197–205. doi: 10.1016/j.bbr.2004.10.017

Prediger, R. D., and Takahashi, R. N. (2005). Modulation of short-term social memory in rats by adenosine A_1 and A_{2A} receptors. *Neurosci. Lett.* 376, 160–165. doi: 10.1016/j.neulet.2004.11.049

Salamone, J. D., and Correa, M. (2002). Motivational views of reinforcement: implications for understanding the behavioral functions of nucleus accumbens dopamine. *Behav. Brain Res.* 137, 3–25. doi: 10.1016/s0166-4328(02)00282-6

Salamone, J. D., and Correa, M. (2012). The mysterious motivational functions of mesolimbic dopamine. *Neuron* 76, 470–485. doi: 10.1016/j.neuron.2012.10.021

Sano, H., Nagai, Y., Miyakawa, T., Shigemoto, R., and Yokoi, M. (2008). Increased social interaction in mice deficient of the striatal medium spiny neuron-specific phosphodiesterase 10A2. *J. Neurochem.* 105, 546–556. doi: 10.1111/j.1471-4159.2007.05152.x

Schiffmann, S. N., Libert, F., and Vassart, G. (1991). Distribution of A_{2A} receptor mRNA in the human brain. *Neurosci. Lett.* 130, 177–181. doi: 10.1016/0304-3940(91)90391-6

Schwarzschild, M. A., Agnati, L., Fuxe, L., Chen, J., and Morelli, M. (2006). Targeting adenosine A_{2A} receptors in Parkinson's disease. *Trends Neurosci.* 29, 647–654. doi: 10.1016/j.tins.2006.09.004

Sebastião, A. M., and Ribeiro, J. A. (2009). Adenosine receptors and the central nervous system. *Handb. Exp. Pharmacol.* 193, 471–534. doi: 10.1007/978-3-540-89615-9_16

Spinetta, M. J., Woodlee, M. T., Feinberg, L. M., Stroud, C., Schallert, K., Cormack, L. K., et al. (2008). Alcohol-induced retrograde memory impairment in rats: prevention by caffeine. *Psychopharmacology (Berl)* 201, 361–371. doi: 10.1007/s00213-008-1294-5

Tanaka, Y., Sakurai, M., Goto, M., and Hayashi, S. (1990). Effect of xanthine derivatives on hippocampal long-term potentiation. *Brain Res.* 522, 63–68. doi: 10.1016/0006-8993(90)91577-4

Thor, D., and Holloway, W. (1982). Social memory of the male laboratory rat. *J. Comp. Physiol. Psych.* 96, 1000–1006. doi: 10.1037/0735-7036.96.6.1000

Varlinskaya, E. I., and Spear, L. P. (2002). Acute effects of ethanol on social behavior of adolescent and adult rats: role of familiarity of the test situation. *Alcohol. Clin. Exp. Res.* 26, 1502–1511. doi: 10.1097/00000374-200210000-00007

Varlinskaya, E. I., and Spear, L. P. (2009). Ethanol-induced social facilitation in adolescent rats: role of endogenous activity at mu opioid receptors. *Alcohol. Clin. Exp. Res.* 33, 991–1000. doi: 10.1111/j.1530-0277.2009.00920.x

Wellmann, K. A., and Mooney, S. M. (2015). Unilateral whisker clipping exacerbates ethanol-induced social and somatosensory behavioral deficits in a sex- and age-dependent manner. *Physiol. Behav.* 148, 166–175. doi: 10.1016/j.physbeh.2014.09.002

Yohn, S. E., Arif, Y., Haley, A., Tripodi, G., Baqi, Y., Müller, C. E., et al. (2016a). Effort-related motivational effects of the pro-inflammatory cytokine interleukin-6: pharmacological and neurochemical characterization. *Psychopharmacology (Berl)* 233, 3575–3586. doi: 10.1007/s00213-016-4392-9

Yohn, S. E., Gogoj, A., Haque, A., Lopez-Cruz, L., Haley, A., Huxley, P., et al. (2016b). Evaluation of the effort-related motivational effects of the novel dopamine uptake inhibitor PRX-14040. *Pharmacol. Biochem. Behav.* 148, 84–91. doi: 10.1016/j.pbb.2016.06.004

Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

frontiers in Behavioral Neuroscience

ORIGINAL RESEARCH
published: 22 February 2017
doi: 10.3389/fnbeh.2017.00025

Nicotine Increases Alcohol Intake in Adolescent Male Rats

Armando Lárraga[1], James D. Belluzzi[1] and Frances M. Leslie[1,2]*

[1] Department of Pharmacology, University of California, Irvine, CA, USA, [2] Department of Anatomy and Neurobiology, University of California, Irvine, CA, USA

Background: Use of alcohol and tobacco, the two most concurrently abused drugs, typically first occurs during adolescence. Yet, there have been no systematic analyses of ethanol (EtOH) and nicotine (Nic) interactions during adolescence. Recent animal studies report that *kappa*-opioid (KOR) receptor activation mediates age differences in drug reinforcement. Our hypothesis is that concurrent self-administration of EtOH and Nic will be greater in adolescent rats because of age differences in KOR function. Furthermore, exposure to alcohol and nicotine during adolescence has been reported to increase EtOH intake in adulthood. We performed a longitudinal animal study and hypothesized adolescent rats allowed to self-administer nicotine would drink more alcohol as adults.

Methods: Adolescent, postnatal day (P)32, and adult (P90) male and female Sprague-Dawley rats were allowed to self-administer EtOH, Nic, or a combination of both, EtOH+Nic, in an intravenous self-administration paradigm. The role of KOR was pharmacologically evaluated with the KOR antagonist, norbinaltorphamine (norBNI) and with the KOR agonist, U50,488H. Alcohol drinking was subsequently evaluated with male rats in a drinking in the dark (DID), 2-bottle choice test.

Results: Concurrent Nic increased EtOH intake in adolescent males, but not in adults or females. Pharmacological blockade of KOR with norBNI robustly increased EtOH+Nic self-administration in adult male rats, but had no effect with female rats. Lastly, in our longitudinal study with male rats, we found prior self-administration of Nic or EtOH+Nic during adolescence increased subsequent oral EtOH intake, whereas prior self-administration of EtOH alone in adults increased subsequent EtOH drinking.

Conclusions: There are major age- and sex-differences in the reinforcing effects of EtOH+Nic. Adolescent males are sensitive to the reinforcing interactions of the two drugs, whereas this effect is inhibited by KOR activation in male adults. Nicotine self-administration in adolescent males also increased subsequent oral EtOH intake. These findings suggest that brain mechanisms underlying the reinforcing effects of EtOH and nicotine are both age- and sex-dependent, and that tobacco or e-cigarette use may increase the vulnerability of teenage boys to alcohol abuse.

Keywords: adolescence, ethanol, *kappa*-opioid receptors, norBNI, 2-bottle choice, sex differences, tobacco, nicotine

Chemical compounds studied in this article:

Ethyl Alcohol (PubChem CID: 702); nicotine (PubChem CID: 89594); norbinaltorphimine (PubChem CID: 5480230); and U50,488H (PubChem CID: 135349).

OPEN ACCESS

Edited by:
Merce Correa, Jaume I University, Spain

Reviewed by:
Laura Elena O'dell, University of Texas at El Paso, USA
Patrick Arthur Randall, Bowles Center for Alcohol Studies, University of North Carolina at Chapel Hill, USA

****Correspondence:***
Armando Lárraga
ArmandoLarraga1@gmail.com

Received: *25 November 2016*
Accepted: *06 February 2017*
Published: *22 February 2017*

Citation:
Lárraga A, Belluzzi JD and Leslie FM (2017) Nicotine Increases Alcohol Intake in Adolescent Male Rats. Front. Behav. Neurosci. 11:25. doi: 10.3389/fnbeh.2017.00025

INTRODUCTION

Tobacco use consistently shows a strong positive correlation with alcohol use. Over 80% of alcoholics smoke (Batel et al., 1995), and alcohol abuse is 10–14 times more common among smokers (DiFranza and Guerrera, 1990). Most people begin drinking and smoking as teenagers (Behrendt et al., 2009), and alcohol and tobacco co-use is higher among younger (18–24 years old) than older age groups (Falk et al., 2006). Those who initiate smoking at age 13 or younger are twice as likely to abuse alcohol as those who start at age 17 or above (Falk et al., 2006). Animal behavior studies have shown adolescents find nicotine more rewarding and less aversive (Belluzzi et al., 2004; Wilmouth and Spear, 2004; Shram et al., 2006; Brielmaier et al., 2007). Adolescent rats are also less sensitive to the sedative and acute withdrawal effects of alcohol than adults (Doremus et al., 2003, 2005; Varlinskaya and Spear, 2004). Therefore, it is important to study and compare alcohol and nicotine[1] interactions in both adolescents and adults, and examine the effects on subsequent alcohol drinking behavior.

