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Critical Review

The “First Hit” Toward Alcohol Reinforcement: Role of Ethanol Metabolites

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This review analyzes literature that describes the behavioral effects of 2 metabolites of ethanol (EtOH): acetaldehyde and salsolinol (a condensation product of acetaldehyde and dopamine) generated in the brain. These metabolites are self-administered into specific brain areas by animals, showing strong reinforcing effects. A wealth of evidence shows that EtOH, a drug consumed to attain millimolar concentrations, generates brain metabolites that are reinforcing at micromolar and nanomolar concentrations. Salsolinol administration leads to marked increases in voluntary EtOH intake, an effect inhibited by mu-opioid receptor blockers. In animals that have ingested EtOH chronically, the maintenance of alcohol intake is no longer influenced by EtOH metabolites, as intake is taken over by other brain systems. However, after EtOH withdrawal brain acetaldehyde has a major role in promoting binge-like drinking in the condition known as the “alcohol deprivation effect”; a condition seen in animals that have ingested alcohol chronically, are deprived of EtOH for extended periods, and are allowed EtOH re-access. The review also analyzes the behavioral effects of acetate, a metabolite that enters the brain and is responsible for motor incoordination at low doses of EtOH. Also discussed are the paradoxical effects of systemic acetaldehyde. Overall, evidence strongly suggests that brain-generated EtOH metabolites play a major role in the early (“first-hit”) development of alcohol reinforcement and in the generation of relapse-like drinking.

Key Words: Acetaldehyde, Salsolinol, Acetate, Reinforcement, Self-Administration, Alcohol Deprivation Effect.

THE READER MAY be interested in use of the term “first hit” in relation to ethanol (EtOH) reinforcement. This term is used for several conditions/diseases, such as cancer, alcoholic liver disease, and neurodegenerative disorders, to indicate the first known factor that contributes to the condition. In this review, it applies to the earliest effect of EtOH leading to increases in voluntary intake of EtOH. As such, it should *be fully or substantially prevented in naïve animals that are initially allowed access to EtOH, such that reinforcement does not occur*. “Other hits” may be involved in perpetuating EtOH self-administration in conditions that *follow* its chronic intake (e.g., physical dependence, conditioning, stress). These “other hits” may or may not constitute a response to the “first-hit” effect of EtOH. The literature shows that a complex “system” develops, involving many neurotransmitter receptors and effectors, various genetic haplotypes and several environmental factors, which constitute an interrelated meshwork that perpetuates chronic EtOH intake (see Kalant, 2009; Koob et al., 1998; Spanagel, 2009). As recently as 2009, these reviews had not commented on the possibility that an EtOH-derived metabolite might actually initiate or perpetuate EtOH reinforcement. In the present review, we analyze studies that indicate that an EtOH metabolite constitutes the most likely “first-hit” candidate leading to chronic EtOH self-administration. In line with a “system” view, it will also be shown that in animals that have previously consumed EtOH chronically, EtOH intake becomes fully independent of the “first hit”; although a period of alcohol abstinence can reset the system and can even amplify the “first-hit” role of EtOH metabolites, thus allowing the original hit to again markedly influence EtOH intake. There are also “hits” unrelated to EtOH metabolites, which appear to result from the interaction of the EtOH molecule per se with hydrophobic pockets in some receptors, which result in anxiolytic effects, motor incoordination, loss of consciousness, anesthesia, and death. Some of these hits may contribute to the reinforcing effects of EtOH (e.g., anxiolytic effects in conflict situations) or may blunt the overall reinforcing effect (e.g., noxious effects at high concentrations).

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While EtOH may generate several metabolites, this review deals primarily with metabolites that have been shown to have behavioral effects: (i) acetaldehyde, the first oxidation product of EtOH; (ii) salsolinol, the product of acetaldehyde condensation with dopamine (DA), and (iii) acetate, the product of oxidation of acetaldehyde. The review starts discussing the metabolism of EtOH and the effects exerted by EtOH metabolites in the brain, an organ that metabolizes EtOH only to a small extent. There is no doubt that most of EtOH consumed is metabolized in the liver and, as will be discussed, important behavioral effects also result from such metabolism. The reader is referred to excellent reviews that cover a myriad of effects of EtOH metabolites (Correa et al., 2012, 2014; Deehan et al., 2013a; Quertemont et al., 2005).

BRAIN-GENERATED METABOLITES OF ETOH

Modification of Brain Acetaldehyde: Pharmacological Approaches

Interest in acetaldehyde effects in promoting EtOH intake started in the late 1970s with the studies of Amit and coworkers in Montreal, who showed that rats self-administer acetaldehyde into the cerebrospinal fluid (Amit et al., 1977; Brown et al., 1979). Questions at that time were as follows: (i) Could acetaldehyde be generated in the brain? and (ii) Can acetaldehyde generated in the periphery cross the blood–brain barrier (BBB)? Cohen and colleagues (1980) asked if EtOH could be oxidized in the brain by the action of catalase, an enzyme present in the brain. Catalase in the presence of hydrogen peroxide generates Complex-I, which reacts with a second molecule of hydrogen peroxide releasing both water and oxygen. The catalase inhibitor 3-aminotriazole binds to Complex-I, inactivating the enzyme. Cohen and colleagues (1980) showed that in vivo EtOH administration prevented the inactivation of brain catalase induced by 3-aminotriazole, which suggested that EtOH would also bind to Complex-I and might also be oxidized by brain catalase. Almost simultaneously, Tampier and Mardones (1979) proposed that catalase would metabolize EtOH into acetaldehyde, which was confirmed by Aragon and colleagues (1992) and Gill and colleagues (1992). Zimatkin and colleagues (2006) showed in brain homogenates that catalase accounts for about 70% of acetaldehyde generated from EtOH, while CYP2E1 was responsible for 15 to 20% and alcohol dehydrogenase may account for 0 to 20%.

