

**EXPLORING THE EFFECT OF BINGE DRINKING USING
DRINKING-IN-THE-DARK DURING EARLY VS. LATE ADOLESCENCE IN FEMALE
RATS**



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RATS**

Senior Comprehensive Project: Behavioral Research on Alcohol

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Abstract

High levels of alcohol consumption in a short period of time, or binge drinking, is known to be greater in adolescents than in adults in both humans and laboratory animals. Adolescence is a unique period of neurodevelopment that is characterized by increased vulnerability to binge alcohol-induced alterations in brain maturation and adult neurobiology. Evidence suggests a diversity of neurodevelopmental differences between early/mid-adolescence and late adolescence/emerging adulthood, indicating these could be unique periods of sensitivity to insults such as alcohol. Notably, recent epidemiological data suggests that binge drinking is on the rise in young women, indicating the need for research in females. The present study evaluated binge-like alcohol drinking in adolescent Sprague-Dawley female rats during two distinct developmental periods: early adolescence (postnatal day [P] 32-46) and late adolescence (P50-64; n=9/group). Rats were exposed to 20% (v/v) ethanol (vs. water) 3 hours into the dark cycle for 2 hours a day for 13 days according to the Drinking in the Dark (DID) model of binge drinking during adolescence. Following the adolescent DID sessions, rats were exposed to 4 weeks of operant conditioning training: the first week was training, the following three weeks were under a FR1 schedule of reinforcement, using 20% (v/v) ethanol for the first two weeks and the same solution sweetened with 0.1% saccharin for the last. While no significant main effect of age of exposure was found neither during the DID nor during the self administration sessions, late adolescent DID exposed rats were found to be more likely to acquire self administration in adulthood when compared to their early adolescents exposed counterparts. Taken together, these findings might suggest that binge-like exposure to alcohol during early adolescence results in deficits in effort-related behaviors in adulthood in female rats. Future directions should focus on

better characterization of the different consequences that binge alcohol exposure in discrete vulnerable stages of adolescence may have in the adult brain.

Introduction

Alcohol has been reported to be part of human culture since the beginning of recorded history (Hanson, 1995). Accordingly, it has been noted that the industrialization and the globalization of alcohol's marketing has caused an increase in both its consumption and its associated harmful effects (Rehm et al., 2009). Alcohol use is, indeed, a major contributor to injuries, mortality and the burden of disease (Rehm et al., 2017). The number of alcohol-attributable diseases, such as cancer, infectious diseases, and mental health diseases, and its effect on mortality has been increasing over the past decades (Rehm & Imtiaz, 2016). Adolescence is a period in which individuals tend to initiate alcohol drinking and, particularly, binge alcohol consumption, seems to be higher in adolescents than in adults in both humans and laboratory animals (Spear, 2018). It has been shown that patterns of binge drinking could be involved in Alcohol Use Disorder (AUD) development later on (Olsson et al., 2016). In humans, binge drinking has been shown to be on the rise in young women (Peltier et al., 2019). It is important to determine to what extent drinking patterns in particularly vulnerable groups such as adolescent females lead to risk of heavy drinking in adulthood. Moreover, the existence of different alcohol-related vulnerable stages during adolescence, and the extent in which sex-differences manifest in these stages, needs to be further characterized. Notably, previous animal research has found significant adult behavioral effects in male rats following alcohol adolescent exposure (Varlinskaya et al., 2014, Varlinskaya et al., 2017, Varlinskaya et al., 2020). In the present study we tested the hypothesis of the presence of different vulnerable

alcohol-related stages in female adolescent rats and their role in influencing adult alcohol-seeking behavior.

Pharmacology of Alcohol

Alcohol is widely used due to its pharmacological effects, though reasons for drinking may change over time due to alcohol-related neuroadaptations (Tambour & Quertemont, 2007). Generally, researchers have theorized four categories of drinking motives: drinking to enhance positive mood or well-being (enhancement: positive, internal), to obtain social rewards (social: positive, external), to attenuate negative emotions (coping: negative, internal), and to avoid social rejection (conformity: negative, external) (Kuntsche et al., 2005). In young people, social motives seemed to be associated with moderate alcohol use, enhancement motives with heavy drinking and coping motives with alcohol-related problems (Kuntsche et al., 2005).

The overall effect that the acute alcohol consumption has on the brain is broad and involves all major neurotransmitter systems (Tambour & Quertemont, 2007). As a central nervous system (CNS) depressant, alcohol reduces overall brain activity, by simultaneous and combined enhancement of GABA activity and inhibition of glutamate activity (Tambour & Quertemont, 2007). Besides these two major pharmacological consequences of alcohol use, the substance has been shown to have potential effects on other neuronal targets, such as serotonin, glycine and acetylcholine receptors (Mihic, 1999; Lovinger, 1994; Aistrup et al., 1999). The studies available, however, describe neurophysiological effects of ethanol on the receptors but do not expand on the systemic consequences of these changes (Mihic, 1999; Lovinger, 1994; Aistrup et al., 1999). Indirect effects such as alcohol-related dopamine release, serotonin and endogenous opioids, could be involved in the reinforcing properties of alcohol and in the development of alcohol dependence (Siggins et al., 2005). In particular, it's the presynaptic

release of dopamine in several brain regions that has been suggested to be critical for the occurrence of the reinforcing effects of alcohol and for the development and the maintenance of voluntary alcohol intake as well (Gonzales et al., 2004). In fact, the mesocorticolimbic dopamine system, whose cell bodies project from the ventral tegmental area (VTA) to various brain regions such as the nucleus accumbens, is the major dopaminergic pathway involved in drug and alcohol addiction (Di Chiara et al., 2004). Nonetheless, the exact neurochemical mechanisms by which alcohol provokes the release of dopamine in the nucleus accumbens are not fully understood (Tambour & Quertemont, 2007).

As for chronic alcohol use, it is essential to specify that the human body reacts differently and tends to develop tolerance or reduced sensitivity to the effects of alcohol (Tambour & Quertemont, 2007). As a matter of fact, chronic alcohol use seems to invert its acute effects, with a general GABAergic downregulation and a glutamatergic upregulation (the opposite of acute effects) that seem to contributed to changes in GABA and NMDA receptor subunit expression (Devaud & Alele, 2004). This is what is believed to lead to neuronal hyperexcitability when chronic alcohol consumption is stopped and might propose a scientific explanation to the etiology of the withdrawal syndrome linked to Alcohol Use Disorder (AUD) (Tambour & Quertemont, 2007). In general, acute withdrawal from drugs of abuse produces process-like changes that oppose the acute hedonic effects of drugs of abuse and lead to negative effects (George et al., 2012). Repeated compromised activity in the dopaminergic system and sustained activation of the corticotropin releasing factors' system with withdrawal episodes are suggested to play a role in the transition to drug addiction (George et al., 2012).

Patterns of alcohol drinking

The fifth edition of the Diagnostic and Statistical Manual of Mental Disorders—DSM-5 (APA, 2013) recently set aside the abuse/dependence dichotomy and combined their criteria (Hasin, 2012). Despite these criteria changing along the years, it is generally believed that two dimensions of alcohol consumption are mostly involved in affecting health: average volume of alcohol consumption and patterns of drinking (Rehm et al., 2009). Researchers have previously reported the possible health benefits of moderate alcohol consumption as linked with reduced risk of heart attack, atherosclerosis, certain types of strokes and osteoporosis in postmenopausal women (Dofour, 1999). Yet, this finding hasn't been supported with more recent evidence. To reduce harmful consequences of alcohol use, USDA alcohol drinking guidelines suggest limiting intake to 2 drinks or less in a day for adult men and 1 drink or less in a day for adult women (USDA, 2020). Heavy drinking, instead, is defined as consuming five or more drinks on any day or 15 or more per week for men, whereas for women, consuming four or more on any day or eight or more drinks per week (NIAAA, 2010). Alcohol misuse -which includes binge drinking and heavy alcohol use-over time increases the risk of alcohol use disorder (AUD) (NIAAA, 2010). In addition, excessive alcohol use can result in brain damage and cognitive deficits (Zeigler et al., 2005).

Specifically, binge-like alcohol drinking modalities, which are the main focus of this project, have been gaining increased relevance as adolescents seem to adopt them more and more, and are often associated with high levels of intoxication in a short period of time (Trantham-Davidson et al., 2017). The National Institute on Alcohol Abuse and Alcoholism (NIAAA) defines binge drinking as a pattern of drinking alcohol that brings blood alcohol concentration to 0.08%—or 0.08 grams of alcohol per deciliter—or higher (NIAAA, 2010). As a

concerning pattern of consumption, periods of binge drinking followed by abstinence may trigger a cycle of responses that leads to increased neurotoxicity and cognitive deficits (Zeigler et al., 2005). Other neurocognitive effects of binge drinking include black out, or losing memory of events that occurred during a drinking episode (Zeigler et al., 2005). Behaviorally, binge drinking has been associated with greater positive expectancies and an increased risk of suicide and attempted suicide (Blume et al., 2003; Windle et al., 2004). Binge drinking has also been shown to lead to fatal alcohol poisoning in some cases (Patrick et al., 2013). On the other hand, binge drinking poses also economic and sexual issues, with more than 70% of the excessive alcohol consumption costs attributed to binge drinking and with earlier sexual activity and more frequent changing of sexual partners associated with it (Sacks et al., 2013; Stolle et al., 2009). Finally, it is relevant to discuss changing patterns in binge drinking as well. It has been shown that binge drinking prevalence tends to escalate after high school, peak during the early 20s, and then decline, but that there are also recurring shifts in the shape of these developmental patterns (Patrick et al., 2019). On top of that, a consistently higher likelihood of binge drinking for women, but not men, in the most recent cohort groups appears to be consistent with the narrowing gender gap in alcohol use that has largely been driven by increases among women (Slade et al., 2016). Looking at the data presented about binge drinking, it is essential to consider the issue both broadly in the population and also in relation to some particularly vulnerable groups that might exist.