Sex differences have also been identified in both alcohol and nicotine addiction research publications. Nicotine has been shown to increase alcohol consumption and enhance arousal in men, while decreasing alcohol consumption and positive mood in women (Acheson et al., 2006). Consistent with this, women, but not men, drink less alcohol after *ad libitum* smoking (Perkins et al., 2000). Conversely, alcohol has been shown to increase smoking behavior in men, but not women (King et al., 2009). These studies suggest that alcohol and nicotine have sex-dependent interactions. Preclinical studies have also reported sex differences in nicotine- or alcohol-induced behaviors; however there have been no studies to date comparing the combination of nicotine and alcohol between sexes. Studies evaluating nicotine have reported female rats acquire self-administration of lower doses of nicotine and have higher breakpoint values at a progressive reinforcement schedule than males (Donny et al., 2000). Studies evaluating alcohol similarly report females display conditioned place preference to lower doses of alcohol than male rats (Torres et al., 2014). These studies suggest females may be more sensitive to the rewarding properties of nicotine and alcohol, respectively. Furthermore, the reported sex differences may be age-dependent, as adolescent rats do not show sex differences in aversion to alcohol (Schramm-Sapyta et al., 2014) or nicotine reward (Chen et al., 2007).

Recent studies attribute *kappa*-opioid receptor (KOR) activation as the mediator of age differences in drug reinforcement. KOR is the opioid receptor that binds and is endogenously activated by dynorphin A (Chavkin et al., 1982). KORs are widely distributed in the brain (Mansour et al., 1995) and have been shown to induce a compensatory decrease in reward state by inhibiting dopamine release in the nucleus accumbens (Zapata and Shippenberg, 2006). KOR agonists have been shown to reduce self-administration of alcohol (Nestby et al., 1999; Lindholm et al., 2001). In contrast, stress-induced KOR activation enhances nicotine reward (Lemos et al., 2012; Smith et al., 2012). Pharmacological blockade of KOR with norbinaltorphimine (norBNI), a KOR antagonist, has been reported to both increase (Mitchell et al., 2005; Anderson et al., 2012; Morales et al., 2014) and decrease (Walker and Koob, 2008; Walker et al., 2011) alcohol reward in rats. Recently, the relationship between drug reward and the *kappa*-opioid receptor (KOR) has been shown to be age-dependent for both alcohol (Anderson et al., 2014) and nicotine (Tejeda et al., 2012).

In the present study, we examined the reinforcing effects of concurrent self-administration of alcohol and nicotine in adolescent and adult, male and female rats. Our laboratory has previously shown adolescent nicotine pretreatment enhances acquisition of EtOH self-administration (Dao et al., 2011), and self-administration of a nicotine + acetaldehyde mixture is enhanced in adolescents (Belluzzi et al., 2005). Hence, our hypothesis was that adolescents would be more sensitive than adults to the reinforcing effects of combined alcohol and nicotine (EtOH+Nic), and that age differences would be more pronounced in males than females. Furthermore, we examined the role of KOR in mediating EtOH and Nic reinforcement with a KOR antagonist (norBNI) and agonist (U50-488H). Lastly, we conducted a longitudinal study to test if *self-administration* of EtOH, Nic, or EtOH+Nic during adolescence influences subsequent alcohol preference in adulthood.

MATERIALS AND METHODS

Animals

Male and female Sprague Dawley rats were obtained from Charles River at postnatal day (P)18 and housed with a dam until weaning (P21). Weaned juveniles and adults (P79) were group housed in an AALAC-accredited vivarium on a 12-h light-dark cycle (7 p. m. to 7 a. m.) with food and water available *ad libitum*. No more than one animal per litter was used per experimental group. All procedures were in compliance with NIH guidelines and were approved by the Institutional Animal Care and Use Committee of the University of California, Irvine. Animal suffering and the number of animals used in this study were minimized as much as possible. All animals were handled daily 3 days prior to surgery and thereafter. Consistent with our other studies (Belluzzi et al., 2005; McQuown et al., 2007; Dao et al., 2011), intravenous self-administration experiments were performed during the light cycle, whereas drinking in the dark (DID) 2-bottle choice experiments were conducted during the dark cycle. **Figure 1** schematically illustrates the experimental protocol.

Drugs

(−)-Nicotine di-(+)-tartrate (Nic) was purchased from Sigma (St. Louis, MO), 100% ethanol (EtOH) from Gold Shield distributors (Hayward, CA), norbinaltorphamine (norBNI), and U50,488H from Tocris Biosciences (Minneapolis, MN), and propofol from Abbot Laboratories (Chicago, IL). All drugs were dissolved in saline and filtered through sterile filters (Millipore Millex Sterile Filters, 0.22 μm pore, 3.3 mm diameter). Nic concentrations (pH 7.4) were calculated as free base, and EtOH was prepared at concentrations no greater than 10% EtOH (v/v).

[1]Abbreviations: Ethanol (EtOH); nicotine (Nic); *kappa*-opioid receptor (KOR); norbinaltorphimine (norBNI).

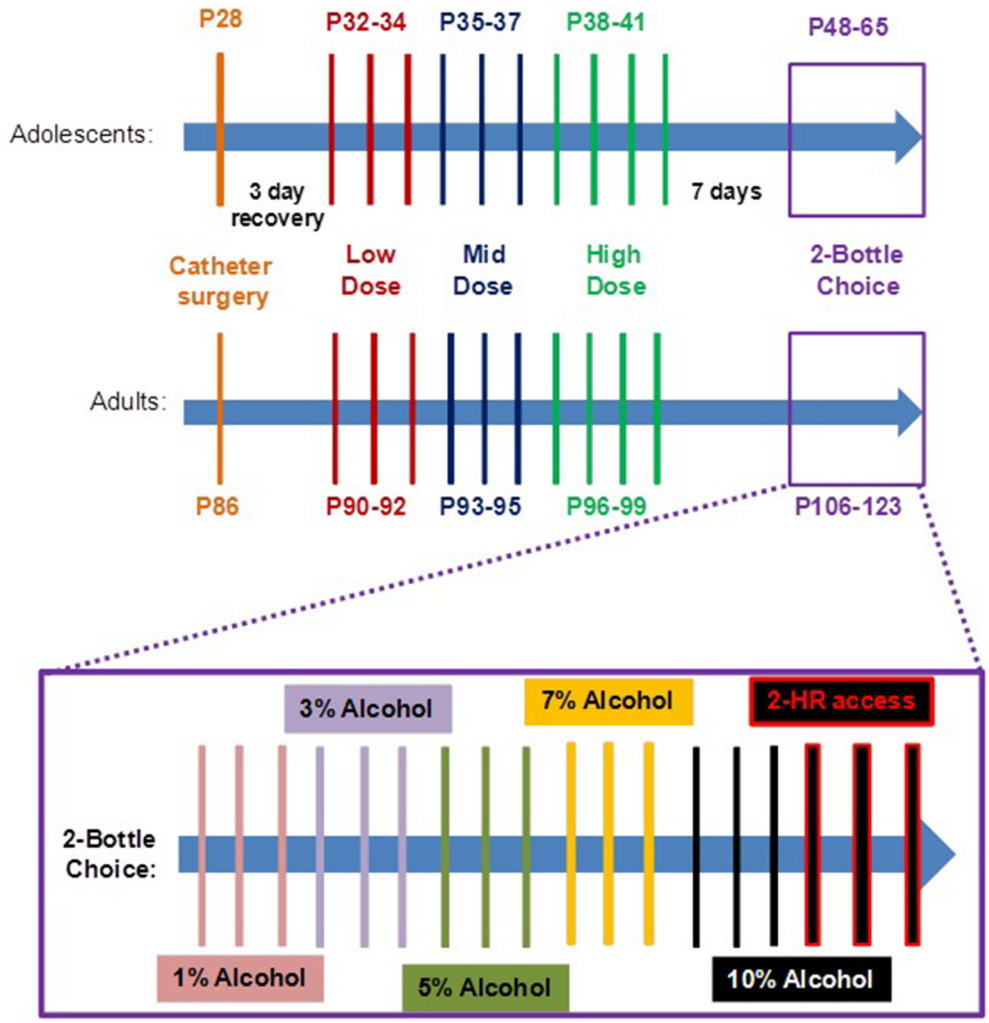

FIGURE 1 | Schematic illustration of the experimental design. Adolescents aged postnatal day (P) 28 and adults (P86) were surgically implanted with an intravenous catheter. At P32 or P90, adolescents and adults, respectively, began intravenous self-administration (IVSA) of EtOH and nicotine (Nic), alone or in combination (EtOH+Nic) at escalating doses over 10 consecutive days. Seven days following the conclusion of IVSA, the same male rats, only, were tested in an overnight 2-bottle choice paradigm with water and increasing concentrations of unsweetened EtOH solution After escalating to a 10% EtOH solution, alcohol preference was measured during a limited 2-h access (7 p. m. to 9 p. m.); all animals had reached adulthood (Age > P60) at this time.

Catheterization

Adolescent and adult rats underwent surgery at P28 and P86, respectively. Animals were anesthetized with Equithesin (0.035 mg/kg, i.p.), and were surgically implanted with a catheter into their right jugular vein (Belluzzi et al., 2005). Rats were given 3 days to recover before beginning experiments. Cannulas were flushed daily with heparinized saline solution to maintain catheter patency. Propofol (5 mg/kg, i.v.) was injected only once, following the last self-administration session; data were discarded from animals that did not display rapid (5–10 s) anesthesia.