As acetaldehyde is highly lipophilic, peripheral acetaldehyde is expected to cross the BBB. However, to do so, acetaldehyde must first enter the (tight-junction) endothelial cells of the BBB which—as all nucleated cells—are rich in mitochondrial aldehyde dehydrogenase-2 (ALDH2), which degrades acetaldehyde with high affinity (K_m 0.2 μM). At the levels present in blood (<20 μM) in Caucasians or in animals administered EtOH, acetaldehyde is not found in the brain compartment. Acetaldehyde is found in the brain only if it is administered exogenously, to reach blood concentrations that exceed 100 μM (Tabakoff et al., 1976).

The studies of Amit and coworkers led a number of investigators to determine whether EtOH drinking by animals could be modified by pharmacological manipulations that either (i) inhibited catalase activity by 3-aminotriazole (Aragon and Amit, 1992; Rotzinger et al., 1994; Tampier et al., 1995); (ii) trapped acetaldehyde by administration of penicillamine, a synthetic sulfur-containing amino acid (Font et al., 2006; Orrico et al., 2013), or (iii) reduced hydrogen peroxide levels (the cosubstrate of catalase) by administration of scavengers of hydrogen peroxide, such as ebselen a seleno-organic drug (Ledesma et al., 2014a). Although these studies support the view that acetaldehyde might constitute the "first hit" in EtOH reinforcement, some of the pharmacological agents used have secondary effects (e.g., inhibition of food intake by aminotriazole [Rotzinger et al., 1994; Tampier et al., 1995] or inhibition of acetylcholinesterase by ebselen [Martini et al., 2014]). Recently, Aragon and coworkers (Ledesma et al., 2014a) administered alpha-lipoic acid, a natural compound with no known secondary effects, which inhibited EtOH voluntary intake by 40%.

A second wave of evidence suggesting that acetaldehyde could be involved in the "first hit" of EtOH reinforcement was contributed by Rodd and colleagues in Indianapolis. Rodd-Henricks and colleagues (2002) and Rodd and colleagues (2005) showed that rats bred as alcohol drinkers (P strain) self-administer acetaldehyde into the posterior ventral tegmental area (pVTA) at concentrations (6 to 20×10^{-6} M) that are 3 orders of magnitude lower than those needed for EtOH self-administration (17×10^{-3} M) in the same area. The question that arises is whether these concentrations of acetaldehyde can be attained endogenously upon EtOH intake, as the measurement of acetaldehyde in the VTA has not been feasible (Buscaglia, 2013). Clearly, a number of drugs of abuse (e.g., morphine, nicotine) are also self-administered into the pVTA (Devine and Weiss, 1994; Ikemoto et al., 2006) but are not generated endogenously. Nevertheless, these drugs are known to induce the release of DA in nucleus accumbens, an effect also shared by EtOH (Bustamante et al., 2008; Di Chiara and Imperato, 1988; Quintanilla et al., 2007) and by acetaldehyde (Deehan et al., 2013a,b).

A third wave of studies supporting the view that acetaldehyde could be involved as a "first hit" in reinforcement was contributed by Karahanian and colleagues (2011, 2015), Quintanilla and colleagues (2012), and Tampier and colleagues (2013) in Santiago. These studies used 2 different approaches aimed at reducing the levels of acetaldehyde in the VTA. Studies of Zimatkin and colleagues (2006) in brain homogenates had shown that while catalase plays a major role in the generation of acetaldehyde, the latter is in turn oxidized into acetate by ALDHs. Thus, the levels of acetaldehyde in a specific brain area will depend on the balance between (i) the generation of acetaldehyde and (ii) its degradation. In a series of studies, Karahanian and colleagues

(2011, 2015) made genetic modifications in the VTA to either (i) inhibit the synthesis of catalase or (ii) increase the synthesis of ALDH2.

Administration of an Anticatalase Lentiviral Vector

A lentiviral vector coding for an anticatalase shRNA (RNAi precursor) or a control lentiviral vector was administered into the VTA of naïve UChB rats bred for their high EtOH preference (see Quintanilla et al., 2006). Four days after the intracerebral administration, animals were allowed free access to 10% EtOH and water on a 24-hour basis (Karahanian et al., 2011). Animals that received a control vector started ingesting 3 g EtOH/kg/d and reached intakes of 8 to 9 g EtOH/kg EtOH/d. Animals that received the shRNA anticatalase vector showed a marked reduction of EtOH intake resulting in an inhibition of 95% versus controls, an effect that lasted for 50 days, the duration of the study (Fig. 1). In animals that received the shRNA anticatalase intra-VTA, catalase activity was inhibited by 75% (Quintanilla et al., 2012).