Adolescence: a period of vulnerability

Adolescence is the period in which individuals commonly appear to initiate alcohol drinking (Spear, 2018). Alcohol is also the most frequently abused substance in adolescence, as it is generally the easiest for adolescents to access (WHO, 2018), and alcohol consumed per

occasion, or binge alcohol consumption, seems to be higher in adolescents than in adults in both humans and laboratory animals (Spear, 2018). Adolescence is also a period of significant brain maturation (Weinberger et al., 2005). The great amount of changes that the adolescent brain undergoes implicate plasticity but also a great vulnerability and a particular susceptibility to neurological insults (Casey & Jones, 2010). For instance, there is strong evidence that stress exposure during adolescence can lead to short- and long-term changes in limbic and cortical structure and function, with important behavioral repercussions (Eiland & Romeo, 2013). In particular, a recent review reports that adolescents are particularly prone to vulnerability to both alcohol- and stress-provoked changes in brain development because the exposure to both of these insults might interfere with necessary neurological maturational processes (Sicher et al., 2022). In fact, late-occurring developmental changes in the PFC throughout adolescence which involve a variety of changes, such as myelination, synaptic pruning, and refinement of several key neurotransmitter systems, likely make the adolescent brain particularly susceptible to exposure to alcohol or stress during this period, with behavioral and structural changes often becoming permanent (Sicher et al., 2022). Many of the major neurotransmitter systems also undergo important changes during adolescence, such as the dopaminergic one that, like other neurotransmitter and receptor systems, may have a U-shaped developmental trajectory (progressively increasing from childhood to adolescence and then declining in adulthood) (Sicher et al., 2022). Based on this evidence, we can certainly conclude that adolescence is a unique period for the brain maturation which deserves particular attention.

For instance, adolescent humans are unique in developing binge drinking induced damage areas that correspond to orbital frontal and temporal cortical areas when compared to adults that showed no binge-induced degeneration in the frontal regions (Crews et al., 2000).

Among the several risk factors, adolescence seems to be specifically characterized by increased vulnerability to binge alcohol-induced alterations in brain maturation and adult neurobiology (Crews et al., 2016). Looking at some data about adolescent alcohol drinking, fifteen years old has been previously reported to be the average age of initiation for alcohol use among adolescents in the US (Aiken et al., 2018). Whereas across Europe, most adolescents initiate alcohol drinking earlier, with an age range that goes from 12 to 16 (WHO, 2018). The use of a substance like alcohol during adolescence might compromise the development processes indicative of that period of life (Lees et al., 2020). More precisely, it has been previously shown a correlation between early age of first drink (15-16) and the incidence of AUD later in life (Dawson et al., 2008).

In general, cognitive features of adolescence include heightened reward sensitivity, sensation seeking and impulsive action, and diminished self-control to inhibit emotions and behaviors as opposed to adulthood (Lees et al., 2020). Importantly, adolescence is characterized by different responses to alcohol, such as less sensitivity to ethanol sedative motor responses, more sensitivity to “ethanol-induced social facilitation” and greater ethanol mediated reinforcement than adults (Crews et al., 2016). In addition to this, it has been shown the existence of the “lock in effect”, according to which there is persistence of some adolescent typical behavioral, cognitive, electrophysiological and neural features in adults after adolescent ethanol exposure (Spear & Swartzwelder, 2014). Permanent changes in adult brain structure after adolescent binge drinking have been demonstrated and could contribute to behavioral changes that alter the life course of individuals, including promoting the progression to alcohol dependence (Monti et al., 2005). With these being the premise, alcohol use, especially binge

drinking, in relation to adolescence seems to be a relevant problem that needs to be addressed and explored through both interventions and research, respectively.

Sex differences in alcohol's effects in humans

Looking at sex differences reported for humans, alcohol use has been suggested to promote later expression of anxiety disorders more frequently in males than in females (Falk, 2008). Nonetheless, in recent years, rates of binge drinking appear to have increased markedly among 21 to 23 year old female college students (Gruza et al., 2008). Sex differences have been shown in human brain development, as female brains develop one to two years earlier than males (Lees et al., 2020). This adds up to the implications about sex differences in adolescent drinking, during which individuals might be at different stages of development depending on their sex. Research on sex differences in response to therapies, development of diseases and, in general, individual's health has increased in the last two decades (Becker et al., 2005). In addition, sex differences in alcohol use consequences have been increasingly investigated. It has been shown in a longitudinal study that more drinking days in the past year predicted a greater reduction in a visuospatial challenge for female adolescents (Squeglia et al., 2009). On the contrary, a study found that adolescent binge drinking impairs immediate and delayed recall in the same way for both males and females (Carbia et al., 2017). The use of alcohol during adolescence might affect the two sexes in different ways and/or only in specific regions. These differences might be more pronounced during adolescence and could be related to the surge of gonadal hormones (Witt, 2007). It has been shown a correlation between early age of first drink (15-16) and incidence of AUD coupled with a greater likelihood of engaging in heavy/hazardous patterns of drinking in adulthood (Dawson et al., 2008). It is essential to better characterize the risk factors of alcohol drinking during adolescence and its consequences later in life. In fact, though it has been argued

that alcohol exposure during adolescence increases alcohol consumption in adulthood, not all studies have shown this relationship (Sicher et al., 2022). Substantial work in preclinical research about use of alcohol in adolescents, especially females, will allow us to address these current problems and to fill out the gaps in our current understanding of these phenomena.

Animal models of alcohol addiction

Whereas human studies of adolescent drinking on adult behavior are necessarily correlational (Monti et al., 2005), preclinical animal models can serve as experimental translational models to try to establish a relationship among these variables, if there is any. Looking at ways in which they have already been useful, preclinical animal models of drug consumption and addiction have been shown to provide optimal predictive validity in alcohol research. Thanks to them, three approved medications (acamprosate, naltrexone, and nalmefene) were developed and were then successfully translated into the clinical situation (Spanagel, 2017). At the same time, as it is assumed that humans and rodents share similar neurochemical mechanisms and neuroanatomical substrates involved in drug-intake behavior, it has been extensively argued whether the difference between voluntary or non-voluntary animal models of drug intake would challenge face validity (Spanagel, 2017). According to a review, animal models of voluntary drug intake, aside from being essential informative tools, provide great face validity (Spanagel, 2017). With regard to binge drinking and its popularity in human adolescents, lots of preclinical studies have been investigating its consequences in adulthood (Monti et al., 2005 Carbia et al., 2017 Coleman et al., 2011, Trantham-Davidson et al., 2017).

Nonetheless, non-voluntary methods of consumption are commonly used to induce binge-like alcohol intake in laboratory animals. Indeed, its aversive taste and pharmacological properties when ingested considerably limit the amount of alcohol that laboratory animals

consume voluntarily (De Guglielmo et al, 2017). A preclinical study tested adolescent rats for binge-like alcohol exposure through a vapor model in which rats were intermittently exposed to high levels of alcohol and found cellular and dopaminergic abnormalities in their adult prelimbic cortex, a subunit of the prefrontal cortex (PFC) (Trantham-Davidson et al., 2017). The PFC develops later than other brain regions and undergoes critical changes during adolescence (Casey & Jones, 2010). Moreover, adolescence represents a critical period for the development of dopaminergic signaling within the PFC, and perturbations of this process induced by alcohol exposure would be expected to result in deficits in prefrontal-mediated cognition that may persist or be expressed in adulthood (Trantham-Davidson et al., 2017). Another study has shown significant changes in the developmental trajectory of neurotransmitter-specific gene expression by intragastrically injecting ethanol in adolescent mice (Coleman et al., 2011). Generally, this is considered a problem because greater levels of neurotransmitter-specific gene expression are found in the adolescent brain rather than in the adult brain, with dopaminergic, cholinergic and other key neurotransmitters peaking during adolescence (Coleman et al., 2011). Hence, preclinical models have shown that disruptions in the brain systems during adolescence caused by insults such as alcohol use seem to suggest long term damages to the brain and its functions. These paradigms of forced-ethanol administration in animals are flawed as models of human binge drinking (hence they do not provide face validity) because consumption cannot be viewed as voluntary and both motivation to consume or the seeking behaviors for the drug can't be assessed (Jeanblanc et al., 2019).