Intravenous Self-administration (IVSA)

At P32 and P90, respectively, adolescent and adult rats initiated intravenous self-administration (IVSA) of EtOH, Nic, EtOH+Nic, or saline in daily 2-h sessions at a fixed ratio (FR) 1-reinforcement schedule. Though intravenous self-administration has been rarely used for alcohol studies, both humans (Plawecki et al., 2013) and animals (Hyytiä et al., 1996; Dao et al., 2011) self-administer alcohol intravenously. Animals were placed in an operant chamber equipped with a house light, two nose-poke holes, and cue-lights directly above each nose-poke hole. Following each infusion there was a 3-sec time out, during which animals could not receive drug. Drug reinforcement was indicated by significant differences between responding at reinforced and non-reinforced holes. Drug doses were escalated over 10 consecutive days. We began Nic at 7.5 µg Nic/kg/infusion (Low dose), a dose that is self-administered by both adolescents and adults (Gellner et al., 2016), and maintained for the first 3 days. We then escalated to standard IVSA Nic doses: 15 µg Nic/kg/infusion (Mid dose) for days 4–6 and 30 µg Nic/kg/infusion (High dose) for days 7–10. For EtOH doses, we began with 1 mg EtOH/kg/infusion as the Low dose, and

increased this dose by log scale to 10 mg EtOH/kg/infusion (Mid dose), and 100 mg EtOH/kg/infusion (High). In order to keep EtOH solutions below 10% (v/v), infusion volumes were increased as drug dose was escalated. However, infusion volumes did not differ between drug groups; animals self-administering Nic alone had the same infusion volumes as those self-administering EtOH and EtOH+Nic at each drug dose.

Two-Bottle Choice

A longitudinal study with male rats was used to evaluate alcohol drinking after adolescent self-administration of EtOH, Nic, or EtOH+Nic. Alcohol drinking was evaluated on the same adolescent and adult male rats that completed the 10-day intravenous self-administration study. To allow the younger experimental rats to mature into adulthood, and allow the drugs from the intravenous self-administration (IVSA) experiments to clear out, the 2-bottle choice paradigm began 1 week after the IVSA study was complete. For 2-bottle choice experiments, male rats were single housed overnight for 12 h and allowed to drink from two bottles: one containing water, and the other EtOH. During the day, while 2-bottle choice experiments were not being conducted, all animals were group-housed in their original cages. Placement of the water and EtOH bottles was rotated each night to prevent a side preference by our animals. Rats were offered escalating EtOH concentrations over 15 consecutive nightly trials as follows: 1% EtOH (v/v) for trials 1–3, 3% EtOH (v/v) for trials 4–6, 5% EtOH (v/v) for trials 7–9, 7% EtOH (v/v) for trials 10–12, and 10% EtOH (v/v) for trials 13–15. These EtOH concentrations are standard for 2-bottle choice experiments. However, saccharine was not used in our 2-bottle choice experiments. Following the 15-trial overnight two-bottle choice procedure, rats underwent a limited access paradigm for 3 consecutive nights. At this point, all animals were over 60 days old and were considered to be adult rats. Each evening at the beginning of the dark cycle, rats were given access to a bottle of water and a bottle of 10% EtOH solution for 2 h only. After 2 h, the bottles were removed and the remaining liquid was measured to determine the amount of fluid the rats drank from each bottle.

Role of *Kappa*-Opioid Receptors (KORs) in Acquisition of EtOH+Nic Self-administration

Separate groups of animals were prepared with jugular catheters, and treated with the irreversible *kappa*-opioid receptor antagonist, norBNI (0, or 10 mg/kg, i.p.), 1 day after surgery. A single dose of norBNI at 10 mg/kg inhibits activation of KOR for more than 21 days *in vivo*, and is dependent on c-Jun N-terminal kinase 1 activation (Melief et al., 2011). Hence, a dose response with norBNI is not necessary. Three days following norBNI or saline pretreatment, at P32 and P90, respectively, adolescent and adult rats were allowed to intravenously self-administer EtOH, Nic, or EtOH+Nic at the Low dose for three consecutive days. To evaluate the effect of direct KOR activation on EtOH+Nic self-administration, a separate groups of adolescent males was catheterized and allowed to self-administer EtOH+Nic for three consecutive days. On the fourth day, the specific KOR agonist, U50,488H (0, 0.3, 1.0, or 3.0 mg/kg, i.v.) was administered immediately before beginning the final EtOH+Nic self-administration session.

Data Analysis

Mean reinforced and non-reinforced responses for the last 2 days of intravenous self-administration of each drug dose were analyzed with a 4-way ANOVA for Age x Drug x Response (Reinforced/Non-Reinforced) x Dose (Low, Mid, and High), with repeated measures on Response and Dose. Data from animals pretreated with norBNI were analyzed by a 4-way ANOVA for Age X Pretreatment X Drug X Response, with repeated measures on Response. Results from the U50,488 experiment were also analyzed by a 2-way ANOVA for Dose X Reinforcement. Total EtOH and Nic intake during intravenous self-administration in male or female rats were analyzed separately by 3-way ANOVA for Age x Drug x Dose. Significant differences were further analyzed with ANOVAs and *post-hoc* Bonferroni-corrected *t*-tests, where appropriate.

For 2-bottle choice data, mean oral alcohol consumption for the three limited access trials was calculated by dividing alcohol intake by total fluid intake (alcohol + water). Mean alcohol consumption was analyzed with a 2-way ANOVA for Drug (prior exposure) X Age. Significant differences within each age were further analyzed with one-way ANOVA and *post-hoc* Dunnett or Bonferroni tests. Age differences were analyzed by Bonferroni-corrected unpaired *t*-tests.

Data from norBNI-treated male and female rats were analyzed separately. Mean reinforced and non-reinforced responses for the last 2 days of intravenous self-administration of each drug group were analyzed with a 3-way ANOVA for Age x Pretreatment x Reinforcement (Reinforced/Non-Reinforced Responses) with repeated measures on Reinforcement. Significant differences were further analyzed with ANOVAs and *post-hoc* Bonferroni-corrected *t*-tests. Significant effects of Reinforcement were analyzed by Bonferroni-corrected paired *t*-tests between Reinforced and Non-Reinforced Responses for each treatment group.

RESULTS

Combining EtOH and Nic Is Reinforcing in Adolescent, but Not in Adult Males

Our initial self-administration experiment was done with adolescent and adult male rats. We observed significant age differences in the acquisition of self-administration of EtOH, and the combination of EtOH+Nic (**Figure 2**). Since overall ANOVA indicated significant Dose interactions with Age [$F_{(2, 98)} = 12.383$, $p < 0.001$], and Response [$F_{(2, 98)} = 4.256$, $p = 0.017$], responses at each Dose were analyzed separately.

At the Low dose (**Figure 2A**), there was an overall Age X Drug interaction [$F_{(1, 49)} = 5.858$, $p = 0.019$]. Adolescent males found EtOH, alone and in combination with Nic (EtOH+Nic), to be reinforcing, as indicated by significant differences between reinforced and non-reinforced responses (**, $p < 0.01$). Adolescents also self-administered significantly more EtOH+Nic than EtOH alone (++, $p = 0.009$). In contrast, adult

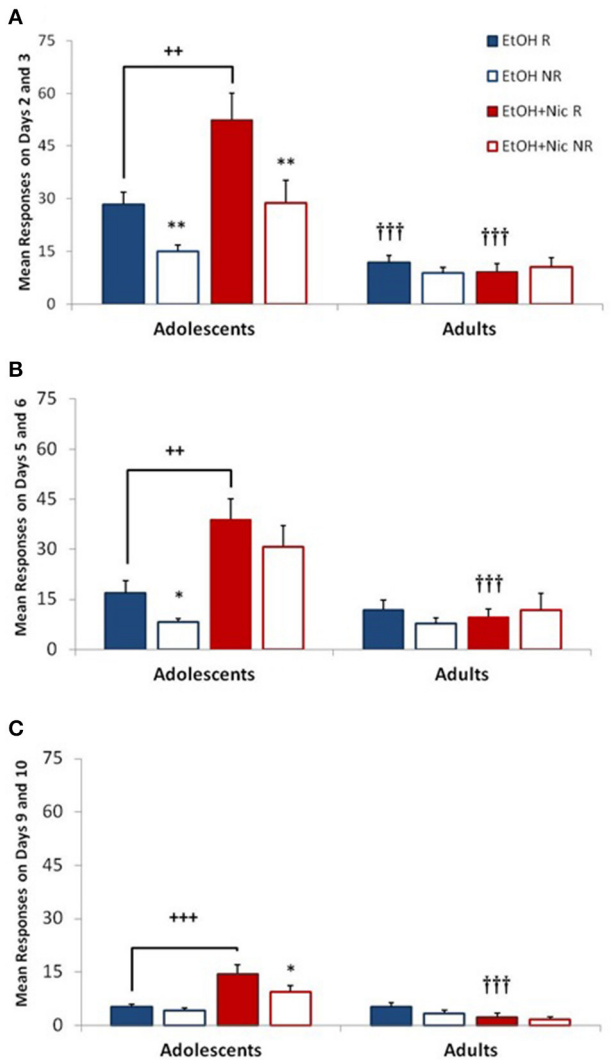

FIGURE 2 | Combining EtOH and Nicotine is reinforcing in adolescent, but not adult males. Adolescent and adult rats self-administered EtOH alone, or in combination with Nic (EtOH+Nic) at **(A)** a Low dose (7.5 μg Nic, 1 mg EtOH/kg/infusion), **(B)** a Mid dose (15 μg Nic, 10 mg EtOH/kg/infusion), and **(C)** a High dose (30 μg Nic, 100 mg EtOH/kg/infusion). A reinforcing effect of drug is indicated by significantly higher reinforced ("R," closed bars) than nonreinforced ("NR," open bars) responses (**, $p < 0.01$; *, $p < 0.05$). Reinforced responses (R) for EtOH+Nic were significantly higher than for EtOH alone in adolescents (+++, $p < 0.001$; ++, $p < 0.01$), but not adults. Reinforced responses in adolescents were significantly higher than adults (†††, $p < 0.001$) for EtOH at the Low dose, and for EtOH+Nic at all three test doses. Data represent mean + SEM responses averaged over the last two days at a given drug dose, $n = 9$–16 males per group.

male rats did not find either EtOH or EtOH+Nic reinforcing, and self-administered significantly less EtOH and EtOH+Nic than adolescents (†††, $p < 0.001$).