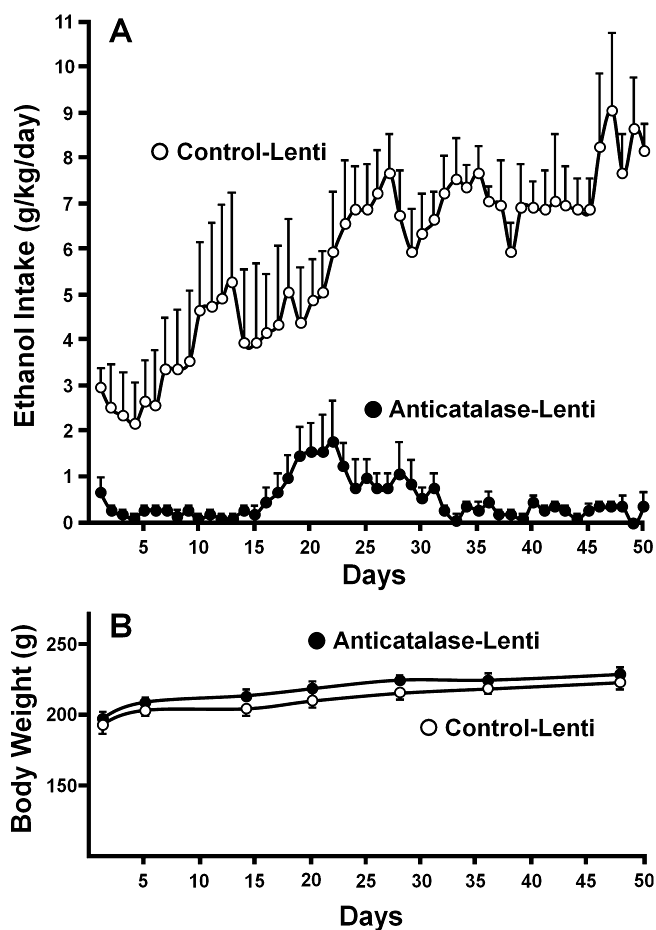

Fig. 1. Virtual abolition of ethanol (EtOH) intake following a single administration of an anticatalase lentiviral vector into the ventral tegmental area (VTA). (**A**) Rats bred for their high EtOH intake received VTA microinjections (1 μl) of 8 × 10^4 particles of a lentiviral vector coding for an shRNA against catalase mRNA. Controls received the empty lentiviral vector. Four days after vector injection animals were allowed 24-hour access to 10% EtOH and water. (**B**) Animal weight was not affected by the anticatalase vector (from Karahanian et al., 2011).

Karahanian and colleagues (2011) also determined whether the anticatalase vector inhibited the EtOH-induced increase of DA in the nucleus accumbens shell, as determined by microdialysis. Animals that received the control vector and were administered 1 g EtOH/kg (intraperitoneal [i.p.]) significantly increased the extracellular levels of DA in nucleus accumbens, while in animals that received the anticatalase lentiviral vector the EtOH-induced DA release was fully inhibited (Karahanian et al., 2011). The effect of the anticatalase vector was specific in inhibiting the EtOH-induced release of DA as it did not affect the release of DA induced by amphetamine or potassium chloride. Still, a question that required addressing was the (unlikely) possibility that secondary effects of a reduction in catalase might be the basis for the reduction in the EtOH intake. It is noted that at variance from other tissues (e.g., liver), brain catalase contributes only minimally to degrading brain hydrogen peroxide (Halliwell, 2006); rather there are 2 other enzymes: peroxiredoxins and glutathione peroxidases that are responsible for brain hydrogen peroxide degradation (Rhee et al., 2005; Turrens, 2003). However, subsequent studies were conducted aiming at increasing the degradation of acetaldehyde in the VTA.

Administration of an ALDH2-Coding Lentiviral Vector

The second approach used by Karahanian and colleagues (2015) was to transduce the VTA with a lentiviral vector carrying a gene that codes for the high affinity ALDH2; an enzyme with a K_m of 0.2 μM for acetaldehyde (Klyosov, 1996). (The in vivo apparent K_m of brain ALDH2 is unknown.) These studies showed that the administration of the lentiviral vector coding for ALDH2 into the pVTA greatly inhibited voluntary EtOH intake, reaching an inhibition of 80 to 90% versus control viral vector-treated animals, an effect which lasted for the 45 days of the study (Fig. 2). EtOH intake in rats that received the control lentiviral vector reached approximately 8 g/kg/d.

Recently Stanford scientists (Chen et al., 2008) discovered that Alda-1, a small molecule, increases ALDH2 activity (including ALDH2-1). It can be hypothesized that administration of Alda-1, by reducing brain acetaldehyde levels, would also inhibit voluntary EtOH intake. Investigators in Santiago have synthesized Alda-1, and studies on the effects of this molecule on voluntary EtOH intake in rats are in progress. Conversely, a recent study showed that increases in brain CYP2E1 by chronic oral exposure to acetone, which would increase brain acetaldehyde levels, lead to increases in *locomotor* activity in mice (Ledesma et al., 2014b). In human and non human primates, the *cyp2e1* gene shows an activating promoter polymorphism such that varied levels of CYP2E1 may be express in different individuals. However, this polymorphism did not correlate with a 3.6-fold difference in voluntary EtOH intake in monkeys (Walker et al.,