Conversely, researchers have been researching ways to simulate voluntary alcohol binge drinking in rats. It has been argued that both non-operant and operant conditions of voluntary drug intake in animal models can provide face validity (Spanagel, 2017). To date, binge drinking

has been induced in preclinical studies using a variety of different techniques including: Drinking in the dark (DID), Two-bottle preference, Scheduled high alcohol consumption, alcohol liquid diet, Intragastric self-administration (IGSA), chronic intermittent alcohol vapor exposure (CIE), sweet solution (“supersac”) binge drinking and schedule-induced polydipsia (Crabbe et al., 2011). Out of all of these, the ones that include a two-bottle choice preference model are the only ones that propose a voluntary model of alcohol intake capable of recreating a binge-like consumption. Intermittent access to alcohol in a two bottle choice (IA2BC), in which rats can choose between alcohol and water for a limited amount of time during the week, yields the highest amount of consumption of the substance (De Guglielmo et al, 2017). Additionally, this method of drinking has been shown to produce escalation of drinking without sweetening or either food nor water deprivation involved (Carnicella et al., 2014; Spear, 2020). However, another voluntary model of alcohol intake in rats where the solution is presented only during a restricted amount of time in the dark hours, called drinking in the dark (DID), produced greater binge-like consumption, particularly during the first 30 min of the drinking session according to another study that compared it to IA2BC (Holgate et al., 2017). On top of this information, it has been demonstrated that oral self administration of ethanol in mice does not achieve clinically relevant blood ethanol concentration unless access is restricted to the beginning of the dark phase (Rhodes et al., 2005). Subsequently, it is suggested to conduct studies during the first half of the dark phase, because rodents, being nocturnal animals, are more active during that time (Butler-Struben et al., 2022). The DID model, commonly used in mice, has been validated for use as a rat model of ethanol binge-like consumption as well (Holgate et al., 2017). Limited exposure to alcohol followed by intermittent periods of abstinence have been shown to yield increased binge-like alcohol seeking behaviors in adolescents more than in adults (Hargreaves et

al., 2009). Moreover, it has been argued that the longer period of access to ethanol provided by the IA2BC model may not provide any additional benefits for studying binge-like consumption or the effects of long-term excessive ethanol consumption as opposed to the DID (Holgate, 2017).

Moving on to operant voluntary models of binge drinking, it has been argued that they provide better information with regard to complex alcohol-associated behaviors when compared to 2-bottle choices paradigm in which the access is direct (Jeanblanc et al., 2019). In fact, in operant procedures, the experimenter can vary how hard the animal must work to obtain alcohol, how frequently alcohol will be available, and how much alcohol can be consumed each time the response requirement is completed (Cunningham et al., 2000). However, it has been also suggested that when dealing with operant voluntary models of alcohol consumption, manipulations, such as the use of any type of sweetener or food deprivation, should be avoided to rule out the possible interactions with neurobiological and behavioral outcomes (Carnicella et al., 2014). The operant paradigm, hence, has been suggested to provide great face validity as models of human binge drinking because it allow the assessment of factors such as loss of control, compulsivity, high seeking (craving), motivation, loss of cognitive flexibility and habit learning that are central to the transition to dependence (Jeanblanc et al., 2019). A review provides different criteria to validate an animal model of binge drinking which include voluntary intake, preference of operant paradigms, absence of sweeteners and food deprivation, sufficient levels of ethanol consumption to achieve significant BEC, drinking episodes should be both intermittent and repeated several days a week and interindividual variability (Jeanblanc et al., 2019). Overall, these are all relevant information to be taken into consideration when recreating a binge drinking model in rats.

Sex differences in alcohol's effects in animals

Focusing on voluntary alcohol consumption in rodents, females have been found to generally consume more alcohol than males (Chester et al., 2006). An empirical study has reported increased self administration and seeking of alcohol in female rats at baseline, and to a greater degree after they had been administered with a pharmacological stressor (Bertholomey et al., 2016). It is important to identify what exactly determines sex differences. In a more recent study, the researchers performed gonadectomy in rats around day P80 to investigate the role of gonadal hormones and, while hormone levels were associated with changes in drinking, removal of gonadal hormones in adulthood did not eliminate the differences between males and females (Bertholomey & Torregrossa, 2019). If adult gonadectomy does not account for the sex differences, it means that the hormones might have acted during early stages of development to cause permanent sex differences (Becker et al., 2005). Thus, these permanent changes in sensitivity to alcohol might be happening earlier in development, potentially around puberty and during adolescence. Nonetheless, it has been found that removal of the gonads prior to puberty did not influence ethanol drinking or preference during adolescence in either males or females using a 2-hour limited-access 2-bottle choice paradigm (Vetter-O'Hagen & Spear, 2011). Contrarily, a successive study conducted by the same researchers found that the pre-pubertal surgical manipulation process itself was responsible for later ethanol intake increase (as the early sham surgery also displayed the same outcomes) in males (Varlinskaya et al., 2013). Although few gonadectomy effects were evident in females (suggesting that it could have been related in part to the relatively high residual estradiol levels seen post-gonadectomy), the effects seen on males seem plausibly associated with the stress of this manipulation among juvenile animals (Varlinskaya et al., 2013). These findings further complicate the interconnectedness between

neural, hormonal and behavioral changes during adolescence and their possible effects, especially if disrupted or altered, on future ethanol consumption.

Separated alcohol-related vulnerable stages in adolescence

Through the use of other animal studies, for instance, it has been possible to highlight evidence of a diversity of neurodevelopmental differences between early/mid-adolescent (P25–45) and late adolescent/emerging adult rats (P45–65) (Spear, 2015). These age spans have been thought to approximately correspond to 10–18 and 18–25 years of age in humans, respectively (Spear, 2015).

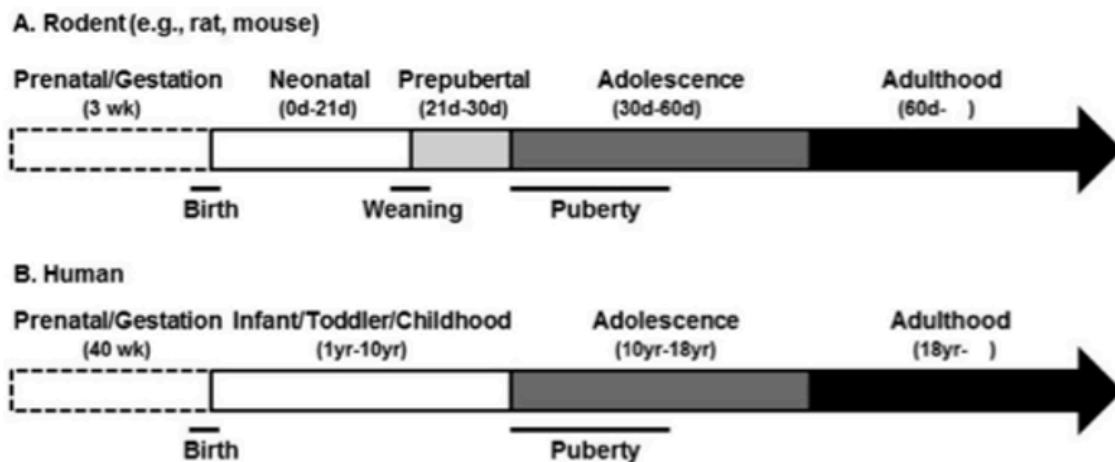


Table 1. Time lines of development in rodents (e.g., rats and mice, **A**) and humans **B**) along with approximate age ranges and commonly used terms for referring to those stages of development. Abbreviations: d, day; wk, week; yr, year. (Eiland & Romeo, 2013).

By making a parallel between rodents and human development, we can relate back to the findings about the increase of binge drinking among 21 to 23 year old female college students, which we can now identify as late adolescents (Grucza et al., 2008), and wonder what specific

effects could binge drinking during that time have on drinking later on in humans, or specifically females in this case. A review suggests evidence of timing-specific vulnerabilities of ethanol exposure within adolescence, even though it is mentioned that substantially more work is needed (Spear, 2015).

Timing-specific consequences of adolescent insults have been investigated recently in rats with a categorical division of adolescence into two distinct periods: early (P25-P45) vs late (P45-P65)(Varlinskaya et al., 2014, Varlinskaya et al., 2017, Varlinskaya et al., 2020). Based on physiological, hormonal and neurobehavioral adolescent-typical characteristics, the period between P25 and P45 has been considered as the pre- and peri-pubertal period of early-mid adolescence and the interval between P45 and P65 as late adolescence/emerging adulthood (Spear, 2015). In a series of studies, the early adolescent male group of rats seem to be the most vulnerable to long lasting adverse effects of chronic ethanol exposure (Varlinskaya et al., 2014; Varlinskaya et al., 2017; Varlinskaya et al., 2020). The findings of a study indicate that many brain regions important for cognition exhibit a sex difference in the number and morphology of microglia that shifts as the brain develops and that their differences are overall affected by sex, age, and the brain region of analysis (Schwarz et al., 2012). It is then particularly relevant to test what does adolescent alcohol exposure during separate periods of adolescence causes in adult females alcohol-seeking behaviors, knowing from pre existing literature that males, specifically those exposed in early adolescence, seem to be the most affected (Varlinskaya et al., 2014; Varlinskaya et al., 2017; Varlinskaya et al., 2020). Taken all together, this evidence in the literature might suggest not only the existence of significant different vulnerable stages during the critical development that happens in adolescence, but also significant sex differences in the same period.