At the Mid doses (**Figure 2B**), there was a significant Age X Drug interaction [$F_{(1, 49)} = 9.149$, $p = 0.004$]. EtOH continued to be reinforcing in adolescent male rats at this higher dose, as indicated by significant differences between reinforced and non-reinforced responses (**, $p = 0.01$). The combination of EtOH+Nic, however, was not reinforcing at the Mid dose. Yet, adolescent rats self-administered larger amounts of the drug combination than EtOH alone; reinforced responses for EtOH+Nic were significantly higher reinforced responses for EtOH in adolescents (++, $p = 0.002$) or for EtOH+Nic in adults (†††, $p = 0.001$). Adult male rats did not show a reinforcing effect to EtOH or EtOH+Nic at the Mid dose.

At the High dose (**Figure 2C**), there was a significant Age X Drug interaction [$F_{(1, 49)} = 13.274$, $p = 0.001$], and significant effect of Responses [$F_{(1, 49)} = 11.365$, $p = 0.001$]. The only drug treatment that was reinforcing was EtOH+Nic in adolescents (*, $p < 0.05$). Adolescent males also self-administered significantly more EtOH+Nic than EtOH (+++, $p = 0.001$). Lastly, reinforced responses for EtOH+Nic were also significantly higher in adolescent than adult males (†††, $p = 0.001$).

Combining Alcohol and Nicotine Increased Drug Intake in Adolescent Male Rats

Since we observed a significant age difference in EtOH+Nic self-administration that persisted over three different dose combinations, we also included female adolescent and adult rats, and compared drug intake between age and sex. Nic significantly increased EtOH intake in adolescent, but not adult males (**Figure 3**). EtOH intake in males showed a significant Dose x Age x Drug interaction [$F_{(2, 96)} = 10.652$, $p < 0.001$]; hence, each dose was analyzed separately. At all three test doses, the adolescent EtOH+Nic group had significantly higher EtOH intake than the adolescent EtOH alone group (**, $p < 0.01$). In addition, adolescent males self-administered more EtOH than adults at the Low, Mid, and High doses of EtOH+Nic, and at the Low dose of EtOH alone (†††, $p < 0.001$; ††, $p < 0.01$ vs. adults). In contrast, combining Nic with EtOH did not increase EtOH intake in females. Three-way ANOVA analysis of Age x Drug x Dose was not statistically significant [$F_{(2, 78)} = 1.143$, $p = 0.324$] and did not show a statistically significant Drug effect ($p = 0.622$) or Age x Drug interaction ($p = 0.448$). Females only showed a significant Age difference at the High dose that was not dependent on Drug (††, $p < 0.01$).

Age Differences in KOR Function Mediate Age-Dependent Effects of EtOH+Nic Reinforcement

To test if age differences in EtOH+Nic self-administration reflect age-dependent KOR activation, we pretreated adolescent and adult, male and female rats, with the standard dose of the irreversible KOR antagonist, norBNI or saline, and compared self-administration of EtOH+Nic at the Low dose (**Figure 4**). Significant overall interactions of Response x Age x Drug x Pretreatment were found for males [$F_{(2, 130)} = 5.651$, $p = 0.004$]. NorBNI pretreatment had a robust effect on EtOH+Nic reinforcement in adult male rats. Males (**Figure 4A**; left panel) showed a significant overall Response X Age X Pretreatment interaction [$F_{(1, 46)} = 6.902$, $p = 0.012$]. EtOH+Nic was reinforcing in norBNI-pretreated adult

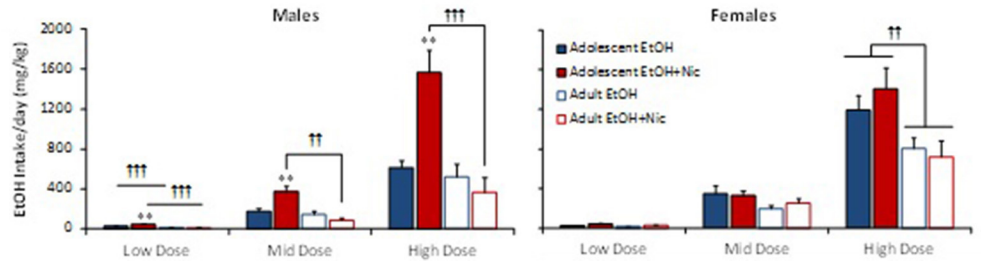

FIGURE 3 | Combining alcohol and nicotine increases EtOH intake in adolescent males, but not adult males or females. EtOH intake of male (left panel) and female (right panel), adolescent (filled bars) and adult (open bars), rats at Low, Mid and High test doses. Combining EtOH and Nic (EtOH+Nic) significantly increased EtOH intake compared to EtOH alone in male adolescent rats (**, $p < 0.01$,) but not in male adult or female rats. EtOH intake is higher in male adolescents than male adults at the Low dose (†††, $p < 0.001$), and at the Mid and High EtOH+Nic doses (††, $p < 0.01$). Female adolescents had a higher EtOH intake at the High dose than female adults (††, $p < 0.01$). Data represent mean + SEM of the last 2 days of intravenous self-administration at each test dose for each group, $n =$ 9–16/group.

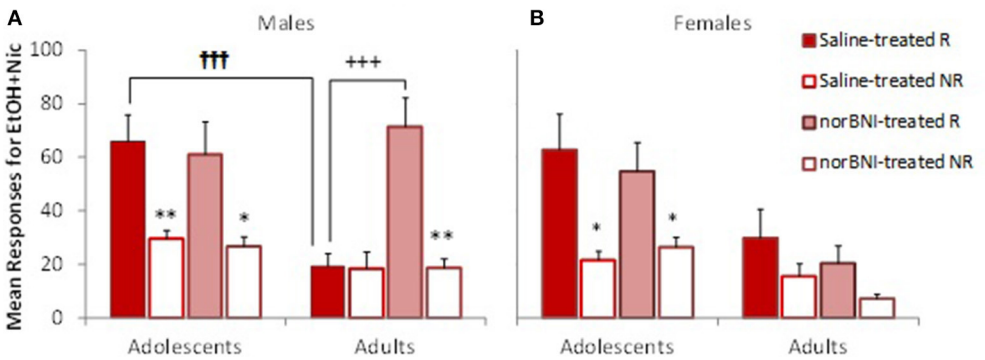

FIGURE 4 | KOR blockade increases EtOH+Nic reinforcement in adult male rats, but not adolescent males or females. (A) Blockade of kappa-opioid receptor (KOR) with norBNI significantly increased EtOH+Nic reinforcement in adult males; reinforced (R) responses were significantly higher than non-reinforced (NR) (**, $p < 0.001$) and saline-pretreated reinforced responses (+++, $p < 0.001$). Saline-treated adolescents males had significantly higher reinforced responses than saline-treated adult males (†††, $p < 0.001$). **(B)** Male and female adolescent rats find EtOH+Nic reinforcing regardless of pretreatment; Reinforced (R) responses were significantly higher than non-reinforced (NR; **, $p < 0.01$; *, $p < 0.05$). No effect of norBNI was observed in females, $n = 7$–13/group.

males: reinforced responses were significantly higher than non-reinforced responses (**, $p < 0.01$), and higher than reinforced responses of saline-pretreated adults (+++, $p < 0.001$). In adolescent males, norBNI pretreatment did not have an effect on EtOH+Nic reinforcement. Consistent with our first experiment, adolescent males found EtOH+Nic reinforcing regardless of pretreatment (**, $p < 0.01$; *, $p < 0.05$). NorBNI pretreatment of male rats increased adult reinforced responses for EtOH+Nic to levels seen with adolescents (**Figure 4A**). In contrast, females showed no effect to norBNI pretreatment (**Figure 4B**; right panel). There was a significant overall Response x Age interaction [$F_{(1, 30)} = 5.542$, $p = 0.016$]; EtOH+Nic was reinforcing in adolescent (*, $p < 0.05$), but not adult females, regardless of pretreatment.

NorBNI pretreatment revealed that KOR activation inhibits EtOH+Nic reinforcement in male adults, but not adolescent males. In order to test whether KOR was functionally active in adolescent males that show a reinforcement effect to the combination EtOH+Nic, we examined the effect of the KOR agonist, U50,488, on a separate group of adolescent male rats (**Figure 5**). Only an overall reinforcement effect was observed [$F_{(1, 43)} = 18.327$, $p < 0.001$], with no significant inhibition of responding at any U50,488 dose.