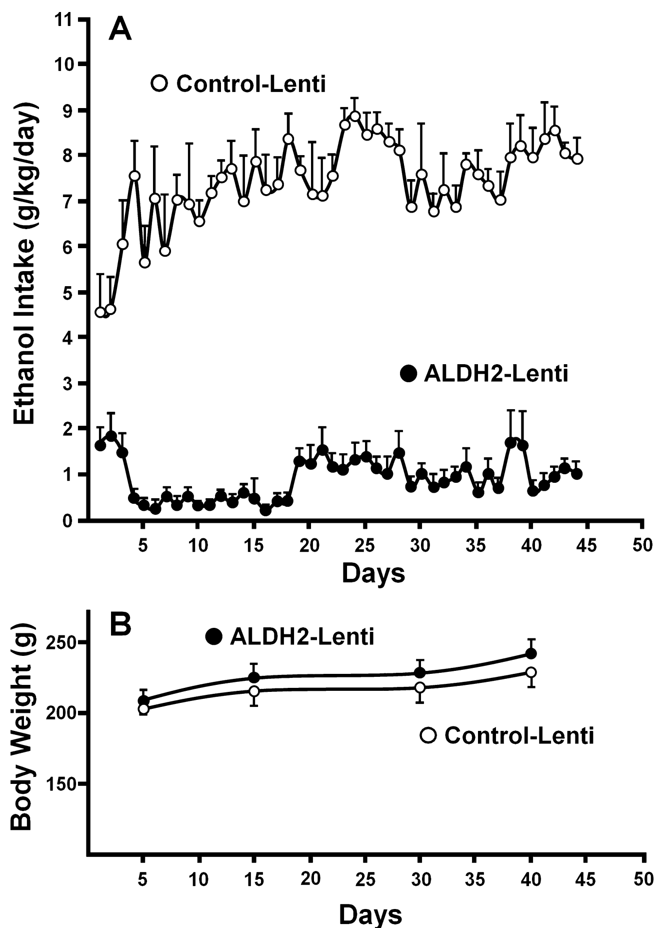

Fig. 2. Long-lasting inhibition of ethanol (EtOH) intake following a single administration of a lentiviral vector coding for aldehyde dehydrogenase-2 (ALDH2) into the ventral tegmental area (VTA). (**A**) Rats bred for their high EtOH intake received VTA microinjections (1 μl) of 8 × 10^4 particles of a lentiviral vector coding for an shRNA against catalase mRNA. Controls received the empty lentiviral vector. Four days after vector injection animals were allowed 24-hour access to 10% EtOH and water. (**B**) Animal weight was not affected by the ALDH2-coding vector (data from Karahanian et al., 2015).

2001) and it did not differentiate alcoholics from nonalcoholics (Maezawa et al., 1995).

Conclusions from Pharmacological, Self-Administration, and Genetic Approaches

The combined 3 waves of studies strongly suggest that acetaldehyde (or a metabolite of acetaldehyde; vide infra) is a strong "first-hit" candidate for alcohol-induced reinforcement. Noteworthy, the acquisition of voluntary EtOH intake by naïve animals was virtually abolished in experimental paradigms designed to either inhibit VTA acetaldehyde generation or to increase its degradation. The question that was addressed subsequently was whether in animals that had consumed EtOH chronically this "first-hit" effect was replaced by a "system" which no longer responds to manipulations of acetaldehyde generation or its degradation.

DISSOCIATING CHRONIC ETOH INTAKE FROM THE "FIRST HIT" ON THE VTA AND ITS RECOVERY

Other Animal Models

A study showing the dissociation between the mechanism(s) leading to EtOH intake in naïve animals from those maintaining EtOH intake in chronically EtOH consuming rats was reported in alcohol-preferring animals (P rats) by the Indianapolis group (Ikemoto et al., 1997). The investigators partially damaged the nucleus accumbens of naïve rats by 6-OH-dopamine administration, which led to 30 to 60% reductions of voluntary EtOH consumption in a 10% EtOH versus water choice paradigm. However, the same damage to the nucleus accumbens in rats that had been allowed to consume EtOH chronically failed to reduce their EtOH intake. Similar results were reported in animals that learned to self-administer EtOH by the sucrose-fading technique (Rassnick et al., 1993). In mice, dissociation between the effects of EtOH on the development of a conditioned place preference was also observed. In nearly naïve animals, a low dose of EtOH (0.2 g/kg) led to marked conditioned preference, an effect that was fully obliterated in DA D2 receptor knockout mice. However, the D2 knockout did not alter the development of a conditioned place preference in mice that had been fed chronically with alcohol-containing liquid diets and were withdrawn (Ting-A-Kee et al., 2009).