Consequences of alcohol exposure during adolescence in animal studies

In multiple studies looking at distinct periods during adolescence, the rats were exposed to chronic intermittent ethanol intragastrically (Varlinskaya et al., 2014, Varlinskaya et al., 2017, Varlinskaya et al., 2020). Anxiety-like behavioral alterations (Varlinskaya et al., 2014), social response-like alterations to repeated restraint (Varlinskaya et al., 2017) and deficits in behavioral flexibility (Varlinskaya et al., 2020) were found in adult male rats exposed to early adolescent intermittent alcohol. These findings seem to suggest an early adolescent male-typical vulnerability to alcohol-induced behavioral impairments in adulthood. As for female rats, they appeared to respond differently to adolescent intermittent ethanol exposure, which, in turn, may have attenuated later stress reactivity only in male rats (Varlinskaya et al., 2017). Again, these findings add up to the significant differences that males and females might display with regard to these delicate periods of development. As for the specific consequences of alcohol exposure during separate periods of adolescence in alcohol consumption in adulthood, a study did not find any main effects of adolescent intermittent ethanol exposure (AIE) on social drinking later in life in rats (Varlinskaya et al., 2017). However, the females were found to drink more than the males regardless of AIE and adult stress exposure conditions, suggesting that females might be more vulnerable to have modifications in their alcohol seeking behaviors rather than in their social-responses later in adulthood (Varlinskaya et al., 2017). It is concluded that different approaches might be needed for female subjects (Varlinskaya et al., 2017). Other researchers, instead found adult male rats that were previously exposed to adolescent intermittent ethanol (AIE), exhibited enhanced levels of ethanol self-administration in comparison with control rats and resistance to extinction of ethanol seeking, a simple measure of behavioral flexibility (Gass et al., 2014). The AIE model of the study was done by using a non-voluntary model of ethanol

consumption, namely four repeated cycles of binge-like intermittent ethanol exposure by vapor inhalation (Gass et al., 2014). Moreover, adding up to the finding of a significant increase in adult ethanol self administration following AIE, the study employed exclusively male rats (Gass et al., 2014). The results, however, seem to suggest that adolescent ethanol exposure enhances drinking in adulthood. Furthermore, the increase in resistance to extinction may indicate that not only a greater alcohol seeking and consumption in the adult, but also a greater difficulty to discontinue drinking once is initiated (Gass et al., 2014).

In the present study, respecting the assumption already done in previous reviews that voluntary alcohol consumption provides better face validity as models of human binge drinking in humans, we propose a voluntary Drinking in the Dark (DID) model of alcohol consumption during adolescence (Jeanblanc et al., 2019). Previously, a study that initiated DID in 5 weeks old male rats found that the rate of ethanol consumed per hour was higher than the IA2BC model when taking blood ethanol measurements after 10 weeks of testing (Holgate et al., 2017). Another study, instead, assigned adult male rats to continuous or intermittent access to alcohol and water in a 2-bottle choice setup in the home cage and then tested them into operant setting right after (Spoelder et al., 2015). The findings revealed that the rats which had been selected on their high alcohol intake in the home cage obtained more rewards during FR1 and PR schedules of reinforcement than rats which had been selected on their low alcohol intake in home cage (Spoelder et al., 2015). Nevertheless, beside the former covering a period that extends from early adolescence until adulthood, and the latter showing that the rats maintained higher intake in both the homecage and operant models, hence both providing important information, each study employed exclusively male rats (Holgate et al., 2017; Spoelder et al., 2015). To our knowledge,

it is not known what changes adolescent voluntary consumption in female rats can cause in adult alcohol-seeking behaviors and specifically self operant administration.

Objective of the study

In the current study, it was investigated to what extent binge-like alcohol drinking during separate periods of adolescence can affect female rats' alcohol-motivated behaviors later in life. Extensive research has shown the pharmacological effects of acute alcohol consumption and neuroadaptive changes that might stem from chronic alcohol exposure in adolescence on behavior in adulthood (Tambour & Quertemont, 2007). While previous studies have investigated forced binge-like exposure, with findings of increased vulnerability in males, the problem of whether voluntary binge-like alcohol drinking during early or late adolescence might influence subsequent alcohol seeking still needs to be addressed. Further, as young women are binge drinking in greater numbers than young men, it was important to model these effects in female rats. In the present study, female rats were tested for binge-like alcohol drinking using the Drinking in the Dark (DID) paradigm during either early (P32-46) or late adolescence (P50-64). These adolescent sessions were followed by alcohol self-administration testing in adulthood using a fixed ratio 1 (FR1) schedule of reinforcement. It was hypothesized that the early adolescent-exposed group would be the most affected by the binge-like alcohol consumption due to the more extensive brain maturation during that time. We predicted that early adolescent-exposed rats would have higher total alcohol consumption across both the DID and self administration sessions with a FR1 schedule, along with a higher amount of active lever presses and reinforcers obtained during the FR1 schedule.

Method

Subjects

Eighteen female adolescent Sprague-Dawley rats were employed for this study. Animals were bred in the Allegheny College general breeding colony located in Carnegie Hall. They had *ad-libitum* access to water and food except when noted. They were triple housed at all times but during the 2 hour sessions of Drinking in the Dark (DID). They were maintained on a reverse 12:12 light dark cycle and the behavioral testing was performed during the first hours of the dark cycle. All procedures were approved by the Allegheny College Animal Research Committee (ARC).

Materials

Drugs

The ethanol solution (EtOH; 20% v/v ethanol) was dissolved in tap water. The solution was sweetened with 0.1% Saccharin when noted.

Operant chambers

Administration of ethanol during adulthood (P112-P139) was performed through the use of operant conditioning chambers (MedAssociates, St. Albans, VT). The apparatus was provided by Allegheny College department of Neuroscience. Two retractable levers with cue lights above each were present inside the operant conditioning chambers between which was present a magazine, into which a dipper arm would deliver 0.1 ml of the 20% ethanol solution.

Procedure

Rats were bred, and then weaned at PostNatal day 21 (P21) in the Allegheny College colony. All the study rats came from two different litters born 2 days apart. They were allowed 8 days to acclimate to the light/dark cycle before the adolescence drinking sessions. Rats

underwent binge-like homecage drinking during either early (P32-46; n=9/group) or late adolescence (P50-64; n=9/group). The DID 2BC model was adapted from Holgate et al. 2017. The bottles (one bottle containing water, the second containing 20% v/v ethanol) were presented daily for 2 h, 3 h following the commencement of the dark cycle. Bottle measurements for both alcohol and water were recorded at the end of the session, and bottle positions were switched every day to reduce the influence of a side preference. Rats in both age groups were exposed for a total of 13 days, with a single abstinence day halfway through (Day 8). At the conclusion of the adolescent drinking period, rats were kept under standard conditions (triple housing and continuous access to water and food) and left undisturbed in the colony (other than routine care) until adulthood was reached.

Once rats have reached P112, they began a food restriction of 15 grams of food pellets per day. They were initially trained with the operant conditioning boxes with 30-min magazine training sessions for 2 days and 30-min remedial training sessions for three days. The dipper alone went up for 30s during the magazine training sessions, whereas it was accompanied by the cue light, the tone sound, and the active lever presentation, followed by its retraction right after during remedial training. Both the training and the testing period always started 1 hour following the commencement of the dark cycle. Starting at P119, rats began 3 weeks of 5-day-per-week sessions on a FR1 schedule of reinforcement in the operant conditioning chambers. Then, only an active lever press produced a 10-s presentation of the ethanol dipper (0.1 mL of 20% ethanol) and the tone + light cue. Inactive lever responses had no programmed consequences. Total ethanol consumption was calculated using magazine entries, ethanol concentration and body weight. Total reinforcers earned, active and inactive levers were also measured. Due to low

levels of acquisition of the operant response, 0.1% saccharin was added to the 20% ethanol solution during the last 5 sessions.

Statistical Analyses

Age of exposure (early P32-46 vs. late adolescence P50-64) was the independent variable and alcohol-related behaviors (g/Kg consumption during DID and self-administration; reinforcers earned, active and inactive levers during self-administration) were the dependent variables. All statistical analyses were conducted using JASP or GraphPad Prism software, with $\alpha < 0.05$, effect sizes calculated, and post-hoc analyses performed where appropriate.

Alcohol Homecage Drinking

During the adolescent DID exposure phase, daily alcohol intake (expressed in g pure alcohol/kg body weight) was statistically analyzed by a mixed factorial 2-way (age of exposure as the between subject factor; time [13 days of exposure] as the within subject factor) ANOVAs .

Alcohol self administration

During self-administration sessions in adulthood, number of responses on active lever and inactive lever presses, amount of reinforcers earned, and ethanol intake (g/Kg) were statistically analyzed by a mixed factorial 2-way (age of exposure as the between subject factor; time [15 days of exposure] as the within subject factor) ANOVAs . An acquisition criterion was calculated looking at the rats who obtained at least five reinforcers in the last five days of self administration and needed to be met in order for rats to be included in the analysis.