Nicotine Self-administration during Adolescence Increases Subsequent EtOH Drinking in Adulthood

In order to test the effect of adolescent Nic and/or EtOH use on EtOH drinking during adulthood, we performed a 2-bottle choice experiment on the same male rats from our initial self-administration experiment. No female rats were tested in our 2-bottle choice paradigm. We found that self-administration of Nic during adolescence increased subsequent oral intake of EtOH in a drinking-in-the-dark paradigm during adulthood (**Figure 6**). Though none of the test groups demonstrated a preference for EtOH over water, a significant Drug x Age (during IVSA) interaction [$F_{(3, 74)} = 5.051$, $p = 0.003$] was seen for EtOH intake as a percent of total liquid consumption. Male rats that, as adolescents, self-administered Nic alone or

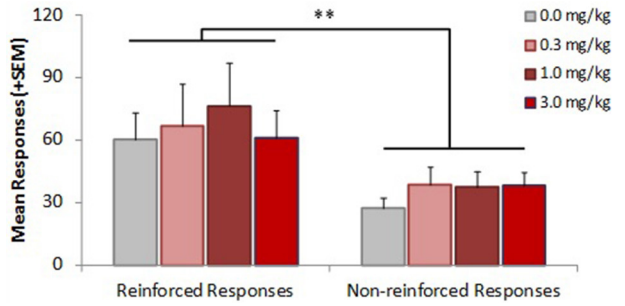

FIGURE 5 | Activating KOR does not inhibit EtOH+Nic reinforcement in male adolescent rats. Adolescent male rats self-administering EtOH+Nic were treated with U50,488 (0, 0.3, 1.0, or 3.0 mg/kg, i.v.) on test day. No statistically significant effect of U50,488 was observed. Overall, reinforced responses were significantly higher than non-reinforced responses (**, $p < 0.01$), $n = 11$–13 adolescent males.

FIGURE 6 | Nic self-administration during adolescence increases subsequent alcohol drinking in adulthood. Data are average 10% EtOH consumption as a percent of total fluid consumption over 3 consecutive trials in a limited 2-hr access 2-bottle choice paradigm with male rats that had previously self-administered saline, Nic, EtOH, or EtOH+Nic as either adolescents or adults. Rats that self-administered Nic or EtOH+Nic as adolescents drank more 10% EtOH solution than saline-control rats (+, $p < 0.05$). Rats that self-administered EtOH as adults drank more 10% EtOH solution than adult saline controls (+, $p < 0.05$) and rats that previously self-administered EtOH as adolescents (†, $p < 0.05$). 10% EtOH consumed as a % of total fluid is shown as mean + SEM for each treatment group, $n =$ 8–13 males/group.

in combination with EtOH (EtOH+Nic), drank more EtOH than saline controls (+, $p < 0.05$). Adolescent rats that intravenously self-administered EtOHalone did not subsequently drink more EtOH than saline-control animals. In contrast, rats that intravenously self-administered EtOH alone as adults did drink more EtOH than saline-controls (+, $p < 0.05$). They also drank more EtOH than their adolescent counterparts (††, $p < 0.01$). Notably, adults that self-administered EtOH+Nic did not increase subsequent oral EtOH intake (**Figure 6**), even though Nic did not significantly alter intravenous EtOH intake in this group (**Figure 2A**). Thus, Nic self-administration in adolescents increases subsequent alcohol drinking, whereas it inhibits the enhancing effects of EtOH self-administration on subsequent alcohol drinking in adults.

DISCUSSION

We have shown major age and sex differences in the behavioral interactions of nicotine and alcohol with a novel self-administration paradigm. Consistent with our earlier study in which nicotine was given as a passive pretreatment (Dao et al., 2011), we now show that concurrent nicotine self-administration enhances alcohol reinforcement and intake in adolescent male, but not adult male or female rats of either age. In contrast, concurrent self-administration of EtOH and Nic in adults activates KOR, which blocks drug reinforcement. With the use of a longitudinal study, we also show that nicotine self-administration during adolescence increases subsequent oral consumption of alcohol. These findings suggest that tobacco use by male teenagers may pose a significant risk factor for subsequent alcohol abuse. The present findings also contribute to a growing literature of substantial interactions between nicotine and alcohol, at both the molecular and circuit level (Doyon et al., 2013; Hendrickson et al., 2013).

Nicotine Increases Alcohol Intake When Combined in Adolescent Males

This is the first study to examine concurrent self-administration of nicotine and EtOH in adolescents. Our intravenous self-administration paradigm was designed to avoid confounds resulting from age differences in response to alcohol taste, and to allow for a direct comparison of nicotine and alcohol reinforcement when the two are combined. Although intravenous self-administration is the standard method used in animal models of addiction, it has been rarely used for alcohol studies. However, both humans (Plawecki et al., 2013) and animals (Hyytiä et al., 1996; Dao et al., 2011) self-administer alcohol intravenously. Lastly, we used nose-pokes, not levers, in our self-administration operant chambers to facilitate spontaneous acquisition of responding with adolescent animals. Although non-reinforced activity is higher with nose pokes than levers (Clemens et al., 2010), we observed immediate acquisition of self-administration in all adolescent groups, as defined by significantly higher reinforced than non-reinforced responses.

We found significant age differences in drug reinforcement and intake with our self-administration experiments. Nic *increased* EtOH reinforcement and intake in *adolescent* males. This finding suggests adolescents have increased sensitivity to alcohol reward, as has been reported previously (Maldonado et al., 2008; García-Burgos et al., 2009); and is consistent with a study where cigarette smoke exposure increased alcohol consumption in adolescent mice (Burns and Proctor, 2013). Our study is also supported by the clinical finding that alcohol and nicotine co-use is higher among younger (18–24) than older age groups (25–44, 25–64, and 65+) (Falk et al., 2006).

Contrary to the males, EtOH intake did not differ by age in females, and was not affected by combining Nic with EtOH. Other studies have also observed sex differences in responses to nicotine and alcohol. A clinical study reported mecamylamine,

a nicotinic acetylcholine receptor (nAChR) antagonist, is more effective in attenuating the positive effects of alcohol in men than in women (Chi and de Wit, 2003). Another group later showed that nicotine increased alcohol consumption in men, but decreased it in women (Acheson et al., 2006). This study also reported that nicotine enhances arousal state in men, but decreases positive mood in women. This suggests that nicotine may alter the motivation to drink differently across sexes. Consistent with this, women have been reported to drink less alcohol after *ad libitum* smoking than males (Perkins et al., 2000). These studies suggest women may not co-use alcohol and tobacco as much as men. Since our findings are consistent with rodent and human literature, we believe our self-administration model is a valid novel model to study EtOH+Nic reinforcement and the first to examine concurrent self-administration of nicotine and EtOH in adolescents.

EtOH and Nic Age- and Sex-Dependently Interact with *Kappa*-Opioid Receptor Function

Age differences in EtOH+Nic reinforcement in male rats were due to differences in KOR activation. Pharmacological blockade of KORs with an irreversible antagonist, norBNI, did not significantly affect responding for EtOH+Nic in females or adolescent males. However, the same treatment induced a very robust increase in EtOH+Nic reinforcement in adult males. This suggests the drug combination of EtOH and Nic induces KOR activation in adults, but not adolescents. Age differences in EtOH+Nic-induced activation of KOR may either reflect functional differences in the receptor, or in release of dynorphin, the endogenous KOR ligand (Chavkin, 2013). In order to evaluate the underlying KOR mechanism, we treated adolescent male rats with the KOR agonist, U50,488H, immediately before beginning an intravenous EtOH+Nic self-administration session. The fact that U50,488H did not inhibit EtOH+Nic reinforcement in adolescent males, suggests that the observed age difference in KOR function reflects an alteration in receptor function rather than an age difference in dynorphin release. This finding complements the conclusions of Tejeda et al. (2012), who showed that chronic nicotine increases KOR function in adults, but not adolescents. However, norBNI has recently been shown to inhibit dynorphin-stimulated G-protein signaling in the absence of KOR (Zhou et al., 2015). Thus, other potential mechanisms involving dynorphin systems should also be considered.

Adolescent Nicotine Self-administration Causes a Lasting Increase in Alcohol Preference

Following the initial intravenous self-administration experiments, the same male rats were allowed to mature and reach adulthood. Then, we allowed them to drink alcohol using a drinking in the dark paradigm (Rhodes et al., 2005; Kamdar et al., 2007). Nicotine self-administration during adolescence was found to increase subsequent EtOH consumption in adulthood. This is the first study to compare the effect of age of onset of Nic and EtOH self-administration on subsequent EtOH drinking later in life. These findings support the "gateway hypothesis" that adolescent Nic use subsequently increases drug reward; specifically, enhancing the vulnerability to develop alcohol abuse problems later in life**.** This long-lasting effect of nicotine on alcohol reward is consistent with the findings of a prior study that reported exacerbated ethanol withdrawal during adulthood in rats exposed to nicotine during adolescence (Riley et al., 2010), but is not consistent with another study that reported periadolescent nicotine treatment does not affect subsequent oral EtOH consumption (Smith et al., 2012). Such differences may reflect methodological distinctions, such as route and duration of nicotine administration, and the impact of choice on oral EtOH consumption. Our finding that adolescent EtOH intake did not affect subsequent oral alcohol preference also differs from other studies that passively administered EtOH, forced EtOH consumption (Pascual et al., 2009; Sherrill et al., 2011), or used EtOH preferring rats (Rodd-Henricks et al., 2002). However, our data are consistent with one study that allowed adolescent rats to voluntarily self-administer EtOH (Vendruscolo et al., 2010). This suggests that voluntary access and forced exposure to EtOH may differentially impact subsequent EtOH reward later in life; voluntary access has no effect, while forced exposure increases reward. Considering that stress has been shown to increase EtOH reward (Matsuzawa et al., 1998), it is possible the stress induced during forced EtOH exposure is the reason for the subsequent increase of EtOH reward in those other studies, not adolescent EtOH exposure itself. Consistent with this hypothesis, prior work has shown that adolescent mice show a long-term increase in EtOH preference after stress exposure (Siegmund et al., 2005). Thus, the stress involved in forced EtOH exposure may impact its effect on developing reward circuitry.

