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Authors

Bowen, Michael T
George, Olivier
Muskiewicz, Dawn E
et al.

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FACTORS CONTRIBUTING TO THE ESCALATION OF ALCOHOL CONSUMPTION

Michael T. Bowen[1,2,*], **Olivier George**[3], **Dawn E. Muskiewicz**[4], **F. Scott Hall**[4]

[1]The University of Sydney, Brain and Mind Centre, Sydney, NSW, 2050, Australia

[2]The University of Sydney, Faculty of Science, School of Psychology, Sydney, NSW, 2006, Australia

[3]Department of Psychology, University of California, San Diego, School of Medicine, La Jolla, CA, 92093, USA

[4]Department of Pharmacology & Experimental Therapeutics, College of Pharmacology and Pharmacological Science, University of Toledo, OH, USA

Abstract

Understanding factors that contribute to the escalation of alcohol consumption are key to understanding how an individual transitions from non/social drinking to AUD and to providing better treatment. In this review, we discuss how the way ethanol is consumed as well as individual and environmental factors contribute to the escalation of ethanol consumption from intermittent low levels to consistently high levels. Moreover, we discuss how these factors are modelled in animals. It is clear a vast array of complex, interacting factors influence escalation of alcohol consumption. Some of these factors act early in the acquisition of ethanol consumption and initial escalation, while others contribute to escalation of ethanol consumption at a later stage and are involved in the development of alcohol dependence. It is apparent from our review that much of the literature examines factors contributing to the acquisition of ethanol consumption and on initial escalation from low levels to pharmacologically relevant levels of consumption. Some models capture escalation associated with the formation of dependence; however, neurobiological studies in these models usually focus on comparisons between the AUD model animals and alcohol naïve animals (or animals from other models), making it difficult to distinguish factors associated with the escalation of interest from those associated with consumption in the model *per se*. There is thus considerable need for more studies examining escalation associated with the formation of dependence as it is of considerable relevance to understanding and treating AUD.

[*]Corresponding Author: Michael T. Bowen, Brain and Mind Centre, The University of Sydney, 94 Mallett Street, Camperdown, Sydney, NSW, 2050, Australia, michael.bowen@sydney.edu.au.

[7]·Declaration of Competing Interest

MTB is listed as an inventor on patents for novel oxytocin-based therapeutic candidates and for therapeutic candidates for alcohol use disorder and is co-founder and chief scientific officer of a company, Kinoxis Therapeutics Pty Ltd, commercialising some of this technology. The other authors declare they have no conflicts of interest.

Keywords

alcohol; ethanol; alcohol use disorder; alcohol dependence; alcoholic; alcoholism; incubation of craving; alcohol deprivation effect; drinking in the dark; sucrose fading; intermittent ethanol access; continuous ethanol access; social isolation; social anxiety

1. Introduction

People with AUD (and those without) do not begin voluntarily consuming alcohol at birth, but rather follow several different trajectories of consumption patterns over their lifespan. Ethanol consumption typically begins in adolescence or early adulthood, and for individuals who develop alcohol use problems there can be several different developmental trajectories. Some of these lead to AUD and alcohol dependence, while some do not. For instance, some include problem drinking during adolescence and early adulthood that does not lead to AUD or dependence in the long-term (Behrendt et al., 2008; Costanzo et al., 2007). Many factors are thought to contribute to the development of an escalating pattern of ethanol consumption, including: certain patterns of drinking, such as binge-drinking (Chassin et al., 2002); early life trauma (Davis et al., 2018; Meyers et al., 2019); early initiation of ethanol consumption (Pfefferbaum et al., 2018); parental alcohol and drug use/abuse patterns (Sternberg et al., 2018; Walden et al., 2007); permissive societal norms and/or regulations governing ethanol consumption, particularly in adolescence (Fairman et al., 2019); whether the individual lives in a rural or urban community (Donath et al., 2011); impulse control and other psychological features (Dick et al., 2009; Hardee et al., 2014); genetic factors (Hendershot et al., 2017); and other factors (Brunborg et al., 2018). Not surprisingly, many of these genetic, psychological and environmental factors interact to affect alcohol use/abuse trajectories (Kendler et al., 2011).

This review will discuss these factors alongside the many ways in which these factors have been modelled in animals. The purpose of this review is to identify and study the factors contributing to the escalation of ethanol consumption under controlled circumstances. We consider changes in consumption at different stages of the development of alcohol consumption. We refer to the change from no drinking, or low levels of consumption, to consumption of pharmacologically relevant amounts of alcohol as acquisition or initial escalation. We refer to increases in consumption that would be considered relevant to the formation of alcohol use disorder and dependence as escalation. As will be seen, the work conducted to date has provided valuable insights into factors that contribute to acquisition, initial escalation, and escalation of consumption, but also the biological mechanisms that change over the course of that escalation, especially in earlier stages. However, it is also clear that a detailed causal understanding of the underlying neurobiology of many of these factors contributing to the escalation of alcohol consumption remains lacking, especially those relating to escalation to consumption to levels associated with the formation of dependence and AUD. Discovering the essential mechanisms underlying escalation of ethanol consumption is of considerable important as it will identify points where intervention might be most effective, provide new targets for the development of interventions for AUD, and may also help provide biomarkers for identifying those most at

risk. The discussion in this review will confirm and assert that understanding the process of escalation is critical to understanding the development of excessive alcohol consumption, alcohol dependence and AUD, and to better treating AUD.

Figure 1 presents a how different factors can contribute to escalation of ethanol consumption at different stages of drinking, aligned to the stages of life in which they usually occur, from initiation of drinking in first-time users to relapse in AUD, and lists some of the key animal models that facilitate exploration of these different factors.

2. The impact of how ethanol is consumed on escalation of consumption

The way in which alcohol is consumed can have a significant impact on acquisition, initial escalation and escalation of alcohol consumption. This includes the solution in which alcohol is consumed, the time of day alcohol is consumed, and the frequency and quantity of alcohol consumed. The way alcohol is consumed can lead to changes in psychological functioning underpinned by alterations to neural systems subserving stress coping and reward, which not only appear to play a critical role in initial escalation of consumption, but also changes in craving that occur during abstinence that can reinvigorate consumption. These effects on both stress coping and reward, as well as other acute and chronic effects of alcohol, suggest the importance of both positive and negative reinforcement in increased ethanol consumption over the course of the development of alcohol dependence and AUD. These effects must also be considered in the context of not just ethanol withdrawal-induced impairments in affective and cognitive function, but also pre-existing psychopathology which could contribute to negative reinforcement upon symptom alleviation by alcohol.

In this section we will explore how sweetened alcohol consumption can facilitate acquisition and initial escalation of alcohol consumption and subsequent escalation to binge-drinking. Similarly, we will discuss circadian factors that appear to confer increased risk for escalation of alcohol consumption, which back-translates to animal models showing increased binge-like consumption when alcohol is provided during certain stages of the light-dark cycle. After acquisition of alcohol consumption and initial escalation, intermittent patterns of alcohol consumption appear to facilitate escalation to levels conferring significant risk of developing AUD. We will discuss studies exploring intermittent alcohol exposure and the neurobehavioural adaptations driving escalation. Finally, we will discuss how repeated cycles of withdrawal and abstinence promote changes that lead to an incubation of craving and a subsequent rebound in consumption that is escalated above baseline levels following a period of abstinence; a phenomenon known as the alcohol deprivation effect.

2.1. Sweetened alcoholic drinks

Most people start consuming alcohol during mid-to-late adolescence and it is common among young users to start consuming alcohol for the first time in sweetened beverages and to later transition to stronger and/or unsweetened alcoholic drinks (Roberts et al., 2015; Rossheim and Thombs, 2013). Some evidence suggests that sweetened alcoholic beverages promote acquisition and initial escalation of alcohol consumption among naïve and inexperienced drinkers (Roberts et al., 2015; Rossheim and Thombs, 2013). For instance, Roberts et al. (2015) found that sweetened alcoholic beverages (often referred to as

"alcopops") were among the most highly consumed alcoholic drinks in youth experiencing negative alcohol-related consequences. This study is also supported by epidemiological data showing that increasing popularity of alcopops in the 1990s and 2000s in a number of countries coincided with significant increases in the average amount of alcohol being consumed by adolescents (e.g. Romanus, 2000). This drove policy changes in many of these countries to reduce the accessibility of alcopops, with some evidence this has led to a reduction in alcohol consumption and alcohol-related harms among adolescents and young adults (Gale et al., 2015; Lensvelt et al., 2016; Mojica-Perez et al., 2020).

While the human data points to a possible connection between sweetened drinks, increased consumption in new drinkers, and transition to consumption of stronger alcoholic drinks, studies in animals provide causal evidence that sweetened alcoholic solutions promote acquisition and initial escalation of consumption. This evidence comes primarily from studies using the sucrose-fading initiation procedure in rats (Grant and Samson, 1985a, b; Samson, 1986; Tolliver et al., 1988). At its core, the procedure involves initiating consumption of ethanol by providing rats with access to ethanol in a palatable sucrose solution, then, once responding has stabilised, reducing the sucrose concentration until the rats are consuming a solution of ethanol in water. The procedure can successfully induce high levels of ethanol consumption (leading to BACs > 0.1 g/dL in some animals) consumed in solutions of up to 40% ethanol in water (Simms et al., 2010). Importantly, rats obtain these high levels of alcohol consumption without food or water deprivation. Interestingly, Tolliver et al. (1988) found that male rats with an initially low preference for ethanol show the greatest increase in ethanol preference following initiation using the sucrose-fading procedure. Thus, sweetened alcoholic drinks facilitate acquisition and initial escalation of ethanol consumption, perhaps especially in individuals who might not otherwise consume high levels of ethanol, consistent with conclusions drawn from the human data with alcopops, discussed above.

2.2. Circadian factors

In both humans and in animal models, circadian factors appear to play a key role in the acquisition and initial escalation of alcohol consumption, and may also contribute to escalation associated with AUD. In humans, circadian changes during late adolescence coincide with the initiation of alcohol consumption, and several sleep and circadian factors have been identified as potential contributors to initial escalation of alcohol consumption (for a recent review see Hasler and Pedersen, 2020). Later circadian timing, circadian misalignment, and a range of associated sleep disturbances have all been associated with increased alcohol consumption and in some studies with AUD (e.g. DeMartini and Fucito, 2014; Hasler et al., 2015). It has been proposed that both positive and negative reinforcement processes can play a role in circadian influences on alcohol consumption. Later circadian timing (associated with increased sensitivity to alcohol reward), perceived facilitation of sleep onset by alcohol, and perceived reductions in anxiety following alcohol consumption are among the most commonly reported reasons for drinking (Hasler and Pedersen, 2020).

In animal models, the critical role played by circadian factors in alcohol consumption is also borne out by the significant influence of *when* alcohol is provided during the light/dark

cycle on acquisition of consumption. Rodents are nocturnal, so they show greater activity and ingestive behaviour during the dark cycle. Not surprisingly, alcohol consumption is higher during the dark phase. One model, in particular, warrants discussion here: the drinking-in-the-dark (DID) mouse model of binge-drinking ((Rhodes et al., 2005; Rhodes et al., 2007) and for review see Thiele et al. (2014)). The key goal of the model is to produce heavy drinking within a short period of time. However, the DID procedure might also be considered as accelerating the process of escalation, especially in susceptible animals. Numerous factors consistent with those discussed above affect DID, although considerable work has focussed upon genetic factors (Crabbe et al., 2009; Rhodes et al., 2007). One of those genetic approaches involved selection of high DID lines of mice. It is interesting to note, in the context of the previous discussion about circadian rhythms, that this selection resulted in alterations in circadian rhythms as well as binge-like alcohol consumption.

A common problem with rodent models of alcohol consumption is that subjects often will not voluntarily consume alcohol to levels of behavioural intoxication (BAC > 0.1 g/dl). The DID model overcomes this problem by capitalising on rodents increased ingestive behaviour about a quarter of the way into the dark cycle, although the limited periods of access during each day are also likely to contribute to these higher levels of consumption. In the most commonly utilised format, the DID model involves providing C57BL/6J mice with only 20% (v/v) ethanol in water for 2 hours, 3 hours into the dark cycle for 3 days in a row, with access provided for 4 hours on the fourth day. The model has high face validity as it produces binge-like drinking with short term access to the point of displaying behavioural intoxication – including impaired performance on the rotarod and balance beam test (Thiele and Navarro, 2014) – without requiring a long-history of alcohol consumption, sucrose-fading or long-term fluid or food deprivation.

As is the case with any model, the DID procedure is not without issues. The time-of-day dependency has led to criticisms it is simply capitalising on circadian factors that influence ingestive behaviour, although as discussed above, circadian factors also appear to play a role in increases in alcohol consumption in humans. The lack of choice, with only one bottle being provided, has also been criticised, as has the strain dependency, with C57BL/6J mice being the only strain reliably showing binge-like consumption in this model. That criticism is, arguably, misplaced, as it is clear in humans that genetic and environmental susceptibility contribute to the development of AUD. To put it another way, not all humans develop AUD after the same early alcohol drinking experiences, and the same should be expected of our models. In that sense, differential susceptibility based upon genetic or environmental predisposition, such as that seen with the DID paradigm, improve the validity of the model rather than diminish it. The predictive validity of the DID paradigm for pharmacotherapies further supports the model having translatability to humans with alcohol problems (Crabbe et al., 2017). Finally, given high, stable levels of consumption are usually established rapidly with the DID paradigm (providing a nice model of binge-like consumption), significant escalation is not commonly observed over time; this makes the model more useful for studying factors resulting in rapid acquisition of high levels of consumption, rather than escalation.

2.3. Intermittent access

Evidence from studies in humans suggests that intermittent exposure to ethanol, primarily binge-like consumption patterns, serves to escalate alcohol consumption over time and contributes to the risk of developing AUD. Binge drinking during adolescence and early adulthood is among the strongest predictors of developing AUD later in life; the earlier the pattern of binge-drinking commences, the greater the risk of later developing AUD (Chassin et al., 2002; Spear, 2015). For instance, a study of 21,137 individuals found binge drinking in high school was one of the strongest predictors of developing AUD by age 35 (Merline et al., 2008). Whilst studies in human populations have established a clear correlation between binge drinking and AUD, they are not able to provide evidence for binge drinking having a causal impact on propensity to develop AUD.

Animal studies help provide an understanding of the causal relationship between patterns of heavy intermittent alcohol consumption and establishment of AUD. Many studies have focused on comparing rodents provided with intermittent ethanol access (IEA) to those given continuous ethanol access (CEA). IEA more closely aligns with human patterns of alcohol consumption, in which ethanol usually completely clears from the drinker's body between drinking sessions. A recent review (Spear, 2020), identified 14 studies that directly compared IEA to CEA in rodents; in all of these studies (Crabbe et al., 2012; Hopf et al., 2010b; Hwa et al., 2011; Hwa et al., 2016; Kimbrough et al., 2017b; Melendez, 2011; Osterndorff-Kahanek et al., 2013; Pinel and Huang, 1976; Rosenwasser et al., 2013; Simms et al., 2008; Sinclair, 1979; Spoelder et al., 2015; Tomie et al., 2006; Wise, 1973) rodents given IEA showed greater escalation of consumption than those given CEA (although note that Crabbe et al., 2012 reported escalation only in one of two strains of mice tested and only under certain conditions). Escalation of consumption with IEA relative to CEA has also been reported in male non-human primates (Lindell et al., 2017), providing further support for the validity of this paradigm and the translatability of findings across species.