Dissociation from Acetaldehyde

The dissociation observed in other animal models is in line with the loss of the putative "first-hit" effect of acetaldehyde on EtOH intake as shown in 2 studies (Karahanian et al., 2015; Quintanilla et al., 2012). UChB rats that had consumed EtOH chronically for 45 or 80 days were microinjected into the VTA, with an shRNA anticatalase-coding viral vector or an ALDH2-coding vector. EtOH intake of rats that had chronically ingested EtOH (reaching 7 to 8 g EtOH/kg/d) remained constant after the intra-VTA administration of either the shRNA anticatalase viral vector or the ALDH2-coding vector. It is unlikely that a constant intake would be due to negative reinforcement, as in these animals, blood EtOH levels of only 20 to 50 mg/dl (mean value of 32 mg/dl) are achieved in the dark period of the circadian cycle (M. Rivera-Meza and M.E. Quintanilla, personal communication). In addition, the unlikely possibility that negative reinforcement might be responsible was further tested by the addition of a small concentration of quinine (0.01%) to the EtOH solution. Quinine adulteration of the EtOH solution fully inhibited EtOH intake in the sRNA anticatalase-treated animals (Quintanilla et al., 2012). Data also indicated that self-perpetuating "systems" of EtOH intake that develop after chronic intake are not permanent, as in these animals, the inhibitory effect of the shRNA anticatalase could

be partially recovered following a 4-week long deprivation from EtOH, which allowed a 50% inhibition of EtOH intake by the anticatalase vector (Quintanilla et al., 2012), thus suggesting that EtOH metabolites in the VTA were again important to maintain intake.

Tampier and colleagues (2013) and Karahanian and colleagues (2015) used a different experimental approach to show that the putative "first-hit" effect of EtOH metabolites on the VTA could be recovered and even enhanced in an experimental condition known as the "alcohol deprivation effect" (see Spanagel and Hölter, 1999) a condition that has been equated with "relapse-like drinking" in alcoholics (Vengeliene et al., 2005). The alcohol deprivation effect was generated in EtOH drinker rats (UChB) following 67 to 80 days of EtOH chronic intake, after which the animals were deprived from EtOH for only 1 or 2 weeks. Re-exposure to EtOH leads to marked increases in EtOH intake, usually lasting 24 to 48 hours, but which are most clearly seen as "binge-like" intake on the first hour of EtOH re-access. It has been shown that animals experiencing the alcohol deprivation effect will work to a greater extent (to a higher break point in progressive ratio schedules) to obtain EtOH (Oster et al., 2006; Rodd et al., 2003; Vengeliene et al., 2009), thus suggesting that the rewarding value of EtOH increases in the postdeprivation and re-access condition. After 2 EtOH deprivation re-access cycles, alcohol intakes in control vector-treated animals reached 2 g EtOH/kg in 60 minutes; while after 3 deprivation re-access cycles, intakes of control vector-treated animals reached 3 g/kg EtOH in 60 minutes. Both the administration of the anticatalase-coding vector and of the ALDH2-coding vector led to inhibitions of EtOH intake of 75 to 80% upon re-access that followed the EtOH deprivation. Thus, EtOH metabolites (acetaldehyde or acetaldehyde-derived metabolites) seem necessary to achieve these high EtOH consumption levels in a relapse-like drinking situation. This view is in line with recent studies in Wistar rats by Orrico and colleagues (2013) who showed that infusion of D-penicillamine (an acetaldehyde binding amino acid) reduced the intake of EtOH in the EtOH deprivation and re-access condition by 40 to 60%. In these studies, EtOH intake of rats prior to experiencing the alcohol deprivation effect was low (below 1 g EtOH/kg/24 h*)* while reaching postdeprivation and re-access intakes of 2.5 g/EtOH/24 h), thus indicating that animal lines or strains selected for their high EtOH intake are not required to demonstrate the participation of an EtOH metabolite to support higher alcohol intakes associated with the alcohol deprivation effect. It is also unlikely that physical dependence would be generated in Wistar rats given access to EtOH solutions and water ad libitum as chronic exposure of animals to EtOH vapor, allowing blood EtOH levels above 150 mg/dl, are needed to generate physical dependence (see Gilpin and Koob, 2010). Thus, negative reinforcement unlikely plays a dominant role in the alcohol deprivation effect.

DELIVERING THE "FIRST HIT"

Role of Acetaldehyde–DA Adducts: Salsolinol and Analogs

Acetaldehyde readily reacts with DA generating 2 adducts: (R/S)-salsolinol and (R/S)-isosalsolinol (King et al., 1974) (Fig. 3). While at a physiological pH both products are formed, at low pH the generation of (R/S)-salsolinol predominates (Bates et al., 1986). An 85% pure salsolinol sold by Sigma (St. Louis, MO) for the past decades contained 10 to 15% of isosalsolinol (Juricic et al., 2012).

Operant self-administration studies by Rodd and associates (Deehan et al., 2013a,b; Rodd et al., 2005, 2008) showed that rats self-administer salsolinol (ca. 85% pure) into the pVTA. The concentrations required for salsolinol self-administration were 1 to 2 orders of magnitude lower (30 to 100 nM) than those required for acetaldehyde (23 μM) self-administration (Rodd et al., 2008). These studies suggest that salsolinol is the molecule that delivers the acetaldehyde "first hit." In line with this view, it has been reported that salsolinol injected into the VTA led to conditioned place preference in rats (Hipólito et al., 2011). Matsuzawa and colleagues (2000) showed that systemic administration of salsolinol (10 mg/kg) also led to the development of conditioned place preference. Furthermore, it has been reported that microinjection of salsolinol into the VTA increases DA release in nucleus accumbens (Deehan et al., 2013a,b; Hipólito et al., 2010), which may be responsible at least in part for the motivational effects.