Lastly, we found adults that previously self-administered EtOH drank significantly more EtOH than saline-control animals, a finding consistent with much earlier reports (Roberts et al., 1996, 2000). Interestingly, combining nicotine during intravenous self-administration experiments eliminated the enhancing effect of EtOH. In contrast to adolescents, self-administration of Nic alone in adults did not change subsequent EtOH consumption. EtOH+Nic was not reinforcing in adult males. Hence, our findings suggest that, in adulthood, concurrent Nic inhibits the immediate and subsequent reinforcing properties of EtOH. Our findings are consistent with an epidemiological study which reported that heavier drinking in teenagers is correlated with smoking and the male gender (Poikolainen et al., 2001).

CONCLUSIONS

Our study revealed age-and sex-dependent interactions of EtOH and Nic in mediating drug reinforcement behavior. Recent mouse studies have indicated that EtOH and Nic interact at α4- and α6-containing nicotinic acetylcholine receptors (nAChR) to mediate reward (Liu et al., 2013a,b). However, our present findings of age- and sex-dependent interactions of EtOH and Nic in mediating drug reinforcement, suggest that a more complex model may apply. Our research supports the concept

that adolescents are less sensitive to KOR activation than adults (Natividad et al., 2010; Tejeda et al., 2012; Anderson et al., 2014; Morales et al., 2014). Furthermore, this is the first study to report an interaction by EtOH and Nic on KOR function, and offers an explanation to why most tobacco-using teenagers also drink alcohol (Orlando et al., 2005). The reported increase in alcohol intake when combined with Nic in male rats is a remarkable finding considering that these animals are voluntarily consuming EtOH. Our findings are supported by clinical studies reporting increases in smoking behavior with alcohol (Friedman et al., 1991; Rose et al., 2004; King et al., 2009; McKee et al., 2010), and conversely, increased alcohol drinking with nicotine (Barrett et al., 2006). Female rats exhibited vastly different behavioral responses to the drug combination than males. The self-administration findings are consistent with those of a clinical study reporting that nicotine increased alcohol consumption in men, but decreased it in women (Acheson et al., 2006). These findings suggest sex-dependent interactions of EtOH and Nic with KOR function.

Our longitudinal analysis of EtOH consumption with a drinking in the dark 2-bottle choice showed significant age of onset of drug use, and drug group differences. We found adolescent use of Nic, but not EtOH, significantly increased subsequent EtOH drinking in adulthood. This supports the "gateway hypothesis" for Nic and suggests that EtOH may not be a "gateway" drug in adolescence. Our findings are supported by an epidemiological study reporting heavier drinking in teenagers is correlated with smoking and the male gender (Poikolainen et al., 2001).

In summary, our study supports the growing body of literature suggesting age- and sex-dependent KOR function. In addition, it illustrates that EtOH and Nic interact with KOR to induce age- and sex-dependent behaviors. Alcohol research and tobacco research have always been considered separate fields. Our work incorporates the two, and suggests they are interconnected as so much clinical data suggest. For years, we have known people begin drinking and smoking as teenagers. These findings propose a biological factor for this highly co-morbid phenomenon; male adolescents are less sensitive to KOR inhibition of reinforcement induced by combining alcohol and nicotine. Our research suggests KOR activation as a novel mechanism mediating age differences in alcohol and tobacco co-abuse, and provides strong evidence that sex and age of first exposure are important determinants of the interactive effects of nicotine and alcohol. Two subtypes of alcoholism have been widely recognized: Type-A, or late onset, alcoholism develops after age 25; and Type-B, or early onset, alcoholism develops before age 25 (Johnson, 2010). Pharmacological treatment for alcoholism depends on severity of use and on age of onset. People who consistently smoke and drink as teenagers, and throughout their early twenties, experience substantially more negative consequences by age 29 than those who occasionally smoke and drink, or drink and not smoke (Orlando et al., 2005). With the recent escalating use of e-cigarettes in school-age children (Arrazola et al., 2015), concern has been raised that these may also increase the risk for alcohol abuse (Hughes et al., 2015; Kristjansson and Sigfudottir, 2015). Our present findings suggest that adolescent nicotine exposure, through use of either conventional tobacco products or e-cigarettes, may not only increase the immediate rewarding effects of alcohol in males, but may also increase long-term susceptibility to alcohol use. Such findings provide further strong arguments for limiting adolescent access to nicotine and tobacco products.

AUTHOR CONTRIBUTIONS

All authors are responsible for the experimental design; AL created the figures, conducted the experiments, literature research, data analysis, wrote the manuscript, and obtained funding; JB consulted on statistical analysis; FL consulted on statistical analysis, wrote the manuscript, is the principal investigator, and the principal recipient for funding for this work. All authors critically reviewed content and approved final version for publication.

FUNDING

This works was supported by Tobacco-related disease research program (TRDRP# 18XT-0085); the National Institutes of Health (DA 040440); and Faculty Mentor Program Fellowship and President's Dissertation Year Fellowship from the University of California, Irvine.

ACKNOWLEDGMENTS

We thank Elias Almaz, Chanel Chew, Christina Gadalla, Prabh Kaur, Maria Soledad Menchaca, Emily Nguyen, Michelle Nguyen, and Matthew Wong for their technical support.

REFERENCES

Acheson, A., Mahler, S. V., Chi, H., and de Wit, H. (2006). Differential effects of nicotine on alcohol consumption in men and women. *Psychopharmacology (Berl).* 186, 54–63. doi: 10.1007/s00213-006-0338-y

Anderson, R. I., Agoglia, A. E., Morales, M., Varlinskaya, E. I., and Spear, L. P. (2012). Stress, kappa manipulations, and aversive effects of ethanol in adolescent and adult male rats. *Neuroscience* 249, 214–222. doi: 10.1016/j.neuroscience.2012.12.028

Anderson, R. I., Morales, M., Spear, L. P., and Varlinskaya, E. I. (2014). Pharmacological activation of kappa opioid receptors: aversive effects in adolescent and adult male rats. *Psychopharmacology (Berl).* 231, 1687–1693. doi: 10.1007/s00213-013-3095-8

Arrazola, R. A., Singh, T., Corey, C. G., Husten, C. G., Neff, L. J., Apelberg, B. J., et al. (2015). Tobacco use among middle and high school students - United States, 2011–2014. *Morb. Mortal Wkly. Rep.* 64, 381–385.

Barrett, S. P., Tichauer, M., Leyton, M., and Pihl, R. O. (2006). Nicotine increases alcohol self-administration in non-dependent male smokers. *Drug Alcohol Depend.* 81, 197–204. doi: 10.1016/j.drugalcdep.2005.06.009

Batel, P., Pessione, F., Maître, C., and Rueff, B. (1995). Relationship between alcohol and tobacco dependencies among alcoholics who smoke. *Addiction* 90, 977–980. doi: 10.1111/j.1360-0443.1995.tb03507.x

Behrendt, S., Wittchen, H.-U., Höfler, M., Lieb, R., and Beesdo, K. (2009). Transitions from first substance use to substance use disorders in adolescence: is early onset associated with a rapid escalation? *Drug Alcohol Depend.* 99, 68–78. doi: 10.1016/j.drugalcdep.2008.06.014

Belluzzi, J. D., Lee, A. G., Oliff, H. S., and Leslie, F. M. (2004). Age-dependent effects of nicotine on locomotor activity and conditioned place preference in rats. *Psychopharmacology (Berl).* 174, 389–395. doi: 10.1007/s00213-003-1758-6

Belluzzi, J. D., Wang, R., and Leslie, F. M. (2005). Acetaldehyde enhances acquisition of nicotine self-administration in adolescent rats. *Neuropsychopharmacology* 30, 705–712. doi: 10.1038/sj.npp.1300586

Brielmaier, J. M., McDonald, C. G., and Smith, R. F. (2007). Immediate and long-term behavioral effects of a single nicotine injection in adolescent and adult rats. *Neurotoxicol. Teratol.* 29, 74–80. doi: 10.1016/j.ntt.2006.09.023

Burns, B. E., and Proctor, W. R. (2013). Cigarette smoke exposure greatly increases alcohol consumption in adolescent C57BL/6 mice. *Alcohol. Clin. Exp. Res.* 37(Suppl. 1), E364–E372. doi: 10.1111/j.1530-0277.2012.01911.x

Chavkin, C. (2013). Dynorphin–still an extraordinarily potent opioid peptide. *Mol. Pharmacol.* 83, 729–736. doi: 10.1124/mol.112.083337

Chavkin, C., James, I. F., and Goldstein, A. (1982). Dynorphin is a specific endogenous ligand of the kappa opioid receptor. *Science* 215, 413–415. doi: 10.1126/science.6120570

Chen, H., Matta, S. G., and Sharp, B. M. (2007). Acquisition of nicotine self-administration in adolescent rats given prolonged access to the drug. *Neuropsychopharmacology* 32, 700–709. doi: 10.1038/sj.npp.1301135