Importantly, the escalation of consumption in IEA rodents is accompanied by other hallmarks of AUD which suggest it is an especially useful model for studying escalation relevant to the development of AUD and alcohol dependence. A major feature of AUD is the continuation of use despite negative consequences. One way this is modelled in animals is through assessment of the effect of adulteration of alcohol with quinine on consumption. In one study male IEA rats develop quinine resistance while CEA rats did not (Hopf et al., 2010a). Moreover, the development of the quinine resistance was linked to the escalation and duration of consumption with rats displaying resistance after 3 months, but not 1.5 months, of IEA. A study by one of the authors of this review provided particularly compelling evidence that binge-like drinking could play a causal role in development of AUD, demonstrating that adolescent male rats that had previously been given IEA showed faster escalation to compulsive alcohol consumption (as measured by progressive ratio responding and consumption of quinine-adultered alcohol) in adulthood using the chronic intermittent ethanol vapour exposure paradigm (Kimbrough et al., 2017b). Similarly, intermittent ethanol exposure in male mice during adolescence resulted in greater attenuation of the response to the aversive components of ethanol consumption in adulthood than those exposed to ethanol for the same duration continuously, as evidenced by greater

attenuation of ethanol-induced conditioned taste-aversion in adulthood (Diaz-Granados and Graham, 2007). Another key indicator that escalation in IEA models is relevant to AUD comes from work demonstrating that male rats that received chronic IEA developed physical dependence and experienced withdrawal symptoms when ethanol was not available (Li et al., 2011).

2.4. Dependence, withdrawal, and negative reinforcement

It has been argued that negative reinforcement mechanisms play a key role in the escalation of alcohol consumption in dependent binge drinkers (Koob, 2013). This is supported by the observation that escalation of voluntary alcohol consumption is much greater in physically dependent rats (Buck et al., 2014; Vendruscolo and Roberts, 2014). Central to the link between physical dependence, intermittent access, and escalation of consumption, is a phenomenon referred to as “kindling”, which, in the context of alcohol withdrawal, refers to the increase in the severity of alcohol withdrawal symptoms that occurs over successive cycles of intoxication and withdrawal (Becker, 1998). Kindling has primarily been studied in the context of physical alcohol withdrawal symptoms, most notably seizures. However, kindling of physical withdrawal symptoms does not appear to greatly contribute to motivation to resume or increase alcohol consumption (Breese et al., 2005; Heilig et al., 2010). A growing body of evidence indicates kindling also occurs with the negative affective symptoms of withdrawal (for reviews see Becker, 1998; Breese et al., 2005; Heilig et al., 2010).

It appears that heightened anxiety, which is present during acute withdrawal and early abstinence, also becomes more severe over repeated cycles of intoxication and withdrawal and eventually leads to a more enduring state of heightened stress reactivity and impaired stress coping, a phenotype which is believed to play a major role in enduring relapse risk during protracted withdrawal (Koob and Le Moal, 2005). In this sense, repeated cycles of withdrawal can be thought of as a form of chronic stress, resulting in dysregulation of stress coping and socioemotional functioning. This greater intensity of negative emotional/motivational signs and symptoms during abstinence from substances of abuse has been coined hyperkatifeia (Shurman et al., 2010). Hyperkatifeia is associated with an increase in anxiety-like behaviours, stress-like behaviours, and pain-related behaviours during abstinence from alcohol and has been validated in both humans and rodents (Koob, 2021).

Negative reinforcement through alleviation of negative emotional/motivational symptoms, particularly anxiety, pain, and heightened stress reactivity, thus likely plays a crucial role in increasing motivation to consume alcohol when there is heavy intermittent exposure, especially in dependent animals. Consistent with this hypothesis, cues associated with negative affective states cause an escalation of alcohol consumption in male rats (Berger et al., 2013), and it is well established that stress, in particular social stress, can lead to escalation of alcohol consumption (which will be discussed in more detail later in this review). Hwa et al. (2016) specifically examined the link between stress, IEA and escalation of consumption in male mice. They found that a combination of social defeat stress and IEA led to the most pronounced escalation of ethanol intake. Newman et al. (2018a), extended on these findings, demonstrating that while socially stressed male mice with CEA reduced 24-

hour alcohol consumption when administered a CRF-R1 antagonist, this effect was absent in stressed mice given IEA, suggesting that IEA leads to more complex neuroadaptations in stress pathways, conferring potential resistance to certain pharmacotherapies.

2.4.1. Biological systems implicated in negative reinforcement driven escalation—Negative reinforcement mechanisms underlying escalation of ethanol consumption may involve the κ-opioid receptor (KOR; *OPRK*) system. Chronic intermittent ethanol vapour exposure sensitizes KORs, and is associated with increased ethanol intake, ethanol preference and anxiety in male mice (Rose et al., 2016). These consequences were reduced by KOR antagonists and associated with a hypodopaminergic state, including reduced dopamine release and increased dopamine uptake. Conditions that induce affective impairments, and the mechanisms that underlie these changes, may be critical for escalation of ethanol consumption as the organism learns that the drug reverses these undesirable subjective states.

The biological changes underlying the development of negative reinforcement likely lie within a broad circuitry involving CRF, particularly those lying within the extended amygdala circuitry (Fig. 2). This circuitry is integrated into mesolimbic dopamine circuits underlying ethanol reinforcement as well as corticostriatal circuits underlying behavioural choice. The role of CRF in alcohol dependence is well-established (for review see Simpson et al. (2020) and Koob (2009)), and linked in part to the role that CRF plays in somatic and affective withdrawal symptoms (Kimbrough et al., 2017a; Schuckit, 2009). Chronic exposure to alcohol leads to down-regulation of periventricular nucleus CRF systems and the HPA axis, but increased CRF function in the extended amygdala (Logrip et al., 2013; Rivier et al., 1984), along with a network of other transcriptional changes (Contet et al., 2011). Moreover, over repeated binge drinking sessions in the DID paradigm, central amygdala CRF neurons become sensitised to ethanol effects in male and female mice (Aroni et al., 2021). At the same time that CRF function is elevated in the extended amygdala, neuroendocrine tolerance might contribute to sensitization of mesolimbic and mesocortical dopamine systems, and consequently to reduced prefrontal inhibitory control of mesolimbic reward and hypothalamic stress systems (for discussion of this theory see Blaine et al. (2016)). A further link in this cascade of events is CRF1A receptor dependent neuroimmune activation (Breese and Knapp, 2016).

One of the important points to bear in mind when considering the state of CRF systems in AUD, is that this state is not constant, as reflected in animal models of ethanol dependence and withdrawal. Thus, CRF levels in the bed nucleus of the stria terminalis are elevated during withdrawal, but normalized by ethanol consumption in male rats (Olive et al., 2002). A 24-hr period of abstinence was associated with increased numbers of Fos-reactive cells in the medial prefrontal cortex (mPFC) and the central amygdala (CeA) in male rats that had been previously exposed using chronic alcohol administration (George et al., 2012). The majority of these Fos positive cells in the CeA are also CRF positive (De Guglielmo et al., 2019). Importantly, changes in the CeA were only observed after intermittent access, and not in animals that had been given continuous access (George et al., 2012). This elevated neuronal activity was normalized by ethanol consumption, so likely represents a counter-adaptation to the higher levels of ethanol consumption during intermittent access,

the associated periods of forced abstinence, or the negative reinforcement involved in ethanol consumption after those periods of abstinence.

Importantly, these changes were found to affect the connectivity between important brain regions subserving alcohol seeking behaviour, as determined by correlations between Fos activity in corticolimbic brain regions. Abstinence after intermittent access was associated with reduced connectivity between the dorsal mPFC (dmPFC) and the CeA, the ventral mPFC (vmPFC) and the CeA, and also between the dmPFC and vmPFC. This contrasts with the strong correlation observed between these structures in rats that had been given continuous access to ethanol and did not show escalation of ethanol consumption. The activated neurons in the mPFC were shown to be GABA- and CRF-positive.

An important idea underlying this research is that coordinated activity within ensembles of neurons underlies responses to stimuli that drives drug-seeking behaviour (George and Hope, 2017). Inactivation of the neuronal ensemble comprised of Fos-positive cells in the CeA after 24h of abstinence using the Daun02 technique in non-dependent male rats with intermittent access to alcohol produced limited reductions in Fos-positive cells and only a transient (24 h) reduction in ethanol consumption (de Guglielmo et al., 2016). This result suggests that in non-dependent rats, the Fos-positive CeA neuronal ensemble activated during abstinence is labile and only partially controls alcohol drinking. To induce a greater degree of dependence, a vapour chamber approach was then used (de Guglielmo et al., 2016), along with intermittent access to operant ethanol self-administration. In this case, the reduction in the number of Fos-positive cells was greater, and the reduction in ethanol self-administration was long lasting (at least 2 weeks), suggesting that in dependent rats, the Fos-positive CeA neuronal ensemble activated during abstinence is stable and fully controls alcohol drinking. Optogenetic inhibition of CRF cells in the CeA that project to the bed nucleus of the stria terminalis (BNST) prevents recruitment of these neuronal ensembles, reduces escalation of ethanol consumption, and decreases somatic signs of withdrawal in male rats (De Guglielmo et al., 2019).

The importance of these CRF-dependent circuits are consistent with numerous studies demonstrating effects of CRF antagonists on ethanol consumption in dependent subjects, but not non-dependent subjects (Funk et al., 2007; Gilpin et al., 2008), including when injected only into the CeA (Funk and Koob, 2007). Moreover, these studies appear to dissociate the role of CRF between drinking after the establishment of dependence and in non-dependent binge-drinking (Ji et al., 2008). Of course, binge-drinking can contribute to the development of dependence and escalation of ethanol consumption due in part to its intermittent aspects, but CRF in the CeA circuit does not appear to be an important regulator of consumption until after dependence has developed. Many studies examining increases in ethanol consumption examine initial escalation in non-dependent individuals, rather than escalation to the point where the subject is physically dependent, although this is not always a line that is clearly drawn experimentally as physical dependence is not assessed in many studies. Withdrawal symptoms certainly can emerge early on in the establishment of addiction, and indeed may be an important part of the development of the dependent state. CRF sensitivity is reduced during withdrawal in BNST neurons projecting to the ventral tegmental area (VTA) (Silberman et al., 2013), while CRF-mediated potentiation of

glutamatergic VTA afferents is enhanced after a binge ethanol procedure (DID) (Sparta et al., 2013). Intra-VTA CRF antagonists reduce ethanol consumption under these conditions, consistent with other observations (Hwa et al., 2013).

Collectively, the literature discussed above suggests that several CRF-modulated neural circuits are involved in alcohol dependence, and potentially have a role in escalation of ethanol consumption. Kimbrough et al. (2020) recently confirmed much of this model using immunolabeling-enabled three-dimensional imaging of solvent cleared organs (iDISCO) in male mice. Like the previous study from this group (De Guglielmo et al., 2019), single cell Fos activation was compared between brain regions, but on a much broader scale facilitated by the use of the iDISCO technique. The primary comparisons were between dependent and non-dependent ethanol exposed animals. This study confirmed many aspects of previous findings in alcohol dependent/abstinent animals, including the importance of changes in the connectivity of the extended amygdala and midbrain striatal modules discussed above, as well as the emergence of a novel cortico-hippocampo-thalamic module (the three-stage theory, Figure 2D). Overall, there was a broad functional reorganization, whereby dependence/abstinence was characterized by larger networks of co-activation within these regions, in contrast to the smaller, more diverse networks of co-activation that characterized non-dependent individuals. Many regions became incorporated into the cortico-hippocampo-thalamic module, becoming co-activated in dependent/abstinent individuals, while this activity was negatively correlated with activity within the extended amygdala module.

2.5. Incubation of craving and the alcohol deprivation effect

Intermittent patterns of ethanol consumption are thought to be at least partly driven by negative reinforcement from alleviation of acute withdrawal induced anxiety and dysphoria. However, whilst relapse risk is usually highest during acute withdrawal, relapse does occur in individuals who have remained abstinent beyond the acute withdrawal period (Kirshenbaum et al., 2009). The discussion above shows that initial escalation appears to occur in non-dependent individuals, but then further changes are necessary for the transition to alcohol dependence. As individuals who relapse beyond one week of entering sobriety are no longer experiencing acute withdrawal symptoms, other factors present during abstinence must play a key role in relapse in these individuals. These factors may include enduring heightened stress reactivity and alterations to reward and motivational systems (for review of the relationship of this protracted withdrawal syndrome to relapse, see Koob and Volkow (2016) and Beracochea et al. (2019)). An important factor that describes aspects of the change in drug seeking phenotypes over prolonged periods of abstinence is called incubation of craving.

Incubation of craving refers to the time-dependent increase in cue-induced craving or drug-seeking observed during abstinence. Numerous studies have shown that exposing individuals with AUD who are abstinent to alcohol-associated cues induces craving (Fox et al., 2007; Petrakis et al., 2001; Sinha and Li, 2007). Moreover, craving is a significant predictor of relapse (Bottlender and Soyka, 2004; Stohs et al., 2019). An important insight of these observations was that craving was often higher after an extended period of abstinence, beyond the period of initial withdrawal, than it was at earlier timepoints. This incubation

of craving during abstinence thus may play an important role in relapse beyond the acute withdrawal period. This extended elevation of the likelihood of relapse suggests the involvement of processes other that just alleviation of acute withdrawal symptoms. Factors influencing this long-term increase in relapse rates have been linked to cue-induced or stress-induced relapse, and thus may involve learning that occurs early in the addictive process, during intermittent periods of consumption and abstinence, occurring before more consistent drug consumption develops. Of course, learning that occurs late in the addiction process is also likely a major contributor to persistent risk of relapse in abstinent individuals, with phenomenon such as conditioned withdrawal not expected in individuals with only limited exposure to alcohol.

Bienkowski et al. (2004) were the first to demonstrate incubation of craving for alcohol. In their study, male rats stably lever pressing for 8% alcohol for 30 days (following an ~20-day training and induction protocol) underwent either 24 h, 28 days, or 56 days of forced abstinence. Rats that underwent 28 days of abstinence showed the highest levels of lever pressing under extinction conditions, whereas those that underwent 56 days of abstinence showed the greatest cue-induced reinstatement of alcohol seeking. More recently, Li et al. (2015) provided the first experimental evidence for incubation of alcohol craving in humans. They assessed cue-induced alcohol craving in adult male inpatients with alcohol use disorder following 7, 14, 20 and 60 days of abstinence. Both between and within subjects, craving was at its highest after 60 days of abstinence, consistent with previous studies suggesting relapse risk remains high around this timepoint (Kirshenbaum et al., 2009). Importantly, this incubation of cue reactivity has been replicated in male alcohol dependent patients, and shown to be reduced by treatment with naltrexone (Bach et al., 2020b).

Given this incubation of craving, it is perhaps not surprising that relapse usually involves not just the rapid resumption of alcohol consumption, but escalation to levels of consumption that are initially above those consumed prior to entering abstinence (Burish et al., 1981; Ludwig and Wikler, 1974; Ludwig et al., 1974; O'Donnell, 1984). This transient escalation of consumption upon relapse is often referred to as the alcohol deprivation effect (ADE). ADE has been most closely studied in animals and has been shown in rats, mice and non-human primates (Sinclair, 1971). However, it should be noted that not all species display an alcohol deprivation effect and there are strain dependencies within species (for a review see Vengeliene et al., 2014). In general, the ADE is less consistently observed in mice than rats (Vengeliene et al., 2014). In at least some species and strains, the magnitude of the ADE increases with repeated phases of deprivation and access (Martin-Fardon and Weiss, 2013; Vengeliene et al., 2014). This suggests repeated cycles of abstinence and relapse can also serve to escalate consumption over time, which fits with the pattern of drinking observed in some humans with alcohol use disorder (Martin-Fardon and Weiss, 2013) and may be driven by strengthening of learning through repetition of negative reinforcement and changes in negative affect, along with underlying neuroadaptations.