Correlations and χ^2 -test

A correlational analysis was conducted between the averaged ethanol consumption (g/Kg) during the average of the last three days of both DID and self administration.. The early adolescent groups and the late adolescent groups were divided into two different correlation

groups to determine differences in the magnitude of the correlation as a function of age of exposure.

Because a number of rats failed to acquire self-administration, a χ^2 -test was run to determine whether early or late adolescent DID-exposed rats were more likely to acquire alcohol self administration in adulthood.

Results

Alcohol Homecage Drinking (Drinking in the Dark)

A repeated-measures ANOVA with adolescent DID exposure (early vs late) as the between-subjects factors and day (1-13) as the within-subjects factor was performed for total ethanol intake (g/Kg) during the DID period. A main effect of day [$F(12, 192)= 5.758$, $p<0.0001$] was found for intake (g/kg), with a general trend of early adolescents to consume more during the first week of DID. A significant day x age interaction [$F(12, 192)= 3.188$, $p=0.0004$] indicated that acquisition curves for early- and late-exposed rats differed. However, no main effect of age was found [$F(1, 16)=0.8218$, $p=0.378$] for total ethanol intake during DID in adolescence (**Fig. 1**).

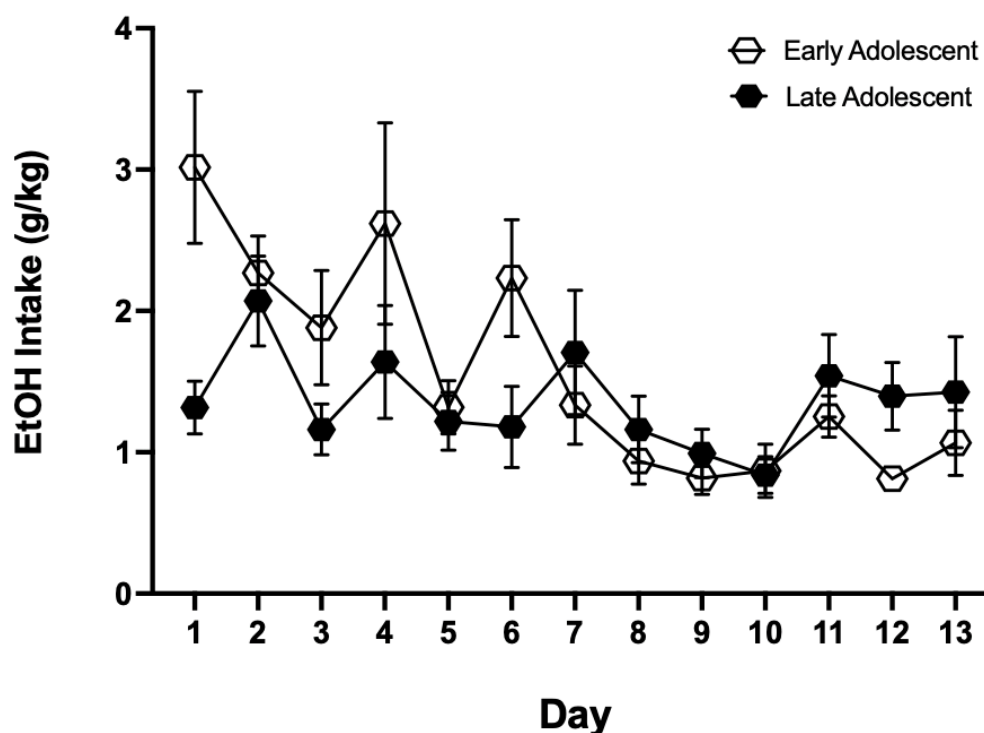


Fig.1 EtOH g/kg consumption during the Drinking in the Dark (DID) period for the early adolescent group (P32-P46) (open hexagons) and for the late adolescent group (P50-P64) (closed hexagons). The 2-way ANOVA repeated measure did not show any significant main effect of age of exposure ($p>0.05$). However, there was a main effect of day ($p<0.001$) and a significant day by age of exposure interaction ($p=0.0004$).

Alcohol self Administration

Separate repeated-measures ANOVAs with adolescent DID exposure (early vs late) as the between-subjects factors and day (1-15) as the within-subjects factor were performed for total ethanol intake (g/Kg), active and inactive lever presses. A main effect of day [$F(14, 224)= 4.410$, $p<0.0001$] was found for alcohol intake (g/kg) across the sessions. A significant day x age interaction [$F(14, 224) = 2.302$, $p=0.0057$] indicated again that acquisition curves for early- and late-exposed rats differed also during self administration. No main effects of age [$F(1, 16)=$

0.3420, $p=0.5668$] were found for total ethanol intake during the self administration in adulthood (**Fig. 2 A**). A significant day x age interaction [$F(14, 224)= 1.696$, $p=0.0577$] and main effects of age [$F(1, 16)= 0.9229$, $p=0.3510$] and day [$F(14, 224)= 2.510$, $p=0.0024$] were found for active lever presses during the self administration in adulthood (**Fig. 2 B**). Main effects of day [$F(14, 224)= 3.878$, $p<0.0001$] was found, whereas no day x age interaction [$F(14, 224)= 0.4896$, $p=0.9373$] or main effects of age [$F(1, 16)= 0.3236$, $p=0.5774$] were found for inactive lever presses during the self administration in adulthood (**Fig. 2 C**).

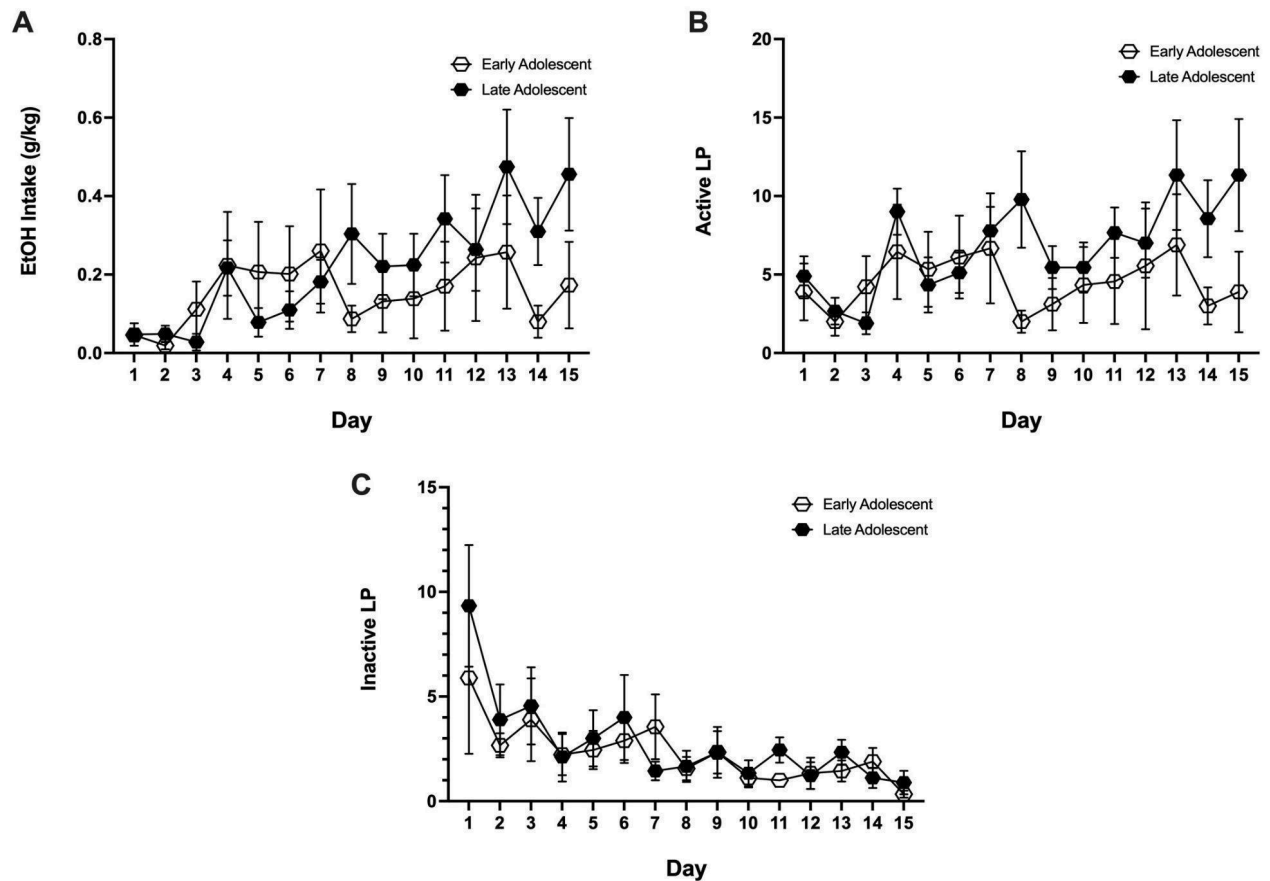


Fig.2 A. EtOH g/kg consumption during the self administration testing under a FR1 schedule of reinforcement for the early adolescent group (P32-P46)(open hexagons) and for the late adolescent group (P50-P64) (closed hexagons). **B.** Number of active lever presses **C.** Number of inactive lever presses. Main effects of day (all $ps<0.05$) were found for all measures, and a day x age interaction was found for g/kg intake ($p=0.0057$).