Chi, H., and de Wit, H. (2003). Mecamylamine attenuates the subjective stimulant-like effects of alcohol in social drinkers. *Alcohol. Clin. Exp. Res.* 27, 780–786. doi: 10.1097/01.ALC.0000065435.12068.24

Clemens, K. J., Caillé, S., and Cador, M. (2010). The effects of response operandum and prior food training on intravenous nicotine self-administration in rats. *Psychopharmacology (Berl).* 211, 43–54. doi: 10.1007/s00213-010-1866-z

Dao, J. M., McQuown, S. C., Loughlin, S. E., Belluzzi, J. D., and Leslie, F. M. (2011). Nicotine alters limbic function in adolescent rat by a 5-HT1A receptor mechanism. *Neuropsychopharmacology* 36, 1319–1331. doi: 10.1038/npp.2011.8

DiFranza, J. R., and Guerrera, M. P. (1990). Alcoholism and smoking: *J. Stud. Alcohol Drugs* 51, 130–135. doi: 10.15288/jsa.1990.51.130

Donny, E. C., Caggiula, A. R., Rowell, P. P., Gharib, M. A., Maldovan, V., Booth, S., et al. (2000). Nicotine self-administration in rats: estrous cycle effects, sex differences and nicotinic receptor binding. *Psychopharmacology (Berl).* 151, 392–405. doi: 10.1007/s002130000497

Doremus, T. L., Brunell, S. C., Rajendran, P., and Spear, L. P. (2005). Factors influencing elevated ethanol consumption in adolescent relative to adult rats. *Alcohol. Clin. Exp. Res.* 29, 1796–1808. doi: 10.1097/01.alc.0000183007.65998.aa

Doremus, T. L., Brunell, S. C., Varlinskaya, E. I., and Spear, L. P. (2003). Anxiogenic effects during withdrawal from acute ethanol in adolescent and adult rats. *Pharmacol. Biochem. Behav.* 75, 411–418. doi: 10.1016/S0091-3057(03)00134-5

Doyon, W. M., Thomas, A. M., Ostroumov, A., Dong, Y., and Dani, J. A. (2013). Potential substrates for nicotine and alcohol interactions: a focus on the mesocorticolimbic dopamine system. *Biochem. Pharmacol.* 86, 1181–1193. doi: 10.1016/j.bcp.2013.07.007

Falk, D. E., Yi, H. Y., and Hiller-Sturmhöfel, S. (2006). An epidemiologic analysis of co-occurring alcohol and tobacco use and disorders: findings from the national epidemiologic survey on alcohol and related conditions. *Alcohol Res. Health* 29, 162–171.

Friedman, G. D., Tekawa, I., Klatsky, A. L., Sidney, S., and Armstrong, M. A. (1991). Alcohol drinking and cigarette smoking: an exploration of the association in middle-aged men and women. *Drug Alcohol Depend.* 27, 283–290. doi: 10.1016/0376-8716(91)90011-M

García-Burgos, D., González, F., Manrique, T., and Gallo, M. (2009). Patterns of ethanol intake in preadolescent, adolescent, and adult Wistar rats under acquisition, maintenance, and relapse-like conditions. *Alcohol. Clin. Exp. Res.* 33, 722–728. doi: 10.1111/j.1530-0277.2008.00889.x

Gellner, C. A., Belluzzi, J. D., and Leslie, F. M. (2016). Self-administration of nicotine and cigarette smoke extract in adolescent and adult rats. *Neuropharmacology*, 109, 247–253. doi: 10.1016/j.neuropharm.2016.06.026

Hendrickson, L. M., Guildford, M. J., and Tapper, A. R. (2013). Neuronal nicotinic acetylcholine receptors: common molecular substrates of nicotine and alcohol dependence. *Front. Psychiatry* 4:29. doi: 10.3389/fpsyt.2013.00029

Hughes, K., Bellis, M. A., Hardcastle, K. A., McHale, P., Bennett, A., Ireland, R., et al. (2015). Associations between e-cigarette access and smoking and drinking behaviours in teenagers. *BMC Public Health* 15:244. doi: 10.1186/s12889-015-1618-4

Hyytiä, C., Schulteis, G., and Koob, G. F. (1996). Intravenous heroin and ethanol self-administration by alcohol-preferring AA and alcohol-avoiding ANA rats. *Psychopharmacology (Berl).* 125, 248–254. doi: 10.1007/BF02247335

Johnson, B. A. (2010). Medication treatment of different types of alcoholism. *Am. J. Psychiatry* 167, 630–639. doi: 10.1176/appi.ajp.2010.08101500

Kamdar, N. K., Miller, S. A., Syed, Y. M., Bhayana, R., Gupta, T., and Rhodes, J. S. (2007). Acute effects of naltrexone and GBR 12909 on ethanol drinking-in-the-dark in C57BL/6J mice. *Psychopharmacology (Berl).* 192, 207–217. doi: 10.1007/s00213-007-0711-5

King, A., McNamara, P., Conrad, M., and Cao, D. (2009). Alcohol-induced increases in smoking behavior for nicotinized and denicotinized cigarettes in men and women. *Psychopharmacology (Berl).* 207, 107–117. doi: 10.1007/s00213-009-1638-9

Kristjansson, A. L., and Sigfusdottir, I. D. (2015). E-cigarette use and relations to tobacco and alcohol use among adolescents. *BMC Med.* 13:103. doi: 10.1186/s12916-015-0339-y

Lemos, J. C., Wanat, M. J., Smith, J. S., Reyes, B. A., Hollon, N. G., Van Bockstaele, E. J., et al. (2012). Severe stress switches CRF action in the nucleus accumbens from appetitive to aversive. *Nature* 490, 402–406. doi: 10.1038/nature11436

Lindholm, S., Werme, M., Brené, S., and Franck, J. (2001). The selective kappa-opioid receptor agonist U50,488H attenuates voluntary ethanol intake in the rat. *Behav. Brain Res.* 120, 137–146. doi: 10.1016/S0166-4328(00)00368-5

Liu, L., Hendrickson, L. M., Guildford, M. J., Zhao-Shea, R., Gardner, P. D., and Tapper, A. R. (2013a). Nicotinic acetylcholine receptors containing the α4 subunit modulate alcohol reward. *Biol. Psychiatry* 73, 738–746. doi: 10.1016/j.biopsych.2012.09.019

Liu, L., Zhao-shea, R., Mcintosh, J. M., and Tapper, A. R. (2013b). Nicotinic acetylcholine receptors containing the α6 subunit contribute to ethanol activation of ventral tegmental area dopaminergic neurons. *Biochem. Pharmacol.* 86, 1194–1200. doi: 10.1016/j.bcp.2013.06.015

Maldonado, A. M., Finkbeiner, L. M., Alipour, K. K., and Kirstein, C. L. (2008). Voluntary ethanol consumption differs in adolescent and adult male rats using a modified sucrose-fading paradigm. *Alcohol. Clin. Exp. Res.* 32, 1574–1582. doi: 10.1111/j.1530-0277.2008.00733.x

Mansour, A., Fox, C. A., Akil, H., and Watson, S. J. (1995). Opioid-receptor mRNA expression in the rat CNS: anatomical and functional implications. *Trends Neurosci.* 18, 22–29. doi: 10.1016/0166-2236(95)93946-U

Matsuzawa, S., Suzuki, T., and Misawa, M. (1998). Conditioned fear stress induces ethanol-associated place preference in rats. *Eur. J. Pharmacol.* 341, 127–130. doi: 10.1016/S0014-2999(97)01456-8

McKee, S. A., Harrison, E. L., and Shi, J. (2010). Alcohol expectancy increases positive responses to cigarettes in young, escalating smokers. *Psychopharmacology (Berl).* 210, 355–364. doi: 10.1007/s00213-010-1831-x

McQuown, S. C., Belluzzi, J. D., and Leslie, F. M. (2007). Low dose nicotine treatment during early adolescence increases subsequent cocaine reward. *Neurotoxicol. Teratol.* 29, 66–73. doi: 10.1016/j.ntt.2006.10.012

Melief, E. J., Miyatake, M., Carroll, F. I., Carlezon, W. A., Cohen, B. M., Grimwood, S., et al. (2011). Duration of Action of a Broad Range of Selective -Opioid Receptor Antagonists is positively correlated with c-Jun N-Terminal Kinase-1 *Activation.* 80, 920–929. doi: 10.1124/mol.111.074195

Mitchell, J. M., Liang, M. T., and Fields, H. L. (2005). A single injection of the kappa opioid antagonist norbinaltorphimine increases ethanol consumption in rats. *Psychopharmacology (Berl).* 182, 384–392. doi: 10.1007/s00213-005-0067-7

Morales, M., Anderson, R. I., Spear, L. P., and Varlinskaya, E. I. (2014). Effects of the kappa opioid receptor antagonist, nor-binaltorphimine, on ethanol intake: impact of age and sex. *Dev. Psychobiol.* 56, 700–712. doi: 10.1002/dev.21137

Natividad, L. A., Tejeda, H. A., Torres, O. V., and O'Dell, L. E. (2010). Nicotine withdrawal produces a decrease in extracellular levels of dopamine in the nucleus accumbens that is lower in adolescent versus adult male rats. *Synapse* 64, 136–145. doi: 10.1002/syn.20713