Withdrawal-dependent plasticity underlies drug relapse behaviour studied in reinstatement and related procedures, including incubation of drug craving (for review see Dong et al. (2017)). These mechanisms include increased cell-surface expression of AMPA glutamate receptors in nucleus accumbens (NAc) medium spiny neurons (Christian et al., 2017;

Werner et al., 2017), elevated glutamatergic synaptic activity (Conrad et al., 2008; Wolf and Tseng, 2012), increased GluA1 translation (Stefanik et al., 2018), and changes in subunit composition (Conrad et al., 2008). Similar changes occur under conditions that produce incubation of cocaine drug-seeking (Ma et al., 2014; McCutcheon et al., 2011). This process appears to be initiated by the early formation of silent synapses during withdrawal that are subsequently activated by increased cell-surface expression of AMPA receptors (Lee et al., 2013; Ma et al., 2014). Over the course of these adaptations in glutamatergic synapses there are also changes in receptor subunit expression, with increases in GluN2B expression in the first week of withdrawal followed by increased expression of GluN3 subunits 1 to 2 weeks later (Christian et al., 2017). Alterations in glutamatergic neurotransmission during abstinence/withdrawal also involve changes in expression of the glutamate transporter GLT1 (Kim et al., 2018). There are also changes in GABA receptors (Purgianto et al., 2016) that are a part of overall circuit-level changes that involve other brain regions, including the amygdala (Lu et al., 2007), ventromedial prefrontal cortex (Shin et al., 2016; Shin et al., 2018) and the strengthening of the prefrontal cortex–nucleus accumbens pathway (Luis et al., 2017). Incubation of cocaine seeking can be attenuated by mGluR2/3 agonist injection in the amygdala (Lu et al., 2007) and an mGluR1-dependent long term depression emerges in the nucleus accumbens after 35 days of withdrawal (Scheyer et al., 2018). The mechanisms of cellular change noted to occur during incubation, primarily in glutamatergic synapses also involve brain derived neurotrophic factor (Grimm et al., 2003; Schmidt et al., 2012), glial derived neurotrophic factor (Lu et al., 2009), and sensitized kinase signalling (Lu et al., 2006; Szumlinski and Shin, 2018).

The mechanisms that have been shown to be involved in incubation of cocaine-seeking behaviour are also thought to underlie incubation of drug-seeking for other drugs, although these mechanisms have not been studied as extensively for other drugs, particularly alcohol. ADE shows many similarities to incubation of drug seeking, as do other experimental procedures that utilize periods of abstinence to induce increases in ethanol consumption. Despite having been studied for much longer, only limited studies have addressed the underlying mechanisms of ADE. Glutamate has been shown to be important in ADE in ways that are very similar to incubation, but evidence is primarily based upon pharmacological approaches. NMDA receptor antagonist treatments reduce ADE-induced ethanol consumption (Holter et al., 1996, 2000; Vengeliene et al., 2005), as does an mGluR5 antagonist (Backstrom et al., 2004), and the AMPA antagonist GYKI 52466 (Sanchis-Segura et al., 2006). Other treatments that modify NMDA receptor activity also reduce ADE, including glycine transporter inhibition (Vengeliene et al., 2010), and inhibition of the kynurenine-3-monooxygenase (Vengeliene et al., 2016). Lamotrigine, a Na-channel inhibitor that reduces glutamate, dopamine and serotonin activity, also reduces ADE-induced ethanol consumption (Vengeliene et al., 2007). Acamprosate also reduces c-fos activation produced by ADE (Putzke et al., 1996). Like incubation, ADE also involves glutamate activity in the PFC as evidenced by the ability of injections of glutamate or acamprosate into the PFC to reduce ADE-induced ethanol consumption (Salimov and Salimova, 1993; Spanagel et al., 1996).

The long-term consequences of over a year of repeated ADE cycles in alcohol-preferring male rats was used to study the mechanisms underlying ADE (Vengeliene et al., 2006).

Comparisons were made between rats that had undergone extensive and repeated ADE cycles, and those that had not been given alcohol; although to better identify factors specifically involved in escalation it would also have been useful to have additional control groups such as a single ADE cycle group with similar duration of alcohol exposure to the repeated cycle group, and a group given alcohol for a similar duration to the ADE groups but never deprived. Gene expression array analysis found 266 differentially expressed genes in the striatum of P rats and 140 differentially expressed genes in the striatum of HAD rats. The full gene list was not reported, unfortunately, but it is likely that there are substantial differences in the expression patterns between these strains as the majority of gene expression changes in P rats involved down-regulation, while the majority of changes in HAD rats involved upregulation. The authors focussed upon changes in dopaminergic system genes, which were substantially altered in P rats, but not HAD rats. Increased *Drd3* receptor mRNA in P rats was confirmed by quantitative reverse transcription polymerase chain reaction (qRT-PCR) in the dorsal striatum, but not the nucleus accumbens. ADE-induced ethanol consumption was also reduced by a DRD3 receptor antagonist or partial agonist. Unfortunately, that study did not report changes in glutamatergic receptors. However, a subsequent study found that long-term ethanol consumption with repeated ADE cycles was associated with a reduction in the ratio of NR2C to NR2A subunit expression and increased NR1 subunit expression in male rats (Raeder et al., 2008). The importance of glutamatergic and dopaminergic systems in ADE was demonstrated by selective elimination of *Grin1* (GluN1) or *Gria1* (GluA1) gene expression in dopamine transporter (DAT) or DRD1 receptor expressing neurons in male mice (Eisenhardt et al., 2015). The genetic manipulations reduced ADE, which was duplicated by administration of NMDA receptor antagonists in the NAC or VTA, effects that could be reversed by potentiation of AMPA receptors.

ADE differs substantially between rats and mice, and among mouse strains (Vengeliene et al., 2014). These data implicate a genetic basis for the propensity to develop ADE. It might be thought that ethanol-preferring strains would be more sensitive to the development of ADE; or alternatively the ADE might exist in these strains prior to the ADE procedure, a type of pre-sensitization. Male ethanol-preferring AA rats did not show ADE in one study (Sinclair and Tiihonen, 1988), although a study from a previous generation of this line showed a longer-lasting ADE effect compared to ANA and outbred rats (Sinclair, 1979). The sP line develops only a short-lasting ADE (Agabio et al., 2000), and this effect does not change with repeated cycles of access and abstinence (Serra et al., 2003). There was also a shift towards a preference for higher ethanol concentrations beginning with the first ADE cycle in that study. Although the ethanol-preferring HAD line does not show an ADE effect after a single deprivation, it does show the effect after repeated deprivations in male rats (Rodd-Henricks et al., 2000). This presents an important link to intermittent access models, which produce escalation and show gradual increases in consumption over repeated periods of consumption and abstinence. Although several studies have not found ADE in C57BL/6J or C57BL/6N mice (Camp et al., 2011; Khisti et al., 2006; Tomie et al., 2013), the observation of ADE is dependent at least in part on experimental parameters (Melendez et al., 2006). A deprivation period of 1 week resulted in ADE in male C57BL/6 mice, but a longer period resulted in reduced ethanol consumption. An additional factor influencing

the observation of ADE is the daily length of the ethanol exposure. An 18 hr availability (with 6 hr periods of no ethanol availability) resulted in ADE after a longer period of forced abstinence in male mice (Khisti et al., 2006), but ADE was not observed when initial ethanol consumption was continuous (Tomie et al., 2013).

One explanation for this lack of ADE effects in C57 strains in many studies is a ceiling effect. This is consistent with a study examining male and female disks large MAGUK scaffold protein 4 *Dlg4* (PSD95) KO mice on a C57BL/6J background (Camp et al., 2011). No ADE was observed in wildtype (WT) mice that had very high levels of initial ethanol consumption, but ADE was observed in both male and female *Dlg4* KO mice that had low initial ethanol consumption. C57BL/6NCrl mice had more moderate levels of ethanol consumption than C57BL/6J mice. C57BL/6J mice did not show ADE after single or repeated ADE cycles. However, C57BL/6NCrl mice did show an ADE effect after the initial deprivation, although the effect was modest, and tolerance developed over repeated ADE cycles rather than sensitization (e.g. escalation). However, the observation of ADE is clearly not dependent on low initial consumption levels alone. DBA/2J mice, which have low initial levels of ethanol consumption, show decreases in consumption after deprivation (Tomie et al., 2013). It may be that there must be a certain threshold level of initial ethanol consumption to see ADE or escalation, for instance consumption at least sufficient to achieve pharmacologically relevant brain levels of ethanol.

One form of the negative reinforcement hypothesis states that alcohol intake may be promoted by higher baseline pathology (such as anxiety) or greater withdrawal-induced anxiety. This hypothesis was explored in high anxiety-related behaviour (HAB) and low anxiety-related behaviour (LAB) rat lines (Henniger et al., 2002). Contrary to expectation, a substantial ADE was observed in both male and female LAB rats, but little or no ADE was observed in HAB rats. However, negative reinforcement may still play a role in ADE in other ways. It is clear that ethanol consumption in this situation is, to some extent, used to self-medicate since non-contingent administration of ethanol (IP) prior to oral access reduced ADE (Vengeliene et al., 2005). Moreover, it may be that contingency of the negative affective state is key as well as contrast to the normal state: i.e. HAB rats experience heightened anxiety all of the time, thus it may be less pronounced a change during withdrawal than for LAB rats.

Collectively, these studies are consistent with two important overall findings that come from animal models of alcohol consumption that are relevant to escalation: 1) paradigmatic differences (e.g. duration of exposure, number of exposures, length of abstinence periods, ethanol concentrations, etc.) have a great deal of influence over whether escalation of ethanol consumption occurs (see summary in Vengeliene et al. (2014)); and 2) genetic contributions to AUD, as seen in animal models, influence very specific phenotypic contributions to AUD. This last idea will be explored in more detail in a subsequent section, but it is made quite clear from a study that examined ADE and stress-induced ethanol consumption (Vengeliene et al., 2003). ADE was observed in Wistar and P rats, but not HAD or AA rats, whereas repeated swim stress increased ethanol intake in Wistar rats, but not in any of the ethanol-preferring strains. Thus, genetic factors can play a key role in determining whether or not environmental risk factors result in escalation

of ethanol consumption. It is apparent, however, that the literature still relies heavily, especially in mechanistic studies, on comparisons between exposed vs not exposed animals or between preferring and non-preferring strains, with few a notable exceptions discussed above providing extremely valuable information on factors that might contribute specifically to escalation associated with the formation of dependence and AUD.

3. Individual and environmental factors that influence escalation of ethanol consumption

In the previous section of this review we focused on how the circumstances surrounding alcohol consumption can influence escalation of alcohol consumption. In this section, we will focus on genetic, biological and socioemotional factors that influence acquisition, initial escalation and escalation of alcohol consumption. The preceding discussion identified that increases in consumption relevant to the development of dependence and AUD can be modelled by repeated periods of consumption and abstinence in rodents. However, it is also clear that escalation of consumption in intermittent access, ADE, and incubation of craving models is not universally observed. Susceptibility to these effects is influenced by genetic and environmental factors, which will be further elaborated upon in this section. Moreover, it is also apparent that the underlying psychological and biological mechanisms mediating escalation are diverse, with multiple pathways facilitating escalation of consumption, and perhaps, requiring different treatment approaches in AUD patients depending upon the underlying mechanisms that are present.

3.1. Genetic factors and differences in response to alcohol

Variation in the function of neural and physiological systems associated with alcohol effects have been consistently shown to be of importance in conferring risk for the development of AUD. This involves alterations in dopaminergic, opioidergic, serotonergic, glutamatergic and GABAergic systems, among others. Current knowledge of the genetic basis of AUD rests on the findings of many genetic approaches that will be briefly considered here, including genetic studies in rodents and humans. Most human genetic studies have compared individuals with AUD (or other alcohol-related diagnoses, such as alcohol dependence) and non-affected individuals. Given the binary nature of many of these comparisons, not all of these findings will necessarily directly relate to escalation of ethanol consumption, but rather might be involved and thus should be considered and studied in the context of escalation in future studies. Similarly, mouse genetic studies have been used to confirm the role of particular genes in phenotypes relevant to AUD but have not always examined phenotypes that would necessarily relate to escalation of ethanol consumption. Nonetheless, these studies provide a starting point for understanding the potential genetic contributions to escalation.

Genetic alterations that are relevant to AUD likely involve additive and multiplicative interactions between multiple levels of regulation: genetic, epigenetic, transcriptional and translational. The advent of genomic studies of AUD clearly have shown that alcohol dependence is associated with many genetic changes, and that this underlying genetic causality is highly polygenic and heterogeneous (Salvatore et al., 2019; Tawa et al., 2016).

Another way to look at the data from genome-wide association studies (GWAS) studies over the last 20 years is that they have been inconsistent; however, there are many reasons for this seeming inconsistency, not the least of which is that AUD is not a disorder with a singular underlying aetiology nor a singular phenotype. Not surprisingly, then, genetic effects in GWAS studies are stronger for more specific phenotypes, and different AUD phenotypes are associated with different underlying genetics. Research in the post-GWAS research era is beginning to overcome the shortcomings that have been recognized in GWAS studies of addiction for some time (for review see Hall et al. (2013) and Hall (2016)). Recent analytical advances allow the examination of multi-level omics datasets, integrating genetic, epigenetic, transcriptional and proteomic levels to identify complex networks of causality (Weighill et al., 2019), the ability to examine networks of related phenotypes (Chhetri et al., 2019), and the ability to examine complex genetic interactions (Joubert et al., 2018). These techniques will likely greatly advance our understanding of the genetics of AUD in coming years, but our present understanding is largely based upon other approaches that will be discussed here.

Efforts to characterize the genetic contribution to alcohol dependence and AUD liability began with comparisons between closely related individuals. Here it must be noted that most human genetic studies, even recent studies, have examined abuse or alcohol dependence, rather that the newer DSM-V diagnosis of AUD. Although these diagnoses are by no means synonymous, they are certainly overlapping, so here we will most often use the term AUD to broadly encompass alcohol use problems, unless otherwise noted. Estimates of the overall heritability of AUD have averaged about 50% (Verhulst et al., 2015). As summarized in an early review by Schuckit (1985), evidence of a genetic contribution to AUD includes 31% of individuals with AUD having a parent with AUD, monozygotic and dizygotic twin concordance rates of 55% and 28%, respectively, and adoption studies showing a 44% higher rate of AUD in the adopted offspring of parents with AUD.

This high (but by no means absolute) transmission of AUD traits among closely related individuals encouraged the use of genetic markers in linkage transmission studies among closely related individuals (for early reviews see Radouco-Thomas et al. (1979) and Jenkins and Thomas (1981)). However, these studies had trouble controlling for the shared environmental influences among closely related family members (Susser, 1985) and were only able to identify large areas of linkage. Candidate gene studies, primarily among unrelated individuals, thus became the dominant approach to studying the contribution of individual genes to AUD prior to the advent of whole genome approaches and continue to be commonly used.

Candidate gene studies have identified a few large genetic contributions to AUD risk from individual gene variants, however, these are limited to particular circumstances and the majority of gene variants have much smaller effect sizes (for review see Edenberg et al. (2019) and (Sanchez-Roige et al., 2020)). The largest gene effects contributing to AUD involve variation within genes for the alcohol metabolizing enzymes alcohol dehydrogenase (ADH) and aldehyde dehydrogenase (ALDH). Although genetic variants are often considered in terms of predisposing individuals to AUD, in this case, based upon the “flushing” response associated with aldehyde accumulation, some alleles can be thought of

as protective against AUD, particularly alleles in the ADH1B and ALDH2 genes ((Couzigou et al., 1994; Higuchi, 1994), and for review see Edenberg et al. (2019)).

Aside from these limited examples of large individual gene effects in AUD, the vast majority of genetic contributions to susceptibility to AUD, and other substance use disorders, are rather small and heterogeneous (for a discussion, see Hall et al. (2013) and Hall (2016)). This creates a problem for both candidate gene studies and GWAS as small effects, particularly if there are numerous different allelic variants within the same gene that may produce similar outcomes, are difficult to detect. Genes identified in candidate gene studies have also largely failed to be identified in GWAS (Olfson and Bierut, 2012) and the degree of replication of candidate genes is often less than is thought (Hall et al., 2013), especially when publication bias for positive associations is taken into account. Candidate gene studies necessitate *a priori* assumptions about the most important genes for AUD, whereas GWAS makes no such assumptions. GWAS findings have thus highlighted the importance of gene classes that have not been widely considered in the genetic variance that accounts for AUD liability.