In all of the above studies, the salsolinol used was ca 85% pure and contained 10 to 15% isosalsolinol (Juricic et al., 2012). However, a place preference/motivational effect of salsolinol was confirmed by the administration of a recently available isosalsolinol-free salsolinol. Marked conditioned place preference was seen whether pure salsolinol was injected intra-VTA or administered systemically (Quintanilla et al., 2014). The concentrations of salsolinol attained in brain microdialysates (neostriatum) of rats that received

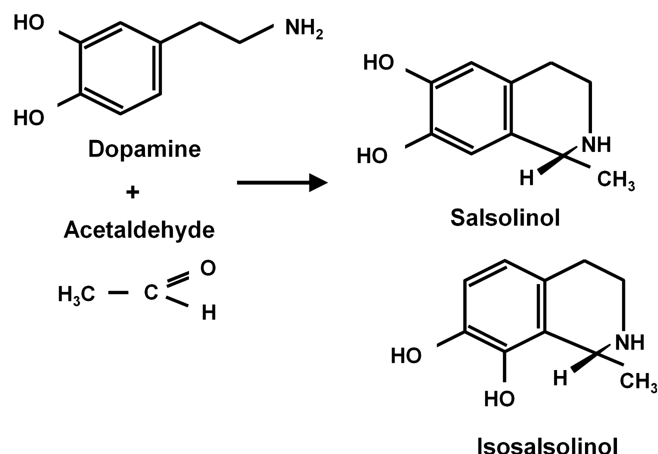

Fig. 3. Acetaldehyde condensation with dopamine. Products formed in the nonenzymatic condensation are (R)- and (S)-salsolinol and (R)- and (S)-isosalsolinol salsolinol (redrawn from Juricic et al., 2012).

salsolinol (10 mg/kg, i.p.) were 30 to 100 nM (Quintanilla et al., 2014) which are within the range of concentrations which rats self-administer salsolinol into the pVTA (Deehan et al., 2013a,b; Rodd et al., 2008). In P rats consuming EtOH chronically, levels of salsolinol in nucleus accumbens are about 1 order of magnitude higher (Starkey et al., 2006); however, much might be bound in vesicles.

As was seen in Fig. 1, rats bred for their preference for alcohol consume lower amounts of alcohol upon the initial days of EtOH access, while intake is increased markedly (doubled or tripled) as their intake continues. Several mechanisms could conceivably account for such increase; 1 is tolerance to any aversive effects of EtOH in the EtOH-versus-water free choice paradigm. Tolerance is primarily a learned phenomenon (Kalant, 1998) that develops to actively compensate for an alcohol-induced loss of a *needed* function. For heavy drinker humans, tolerance is most relevant in their path to alcohol dependence, as it allows individuals to consume large amounts of alcohol while maintaining normal (or near normal) motor or socially required functions/behaviors. In rats that are isolated or are not actively required to compensate for a loss of function (see Chen, 1968; LeBlanc et al., 1973), tolerance may play a smaller role in the escalation to high alcohol intakes, although studies are needed to determine whether tolerance in the animals may occur from a possible self-awareness of dysfunction or a biological effect of EtOH per se.

In addition to tolerance, the development of sensitization to the reinforcing effect of EtOH (i.e., an increase in the hedonic/rewarding value) appears important. If salsolinol was the molecule that delivers the "first hit," chronic administration of salsolinol might per se lead to sensitization to the reinforcing effect of EtOH. This was indeed shown by the studies of Myers and Melchior (1977) who demonstrated that commercial salsolinol (ca. 85% pure) injected into the cerebrospinal fluid of Sprague–Dawley rats led to a several-fold increase in EtOH intake. The increases of EtOH intake were also observed 30 days after discontinuing the administration of salsolinol. Duncan and Deitrich (1980) confirmed, in Sprague–Dawley and Long–Evans rats, these protracted increases (up to 5-fold) in EtOH intake after the chronic administration of salsolinol into a brain ventricle.

The reader may ask why the excellent studies indicated above were not actively pursued, and it has taken over 20 years to renew an interest in these. The view at that time was that these marked long-term effects might be due to nonspecific and permanent brain damage induced by contaminants in salsolinol preparations (or by salsolinol itself) which could increase EtOH intake due to a reduction of any aversive or noxious effects of EtOH in the low intake Wistar and Long–Evans rats. Recently, Quintanilla and colleagues (2014) demonstrated in naïve UChB rats bred for their high EtOH intake that the administration of 4 doses of a newly available isosalsolinol-free salsolinol (+99% purity) administered intra-VTA or administered systemically (10 mg/kg, i.p.) every 3 days, increased EtOH intake by 200 to 250%, such that the rats starting consuming 2.5 g EtOH/kg in 60 minutes. This effect remained constant for 1 week (the duration of the follow-up) after salsolinol administration was discontinued. The authors demonstrated that brain salsolinol has a half-life of 30 to 60 minutes such that the effect of salsolinol is seen primarily as a protracted sensitization of EtOH reinforcement rather than a potentiation of EtOH reinforcement by the salsolinol molecule per se. As will be seen below, knowledge of the action of salsolinol makes it most unlikely that damage was responsible for the increases in EtOH intake induced by salsolinol. While the mechanism of this effect is unknown, these long-term effects of salsolinol are suggestive of "system" adaptations.