Nestby, P., Schoffelmeer, A. N., Homberg, J. R., Wardeh, G., De Vries, T. J., Mulder, A. H., et al. (1999). Bremazocine reduces unrestricted free-choice ethanol self-administration in rats without affecting sucrose preference. *Psychopharmacology (Berl).* 142, 309–317. doi: 10.1007/s002130050894

Orlando, M., Tucker, J. S., Ellickson, P. L., and Klein, D. J. (2005). Concurrent use of alcohol and cigarettes from adolescence to young adulthood: an examination of developmental trajectories and outcomes. *Subst. Use Misuse* 40, 1051–1069. doi: 10.1081/JA-200030789

Pascual, M., Boix, J., Felipo, V., and Guerri, C. (2009). Repeated alcohol administration during adolescence causes changes in the mesolimbic dopaminergic and glutamatergic systems and promotes alcohol intake in the adult rat. *J. Neurochem.* 108, 920–931. doi: 10.1111/j.1471-4159.2008.05835.x

Perkins, K. A., Fonte, C., and Grobe, J. E. (2000). Sex differences in the acute effects of cigarette smoking on the reinforcing value of alcohol. *Behav. Pharmacol.* 11, 63–70. doi: 10.1097/00008877-200002000-00007

Plawecki, M. H., Wetherill, L., Vitvitskiy, V., Kosobud, A., Zimmermann, U. S., Edenberg, H. J., et al. (2013). Voluntary intravenous self-administration of alcohol detects an interaction between GABAergic manipulation and GABRG1 polymorphism genotype: a pilot study. *Alcohol. Clin. Exp. Res.* 37(Suppl. 1), E152–E160. doi: 10.1111/j.1530-0277.2012.01885.x

Poikolainen, K., Tuulio-henriksson, A., Aalto-Setälä, T., Marttunen, M., and Lönnqvist, J. (2001). Predictors of alcohol intake and heavy drinking in early adulthood : a 5-year follow-up of 15 – 19-Year-old finnish adolescents. *Alcohol* 36, 85–88. doi: 10.1093/alcalc/36.1.85

Rhodes, J. S., Best, K., Belknap, J. K., Finn, D. A., and Crabbe, J. C. (2005). Evaluation of a simple model of ethanol drinking to intoxication in C57BL/6J mice. *Physiol. Behav.* 84, 53–63. doi: 10.1016/j.physbeh.2004.10.007

Riley, H. H., Zalud, A. W., and Diaz-Granados, J. L. (2010). The influence of a chronic adolescent nicotine exposure on ethanol withdrawal severity during adulthood in C3H mice. *Alcohol* 44, 81–87. doi: 10.1016/j.alcohol.2009.10.013

Roberts, A. J., Cole, M., and Koob, G. F. (1996). Intra-amygdala muscimol decreases operant ethanol self-administration in dependent rats. *Alcohol. Clin. Exp. Res.* 20, 1289–1298. doi: 10.1111/j.1530-0277.1996.tb01125.x

Roberts, A. J., Heyser, C. J., Cole, M., Griffin, P., and Koob, G. F. (2000). Excessive ethanol drinking following a history of dependence: animal model of allostasis. *Neuropsychopharmacology* 22, 581–594. doi: 10.1016/S0893-133X(99)00167-0

Rodd-Henricks, Z. A., Bell, R. L., Kuc, K. A., Murphy, J. M., McBride, W. J., Lumeng, L., et al. (2002). Effects of ethanol exposure on subsequent acquisition and extinction of ethanol self-administration and expression of alcohol-seeking behavior in adult alcohol-preferring (P) rats: I. Periadolescent exposure. *Alcohol. Clin. Exp. Res.* 26, 1632–1641. doi: 10.1111/j.1530-0277.2002.tb02465.x

Rose, J. E., Brauer, L. H., Behm, F. M., Cramblett, M., Calkins, K., and Lawhon, D. (2004). Psychopharmacological interactions between nicotine and ethanol. *Nicotine Tob. Res.* 6, 133–144. doi: 10.1080/14622200310001656957

Schramm-Sapyta, N. L., Francis, R., Macdonald, A., Keistler, C., O'Neill, L., and Kuhn, C. M. (2014). Effect of sex on ethanol consumption and conditioned taste aversion in adolescent and adult rats. *Psychopharmacology (Berl).* 231, 1831–1839. doi: 10.1007/s00213-013-3319-y

Sherrill, L. K., Koss, W. A, Foreman, E. S., and Gulley, J. M. (2011). The effects of pre-pubertal gonadectomy and binge-like ethanol exposure during adolescence on ethanol drinking in adult male and female rats. *Behav. Brain Res.* 216, 569–575. doi: 10.1016/j.bbr.2010.08.048

Shram, M. J., Funk, D., Li, Z., and Lê, A. D. (2006). Periadolescent and adult rats respond differently in tests measuring the rewarding and aversive effects of nicotine. *Psychopharmacology (Berl).* 186, 201–208. doi: 10.1007/s00213-006-0373-8

Siegmund, S., Vengeliene, V., Singer, M. V., and Spanagel, R. (2005). Influence of age at drinking onset on long-term ethanol self-administration with deprivation and stress phases. *Alcohol Clin. Exp. Res.* 29, 1139–1145. doi: 10.1097/01.ALC.0000171928.40418.46

Smith, J. S., Schindler, A. G., Martinelli, E., Gustin, R. M., Bruchas, M. R., and Chavkin, C. (2012). Stress-induced activation of the dynorphin/κ-opioid receptor system in the amygdala potentiates nicotine conditioned place preference. *J. Neurosci.* 32, 1488–1495. doi: 10.1523/JNEUROSCI.2980-11.2012

Tejeda, H. A., Natividad, L. A., Orfila, J. E., Torres, O. V., and O'Dell, L. E. (2012). Dysregulation of kappa-opioid receptor systems by chronic nicotine modulate the nicotine withdrawal syndrome in an age-dependent manner. *Psychopharmacology (Berl).* 224, 289–301. doi: 10.1007/s00213-012-2752-7

Torres, O. V., Walker, E. M., Beas, B. S., and O'Dell, L. E. (2014). Female rats display enhanced rewarding effects of ethanol that are hormone dependent. *Alcohol. Clin. Exp. Res.* 38, 108–115. doi: 10.1111/acer.12213

Varlinskaya, E. I., and Spear, L. P. (2004). Acute ethanol withdrawal (hangover) and social behavior in adolescent and adult male and female Sprague-Dawley rats. *Alcohol. Clin. Exp. Res.* 28, 40–50. doi: 10.1097/01.ALC.0000108655.51087.DF

Vendruscolo, L. F., Gueye, A. B., Vendruscolo, J. C. M., Clemens, K. J., Mormède, P., Darnaudéry, M., et al. (2010). Reduced alcohol drinking in adult rats exposed to sucrose during adolescence. *Neuropharmacology* 59, 388–394. doi: 10.1016/j.neuropharm.2010.05.015

Walker, B. M., and Koob, G. F. (2008). Pharmacological evidence for a motivational role of kappa-opioid systems in ethanol dependence. *Neuropsychopharmacology* 33, 643–652. doi: 10.1038/sj.npp.1301438

Walker, B. M., Zorrilla, E. P., and Koob, G. F. (2011). Systemic κ-opioid receptor antagonism by nor-binaltorphimine reduces dependence-induced excessive alcohol self-administration in rats. *Addict. Biol.* 16, 116–119. doi: 10.1111/j.1369-1600.2010.00226.x

Wilmouth, C. E., and Spear, L. P. (2004). Adolescent and adult rats' aversion to flavors previously paired with nicotine. *Ann. N. Y. Acad. Sci.* 1021, 462–464. doi: 10.1196/annals.1308.065

Zapata, A., and Shippenberg, T. S. (2006). Endogenous kappa opioid receptor systems modulate the responsiveness of mesoaccumbal dopamine neurons to ethanol. *Alcohol. Clin. Exp. Res.* 30, 592–597. doi: 10.1111/j.1530-0277.2006.00069.x

Zhou, L., Stahl, E. L., Lovell, K. M., Frankowski, K. J., Prisinzano, T. E., Aubé J., et al. (2015). Characterization of kappa opioid receptor mediated, dynorphin-stimulated [^{35}S] GTPγS binding in mouse striatum for the evaluation of selective KOR ligands in an endogenous setting. *Neuropharmacology* 99, 131–141. doi: 10.1016/j.neuropharm.2015.07.001

Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Advantages of publishing in Frontiers

OPEN ACCESS
Articles are free to read for greatest visibility and readership

FAST PUBLICATION
Around 90 days from submission to decision

HIGH QUALITY PEER-REVIEW
Rigorous, collaborative, and constructive peer-review

TRANSPARENT PEER-REVIEW
Editors and reviewers acknowledged by name on published articles

Frontiers
Avenue du Tribunal-Fédéral 34
1005 Lausanne | Switzerland

Visit us: www.frontiersin.org
Contact us: info@frontiersin.org | +41 21 510 17 00

REPRODUCIBILITY OF RESEARCH
Support open data and methods to enhance research reproducibility

DIGITAL PUBLISHING
Articles designed for optimal readership across devices

FOLLOW US
@frontiersin

IMPACT METRICS
Advanced article metrics track visibility across digital media

EXTENSIVE PROMOTION
Marketing and promotion of impactful research

LOOP RESEARCH NETWORK
Our network increases your article's readership