We will not summarize all genes suggested to be associated with AUD, either based upon candidate gene studies or GWAS, and refer readers to recent reviews for a more thorough survey (Sanchez-Roige et al., 2020; Schuckit, 2018). However, a few points need to be made regarding these approaches. Clear associations in GWAS can only be seen with very large datasets (Visscher et al., 2017). However, numbers alone are insufficient. Even a study that included over 250,000 subjects only found 10 positive associations with AUD (Kranzler et al., 2019). Several of these were consistent with previous findings from candidate gene studies or GWAS, including several positive signals in ADH genes, as well as the dopamine *DRD2* receptor, but many were novel. Moreover, findings in European Americans were not completely replicated in other ethnicities, who had even fewer positive associations identified. Techniques that allow examination of multiple “omic” levels simultaneously and interactive gene effects (Joubert et al., 2019; Joubert et al., 2018; Weighill et al., 2019) will help to unravel the genetics underlying AUD susceptibility.

These types of analyses have yet to be done for AUD, but they may also allow us to address another issue – the issue of which phenotypes should be used in association analyses. AUD is arguably a poor phenotype to examine in GWAS as it is a heterogeneous diagnosis. Associations may be much stronger if sub-phenotypes, or endophenotypes, are used. For example, Kranzler et al. (2019) found partially overlapping, but substantially different, genetic associations with AUD and alcohol consumption measures. This begs the question: should more specific symptoms or traits be examined in genetic studies, rather than the broad diagnoses that have primarily been used to date? In essence, this is part of the same question that underlies efforts to reconsider psychiatric diagnoses using Research Domain Criteria (Young et al., 2017) to better recognise their heterogeneity in both aetiology and presentation.

This heterogeneity is reflected in the wide range of neural and physiological systems that have been explored for their role in AUD. Candidate gene studies have chosen genes for study based upon *a priori* considerations about susceptibility to AUD (Hall et al.,

2013) and this same logic has guided preclinical studies. However, human genetic findings present another issue for preclinical studies – if the contribution of any particular genetic variant is small, will this also mean preclinical manipulations of the same targets will have small effects? Luckily, this does not seem to be the case in many instances, but this may be because the genetic manipulations (most often homozygous gene knockouts) used in preclinical studies produce greater consequences than most genetic variants in humans, which may alter protein functioning or gene expression, but not entirely eliminate expression. This does raise a question of translational validity, but at least makes the experiments tractable.

Bearing these general caveats in mind, the following sections consider many of the systems that have been associated with AUD or alcohol-related phenotypes in human and preclinical genetic studies. Because of the nature of escalation, human studies cannot directly assess the phenomenon as they study AUD after this process occurs, but without doubt at least some of the systems identified contribute to escalation. It is important to note that although positive findings are referenced for many genes/gene variants, there are also many examples of negative findings for the same genes/gene variants. Most preclinical studies in this area also have not directly examined the factors that may contribute to escalation. Thus, these human and preclinical studies may provide some insight into mechanisms that should be examined specifically for their role in the escalation of ethanol consumption in future work.

3.1.1. Metabolic Enzymes—Polymorphisms in the alcohol metabolizing enzymes ADH and ALDH have been consistently associated with AUD ((Chen et al., 1999; Couzigou et al., 1994; Higuchi, 1994), and for review see Edenberg et al. (2019)). Moreover, variation in multiple alcohol metabolism genes combine to contribute to overall risk/protection phenotypes (Chen et al., 1999). These genes not only affect ethanol metabolism and ethanol blood levels, but also subjective effects of ethanol and long-term outcomes of ethanol intake (Agarwal and Goedde, 1992). Much of the focus in this research has been on the negative effects of ethanol, in particular the “flushing” responses resulting from accumulation of acetaldehyde (Harada et al., 1981). The presence of these negative effects is protective, with homozygosity of an *ADH1B* variant, most often identified in East Asian populations, reducing the risk of alcohol dependence by 8-fold and homozygosity of an *ALDH2* variant further enhancing this protective effect (Peng and Yin, 2009). More recent evidence has also identified this protective effect in people of European and African ancestry (Gelernter et al., 2014).

Some animal models have supported the idea that genetic variation in alcohol metabolism genes contributes to alcohol consumption phenotypes. An example of the evidence comes from studies in males and females from the selectively-bred UChA (low-consuming) and UChB (high-consuming) rat lines (Quintanilla et al., 2006). Of particular interest is the description in that paper that high-consuming lines “learn” to drink higher amounts, progressing from low levels of ethanol consumption to higher levels of consumption (i.e. escalation). This escalation may at least partly result from faster acquisition of tolerance in UChB rats and greater ability to metabolize acetaldehyde. Some other selectively-bred ethanol-preferring rat lines also show differences in ethanol metabolism that involve ADH and ALDH activity (Koivisto and Eriksson, 1994; Lodge and Lawrence, 2003a),

although such changes in metabolism are accompanied by other wide ranging changes in gene expression (Ciccocioppo et al., 2006). Among the mutations in the human ADH1B gene is a point mutation resulting in an amino acid substitution (Arg47His) that greatly increases enzyme activity and is protective against AUD (Whitfield, 1997, 2002). The rat homologue of this mutation was introduced into UChB rats and produced a similar pattern of phenotypes, including increased liver ADH activity and reduced ethanol consumption in female rats (Rivera-Meza et al., 2010).

Like UChA rats, *Aldh2* KO mice show reduced ethanol consumption and increased behavioural responses to ethanol associated with their increased relative acetaldehyde levels (Fernandez et al., 2006; Isse et al., 2005a; Isse et al., 2002; Isse et al., 2005b). Reductions in ethanol consumption in global *Aldh2* KO mice were only partially recapitulated by hepatic specific *Aldh2* KO or hepatic specific shRNA knockdown (Guillot et al., 2019). This clearly indicates that *Aldh2* expression outside of the liver contributes to the overall effects of global *Aldh2* reduction.

Adh gene mutants should also greatly affect ethanol metabolism and blood ethanol levels in mice. Notably, although the human *ADH1* gene family consists of 3 genes, *ADH1A*, *ADH1B*, and *ADH1C*, the mouse has only one *Adh1* gene. Male *Adh1* KO mice have been shown to have greatly increased blood ethanol levels and reduced metabolism after a bolus ethanol injection (Okuda et al., 2018). A follow-up study examined chronic continuous access to 10% ethanol over a one-month period (Haseba et al., 2020). Male *Adh1* KO mice had much higher blood ethanol levels despite reduced consumption. Moreover, after two weeks of ethanol exposure these mice began dying, with 100% mortality observed by the end of the 8-week period.

As previous discussion in this review has shown, it is likely that escalation requires the attainment of sufficient blood ethanol levels, and consequent behavioural effects, in order to produce counter-adaptations, tolerance, withdrawal and negative reinforcement. It seems likely that differences in the activity of alcohol metabolism genes would contribute to this process, but their specific contribution to escalation of ethanol consumption remains poorly defined.

3.1.2. Dopaminergic systems—Dopamine (DA) is involved in the rewarding and reinforcing effects of ethanol and variation in dopaminergic genes have been thought to modulate these effects, thereby contributing to AUD. Polymorphisms in genes such as *SLC6A3*, which encodes the dopamine transporter (DAT) protein, have been implicated in the propensity to develop alcohol dependence. A9 carriers of the 40-basepair variable number of tandem repeats (VNTR) polymorphism (rs28363170) were found to have higher synaptic dopamine levels than A10 homozygotes (Fuke et al., 2001), and this polymorphism has been associated with severe alcohol dependence (Du et al., 2011; Köhnke et al., 2005). Consistent with the polygenic nature of the disease, *SLC6A3* A10 homozygotes who are also μ-opioid receptor gene (*OPRM1*) G-allele carriers report steeper increases in the effect of alcohol dosage on stimulation and positive mood (Ray et al., 2014). Therefore, variation in this dopamine transporter gene, in combination with others, influences responses to alcohol and AUD liability.

In addition to *SLC6A3* variants, polymorphisms of *DRD2* have also been associated with alcohol abuse liability. For instance, an E8 SNP in the 3' untranslated region of *DRD2* and the A/A genotype at this locus have been associated with increased daily alcohol intake and reduced DRD2 function (Finckh et al.; Lucht et al., 2001). Again exemplifying the polygenic nature of AUD, haplotypes composed of both *DRD2* and *ANKK1* polymorphisms may pre-dispose alcohol dependent individuals to greater incidence of delirium and seizures during withdrawal (Kucharska-Mazur et al., 2012). Collectively, these polymorphisms may affect abuse liability and escalation, especially considering the role that withdrawal symptom severity can play in the escalation of ethanol consumption through negative reinforcement, discussed earlier in this review.

Studies using DAT (*Slc6a3*) KO mice have provided some information about the potential role of the DAT gene in alcohol dependence, but the results have been somewhat contradictory. A two-bottle choice paradigm that presented increasing concentrations of ethanol (0%, 3%, 6%, 10%, and 15%) showed no difference in ethanol preference or consumption between female heterozygous DAT KO mice and wildtype (WT) mice, but female homozygous DAT KO mice had reduced consumption and preference (Savelieva et al., 2002). In contrast, Hall et al. (2003) found that heterozygous and male homozygous DAT KO mice had greater preference and consumption of ethanol at higher concentrations. Morice et al. (2010) found that DAT KO mice show increased behavioural sensitization to the locomotor stimulant effects of ethanol. Together, these studies indicate that altered DAT expression may affect consumption and other behavioural effects of ethanol, although the results are not entirely consistent. This may indicate that there are additional mediating factors affecting the role of DAT in ethanol consumption and AUD-related phenotypes. The different methods of assessing ethanol consumption, including duration of exposure and ethanol concentration, could explain some differences between these studies of ethanol consumption in DAT KO mice. It is interesting to note that these are also key factors regulating escalation of ethanol consumption.

Dopamine D2 receptors (DRD2) have long (DRD2L) and short (DRD2S) isoforms that are thought to influence motivation and reinforcement for many drugs of abuse. Both male and female DRD2L KO mice have been shown to drink significantly more ethanol in a 4-day DID paradigm, but were less active, leading to the conclusion that the overrepresentation of DRD2S, relative to DRD2L, in DRD2L KO mice contributes to increases in ethanol intake (Bulwa et al., 2011). Stress, specifically chronic mild stress (CMS), significantly increases ethanol intake and preference in male DRD2 −/− and DRD2 +/− mice (Delis et al., 2013). Moreover, ethanol was shown to reverse CMS-induced immobility during a forced swim test in DRD2 +/− mice, but not DRD2 −/− or WT mice. Furthermore, a study measuring receptor levels in the basal forebrain of DRD2 KO mice found that D2 receptor levels were higher in the lateral and medial striatum of WT mice after CMS in mice previously exposed to ethanol than in non-stressed controls that had also been exposed to ethanol. This indicates that chronic exposure to ethanol can prime individuals to changes triggered by other events. Importantly, DRD2 levels were negatively correlated with ethanol intake in male WT mice (Delis et al., 2015), suggesting that the ability to up-regulate DRD2 expression may be adaptive.

3.1.3. Opioidergic systems—Additional potential genetic modulators of the response to alcohol include opioidergic genes such as *OPRM1*, which encodes the μ opioid receptor. For instance, some studies have associated a SNP (A118G, rs1799971) in *OPRM1* with increased susceptibility to alcohol dependence (Bart et al., 2005; Town et al., 1999), although a meta-analysis found no effect of this SNP on risk of alcohol or other substance dependence (Arias et al., 2006). Nonetheless, G-allele carriers were reported to have significantly greater alcohol-induced stimulation, vigour, and positive mood than A-allele homozygotes, supporting the idea that these individuals display greater sensitivity to the hedonic effects of alcohol (Ray et al., 2014; Ray and Hutchison, 2004; Ray et al., 2010), which could be especially relevant for acquisition and initial escalation of consumption. More recent evidence has not shown a link between the A118G SNP and the subjective response to intravenous alcohol, although G-allele carriers made significantly more alcohol requests than A-allele homozygotes when allowed to self-administer alcohol (Hendershot et al., 2016).

Several mouse genetic models have been used to study the role of opioidergic systems in AUD. *Oprm* KO mice on a 129/Sv x C57BL/6J background have been consistently shown to have decreased ethanol consumption using a number of ethanol consumption procedures (Becker et al., 2002; Hall et al., 2001; Roberts et al., 2000), although effects on two-bottle continuous access ethanol preference are also dependent on genetic background and sex (Hall et al., 2001). *Oprm* KO also affects ethanol induced dopamine release, which is also dependent on the genetic background of the mice (Job et al., 2007; Ramachandra et al., 2011), suggesting that genetic differences, whether resulting from direct manipulation or genetic background, are highly interactive. Another study found that ethanol consumption was altered in *Oprm* KO mice, but in a manner that was dependent on early rearing experience and sex (Moriya et al., 2015). Ethanol consumption was increased in male isolation-reared *Oprm* KO mice, but this effect was the opposite in female mice, in which socially-reared KO mice had increased ethanol consumption. It is clear from these studies that effects of *Oprm* deletion on ethanol consumption are not always observed, and tend to interact substantially with other factors, either characteristics of the subjects, characteristics of the ethanol exposure, or other environmental factors.

Oprm KO mice have not been explicitly studied under conditions that are likely to show escalation of ethanol consumption. The Moriya et al. (2015) study, like others that have utilized an ascending presentation of ethanol concentrations under continuous access conditions, do not generally show any type of escalation, although this might also be obscured by the procedure. Increases in consumption are observed from low to high doses, which is assumed to result from changing concentrations, but this is also confounded with the length of overall access to ethanol. It will be interesting to determine if escalation is affected in these mice. Some observations suggest that this may be the case. LaBuda et al. (2000) found that *Oprm* KO mice have blunted anxiolytic responses to ethanol and exhibit withdrawal symptoms earlier than WT mice after deprivation, while another study found greater anxiety-like responses during ethanol withdrawal (Ghozland et al., 2005). During an intermittent access procedure, such effects might promote increases in consumption over time.

Based on the findings discussed above, genetic alterations affecting levels of the primary endogenous ligand for *Oprm*, β-endorphin, should also be expected to affect ethanol consumption. Homozygous male and female β-endorphin KO mice on a C57BL/6J background showed increased consumption at a low ethanol concentration of 7% (Grisel et al., 1999), with greater intake occurring during a daily 2-hour test and after 2 days of alcohol deprivation in comparison to controls, but there was no difference during 28 days of two-bottle continuous access (Grahame et al., 2000). Although escalation of ethanol consumption was not explicitly studied, this modest ADE suggests that greater effects might be seen with longer or repeated periods of deprivation. Curiously, heterozygous β-endorphin KO mice showed increased drinking at all concentrations in a two-bottle continuous access preference test. This clearly shows that not only are there genetic contributions to ethanol drinking under different circumstances, but also that different degrees of alteration of the same genes can have different effects. Whether this is a result of differential receptor reserve, as has been suggested for some other effects of opioids in *Oprm* KO mice (Sora et al., 2001), or a matter of different degrees of compensatory changes, is uncertain. However, these types of functional and adaptive complexities are likely to play a role in the consequences of allelic variation on responses to alcohol and the development of AUD in humans.

Thus far, manipulations of dynorphin signalling appear to have inconsistent effects on ethanol consumption. Male and female mice lacking the dynorphin receptor (κ-opioid, *Oprk*) had reduced ethanol intake in a two-bottle continuous access procedure (Kovacs et al., 2005). In contrast, one study reported increased ethanol consumption in male prodynorphin (*Pdyn*) KO mice (Femenia and Manzanares, 2012), but the increase was accompanied by compensatory changes in both dopaminergic and opioidergic systems that might account for these effects. The problem of compensatory alterations has been a significant issue for studies in genetically modified mice, particularly homozygous gene KO mice. In any case, another study found that ethanol consumption and preference were reduced in female, but not male, *Pdyn* KO mice (Blednov et al., 2006), adding further to this inconsistent picture. An additional factor influencing the observation of effects in genetically modified mice is genetic background (the collective genetic variants against which the gene of interest is studied), which differed in these studies. Genetic background is certainly also an issue in human genetic studies, and gene-gene interactions based on differences in genetic background likely account for some of the apparent inconsistencies of genetic effects in humans as well as in mouse models.