A Putative Receptor to Take the "First Hit"

Early studies by Matsuzawa and colleagues (2000) suggested that salsolinol (ca. 85% pure) could generate its motivational effects via the mu-opioid receptor. These authors reported that conditioned place preference induced by systemic salsolinol administration (10 mg/kg, i.p.) was blocked by naloxone, a nonspecific mu-opioid receptor antagonist, while the more selective mu-opioid antagonist β-funaltrexamine significantly attenuated the salsolinol-induced locomotor stimulation (Hipólito et al., 2010). Quintanilla and colleagues (2014) administered isosalsolinol-free salsolinol (10 mg/kg, i.p.) which led to conditioned place preference and to marked increases in EtOH intake; both of which were fully blocked by the intra-pVTA administration of naltrexone, thus strongly suggesting that the effect of salsolinol (even when administered systemically and reaching all brain areas) occurs primarily (or via) the pVTA and is dependent on opiate receptor(s).

The neurocircuitry of the VTA becomes important to understand the possible mode of salsolinol action. The body of dopaminergic neurons expresses GABA receptors and are hence hyperpolarized (inhibited) by GABA released from GABAergic neurons. The latter, in turn, present mu-opioid (inhibitory) receptors. There are studies (vide infra) that suggest that salsolinol binds to mu-opioid receptors on GABA neurons inhibiting an inhibitory effect on DA neurons, resulting in an increase of DA neuron firing. Such an effect would lead to an increase in DA release by dopaminergic axons in the nucleus accumbens. It should be noted that no specific studies of salsolinol binding to mu-opioid receptors have been conducted to show its intrinsic efficacy and that an EtOH-induced release of beta-endorphin by GABAergic interneurons might generate a similar effect. EtOH has been reported to release beta-endorphin into the VTA of adult rats (see Palm and Nylander, 2014). Regardless of the initial effect of salsolinol on the GABA neuron, patch clamp studies of midbrain slices showed that salsolinol dose-dependently stimulates DA neurons in the p-VTA partly by reducing the firing rate of GABAergic neurons (Xie et al., 2012), thus constituting the likely mechanism that leads to increases of

DA release in the nucleus accumbens (Deehan et al., 2013a, b; Hipólito et al., 2010).

ALCOHOL METABOLITES: A CONSISTENT "FIRST-HIT" VIEW

The studies reviewed above strongly suggest that (i) the metabolism of EtOH into acetaldehyde in the pVTA and the maintenance of acetaldehyde levels are required to generate the initial reinforcing effect of EtOH; (ii) EtOH intake becomes dissociated from pVTA acetaldehyde in animals that have consumed EtOH chronically; (iii) in animals experiencing the EtOH deprivation effect (where EtOH availability is discontinued after chronic intake), binge-like drinking is observed and it again becomes dependent on pVTA acetaldehyde; (iv) salsolinol—a condensation product of acetaldehyde and DA increases DA release in nucleus accumbens and is accompanied by the generation of motivational effects as seen in conditioned place preference paradigms; (v) both chronic salsolinol administration and EtOH chronic availability lead to marked increases in EtOH intake (sensitization); and (vi) the effects of chronic salsolinol administration on EtOH intake and conditioned place preference are fully blocked by the intra-pVTA administration of naltrexone. Overall, these data strongly support a primary effect of EtOH metabolites (acetaldehyde and salsolinol) in the initiation of chronic EtOH consumption, the development of conditioned place preference and an increased reinforcing effect of EtOH.

ACETATE: BEHAVIORAL EFFECTS

Most of the acetate generated in the hepatic metabolism of EtOH exits the liver (Lundquist et al., 1962) as the ability of the liver to oxidize the NADH generated in the 2 dehydrogenase steps (ADH and ALDH) uses 70 to 75% of the oxidative capacity of the organ (see Britton et al., 1984), such that little acetate generated from EtOH can be oxidized. This is in line with studies in perfused livers by Forsander and colleagues (1960) who showed that only 2 to 7% of the total ^{14}C-EtOH oxidized by the liver appears as $^{14}CO_2$. Upon EtOH administration, blood acetate concentrations rise from basal levels of 0.2 μM to 1.2–1.5 μM (Carmichael et al., 1991; Nuutinen et al., 1985). These levels are attained at a dose of 0.5 g EtOH/kg, while higher doses do not further increase blood acetate levels (Carmichael et al., 1991). This dose of EtOH has been shown to significantly reduce motor coordination (Lê and Israel, 1994), an effect that is *fully* inhibited by the A1-A2 adenosine receptor blocker 8-phenyl theophylline, while only partially reducing the motor incoordination generated by high doses of EtOH (Carmichael et al., 1991). Studies of Correa and coworkers have also shown motor inhibitory effects of acetate in several experimental paradigms (Correa et al., 2003; McLaughlin et al., 2008; Pardo et al., 2013).

Two mechanisms by which EtOH can increase tissue adenosine levels have been described. First, acetate is metabolized into acetyl-CoA by a mechanism that utilizes ATP, releasing AMP which is readily dephosphorylated generating adenosine (see Israel et al., 1994; Pardo et al., 2013). A second mechanism that also leads to increases in brain extracellular adenosine is an inhibition of adenosine re-uptake by EtOH (Clark and Dar, 1989; Nagy et al., 1990).