Correlations and χ^2 -test

Separate correlation analyses were conducted between the averaged ethanol consumption (g/Kg) during the last three days of both DID and self administration. No significant correlation [$r=-0.2272$, $p=0.5567$] was found between the alcohol consumption (g/Kg) of early adolescents during DID and during self-administration (**Fig.3,A**). No correlation [$r=0.3023$ $p=0.4291$] was found between the alcohol consumption (g/Kg) of late adolescents during DID and during self-administration (**Fig.3,B**). However, the magnitude and the direction of the correlation were opposite in the two groups (**Fig.3,A&B**).

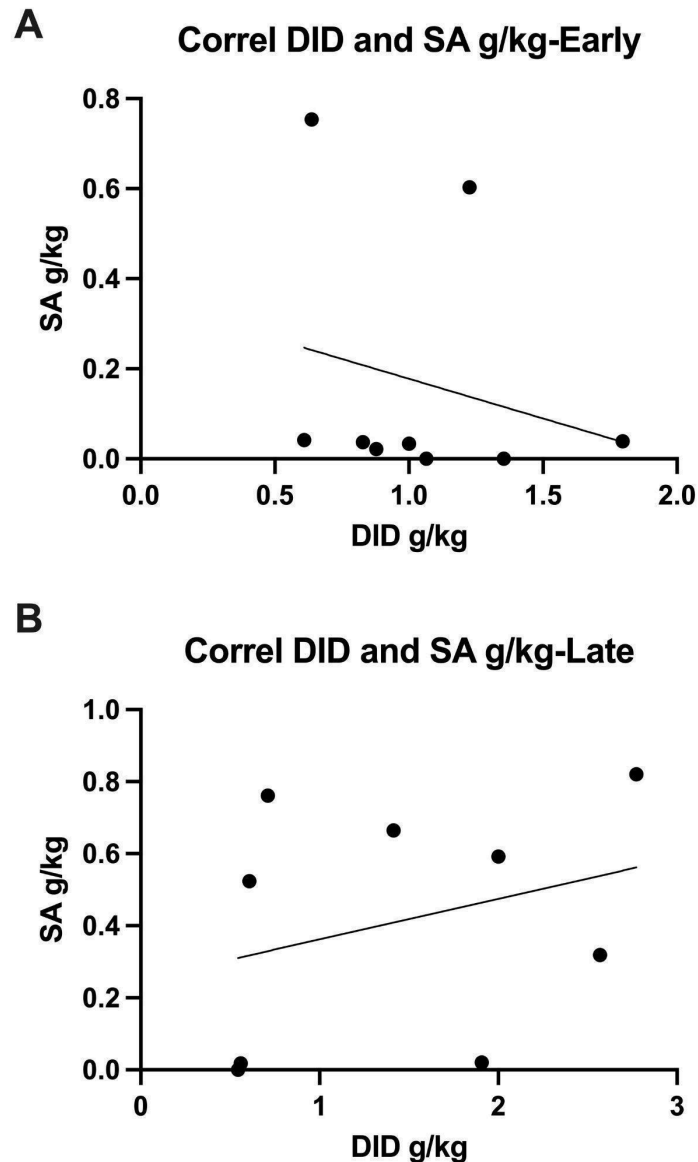


Fig.3 A. Negative correlation between DID ethanol consumption (g/Kg) and self administration in adulthood for early adolescent exposed rats ($r=-0.2272$). **B.** Positive correlation between DID ethanol consumption (g/Kg) and self administration in adulthood for late adolescent exposed rats ($r=0.3023$).

A chi-square test of independence was performed to examine the relation between early and late DID exposure to self administration acquisition in adulthood. The relation between these variables was significant, $\chi^2 (1, n=18) = 4.59, p < .05$. Late adolescent DID exposed were more likely to acquire the self administration task later in adulthood.

Percentage acquisition in EARLY ADOLESCENT-EXPOSED during adult self administration

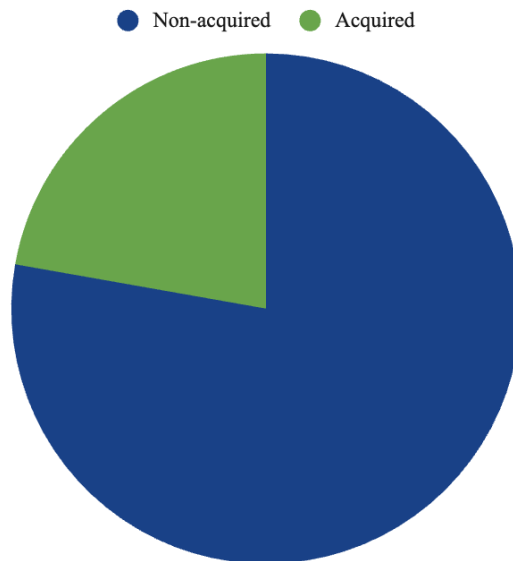


Fig.4 Percentages of acquisition of self administration behavior during adulthood in early adolescent-exposed rats. 22.3 % (n=2, green) among the early adolescent-exposed rats met the criteria for acquisition whereas 77.7% (n=7, blue) did not meet the criteria.

Percentage acquisition in LATE ADOLESCENT-EXPOSED during adult self administration

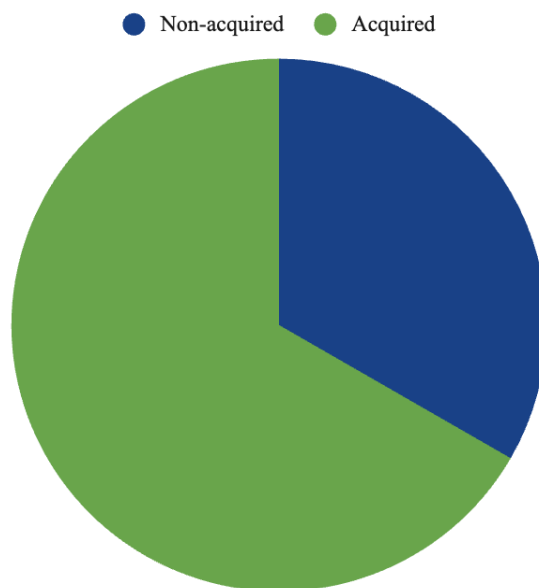


Fig.5 Percentages of acquisition of self administration behavior during adulthood in late adolescent-exposed rats. 66.6 % (n=6, green) among the late adolescent-exposed rats met the criteria for acquisition whereas 33.3 % (n=3, blue) did not meet the criteria.

Discussion

The present study was conducted to determine the effects of adolescent alcohol binge drinking during either early or late adolescence on alcohol self administration, and consequently operant conditioning learning behaviors, during adulthood. During adolescent binge drinking, total ethanol consumption was not found to be statistically different between early and late adolescents, though early adolescent-exposed rats tended to drink more at the beginning of the DID period, whereas late adolescent-exposed rats had more consistent intake (**Fig.1**). The current study also did not show any overall significant differences between the total alcohol consumption, or active or inactive lever presses of the two groups in adult self administration (**Fig.2**). On the other hand, the averaged value of ethanol consumption (g/Kg) during the last three days of both DID and self administration showed patterns of different correlations when early vs late DID groups were compared (**Fig.3**). The early adolescent exposed had a tendency towards a negative correlation between their consumption in adolescence and their consumption in adulthood, whereas the late adolescent exposed rats showed a tendency towards a positive correlation between their respective consumption in adolescence and their consumption in adulthood (**Fig.3**). Nonetheless, neither correlation was strong enough to be considered significant, indicating that further research is needed to determine the actual correlation between drinking in early versus late adolescence and their respective effects in adult self administration. In addition, a chi-square test was conducted to find out whether there was a significant difference in adult acquisition of operant conditioning behaviors between early and late adolescence-exposed. The chi-square test revealed significance, showing that late adolescence-exposed rats were more likely to learn the behavior in adulthood compared to the early adolescence-exposed (**Fig. 4 & 5**) With the early-exposed rats acquiring at lower levels,

this finding likely suggest a contribution to the general lack of a correlation between DID and self administration total ethanol consumption, especially in that age group.