3.1.4. Serotonergic systems—Genetic variation in serotonergic system genes have been widely implicated in AUD, either through their effects on responses to ethanol (Matsushita and Higuchi, 2014) or through their effects on traits that may contribute to AUD (Oreland et al., 2018). However, although candidate gene studies have suggested a role for serotonergic genes in AUD, GWAS have generally failed to confirm these findings. Well-established genetic polymorphisms are related to the effects of tryptophan hydroxylase 1 (*TPH1*) gene variants on suicidality, impulsivity and AUD (Nielsen et al., 1994; Nielsen et al., 1998). The relationship of these gene polymorphisms to central serotonin function is uncertain since the more brain specific TPH gene, *TPH2*, does not appear to be associated

with AUD (Plemenitas et al., 2015). Nonetheless, alterations in serotonin homeostasis, often measured in terms of reduced cerebrospinal fluid levels of 5-hydroxyindoleacetic acid (5-HIAA), are related to AUD. Perhaps not surprisingly, the serotonin transporter (SERT; *SLC6A4*) has also been widely associated with AUD, although a recent meta-analysis did not support this assertion (Villalba et al., 2015). As for other genetic findings, there may be a variety of reasons for this inconsistency, including that differences in *SLC6A4* function may occur only in certain individuals with AUD, or only affect certain AUD- or alcohol-related phenotypes. For example, the *5-HTTLPR* polymorphism of *SLC6A4* yields two functionally different alleles, a long (L) and a short (S) allele, the latter of which has been associated with increased risk of alcohol-related delirium and seizures (Sander et al., 1997). In the context of escalation of ethanol consumption, greater severity of withdrawal symptoms might promote self-medication and escalation through greater negative reinforcement. Moreover, consistent with the complex nature of genetic causality in AUD, interactions between *5-HTTLPR* and *DRD2* exon 8 SNP (*DRD2 E8*, rs6276) influence the likelihood of delirium tremens during alcohol withdrawal (Karpyak et al., 2010). This is just a small number of examples of serotonergic genes associated with AUD, and it remains to be seen which of these may contribute to the escalation process during the development of AUD. Given the nature of genetic studies in humans the role of serotonergic gene polymorphisms specifically in escalation of ethanol consumption is difficult to study, although utilisation of some of the approaches for defining trajectories of addiction would allow useful insights to be gained.

Perhaps not surprisingly given the discussion above, male SERT KO mice have reduced ethanol consumption (Kelai et al., 2003), although only modestly, and this may actually result from increased ethanol sensitivity (Boyce-Rustay et al., 2006). In an operant ethanol self-administration study male SERT KO mice had lower breakpoints when tested on a progressive ratio (Lamb and Daws, 2013). Although much remains to be explored here, this suggests that greater differences may emerge in these mice under certain conditions, such as intermittent access, which remain to be examined. Deletion of the MOA A gene (*MAOA*) in male mice does not affect free-choice ethanol consumption, but does reduce ethanol-induced sleep and hypothermia (Popova et al., 2000). Such effects are sometimes referred to as “innate tolerance” and might lead to greater or more rapid escalation of ethanol consumption, although this remains to be explored in these mice.

One problem with these sorts of studies is that the extreme nature of a homozygous KO may not closely replicate the sort of variation in gene function that results from the human gene variants associated with AUD, which are generally more subtle. Thus, one approach would be to “knock-in” a human gene variant, or modification similar to such a variant. Even just examining heterozygous KO mice might provide some insight into the effects of more subtle modifications, but most studies tend to ignore the heterozygous condition. One example of this type of model is the insertion of a hypofunctioning R439H *Tph2* mutation in a knock-in mouse line, analogous to the R441H polymorphism in humans (Zhang et al., 2005) that reduces brain serotonin levels in mice by 60–80% (Sachs et al., 2014). These mice showed reduced initial ethanol-induced ataxia and tolerance, as well as increased ethanol consumption and preference. Although longer-term ethanol consumption was not examined, differences in initial responses and tolerance suggest that this polymorphism might affect at least initial escalation of ethanol consumption.

Another important regulator of 5-HT homeostasis is the serotonin 5-HT1B receptor (*Htr1b*). An initial study reported that male and female *Htr1b* KO mice had increased ethanol consumption compared to WT mice, which was associated with reduced ethanol-induced ataxia and tolerance (Crabbe et al., 1996). This is slightly surprising since *Htr1b* KO was shown to reduce the rewarding effects of ethanol (Risinger et al., 1996), although increased consumption could also be interpreted as a compensatory response for reduced rewarding effects of ethanol. In any case, a subsequent operant self-administration study, which largely maintained continuous ethanol access (e.g. 23 h/day)), did not find effects of *Htr1b* KO across most test conditions (Risinger et al., 1999). Studies also failed to find differences in ethanol consumption under continuous access conditions (Bouwknecht et al., 2000; Gorwood et al., 2002).

Although these findings would seem to suggest that *Htr1b* does not have a role in ethanol-mediated effects or phenotypes relevant to AUD, these few studies are not a sufficient test of this relationship – something that plagues this field in which the simplest situations are generally examined first, and then subsequent studies looking at more complex situations are not conducted because of the initial negative findings. In particular, no studies examining *Htr1b* KO have examined more chronic ethanol exposure, particularly under circumstances that might produce escalation of ethanol consumption in a manner that is more relevant to the development of AUD. This is true of most other studies of 5-HT receptor gene modifications in mice, including the 5-HT6 (*Htr6*) receptor KO mouse (Bonasera et al., 2006), and mice over-expressing the 5-HT3A (*Htr3a*) receptor overall (Metz et al., 2006), or selectively in the forebrain (Engel et al., 1998). Although the studies by Bonasera et al. (2006) and Metz et al. (2006) did find reduced ethanol consumption.

Genetic studies of the 5-HT1A receptor (*Htr1a*) are lacking, although a post-mortem human brain study found altered 5-HT1A receptor binding was associated with AUD in those who had committed suicide (Underwood et al., 2018). Consistent with this, and suggestive of a potential role in escalation, changes in 5-HT1A receptor expression, especially downregulation in the hippocampus, have been associated with the emergence of alcohol withdrawal-induced anxiety and anxiety during protracted withdrawal following chronic forced alcohol exposure in mice (Breese et al., 2004; Lowery-Gionta et al., 2015; Overstreet et al., 2006; Wills et al., 2009). Another study found a 5-HT1A receptor partial agonist prevented alcohol withdrawal-induced anxiety in mice undergoing chronic binge-like alcohol consumption in the DID paradigm and reversed deficits in hippocampal neurogenesis following chronic DID in male C57BL/6 mice (Belmer et al., 2018). Taken together, these findings suggest changes in 5-HT1A signalling may contribute to escalation-relevant alterations in emotional regulation linked to chronic binge-drinking and withdrawal.

3.1.5. GABAergic systems—Several behavioural effects of alcohol involve gamma-aminobutyric acid, type A receptors ($GABA_A$) (Davies, 2003; Lobo and Harris, 2008) and genetic variance in this receptor has been investigated for its effects on alcohol responses and its role in AUD. A SNP in the gene encoding the GABA α2 subunit (*GABRA2*) has been associated with alcohol dependence (Edenberg et al., 2004; Vengeliene et al., 2014). Moreover, the synonymous A-to-G substitution in exon 4 of *GABRA2* (rs279858) has been associated with differences in the subjective effects of alcohol, including greater stimulatory

and euphoric effects in carriers of the C allele (Arias et al., 2014). However, another study reported that homozygotes for the major A allele of rs279858 had greater subjective effects of ethanol (Pierucci-Lagha et al., 2005). Carriers of the minor allele of *GABRA2* SNPs rs279858, rs279844, rs279845, rs279826, rs279828, and rs279836 have also been reported to have reduced aversive effects of alcohol (Uhart et al., 2013).

Some of these gene variants may ultimately affect levels of mRNA expression, as *post mortem* hippocampal samples from people who had AUD also show lower expression of multiple $GABA_A$ and $GABA_B$ subunit genes including *GABBR1*, *GABRA2*, *GABRG1*, and *GABRG2* (Enoch et al., 2012; Zhou et al., 2011). Differential expression of GABA receptor subunits has also been reported in *post mortem* prefrontal cortex samples (Farris and Mayfield, 2014). Genes implicated in GABA synthesis and transport, including *GAD1*, *SLC6A1*, *PRAF2*, and *GPHN*, are also down-regulated in hippocampal tissue of humans with AUD (Enoch et al., 2013; Enoch et al., 2012; Zhou et al., 2011). Whether these *post mortem* observations reflect pre-existing differences, or differences that develop over the course of the disease cannot be determined from these studies. Nonetheless, although this story is certainly complex, it is clear that genetic variation in GABAergic genes has some role in AUD and responses to alcohol.

The involvement of the $GABA_A$ receptor in responses to ethanol is also seen in rodent genetic models. Increased ethanol intoxication is observed in male and female long-sleep mice and also in mice with reduced levels of protein kinase C (Harris et al., 1995). Long-sleep mice have an increased latency to regain the righting reflex following acute ethanol injections, and elevated ethanol induced Cl- uptake (Allan and Harris, 1986). Indeed, many of the top candidate genes implicated in AUD code for particular $GABA_A$ receptor subunits, including the α_2, α_6 and γ_2 subunits ((Li et al., 2014); and see review by Trudell et al. (2014)).

There are mutant mouse lines available for several $GABA_A$ receptor subunits (Boehm II et al., 2004), including null and overexpressing transgenic lines. Deletion of the α_1 subunit in male and female mice has been shown to decrease alcohol consumption in an operant self-administration task and to reduce ethanol consumption under limited access conditions (June et al., 2007). However, as noted previously, operant procedures generally involve limited access, and the sucrose-fading procedure used to initiate consumption might have obscured any differences in acquisition or initial escalation (see section 2.1 for a review of sucrose fading procedures and effects on acquisition and initial consumption). Indeed, this approach is somewhat typical of these types of procedures, particularly for operant approaches, in which observations are made only after “stabilization” of responding or intake. In conducting the experiments in this way one of the most important portions of the data may be lost, that which occurs over the course of acquisition and initial escalation, showing the transition from low levels of consumption to pharmacologically relevant levels of consumption.

Other genetically modified mouse lines have also been studied, and the results suggest potential roles for many $GABA_A$ receptor subunits in responses to ethanol. However, effects are often complex and interactive. Knockdown of α_5 $GABA_A$ receptor subunits

reduces consumption in male (Boehm II et al., 2004), but not female (Stephens et al., 2005) mice. Deletion of δ or ρ_1 subunits also decreases ethanol drinking in a two-bottle choice continuous access procedure (Blednov et al., 2014; Mihalek et al., 2001). Deletion of glutamic acid decarboxylase (GAD) increases consumption in mice on a mixed C57BL/6J x 129/SvJ (N2) background, but not in mice on a congenic C57BL/6J background (Blednov et al., 2010), as assessed in a two-bottle IEA procedure. Ethanol-insensitive α_1 knock-in mice did not differ from WT mice in ethanol consumption tested through a continuous two-bottle access procedure (Werner et al., 2006), but mice with α_2 subunit knock-in drank more in a two-bottle IEA procedure (Blednov et al., 2011). All of these effects might impact escalation of ethanol consumption, although this has not been explicitly examined for any of these genetically modified mouse lines.

3.1.6. Glutamatergic systems—Ionotropic and metabotropic glutamate receptors are important mediators of the actions of alcohol (D'Souza, 2015) and likely contribute to a broad network of genes involved in AUD (Spanagel et al., 2010). Hypotheses suggesting the fundamental importance of glutamatergic dysfunctions in AUD have existed for some time (see Dodd et al. (2000)). Evidence from many types of studies supports this view, including post-mortem transcriptomic analyses. For instance, a transcriptomic analysis of prefrontal cortex samples from people who had AUD found that the ionotropic receptor NMDA Type Subunit 2B (*GRIN2B*) and AMPA Type Subunit 1 (*GRIA1*) were hub genes in a network of transcriptomic changes (Farris and Mayfield, 2014). Moreover, *GRIN2B* expression is specifically dysregulated in the hippocampus of AUD patients (Zhou et al., 2011). Analysis of frontal cortex modules of AUD patients revealed an upregulation of genes involved in synaptic transmission at glutamatergic synapses, including *GRIN1*, dynamin (*DNM1*), syntaxin 1A (*STX1A*), synapsin 1 (*SYN1*), synaptophysin (*SYP*), and the vesicular glutamate transporter 1 (*VGLUT1, SLC7A7*) (Ponomarev et al., 2012). In another study of *post mortem* hippocampal tissue samples from AUD patients expression of *GRIN2B* (encoding GluN2B), *GRIA4* (encoding GluA4), *GRIK3* (GluR7), *GRM3* (mGluR3), and *GRIN2D* (encoding GluN2D) were upregulated (Enoch et al., 2014). These differences in gene expression no doubt reflect mechanisms that are relevant to AUD, but they do not indicate how this came to pass; whether these effects are the result of genetic differences in these individuals to begin with, the effects of some experience on gene expression, or the effect of the long-term exposure to alcohol. In the context of the present discussion, it is impossible to know how the expression of these genes changed over the course of the development of AUD.

Although changes may be occurring at multiple levels, there is some evidence for genetic differences in some of the genes mentioned above. For instance, a functional polymorphism (Ser310A1a) of the *GRIK3* gene, which encodes glutamatergic kainate receptor subunit GlurR7, is associated with delirium tremens during alcohol withdrawal (Preuss et al., 2006). A *GRIN2B* polymorphism is also associated with earlier onset of alcohol withdrawal symptoms, which may reflect an accelerated trajectory of the development of alcohol dependence (Paul et al., 2017), i.e. faster escalation. Other gene differences, such as the rs6465084 and rs1468412 polymorphisms of the *GRM3* gene may result in AUD-associated prefrontal cortical functional changes that result in executive function deficits, which might

contribute to escalation of alcohol consumption (Xia et al., 2012). Some polymorphisms in glutamatergic genes associated with AUD also interact with stressful life events (Vrettou et al., 2019), suggesting that gene-environment interactions may underlie some contributions of changes in glutamatergic systems to the development of AUD.

Several studies have used genetically modified lines of mice to examine the role of glutamate in alcohol consumption. Male mice with a genetic deletion of the metabotropic glutamate receptor 2 gene (*Grm2*) had increased consumption and preference at high concentrations of ethanol in a 2-bottle choice procedure that gradually increased ethanol concentration from 3 to 17% over 80 days (Zhou et al., 2013). As has been mentioned before, because this type of approach uses an ascending presentation of concentrations it confounds consumption at higher concentrations with the potential escalation of consumption occurring over time. The increased consumption seen in *Grm2*KO mice was seen both at higher concentrations and after a long period of ethanol consumption. This was a long version of this protocol (80 days). Another study using a similar procedure, but with fewer ethanol concentrations (3–9%), did not find differences in consumption or preference between *Grm5*KO mice and WT mice (Blednov and Harris, 2008). The length of testing was not clearly stated in that study, so it is difficult to compare these two studies. Additionally, these studies showed quite low levels of ethanol consumption overall, which may have influenced the nature of the increase, drawing into question the relevance to understanding escalation pertaining to AUD as well as the interpretation of the observed genotypic effects. This low level of consumption is likely due to the background strain of the mice being used in each case: CD1 (Zhou et al., 2013) and a mixed 129/SvJ-C57Bl/6J background (Blednov and Harris, 2008). This is an important question in evaluating the consequences of genetic manipulations on ethanol consumption overall: whether the procedure used produces escalation in control mice. Genetic background clearly affects ethanol consumption (Belknap et al., 1993), although it is less clear how it affects escalation. If escalation is dependent on initial levels of consumption, it would be clear that consequences of genetic manipulations will only be seen when they exist on particular genetic backgrounds. It remains to be seen how genetic background might interact with genetic manipulations to affect ethanol consumption or escalation, although it is clear that genetic background not only influences the observation of phenotypes, but at times can reverse the direction of effects, as shown in the seminal study by Sittig et al. (2016).