An exciting new area of research on brain acetate effects indicates that acetate is metabolized in the brain both in animals and in humans by displacing glucose metabolism (Pawlosky et al., 2010; Volkow et al., 2013; Wang et al., 2013), leading to increases in the levels of glutamate and GABA. Heavy drinkers display an added utilization of brain acetate, in part due to a faster uptake of acetate from the systemic circulation, which is accompanied by increased levels of brain glutamate (Jiang et al., 2013), likely due to transamination of (TCA cycle) alpha-ketoglutarate. Jiang and colleagues (2013) suggest that in alcoholics the recovery of elevated brain adenosine levels by acetate generation upon drinking may be rewarding. These authors also indicate that in malnourished alcoholics with low glucose levels, brain utilization of acetate will serve as an additional source of calories to maintain metabolism.

SYSTEMIC ACETALDEHYDE: PARADOXICAL EFFECTS

Aversion in Naïve Animals but Inert After Chronic EtOH Intake

Among the first studies that described a powerful aversive effect of systemic acetaldehyde were those in subjects of East Asian origin. Studies in the 1980s demonstrated that 30 to 40% of Asians are protected against alcoholism as they experienced overt facial flushing, tachycardia, vasodilation, and nausea (Mizoi et al., 1983). These subjects carry a dominant negative point mutation in the enzyme that codes for aldehyde dehydrogenase (ALDH2*2) which leads to greatly elevated acetaldehyde levels upon EtOH intake. When consuming alcohol, acetaldehyde generated by hepatic alcohol dehydrogenase in these subjects leaves the liver into the systemic circulation, reaching venous blood levels of 60 to 100 μM, while concentrations in subjects carrying the active ALDH2*1 are below 10 μM.

Protection from alcoholism in subjects who carry 1 or 2 inactive *ALDH2*2*-coding alleles is of the order of 66% to 99%, respectively (Chen et al., 1999; Harada et al., 1982; Higuchi, 1994; Luczak et al., 2006; Thomasson et al., 1991; Tu and Israel, 1995; Zintzaras et al., 2006). It is important to note that this aversion will be perceived already on their first drink in young individuals. As will be discussed below, a drug such as disulfiram, which inhibits ALDH2, is administered to alcoholics *after* they have engaged in chronic EtOH consumption.

Disulfiram, described in Denmark as an "anti-alcohol" drug in 1949, markedly elevates blood acetaldehyde levels upon EtOH intake both in animals and in humans (see Hine et al., 1952; Newman, 1950). In rats that initiate their alcohol intake, disulfiram greatly elevates blood acetaldehyde levels and markedly reduces their voluntary EtOH intake (Tampier et al., 2008). Escrig and colleagues (2012) have shown that acetaldehyde is anxiogenic and induces endocrine stress responses. However, a puzzling new finding indicates that in rats that have ingested EtOH chronically, disulfiram although having an identical effect in markedly elevating blood acetaldehyde levels is *fully* ineffective in reducing EtOH intake (Tampier et al., 2008).

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 EtOH relapse in alcoholics (Skinner et al., 2014; Yoshimura et al., 2014). These studies might be taken as an indication that following chronic EtOH intake a *systemic* elevation of acetaldehyde does not inhibit EtOH consumption. This is, however, not correct because increases in systemic acetaldehyde following the administration of a *liver specific* vector coding for an antisense anti-ALDH2 synthesis (which does not enter the brain) markedly inhibited (50 to 65%) voluntary EtOH intake of rats that had ingested EtOH chronically for 60 to 75 days (Ocaranza et al., 2008; see also Rivera-Meza et al., 2012). Rather, the lack of disulfiram effect on EtOH intake in animals fed alcohol chronically (and in alcoholics) may stem from the fact that disulfiram crosses the BBB and also inhibits ALDH2 (Hellström and Tottmar, 1982). Thus, in (sensitized) EtOH fed rats brain disulfiram might contribute an added hedonistic effect of EtOH to counter the aversive effects of acetaldehyde in the periphery. An increase in brain salsolinol which activates chronic EtOH intake or the synthesis of tetrahydropapaveroline (Davis and Walsh, 1970), another strong sensitizing agent (Duncan and Deitrich, 1980; Myers and Melchior, 1977) could be considered.

We quote the experience of Chevens (1953, pp. 1450–1451) in the *British Medical Journal*:

> All those who deal with alcoholics have observed the variability of the Antabuse-alcohol reaction. This variability is to be expected, for the aversion effect of antabuse therapy does not depend on physical symptoms when alcohol is taken—the vasodilatation, headache, tachycardia and nausea- … but on the feeling of impending personal catastrophe. A great many psychopathic drinkers show a magnificent tolerance for acetaldehyde and considerable bravado. They often claim that a small gin makes them feel like several doubles when on Antabuse.

Correa and colleagues (2014) have indicated that it has taken us several decades to accept the role of acetaldehyde as a psychoactive agent. The present review further places this metabolite of EtOH and its condensation product with DA *at the start of sequence of events* that leads to chronic alcohol intake and likely to alcoholism.

AN APOLOGY

The alcohol field has generated extensive and valuable information in different areas which could not all be covered; also many mechanisms that maintain EtOH intake after reinforcement have been proposed. The effect of the EtOH molecule per se on specific neurotransmitter function may contribute to the "first hit" for some individuals or in some experimental conditions.

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