Drinking in the Dark (DID) during adolescence

No significant main effect of age of exposure was found in the ethanol consumption (g/Kg) during the Drinking in the Dark (DID), whereas a significant day by age of exposure interaction suggested that early adolescent-exposed rats tended to drink more during the first week of Drinking in the Dark (DID) compared to late adolescent-exposed rats, in line with our predictions (**Fig.1**). However, during the second week of DID, from day 7 to 13, the difference between early and late adolescent-exposed rats was reduced, as both groups' consumption of ethanol appeared to be similar (**Fig.1**). Despite a lack of statistically significant differences as a function of age of exposure, overall, the adolescent DID showed that the rats were consuming more than 1 g/Kg during almost every session; typically, intakes around 1.5 g/Kg lead to blood alcohol levels at the “binge” criteria of 0.08 g/dl (Gilpin et al., 2012). Our results are close but it is not clear whether we could consider what we observed as binge drinking. The DID 2BC model used in this study was adapted from Holgate et al. 2017 and modified to a 2 hours daily access procedure. The DID limited access model had been considered suitable for studying binge-like ethanol as the rate of ethanol consumption declines as access time increases (Holgate et al., 2017). In that study, in which male rats were either exposed to DID or Intermittent access two-bottle choice (IA2BC), the DID sessions lasted 4 hours a day, 5 days a week for 10 weeks (Holgate et al. 2017). While, in contrast, our study proposed DID sessions of 2 hours a day, 13 days in a row with an abstinence day at day 8. Nonetheless, their findings indicated that DID rats consumed more ethanol than IA2BC rats at 30 min but that, at 2 hours, their consumption was similar (Holgate et al. 2017). The latter information, paired with aspects concerning feasibility

and efficiency, were the reason why we decided to shift the DID model to 2 hours rather than 4 hours a day. Moreover, we are able to make comparisons to the previous study as it adds up to the literature about voluntary alcohol consumption during adolescence and shows that it's possible to generate binge-like drinking under these conditions (Holgate et al., 2017). It is also important to note that the previous study started the DID in adolescence but the rats kept drinking for a period of 10 weeks reaching adulthood (Holgate et al., 2017). On the contrary, the rats in this study stopped DID after 2 weeks (13 days) and did not drink any alcohol until they had all reached adulthood. These factors might have contributed to the lack of significant results and considerable alcohol consumption in the rats during DID.

Although previous studies have conducted blood ethanol measurements, hence having a more accurate idea of the actual alcohol levels circulating in the body, the consumption data from one of those studies seems to be in accordance with our findings of adolescent female rats almost consuming binge-like levels of alcohol when the latter is presented only for a restricted amount of time (Holgate et al., 2017). In the previous study, it was estimated that the DID alcohol consumption that 5 week old male Wistar rats consumed after 2 h was 1.23 ± 0.093 g/kg suggesting that the procedure effectively reproduced a model binge drinking (Holgate et al., 2017). In this present study, the results show oscillating ethanol consumption values close to 1 g/kg with few expectations (Fig.1). Nonetheless, a limitation of this present study is the absence of blood alcohol concentration (BAC) measurements which were not possible for technical and cost reasons, as the standard analyzers and reagents are costly. The results from this study, despite not measuring BACs to verify, showed that the intake levels of the rats in the present study are consistent with a binge pattern (**Fig.1**). Despite our findings being in line pre-existing literature, the absence of BAC measurements complicates the situation. In the same

study mentioned above, the researchers stated that they were unable to achieve the second NIAAA criterion for binge drinking: BECs at or above 80 mg/dL (Holgate et al., 2017).

In humans, binge drinking is defined as a pattern of drinking alcohol that brings blood alcohol concentration to 0.08%—or 0.08 grams of alcohol per deciliter—or higher (NIAAA, 2010).

Laboratory studies that established the threshold BAC required for behavioral intoxication in rats haven't been identified (Crabbe et al., 2011). Nonetheless, while this threshold was developed following human standards, some authors declared that the NIAAA level set for humans of 80 mg% (or 0.08 g/dl) can be considered to represent “excessive” drinking in rats as well (Bell et al., 2006). On the other hand, it has been suggested that 100 mg% is a realistic criterion for mice that metabolize ethanol somewhat more quickly than rats and which require higher ethanol doses and BACs than rats to show equivalent behavioral responses (Crabbe et al., 2011). Future studies should direct the efforts towards a more accurate achievement of binge drinking in adolescent rats while potentially tracking both the ethanol consumption (g/Kg) and BAC (g/dl) to make better estimates.

Self administration during adulthood

No significant main effects of ethanol consumption (g/Kg), active and inactive lever presses were observed between early and late adolescent ethanol-exposed rats when tested with self-administration of the same solution in adulthood (**Fig.2**). Previously, a study tested rats who displayed high and low intake during a 2-bottle choice setup in the home cage into operant settings (Spoelder et al., 2015). Even though the rats were tested from adulthood on, the findings revealed that the rats which had been selected on their high alcohol intake in the home cage obtained more rewards during FR1 and PR schedules of reinforcement than rats which had been selected on their low alcohol intake in home cage (Spoelder et al., 2015). The study, then,

proposes a model that bypasses the period of adolescence and simply investigates consequences that prior intermittent alcohol access has on future operant self administration (Spoelder et al., 2015). Hence, the study's goals and methods differed from ours but highlighted important information about the possible shift in alcohol-seeking behaviors and motivation to obtain it over time when open access is transitioned to an effort-related choice model. Furthermore, in the present study we were not able to differentiate groups of rats between high and low intake of ethanol during homecage adolescent DID. In fact, we didn't have a high enough sample size to determine such differences nor did we have long-term alcohol exposure, two factors that resulted important for previous researchers that were able to identify high, medium and low drinkers after 2 months of either continuous or intermittent ethanol access (Spoelder et al., 2015).

Despite a trend for early adolescent-exposed rats to tend to drink more during the first week of Drinking in the Dark (DID)(**Fig.1**), early adolescent ethanol-exposed rats did not display any significantly different behavior during the self administration period in adulthood when compared to the late adolescents (**Fig.2**). The main objective of self administration in adulthood was to assess the potential alterations that either early or late adolescent alcohol exposure had caused. A study defined their Fixed Ratio use as capable of assessing effort-related choice behavior in rats (Nunes et al., 2014). Nonetheless, the study had assessed this kind of behavior through a fixed ratio 5 (FR5) with respect to obtaining food reinforcers, which might not entirely compare to our alcohol-related behaviors (Nunes et al., 2014). In consequence, it is hard to determine whether the FR1 used in the current study was a valid measure to directly assess motivation or effort-related choice behavior. Other researchers have previously found that adult male rats previously exposed to adolescent intermittent ethanol (AIE) exhibited enhanced levels of ethanol self-administration and higher resistance to extinction of ethanol seeking in

comparison with control rats (Gass et al., 2014). The AIE model of the study was done by using a non-voluntary model of ethanol consumption, whereas the self administration was done using a FR1 schedule of reinforcement that was later followed by progressive ratio (PR) and extinction. Besides the use of FR1 and the results showing its increase in adulthood following adolescent ethanol exposure, the study employed exclusively male rats and a non-voluntary model of adolescent alcohol exposure (Gass et al., 2014). Thus, our study, even if not conclusive, results as a novel attempt to measure similar parameters but in female rats and while proposing voluntary adolescent exposure. It has been reported in multiple reviews, indeed, that DID and, in general, voluntary limited-access binge models, present themselves with significant face validity with respect to binge drinking in humans (Crabbe et al., 2011; Jeanblanc et al., 2019). More efforts should be directed towards the characterization of the effects in alcohol motivation or effort-related choice behavior in female rats following voluntary binge drinking in discrete periods of adolescence.

Patterns of drinking

Two correlation tests were conducted to investigate any possible relationship between alcohol consumption (g/Kg) in the DID sessions during adolescence and in the self administration sessions during adulthood in both early and late adolescent exposed rats (**Fig.3**). Neither correlation of -0.2272 and 0.302, respectively for the early and late adolescent groups, was strong enough to be significant but the magnitude and direction of the correlations turned out to be opposite (**Fig.3 A,B**). More precisely, early adolescent exposed rats displayed a trend towards a negative correlation between consumption of alcohol in the DID session and in the self administration in adulthood (**Fig.3,A**). Late adolescents exposed rats displayed a trend towards positive correlation between consumption of alcohol in the DID session and in the self

administration in adulthood (**Fig.3,B**). Overall, however, the trend of the negative correlation for the early adolescents was not in line with our original prediction. The correlation data suggest there was a tendency for higher early adolescent binge drinkers to be lower adult self administration acquirers. On the other hand, the higher late adolescent binge drinkers tended to be higher self administration acquirers as well (**Fig.2 & Fig.3**). These findings may suggest an impaired or reduced ability to learn operant conditioning behaviors in adulthood for female rats exposed to alcohol insults during early adolescence.