In addition to the influence of genetic background on the observation of effects after genetic manipulations, factors involved in different ethanol consumption procedures clearly are important. The Blednov and Harris (2008) study is particularly interesting in this regard, as it is one of the few genetic studies to extensively examine ethanol consumption using multiple procedures, including a 2-bottle choice continuous access procedure using ascending ethanol concentrations (3–12% EtOH), a 4-bottle continuous access procedure (0, 4, 8 and 12% EtOH), a two-bottle DID procedure (15%), a one bottle DID (15% EtOH), and a limited access procedure with fluid deprivation (5% EtOH). No differences were observed in the 2-bottle choice experiment, the one-bottle DID or the limited access procedure with fluid deprivation, and none of these procedures showed clear escalation in WT mice. In the 4-bottle continuous access procedure there was a slight reduction in ethanol consumption and preference in *Grm5* KO mice, which contrasted to the escalation observed in WT

mice. More robust escalation was observed in WT mice in the 2-bottle DID, and again this escalation was eliminated in *Grm5* KO mice. Another study found reduced consumption and preference for 5 or 10% ethanol in a 2-bottle continuous access consumption model (Bird et al., 2008) in male *Grm5* KO mice compared to WT mice. Reduced consumption coincided with observations of increased sensitivity to ethanol in conditioned place preference and loss of righting assays. Reduced initial consumption might prevent escalation, or perhaps itself reflect reduced escalation, although the conditions used here are not generally conducive to escalation.

Other glutamate receptor subunit mutants have also been studied, although less extensively than *Grm5* mutants. Male *Gria1* KO mice did not differ from controls in voluntary ethanol consumption, stress induced drinking, or ADE (Cowen et al., 2003). Similarly, male *Gria3* KO mice did not differ from WT mice in voluntary ethanol consumption or ethanol preference (2-bottle choice, continuous access), or in baseline responding in an operant self-administration task (Sanchis-Segura et al., 2006). However, while having no effect under baseline conditions, *Gria3* KO blunted consumption after ethanol deprivation (ADE) and reduced cue-induced reinstatement in the operant task. Projected over repeated experiences, these findings might lead to reduced escalation.

Taken together, this research in genetically modified mice supports the extensive evidence of an important role for glutamate in responses to ethanol and the propensity to develop AUD. Moreover, some of the findings point to differences in the expression of particular glutamate receptor subunits potentially being involved in individual differences in the escalation of ethanol consumption.

3.1.7. Stress-related genes—Many polymorphisms in stress-related genes have been associated with AUD or related phenotypes, including many in the noradrenergic system (Clarke et al., 2012; Preuss et al., 2013), particularly the high-activity COMT allele (Kauhanen et al., 2000; Nakamura et al., 2001; Sery et al., 2006; Tiihonen et al., 1999). However, the most consistently observed stress-related genes associated with AUD are in the CRF system. The role of CRF in responses to alcohol and changes in CRF in response to chronic alcohol exposure have already been discussed 2.4.1.. Given these findings, it is not surprising that variation in CRF system genes, or other stress-related systems, are associated with AUD. Variations in the CRF receptor 1 gene (*CRHR1*) are associated with several AUD-related phenotypes, including binge drinking in adolescents (Chen et al., 2010; Treutlein et al., 2006). The binge-drinking phenotype is particularly relevant to much of the literature on the effects of intermittent alcohol access discussed in this review. Moreover, given the role of CRF systems in both neural and endocrine responses to stress, and the inter-relationships between chronic stress and AUD, it is not at all surprising that there are genotype x environment interactions for CRF system genes, notably between *CRHR1* polymorphisms and trauma (Ray et al., 2013). The association of *CRHR1* polymorphisms and AUD were also confirmed by GWAS (Gelernter et al., 2019).

Genetic manipulations in mice have confirmed a functional role for many of the genes identified in human studies of AUD-related phenotypes. CRF-deficient male mice consume much more ethanol than control mice, but this is associated with reduced locomotor

stimulant and reinforcing effects of ethanol (Olive et al., 2003). As would be expected based on that result, over-expression of CRF in male mice decreases ethanol consumption (Palmer et al., 2004). Alcohol self-administration is not increased after a period of abstinence in *Crhr1* KO male mice, as it is in WT mice (Chu et al., 2007). These mice also fail to show sensitization of locomotor responses to ethanol (Pastor et al., 2008). An interesting manipulation involved selective elimination of *Crhr1* from the brain of male mice (Molander et al., 2012). Reduction in brain *Crhr1* did not affect baseline ethanol consumption, and only slightly reduced stress-induced ethanol consumption. A subsequent period of alcohol deprivation did produce an ADE, but this was not affected by the genetic manipulation. This study also examined complete *Crhr1* KO, which similarly did not affect basal consumption, nor did it affect ADE or swim stress-induced increases in ethanol consumption, but it greatly reduced consumption of ethanol after a social stressor. In a final experiment in that study, mice were allowed free access to 8% ethanol for 3 months and then exposed to 4 repeated cycles of ethanol vapour exposure (16 hrs) and withdrawal (8 hrs), followed by a return to free-access 24 hrs later. The vapour exposure was associated with a large increase in ethanol consumption, but this increase was eliminated in *Crhr1* KO mice. These studies demonstrate a clear role for *Crhr1* in stress- and repeated-withdrawal-induced escalation of ethanol consumption in mice. Moreover, they clearly demonstrate the need for detailed assessments of gene effects in ethanol consumption procedures that produce escalation and dependence.

3.1.8. Circadian clock genes—An interesting class of genes involved in AUD are circadian clock genes (Takahashi et al., 2008). From the point of view of escalation this class is interesting because part of the underlying changes that lead to escalation may involve alterations in circadian rhythms, either as a pre-existing risk factor or as something that develops over the course of disease progression. Supporting this idea, alterations in circadian period have been associated with AUD symptom severity (McCarthy et al., 2013) and several circadian clock genes have been associated with AUD or associated phenotypes (Kovanen et al., 2010; Spanagel et al., 2005).

Mice with mutations of the period genes that prevent normal circadian function ($Per1^{BRDM1}$, $Per2^{BRDM1}$ and $Per3^{BRDM1}$ mice) have been studied in multiple alcohol consumption procedures. $Per1^{BRDM1}$ mice showed no differences in baseline consumption, self-administration, reinstatement or ADE-induced increases in ethanol consumption in an initial study (Zghoul et al., 2007), but showed increased consumption after social stress (Dong et al., 2011b). Moreover, an association between heavy drinking and *hPer1* SNP rs3027172 was identified in human adolescents who had suffered psychosocial adversity (Dong et al., 2011a). It is also important to note that alcohol consumption can have significant impacts on circadian rhythms, potentially creating a vicious cycle (Ruby et al., 2009). However, a subsequent study showed that both $Per1^{BRDM1}$, $Per2^{BRDM1}$, and double mutant male and female mice have increases in ethanol consumption in a standard two-bottle test assessing consumption of ascending concentrations of ethanol, as well as slightly increased ethanol conditioned place preference (Gamsby et al., 2013). These changes were also associated with alterations in ethanol metabolism, primarily in females.

3.2. Sex Differences

There are clear sex differences in the presentation of AUD (Flores-Bonilla and Richardson, 2020), which are also observed in animal models of alcohol consumption and alcohol dependence (Bell et al., 2017). Moreover, factors related to sex influence different stages of the addiction process, including acquisition, initial escalation and escalation associated with dependence (Carroll et al., 2004). In many sections throughout this review we have highlighted sex differences, or lack of sex differences, when both males and females have been studied, but in this section we specifically focus on sex as a biological variable in relation to changes in ethanol consumption.

Female rodents are very well known to consume more ethanol than males (see summary table of this literature in Priddy et al. (2017)), but generally the ethanol consumption models that have shown this relate to early stages of the addiction process, and may primarily involve initial differences in the pharmacological responses to ethanol (Blanchard et al., 1993). It has been proposed that males are more driven by positive reinforcement from ethanol and females more by negative reinforcement from ethanol, and that these differences in motivation might contribute to differences in consumption at different stages of the alcohol addiction cycle and in different models (Varlinskaya et al., 2015b). For instance, this difference would impact observations of ethanol consumption under “basal” conditions in males and females since almost all methods involve social isolation, which may tend to potentiate consumption in females but not males. With regard to changes over time, some studies report accelerated or greater escalation of ethanol consumption in females under some intermittent access conditions (Varlinskaya et al., 2015a). However, another study found that males showed escalation of ethanol consumption during an intermittent access procedure, while females did not, and that consumption was increased further in males, but not females, after a period of vapour ethanol exposure (Morales et al., 2015). It is uncertain to what extent this lack of escalation in females was a result of their initially higher levels of ethanol consumption.

This type of pattern, in which males initially consume lower amounts of ethanol, but when tested for an extended period reach levels equal to females, is often observed. For example, Moore and Lynch (2015) showed escalation in males under continuous access 3-bottle choice conditions. Moreover, ethanol preference increased even more than consumption over this period, eventually reaching levels higher than females. A two-bottle choice procedure with 8% ethanol showed much more continuous escalation over the entire period (90 days); again, only in males. When tested in an operant (limited access) procedure, both males and females showed initial escalation over the first two weeks, and then consumption was maintained at the same level for the rest of the experiment. These effects during and soon after acquisition are clearly relevant to acquisition and initial escalation, but it is not clear whether they are relevant to escalation associated with the formation of dependence and AUD. Moreover, they are dependent on initial levels of consumption, and as such the sex differences that are seen may have more to do with those initial levels of consumption than in differences in the process of escalation itself.

Initial levels of consumption are not the only determinant of escalation. Hwa et al. (2011) used a method in which the ethanol concentration was gradually increased over the first

week of exposure from 3 to 20%, and thereafter kept at 20%. These authors compared continuous access and intermittent access, with intermittent access achieving much higher levels of ethanol consumption, although consumption stabilized once the concentration was maintained at 20% and did not increase further. Of relevance to the topic of sex differences, although males and females consumed similar amounts initially during IA, females had faster initial escalation and stabilized at a higher level of consumption. Given that the study confounded time and concentration preference it is difficult to say whether this just represents greater consumption at the higher concentrations in females or greater initial escalation.

Much work remains before it can be determined whether there are sex differences specifically in the escalation of ethanol consumption, especially to levels most relevant to AUD, and what factors determine such differences.

3.3. Socioemotional factors

3.3.1. Social isolation and social support—Studies in human populations have long pointed to social isolation and subsequent loneliness being important factors contributing to the establishment and maintenance of problematic alcohol use (for a review of early work in this area, see Åkerlind and Hörnquist, 1992). A study of elderly Germans found that living alone was associated with increased alcohol use (Du et al., 2008) and another study found that loneliness was a significant predictor of problem drinking in middle to late life (Kuerbis et al., 2018). This association does not appear to be restricted to older individuals, with a systematic review exploring alcohol use among adolescents in Brazil finding loneliness was associated with increased risk for heavy alcohol use (Barbosa Filho et al., 2012). Moreover, systematic reviews examining the impact of social isolation and loneliness on health also identified excessive alcohol consumption as a potential consequence (Leigh-Hunt et al., 2017). These findings linking loneliness to increased alcohol consumption have been further supported by emerging data from the COVID-19 pandemic. One study found that adults in the USA who felt, on average, lonelier during the COVID-19 restrictions of the 2020 northern hemisphere summer consumed significantly more alcohol each day (Bragard et al., 2021). Another study found 65% of participants reported increased loneliness during the pandemic and 58% of these participants reported increased drinking, with change in loneliness and change in consumption significantly correlated (Horigian et al., 2021). However, analysis of data collected in 2004–05 from over 30,000 subjects in the National Epidemiologic Survey on Alcohol and Related Conditions highlights the complex nature of the interaction between social isolation and alcohol consumption. Unexpectedly, the study found that less frequent contact with close friends was associated with *reduced* risk of AUD. In contrast, less frequent contact with members of their religious group within their social network was associated with *increased* risk of AUD. The authors argue that the nature of the relationship is likely a key determinant of the outcome, as individuals with already established AUD tend to have fairly large social networks, with over half of those also having AUD (Chou et al., 2011). Therefore, increased contact with those friends may increase opportunities for drinking. A critical emergent factor across these studies is perhaps the lack of a positive social support network increases risk of developing an AUD.

These human studies have emphasized the relationship between current social circumstances and problem drinking, yet problem drinking is most likely to develop as a result of longer-term issues, often beginning before adulthood. Problem drinking most often has its roots in adolescence, and adolescent social isolation has most often been associated with increased risk of AUD (for review see (Butler et al., 2016; Spear, 2015)). Social isolation at different ages has been extensively studied in rodents.

Social isolation induces many phenotypes that are relevant to AUD, and the specific phenotype is highly dependent on age and the nature and duration of the experience (Hall, 1998). Although often discussed in terms of “social stress”, social isolation early in life is best considered as the interruption of a developmental epigenetic program that guides neural and physiological changes in response to social experience, with the “isolation phenotype” being a pre-programmed alternative to the “social phenotype” (for a discussion of this view of isolation see Hall and Perona (2012)).

In contrast to isolation in adulthood, social isolation beginning in adolescence in rodents (often termed isolation rearing) has been consistently found to increase ethanol consumption (Deehan et al., 2007; Deehan et al., 2011; Hall et al., 1998b; Lopez et al., 2011; McCool and Chappell, 2009; Schenk et al., 1990; Wolffgramm, 1990), and persists even if the isolation is limited to adolescence (Lesscher et al., 2015). In adult rodents, isolation usually has little effect on alcohol consumption (Andreas et al., 1985; Schenk et al., 1990), and under some circumstances actually reduces consumption (Doremus et al., 2005). Increased ethanol consumption after chronic adolescent social isolation, usually assessed in adulthood, is associated with increased reward sensitivity and anxiety, which are potential mediators. For example, isolation-rearing increased ethanol preference in ethanol preferring male fawn hooded rats (Hall et al., 1998b; Lodge and Lawrence, 2003c), which was associated with anxiety (Djouma et al., 2006; Hall et al., 1998a). Consistent with this role for isolation-induced increases in anxiety driving ethanol consumption in these rats, both diazepam and the CRFR1 Antagonist CP-154,526 reduced ethanol consumption in isolation-reared fawn hooded rats (Lodge and Lawrence, 2003b).

Several factors may affect how rodents respond to social isolation, and these overlap with factors influencing susceptibility and resilience to the development of AUD in humans. As previously noted, factors may contribute differently to alcohol consumption and dependence in males and females, which may interact with aspects of experimental procedures used to examine ethanol consumption in animal models. Isolation-rearing increased ethanol consumption in males but not females in one study (Lopez et al., 2011). Interestingly, in the same study, isolation of adult animals increased ethanol consumption in females but not males. In this study, as in most of the studies mentioned previously, ethanol consumption was measured after a period of chronic social or isolation housing by subsequently isolating both groups. It is important to note that allowing animals to consume alcohol in social circumstances affects ethanol consumption, particularly in adolescent mice. Thus, adolescent male mice, but not females or adult male mice, were found to drink more when consumption occurred in the presence of conspecifics (Logue et al., 2014). As is clear from some of the studies discussed in a previous section, genetic predisposition also clearly affects the outcome of social deprivation. For instance, one study found increased ethanol consumption

after isolation rearing only in alcohol-preferring P rats (Ehlers et al., 2007). Another study found that ethanol consumption was altered in *Oprm* KO mice in a manner dependent on isolation rearing and sex (Moriya et al., 2015). Ethanol consumption was increased in isolation reared male *Oprm* KO mice, but decreased in isolation reared female KO mice.

The ethanol consumption studies mentioned above primarily used continuous access paradigms, assessed initial phases of the acquisition of ethanol consumption, and did not examine conditions that tend to produce escalation of ethanol consumption relevant to the formation of dependence. One study did examine a 2-bottle choice DID procedure over an extended period of time with alternating 2-day periods of forced abstinence and 5-day periods of DID access (Holgate et al., 2017). This study utilized the IntelliCage apparatus which uses subcutaneous transponders to individually identify socially housed mice so that consumption can be assessed without short-term isolation for measurement of ethanol consumption. Socially isolated male mice had much greater initial ethanol preference than social housed mice, with testing beginning in late adolescence (6 weeks of age). Moreover, physical environmental enrichment, in addition to social enrichment, further reduced initial ethanol preference. Isolated mice showed a slight increase in ethanol preference over the four weeks, whereas preference mice in the enriched environment decreased their preference. Of most relevance to escalation, when mice consuming ethanol in social housing were moved to isolated housing they showed a rapid and drastic increase in ethanol preference, consistent with the human literature suggesting that social isolation can be a trigger for substantial increases in consumption. In contrast, mice moved from isolated to enriched housing rapidly and drastically reduced their ethanol preference. These findings clearly highlight a potentially significant interaction between social experience/ circumstances and alcohol consumption, and future work would might examine whether the switch from social to isolated housing is sufficient to escalate established consumption to levels that induce dependence and other features relevant to AUD.

As some of the studies discussed above suggest, the impact of social isolation on alcohol consumption extends beyond possibly facilitating escalation of consumption to hindering cessation of problematic use. Here, social support, or more precisely, a lack thereof, appears critically important. Greater social support is consistently found to be a predictor of recovery from excessive alcohol consumption and protective against escalation of consumption (Fuehrlein et al., 2018; Weitzman and Chen, 2005). Moreover, numerous studies point to an individual's level of engagement in the positive social support network provided by programs like Alcoholics Anonymous as the most important predictor of a successful treatment outcome (Bond et al., 2003; Groh et al., 2008; Kaskutas et al., 2002; Longabaugh et al., 1998; Nealon-Woods et al., 1995; Timko et al., 2015). It is important to note here that heavy alcohol consumption can alienate positive social supports and mounting evidence points to disruption of social motivation and capacity for normal social interactions with chronic heavy alcohol use (Moos et al., 2010; Trezza et al., 2014; Zou et al., 2009).

In light of this observation, there is growing interest in how drugs that act to enhance social motivation and facilitate functional social interactions might play an important role in treating AUD (Baskerville and Douglas, 2010; Bowen et al., 2016; Bowen and Neumann, 2017, 2018; McGregor and Bowen, 2012; McGregor and Bowen, 2013). The brain oxytocin

system has received considerable interest in this regard. Among other things, oxytocin plays a critical role in the regulation of social behaviours, anxiety, and stress and fear responses (Jurek and Neumann, 2018). Of specific relevance here, *OXT* genotype appears to moderate the effect of social support on psychological distress in patients with AUD (Love et al., 2018) and chemogenetic activation of hypothalamic OXT neurons was sufficient to inhibit binge-like alcohol consumption in male mice in the DID model (King et al., 2021). Importantly, both clinical and preclinical studies highlight that targeting the brain oxytocin system pharmacologically has potential to prevent the escalation of alcohol consumption, reduce established alcohol consumption, inhibit relapse and interfere with effects of ethanol on neurotransmitter systems involved in ethanol reward and intoxication (Bach et al., 2020a; Bach et al., 2019; Betka et al., 2018; Bowen et al., 2011; Bowen et al., 2015; Dannenhoffer et al., 2018; Hansson et al., 2018; King and Becker, 2019; King et al., 2017; MacFadyen et al., 2016; McGregor and Bowen, 2012; Mitchell et al., 2016; Pedersen et al., 2012; Peters et al., 2013; Peters et al., 2017; Stevenson et al., 2017; Tunstall et al., 2019; Walcott and Ryabinin, 2020). One study that is particularly relevant to the effects of OXT on escalation (Bowen et al., 2011) found that male rats sub-chronically pre-treated with OXT showed reduced initial escalation of ethanol consumption over the course of a 25 day continuous access two bottle paradigm. Moreover, the pre-treatment with OXT was associated with a phenotype characterised by reduced anxiety-like behaviour and increased social interaction.

3.3.2. Social anxiety—There is a clear association between alcohol consumption and social anxiety. As many as 10% of those with AUD have comorbid social anxiety disorder (Gabriels et al., 2019) and those with social anxiety disorder are up to 4.5 times more likely to have AUD (Buckner et al., 2008). Some studies suggest that use of alcohol to cope with social anxiety may contribute to the establishment of problematic alcohol use. For instance, coping with social anxiety symptoms has been identified as a major motivation for alcohol consumption, especially among adolescents and young adults (Caruso et al., 2018; Simons et al., 2017). Further supporting a role for social anxiety in escalation of alcohol consumption, social anxiety disorder occurs first in 80% of comorbid cases of social anxiety disorder and AUD (Schneier et al 2010). A recent human laboratory study found that participants without AUD reported increased craving for alcohol after performing the Trier Social Stress Test (Clay et al., 2018). However, another study found this effect was absent in participants with AUD (Bacon and Thomas, 2013). Together, these studies suggest that acute social anxiety might play a role in driving initial escalation of alcohol consumption, and perhaps establishment of AUD, but that once an individual has AUD it has less influence on consumption.

Due to the challenges in identifying appropriate placebos for alcohol studies in humans, it is difficult to ascertain whether alcohol has any real impact on social anxiety through its pharmacological actions, or whether people merely have an expectancy bias due to commonly held beliefs about alcohol's social lubricant effects (Raymond et al., 2019). In animal studies, the effects of alcohol on social behaviour vary, with the observation of social inhibition or social facilitation depending on a variety of factors, including sex, age and context (Raymond et al., 2019; Varlinskaya and Spear, 2004, 2006). Although these studies provide insight into ethanol interactions with social anxiety-like behaviour, the

stress-paradigms used to induce social fear can also induce non-social-specific changes in behaviour, confounding interpretations.

To address this, Raymond et al. (2019) used a social fear conditioning paradigm that models the acute social avoidance and social fear aspects of social anxiety disorder, while addressing the issue of non-specific effects found in other models (see Toth et al., 2012 for more information on the social fear conditioning paradigm). Raymond et al. (2019) found that alcohol reduced social avoidance in socially fear conditioned adolescent male mice (Figure 3), but only at a low dose. In contrast, the low dose of ethanol had no effect in adult mice and a high dose inhibited social interaction irrespective of social fear status. The results were social specific as they were not observed when mice were conditioned to a non-social stimulus using the same procedure. The reduction of social avoidance only in the adolescent mice aligns with the human literature discussed above, suggesting adolescence is a period during which alleviation of social anxiety symptoms is an important driver of alcohol consumption.

In subsequent work, the same group showed that socially fear conditioned male mice maintain binge-level alcohol consumption in the DID paradigm when a social stimulus is placed into their cage during a drinking session, whereas unconditioned mice reduce their consumption to non-intoxicating levels (Figure 3). Conditioned social fear thus appears to eliminate social buffering of alcohol consumption in this model.

Taken together, these findings exploring the relationship between social anxiety and alcohol consumption in both humans and animal models suggest alcohol might initially serve to alleviate social anxiety symptoms, which might facilitate the establishment of problematic patterns of alcohol use through negative reinforcement. Moreover, under normal conditions, social interactions may serve to inhibit excessive alcohol consumption, which is consistent with some of the literature exploring the effects of social isolation on escalation of alcohol consumption, discussed above. However, more work needs to be conducted in models of consumption that capture escalation associated with the formation of dependence and AUD.

3.3.3. Trauma—Trauma is consistently linked with increased risk of developing AUD. A comorbidity study of 5,877 individuals found that those with post-traumatic stress disorder (PTSD) were twice as likely to have AUD as those without (Kessler, 1995). A systematic review examining comorbidity between AUD and PTSD found 9.8 to 61.3% of those with PTSD misused alcohol, with odds ratios as high as 4.87 (Debell et al., 2014). A recent review of the epidemiological data noted the consistency of the comorbidity between AUD and PTSD across different populations and over time (Smith and Cottler, 2018). Of interest, not only do those with PTSD have an increased likelihood of developing AUD, possibly to self-medicate, those with AUD also appear to have an increased likelihood of developing PSTD, perhaps through increased likelihood of being exposed to traumatic situations (Debell et al., 2014; Smith and Cottler, 2018).

Social defeat stress is commonly used in animal models to explore the impact of social trauma and stress on alcohol consumption. Studies in several rodent species and non-human primates report long-lasting elevations in voluntary alcohol consumption following exposure

to chronic, continuous social defeat stress (for a review see Newman et al., 2018b). In these models, the impact of social defeat stress on alcohol consumption appears independent of baseline levels of consumption prior to stress exposure. Other studies use models which involve exposing subjects to short periods of social defeat stress intermittently over a period of days or weeks. The effect of defeat stress in these models is more subtle than in models involving continuous chronic exposure, with stressed subjects initially consuming *less* alcohol, possibly due to suppression of ingestive behaviour by autonomic nervous system arousal, but then developing escalated consumption from days up to weeks after the cessation of stressor exposure (Newman et al., 2018b). It is also important to note that the relationship between alcohol and social defeat stress is bidirectional, with alcohol exposure increasing sensitivity to social defeat stress (Nelson et al., 2018; Nennig et al., 2020), which is consistent with the human literature on PTSD and AUD discussed above. Finally, recent work by Newman et al. (2021) suggests changes in CRF may be involved in effects of social stress on escalation of alcohol consumption. The study identified a population of CRF positive neurons in the anterior central medial thalamus that are active during social interactions in non-stressed but not socially stressed female mice. Optogenetic activation of these cells in stressed and non-stressed female mice inhibited abstinence-escalated drinking.

Whereas social defeat stress is used to model social trauma, non-social stressors are also used in models of trauma. Two commonly used non-social stressors are restraint stress and predator odour. Evidence for escalation of alcohol consumption in rodents following exposure to restraint stress is inconsistent (for a review see Suh and Ressler, 2018). In contrast, escalation of alcohol consumption has been more consistently demonstrated in rodents following exposure to predator odour stress, although variables that appear to mediate escalation of consumption in response to predator odour are not always consistent. Edwards et al. (2013) found increased escalation of alcohol consumption from baseline levels in passive, but not active, stress coping male Wistar rats following exposure to predator odour (bobcat urine), an effect which the group has replicated (Weera et al., 2020). It should be noted that these individual differences should be expected from a valid animal model for exploring the effect of trauma on alcohol consumption – just as not all humans who experience trauma develop AUD, we do not expect all outbred rats to escalate alcohol consumption in response to a stressor – and stress coping style is a mediator of this relationship consistent with the human literature. In contrast to Edwards et al. (2013), Ornelas et al. (2021) reported increased escalation of ethanol consumption in active, but not passive, female, but not male, Long-Evans rats following exposure to the predator odour TMT. Edwards et al. (2013) did not test females so it is possible they also would have observed this pattern in female rats. Moreover, the two studies used different protocols, different strains of rats, and different predator odours. Some research suggests that TMT is not a true predator odour, but rather an aversive smell (McGregor et al., 2002). However, another recent study did report increased alcohol consumption in male rats following exposure to TMT (Makhijani et al., 2021). In mice, predator odour exposure (dirty rat bedding) resulted in escalation of alcohol consumption in both males and females; however the stressor-induced escalation was only observed in males with prior binge-like drinking experience and only in females with low baseline levels of consumption (Finn et al., 2018).

The involvement of hyperactivity and hyperreactivity of the amygdala in the neurobiology of PTSD is well-established. However, few human studies have examined the amygdala in the context of comorbid PTSD and AUD, and well-controlled studies are even scarcer. One neuroimaging study found a combination of low reward-related activity in the ventral striatum and high reactivity to threat in the amygdala was associated with problem drinking (Nikolova et al., 2016). Supporting a causal role of the amygdala in escalation of alcohol consumption, a recent preclinical study found that escalation of alcohol consumption in passive stress-coping male rats following predator odour exposure was associated with increased fos immunoreactivity in CRF-positive cells in the central amygdala and that infusion of a CRFR1 antagonist into this region reversed escalation of alcohol consumption (Weera et al., 2020). In another study, infusion of a CRFR1 antagonist into the ventral tegmental area was able to reverse social-defeat stress-escalated alcohol consumption in male mice (Newman et al., 2018a). Several other regions and pathways have been identified as potential mediators of the link between PTSD and AUD: the medial PFC, and specifically lack of inhibition of the amygdala by the mPFC; complex changes in HPA axis function; elevated noradrenergic signalling; hippocampal dysfunction; and mesolimbic reward pathway hypoactivity. However, causal evidence for their role specifically in escalation of alcohol consumption in response to trauma is currently lacking and thus they are not discussed in detail here (for an excellent review see Gilpin and Weiner, 2017).

There is a strong link between childhood adversity, specifically, and a range of psychiatric disorders in adulthood, including AUD (for reviews of the human literature see Brady and Back, 2012; Keyes et al., 2011). Maternal separation is a commonly used technique to model early-life adversity in rodents. Maternal separation causes escalation of alcohol consumption in rodents and, not surprisingly, longer periods of maternal separation more reliably cause increases in consumption. Interestingly, several studies report that heightened alcohol consumption in maternally separated rodents only emerges when they reach adulthood. Male mice who were maternally separated showed more pronounced escalation of alcohol consumption in response to stress and a greater ADE (Portero-Tresserra et al., 2018). For an in-depth review on the impact of early life stress on susceptibility for substance use disorders with a particular focus on animal models, see Baracz et al. (2020).

One study found maternally separated male and female mice had altered gene expression in the serotonin, reward and HPA axis systems that was associated with increased alcohol consumption (De Almeida Magalhães et al., 2018). Ethanol consumption reversed heightened stress behaviour and altered gene expression in some parts of the aforementioned systems, suggesting that escalation of consumption may be driven by self-medication under some circumstances. Another study reported that ethanol exposure resulted in greater elevations of plasma corticosterone and brain monoamines in mice that had been maternally separated, with the latter effect being sex-dependent (Kawakami et al., 2013). Elevated CRF and GABA-A receptor α_2 subunit expression have been reported in stress and reward nuclei following maternal separation (Gondré-Lewis et al., 2016). Infusion of either a CRF1 receptor antagonist or negative allosteric modulator of ethanol effects at α2 subunit containing GABA-A receptors prevented maternal separation-induced binge-like ethanol consumption, suggesting mechanisms which drive escalation in other models may also be involved in heightened consumption in maternal separation models.

Several studies in rodents point to alterations in the endocannabinoid system being involved in escalated alcohol consumption following maternal separation. However, studies demonstrating a mechanistic link are lacking. Male mice that underwent maternal separation and early weening showed increased escalation of alcohol consumption in at two week DID procedure, which was associated with reduced endocannabinoid levels in the PFC, reduced endocannabinoid and monoamine levels in the striatum, and reduced sensitivity to the rewarding effects of ethanol (Portero-Tresserra et al., 2018). Another study reported an association between increased cannabinoid type 1 receptor expression in the nucleus accumbens and elevated baseline ethanol consumption as well as greater escalation of ethanol intake in male rats that underwent maternal separation relative to controls (an effect further exacerbated by isolation housing during adolescence) (Amancio-Belmont et al., 2020). More studies examining the biological basis of the link between early life adversity and alcohol consumption in clinical populations are required, especially those with a specific focus on the link between early life adversity and escalation of alcohol consumption. The preclinical literature provides some guidance, although studies establishing causal links between early life trauma and escalation of alcohol consumption are few.

4. Summary and Future Directions

Many studies, especially those examining mechanisms, focus on examining factors that influence ethanol consumption in limited access paradigms in animals that likely do not form dependence or other hallmark features of AUD. These studies, which often use sucrose fading, and/or short-term operant or free consumption procedures, have provided valuable insights into acquisition of ethanol consumption and initial escalation to pharmacologically relevant levels of consumption. However, these studies do not provide clear insight into factors that specifically contribute to escalation in latter stages, which are arguably most relevant to better understanding and treating dependence and AUD.