Further analysis of our results prompted us to look at the issue under different perspectives. For instance, a chi-square test indicated that the late adolescents were more likely to acquire the self administration behavior in adulthood rather than the early adolescents (**Fig. 4 & 5**). Altogether, these findings add up to the potential explanations proposed for the correlations' trends. As a matter of fact, these data also may suggest that the learning ability of the early adolescent exposed rats might be impaired following the early DID sessions but not the one of late adolescents. The present results, even if not significant, might work as preliminary evidence that early adolescence exposure to alcohol might cause general impairments, such as learning impairments, when compared to late adolescence exposure. Previous research suggests that impairments in anxiety-like behaviors alterations (Varlinskaya et al., 2014), social response-like to repeated stress (Varlinskaya et al., 2017) and behavioral flexibility (Varlinskaya et al., 2020) found in early adolescent male rats exposed to intermittent ethanol were not found in the female counterparts. Looking at ethanol consumption, early and late adolescent ethanol-exposed females have been shown to display no significant alterations in sensitivity to acute ethanol challenge when compared to their male counterparts (Varlinskaya et al., 2014). Male rats exposed to late adolescent intermittent alcohol did not result as impaired as their early

exposed counterparts in a variety of behavioral assessments done in adulthood (Varlinskaya et al., 2014, Varlinskaya et al., 2017, Varlinskaya et al., 2020). Contrarily, no significant differences between early and late adolescent intermittent alcohol exposed females were found in a series of behavioral assessments done in adulthood (Varlinskaya et al., 2014, Varlinskaya et al., 2017, Varlinskaya et al., 2020). Nevertheless, with specific regard to the possible learning impairments in the early adolescent exposed females of the current study, another study revealed reductions in the volume of several brain regions that are thought to be important components of a distributed cognitive network of the PFC following adolescent ethanol exposure (Gass et al., 2014). Again, this study used exclusively male rats to make their assessment which might add to the implications of these potential cognitive changes happening in early adolescent females as well when exposed to ethanol. In general, the study concluded that alcohol abuse during adolescence might result in alterations in the executive functioning in adulthood, as adolescent exposed rats had more difficulty relative to control rats when testing for behavioral flexibility as well (Gass et al., 2014). Altogether, the current existing data could serve as a further reason to investigate the diversity of neurodevelopmental differences between early adolescent and late adolescent female and male rats, as previously reported. As a matter of fact, these two cohorts of rats, which were equally represented across the early vs late groups, despite being born within two days of difference, displayed different trends in alcohol-seeking behaviors when exposed to alcohol in early adolescence, late adolescence and adulthood. The trends shown in the current study could be used as inputs for future and more targeted research on the issue of vulnerable periods in female adolescents when compared to male adolescents. As a matter of fact, the specific age ranges picked for this study were chosen according to previous knowledge regarding the surge of gonadal hormones (Bell, 2018) and according to previous research that used similar

methodology (Varlinskaya et al., 2014). This suggests that extending exposure time earlier or later could potentially generate different effects with regard to alcohol-related behaviors both in adolescents and their consequences in adulthood.

Sex differences

The current study used exclusively female rats for both DID during adolescence and self administration in adulthood. This choice reflects an attempt at filling the gap in the knowledge about voluntary binge drinking in females adolescents and its consequences on the female adult alcohol-seeking behaviors. Previous studies focused on voluntary alcohol consumption have shown that DID lead to binge-like voluntary alcohol consumption in male adolescents and then adult rats (Holgate et al., 2017). Nonetheless, the previous study did not use any females (Holgate et al., 2017). Impairments in anxiety-like behaviors alterations (Varlinskaya et al., 2014), social response-like to repeated stress (Varlinskaya et al., 2017) and behavioral flexibility (Varlinskaya et al., 2020) found in early adolescent male rats exposed to intermittent ethanol did not correspond to the same findings in the female counterparts. On the other hand, a recent study that exposed to DID both early and late adolescents, males and females, showed that early adolescents male and female had faster acquisition of associative fear, suggesting an altered learned response to stimuli (Grizzell et al., 2023). Altogether, these findings confirm not only that the early adolescent period might be the most vulnerable to alcohol insults but it also suggests that there isn't consistent evidence of sex differences in alcohol-related behaviors during those periods.

Here we show that early female adolescents exposed to voluntary binge-like drinking might have impaired ability in learning self administration behaviors in adulthood when compared to their late adolescent exposed counterparts. The reasons why this might have

happened could be related to a variety of factors. First of all, it is unclear the extent to which ovarian hormones might be influencing drinking in the early vs. late adolescent exposed females, and/or that they were all fully adults during self-administration. A study that compared postnatal development of gonadal hormones indicated that gonadal hormone surge (puberty) in females begins around P35 but is subject to increases until P60 (Bell, 2018). After that development stage, the study shows steady levels of gonadal hormones in adulthood (Bell, 2018). This information suggests that the early and late adolescent females in this study were fundamentally different according to their hormone levels, and that, following post natal day 112 (in which they started self administration training), they could have been actually considered adults and possessing equal amounts of circulating hormones. Even though it might be that the fluctuation of gonadal hormones between P35 and P60 highlighted by Bell et al., 2018 could be playing a role in the different self-administration learning impairments that we found in adulthood, it has been shown before that removal of the gonads prior to puberty did not influence ethanol drinking or preference during adolescence in either males or females using a 2-hour limited-access 2-bottle choice paradigm (Vetter-O'Hagen & Spear, 2011). Another study, instead, found that the pre-pubertal surgical manipulation process itself was responsible for later ethanol intake increase (as the early sham surgery also displayed the same outcomes) in males but not in females (Varlinskaya et al., 2013). Taken all together, this information adds to the uncertainty associated with the relationship between sex differences in vulnerable stages of adolescence and alcohol-related behaviors and suggests a potential explanation to our findings related to different circulating hormones levels in early vs late adolescent females.

In addition, when looking at females only, another important consideration is the estrous cycle (Bell, 2018). Previous studies found no relationship between estrous cycle and self

administration (Bertholomey & Torregrossa, 2019). Nonetheless, these findings were specific for adult female rats and self administration, which might suggest different implications when dealing with adolescent rats and DID alcohol consumption used in this study. Although these considerations are relevant, our study did not focus on the detection of either gonadal hormones or estrous cycle activity. Thus, our study could simply work as a starting point for further understanding of these variables with relation to binge alcohol drinking in adolescence and adult impairments.

Conclusions, limitations of the study and future directions/research

Our results indicate no significant differences in total alcohol consumption during DID nor during adult self administration under a FR1 schedule of reinforcement between early and late Drinking in the Dark (DID) exposed female rats. At the same time, we show significant differences in the number of acquirers of self administration behaviors in adulthood between early and late adolescent DID exposed, with the late adolescents being more likely to assimilate them. These results suggest that, even though we specifically predicted higher levels of consumption in early adolescent exposed rats during DID and self administration, our broad hypothesis which contemplated the idea that the early exposed rats would have simply been the most affected by alcohol exposure, could be considered as partially supported.

The present studies also offer a model to produce binge-like voluntary drinking in adolescence, although it is hard to assume that the alcohol consumption shown in g/Kg (**Fig.1**) exactly resembles the blood alcohol concentration. The differences that we have found in self administration acquisition in adulthood gives us reason to believe that, even if not considered binge drinking, any amount of alcohol consumed in specific moments during adolescence could pose a challenge to adult behaviors. In fact, given the intersection between the complex neural

reorganization and alcohol use initiation during adolescence, it is extremely relevant to consider what impact this interaction may have on neurodevelopment (Hamidullah et al., 2020).

Overall, further research is needed to validate these present findings. For instance, our current study lacks a control group. Both groups of early (n=9) and late (n=9) adolescents were exposed to binge-like consumption during their respective ages using a DID model. Our interpretation of the self administration behaviors in adulthood is limited, then, because we couldn't compare it to a group that was not exposed to either early or late adolescent DID nor a group that was alcohol naive and, in this way, see if there was a true difference of binge-like adolescent exposure. This option, which would have required a larger amount of animals and time availability, was not chosen because it was not feasible due to costs, people involved and time. In fact, the small sample size could be considered another important limitation of the present study. Not only a small number of animals could generally result as an obstacle to the significance of the study, here we show that it was difficult for many of our rats to acquire self administration in adulthood. Only 2 among the early adolescents acquired, whereas 6 of the late adolescents respected the acquisition criteria. Our acquisition criteria could also result as a further limitation of the study: rats which pressed at least 5 times the active lever in the last 5 days of self administration were considered acquirers. To our knowledge, acquisition criteria vary from study to study.

The assessment of motivation to obtain alcohol was made by using a FR1 schedule of reinforcement. However, even though it has been considered a good measure to assess effort-related behavior, it is unknown to what extent it can directly measure motivation (Jeanblanc et al., 2019). Despite existing literature suggesting that when dealing with operant voluntary models of alcohol consumption, use of sweeteners should be avoided to rule out the

possible interactions with neurobiological and behavioral outcomes (Carnicella et al., 2014), we decided to sweeten the ethanol solution during the last 5 days of self administration due to the low acquisition. The decision was taken in order to assess whether the solution's taste was too aversive or the rats themselves were not picking up the behavior. We didn't notice any particular differences during the last 5 days of self administration, showing that overall the rats were not learning the behavior. Moreover, the original plan of the current study was to follow the FR1 sessions with a progressive ratio session. Progressive ratio (PR) has also been used in drug self administration paradigms to assess reward strength by measuring the maximum number of responses that an animal will make in order to get a reinforcer (Olarde-Sánchez, 2012, p.39-40). However, without adequate acquisition of FR1, we decided to stop the experiment and focus on the data we had already collected.

Taken together, this present study represents a small step towards the characterization of discrete vulnerable stages in adolescence of female rats and the importance of alcohol-related insults happening in this age in relation to adult alcohol-seeking behavior. We hope that further efforts in research will be dedicated to the deeper understanding of this issue because of its clear harmful implications in human beings and because of its potential of unveiling useful information needed to prevent alcohol-related illness.

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