Dependence was identified as a particularly important factor from our review. Studies which involve IEA combined with vapour chamber ethanol exposure have been especially useful in identifying repeated cycles of withdrawal and abstinence as a likely key driver of escalation in latter stages of AUD as well as highlighting the potential involvement of numerous biological mechanisms in this process. More studies are needed that focus on comparing changes at different stages of the cycles, and how the repeated cycling itself is driving changes that differ from change resulting simply from cumulative ethanol exposure over time. Yoked control groups that receive similar ethanol exposure without the repeated cycling would be particularly useful, especially in mechanistic studies. This will facilitate identification of mechanisms involved specifically in the repeated cycling that seems to drive escalation and formation of an AUD-like phenotype.

Studies on the involvement of specific genes in escalation are challenging as they often rely on a comparison between, for example, subjects with a particular gene or gene variant, and subjects without. These studies have provided a wealth of insights, including identifying particular genes associated with differences in alcohol consumption, dependence and withdrawal. However, the constitutive gene expression in these studies makes it challenging to assess the contribution of these genes specifically to escalation and factors that contribute

to escalation. Here, preclinical studies in inducible lines, or using other techniques with appropriate temporal precision, will allow manipulation at key periods involved in escalation (e.g. during repeated cycles of withdrawal and abstinence) which could help provide a more precise exploration of the involvement of genes in escalation.

Across models, chronic IEA is consistently the most reliable means to induce escalation that is relevant to AUD. Given the importance of dependence, and the subsequent cycles of withdrawal and abstinence, in escalation during latter stages, it of considerable interest to identify what factors result in animals escalating *voluntary* consumption to levels where they form dependence and experience withdrawal. In this regard, there should be more emphasis in future studies on assessing physical dependence alongside escalation of consumption. This should be done in chronic IEA paradigms, and other models of interest, that do not involve forced induction of dependence.

Finally, studies examining sex as a biological variable influencing escalation are lacking. The majority of preclinical studies reviewed used male subjects only, some used both, a small number used female subjects only, and a small number did not specify the sex of the subjects used. The bias toward male subjects was far less prevalent amongst the human studies, although a small number of studies only included male subjects.

5. Conclusions

As is clear from the literature explored in this review, a vast array of complex, interacting factors influence alcohol consumption and the development of AUD. Some of these factors act early in the acquisition and initial escalation of ethanol consumption, while others contribute to escalation of ethanol consumption at a later stage and are involved in the development of alcohol dependence and AUD. Many of these processes can now be modelled in animals, which provides a pathway for not only probing genetic and neurobiological substrates involved in these factors, but also a platform for screening novel compounds to treat AUD. From a clinical perspective, appreciation of the different factors that contribute to escalation of alcohol consumption, and maintenance of escalated consumption, could provide important insights into the most appropriate treatment approach to use for a particular individual. Unfortunately, as is clear from the discussion of biological and environmental factors that may contribute to escalation of alcohol consumption, much of this research has yet to be done. Preclinical research investigating genetic mechanisms has tended to use primarily the simplest models of ethanol consumption that produce neither escalation of ethanol consumption nor alcohol dependence. These studies have managed to capture certain factors, including the contribution of underlying co-morbidities, like anxiety, to the development of high levels of alcohol consumption. Nonetheless, they have largely failed to capture more meaningful aspects of negative reinforcement or self-treatment.

Different models capture different aspects of acquisition and escalation of alcohol consumption that can be relevant to different stages of the dependence process, as well as different demographics. For instance, sucrose fading is a relevant approach for exploring factors relevant to increases in consumption from non-pharmacologically relevant levels to pharmacologically relevant levels in those who might otherwise not consume high levels of

alcohol. In contrast, intermittent access and binge paradigms are particularly relevant for exploring the transition from social drinking to excessive alcohol consumption. Studying the impact of dependence on consumption, especially repeated cycles of withdrawal and abstinence, as well as incubation of craving and ADE, has provided insights into the transition to AUD and to more severe AUD-like phenotypes. These approaches can now be used to better understand the biological changes that occur during the process of escalation, paving the way for identifying biomarkers for escalation risk and providing the opportunity to develop more targeted therapeutics for AUD.

We have learnt much about the factors that lead to the initiation of consumption and escalation from low, non-intoxicating to intoxicating levels. In comparison, far fewer studies have been able to capture the escalation of consumption to levels that result in physical dependence and that truly model latter stage AUD. However, suitable models have now been identified and breakthroughs, especially in our understanding of genetic and biological mechanisms driving escalation, will depend on utilising these models with appropriate controls and techniques that allow factors specifically involved in escalation relevant to dependence and AUD to be explored.

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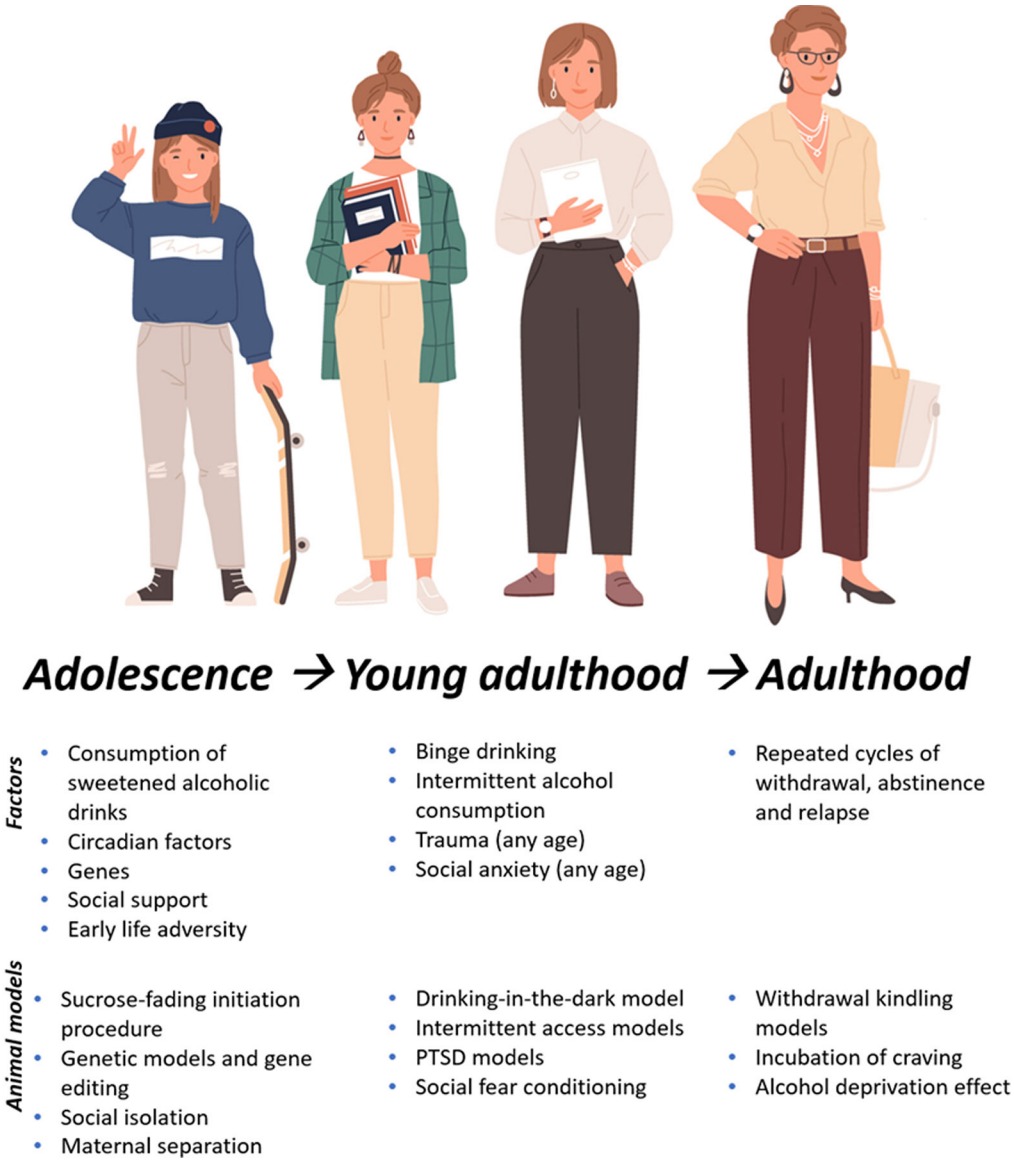

Figure 1.

Factors contributing to escalation of alcohol consumption throughout the lifespan and at different stages of drinking, and animal models.

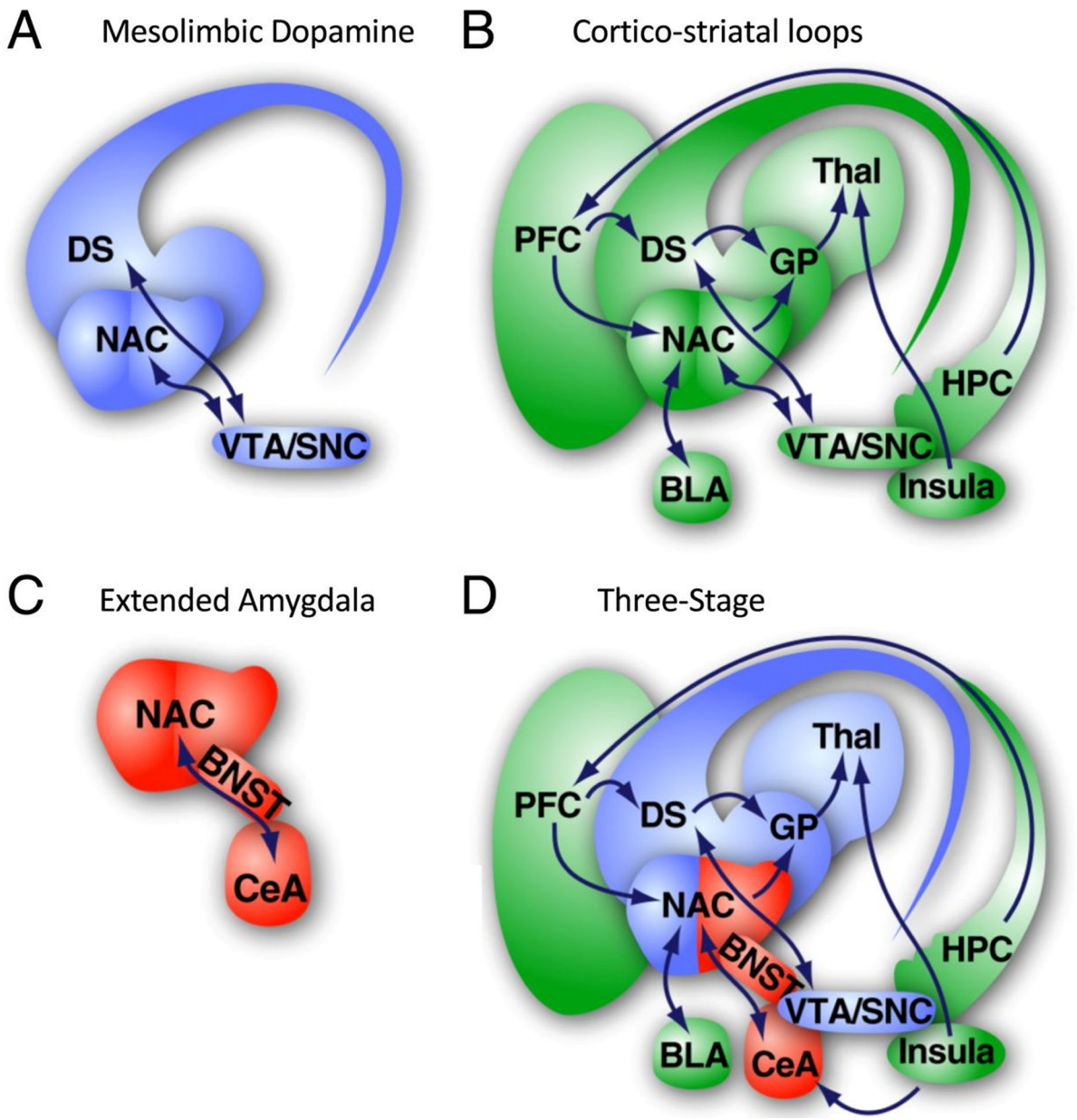

Figure 2. Theories of brain regions that are involved in the neurobiology of alcohol use disorder.
A. Brain regions of the mesolimbic dopamine system. **B**. Brain regions consisting of cortico-striatal loops. **C**. Extended amygdala brain regions. **D**. Three-stage theory. Reprinted from Kimbrough et al. (2020).

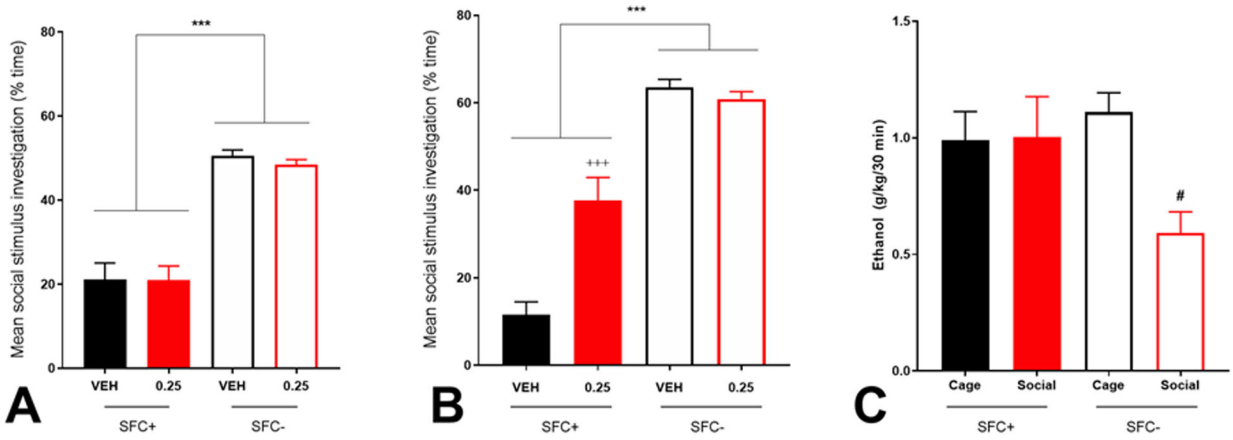

Figure 3. Exploring the interactions between alcohol and social anxiety using murine models.

A. When adult male C57BL/6 mice were administered 0.25 g/kg ethanol i.p. prior to an extinction session after undergoing social fear conditioning (SFC+) or no fear conditioning (SFC-), it had no impact on social investigation time in either the conditioned or unconditioned mice. **B**. In contrast, in adolescent mice, the same dose of ethanol significantly inhibited social avoidance in the conditioned mice, while having no impact on social investigation time in unconditioned mice. A and B are adapted from Raymond et al. (2016). **C**. N = 10 male C57BL/6 mice underwent daily (Monday – Friday) 2 h DID sessions two hours into the dark cycle with 20% ethanol available and an empty wire mesh stimulus cage (7 × 7 × 6 cm) in the back corner of their home cage. After two weeks, mice underwent the social fear conditioning procedure, with half receiving conditioning (SFC+) and half not (SFC-). DID resumed as normal the following day. The next day (~42 h after social fear conditioning), mice underwent DID with a social stimulus (novel mouse inside a cage) for the duration of the drinking session. Cage in the graph is the average consumption on the three days when only the cage was present in the home cage during the drinking session. Consumption was measured 30 min into each session and 2 h into each session. Only data for the first 30 min is shown as the manipulation only impacted consumption in the first 30 min. Conditioned mice showed no difference in ethanol consumption during the first 30 min of their drinking session whether a social stimulus or stimulus cage was present. In contrast, unconditioned mice reduced their consumption of ethanol in the first 30 min of the drinking session when a social stimulus was present (SFC- cage vs social $p < 0.05$). This indicates that induction of social anxiety-like behaviour in mice removes the social buffering effect of social interactions on alcohol consumption in the first 30 min of DID.