EFFECTS OF ALCOHOL AND Δ^9-TETRAHYDROCANNABINOL ON ENERGY BALANCE AND COGNITIVE BEHAVIORS

BY

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DISSERTATION

Submitted in partial fulfillment of the requirements
for the degree of Doctor of Philosophy in Neuroscience
in the Graduate College of the
University of Illinois at Urbana-Champaign, 2019

Urbana, Illinois

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ABSTRACT

Habitual consumption of energy-dense foods is a notable contributor to the high incidence of obesity and metabolic syndrome worldwide. The extent to which calories from alcohol contribute to overweight and obesity is debatable. Δ^9-tetrahydrocannabinol (THC), the main psychoactive constitute of marijuana, mainly activates cannabinoid subtype 1 and 2 receptors (CB_1R and CB_2R) to modulate many behavioral and metabolic processes, including feeding and glucose homeostasis. For several reasons, reports on the long-term effects of alcohol and THC use on energy balance, glucose homeostasis, and cognitive behaviors in both human and rodent studies are fraught with inconsistencies. Further, alcohol and marijuana co-use is believed to be more detrimental to health and safety, yet surprisingly little is known about the specific physiological domains impacted by such a pattern of drug use. There is also a dearth of research on the neurobiological mechanisms that mediate the unique effects of joint alcohol and THC use on metabolism and behavior. To bridge the gap in our understanding of the effects of separate or combined alcohol and THC use on feeding behavior, cognitive function, and metabolic fitness of young adults, we implemented three studies using relatively young Long-Evans rats.

Chapters 1 and 2 reviewed published reports on this topic and outlined the Specific Aims of this dissertation, respectively. Chapter 3 investigated the effects of chronic moderate alcohol consumption on energy balance, cognitive behaviors, and glucose homeostasis in young male and female rats. We observed that rats who voluntarily consumed moderate doses of alcohol compensated for the calories supplied by the beverage. Intermittent moderate alcohol consumption (for 14 weeks) did not appreciably alter object recognition memory and emotion-like behavior in both sexes of rats. But such drinking pattern and duration altered glucose homeostasis in males. Chapter 4 established two rodent models of alcohol and THC co-use that differed in terms of the route of THC administration. In the first model, adolescent male rats were entrained to consume a sweetened alcohol solution immediately after receiving increasing subcutaneous THC doses. To closely capture the voluntary nature of human drug co-use and the trendy consumption of THC via edibles, we implemented a second co-use model where rats voluntarily consumed a sweetened alcohol solution and THC-laced cookies. In the first model, moderate alcohol consumption attenuated the immediate hyperphagic effect of subcutaneous THC. Separate or combined alcohol and THC use under both models did not alter daily caloric

intake and learning and memory, but differently affected alcohol intake, weight gain, and sucrose consumption during abstinence. Western blot analyses uncovered insignificant changes in CB_1R protein expression and basal glycogen synthase kinase-3 beta (GSK-3β) activity in the medial prefrontal cortex (mPFC; center for executive function control), mediobasal hypothalamus (MBH; energy balance regulator), and hippocampus (HIP; involved in spatial learning) during drug abstinence. GSK-3β is a ubiquitously-expressed signaling molecule in the central nervous system. CB_1R and GSK-3β activity help regulate cellular homeostasis. Finally, subcutaneous THC and alcohol co-exposure modestly improved peripheral glucose homeostasis. Chapter 5 examined whether a metabolic challenge using high-fat diet (HFD; 45% kcal from fat) exposure during young adulthood would help uncover robust metabolic effects of adolescent alcohol and THC consumption. There were no effects of oral drug treatment and no interaction between drug treatment and later dietary fat consumption on all the metabolic measures examined. Interestingly, rats exposed to HFD for a short duration demonstrated a hyperphagic response that produced modest weight gain. Four days of HFD feeding altered systemic glucose tolerance as reflected by the modest hyperglycemia and hyperinsulinemia both during a brief fast and in response to an oral glucose load. Although the HFD consumers had a higher basal blood glucose concentration following a mild fast, percent visceral adiposity, and plasma leptin content compared with the chow group, both diet groups responded similarly to exogenous insulin.

Collectively, this set of experiments indicates that separate or combined chronic moderate alcohol and THC use can alter energy balance and glucose homeostasis under different experimental conditions. While we detected no long-term changes in cognition, emotion-like behaviors, and neurobiology (CB_1R expression and GSK-3β activity) following periods of separate or combined alcohol and THC use, studies with more sensitive analytical assays may reveal subtle detrimental effects of drug use. The adolescent alcohol and THC co-use model developed in this dissertation is the first to utilize an edible THC product to truly capture the growing pattern of human alcohol and THC co-use. Considering the lenient regulation of alcohol, the ongoing societal acceptance of marijuana, and the burden of metabolic syndrome on public health, more research to decipher the neurobiological mechanisms that mediate the effects of alcohol and cannabinoid co-use on metabolism and behavior are needed.

To my family and well-wishers, especially the late Mrs. Rhoda Ifejiagwa Nelson (1915–2016)

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CHAPTER 1: LITERATURE REVIEW

Metabolic syndrome: an overview

The World Health Organization (WHO) and other health care governing boards define metabolic syndrome as a constellation of clinical conditions that include hypertension, insulin resistance, high blood sugar, unhealthy lipid profiles, abdominal or central obesity, leptin resistance, and systemic inflammation that increase an individual's risks for cardiovascular diseases, stroke, cancer, diabetes, subfertility, neurological and mental illness, and death (Eckel et al. 2005; Mensah et al. 2004). Data from the National Health and Nutrition Examination Survey (NHANES) show that the prevalence of metabolic syndrome among U.S. adults increased between the year 2003 (32.9%) and 2012 (34.7%) (Aguilar et al. 2015). Metabolic syndrome is a complex disorder that is not confined to Westernized societies or only associated with adulthood or obesity. Some facets of metabolic syndrome, especially diabetes and cardiovascular diseases, afflict otherwise lean or normal weight inhabitants of developing countries at an alarming rate (Atun et al. 2017). There is also a growing rate of obesity and metabolic syndrome among pediatric and adolescent populations worldwide (ACOG 2017; Ogden et al. 2014). The latter point is distressing because childhood obesity is often comorbid with psychiatric and behavioral disorders that can persist into adulthood (Kalarchian and Marcus 2012). Management of metabolic syndrome strains the economies of many countries in the forms of exorbitant health care spending and lost productivity (Atun et al. 2017; Mensah et al. 2004; Ricci et al. 2017). The importance of research efforts aimed at uncovering the predisposing factors for metabolic syndrome cannot be overemphasized.

In addition to genetic and epigenetic influences, several environmental and lifestyle factors are culpable for the burden of metabolic syndrome, including consumption of energy-dense foods and alcohol in Western diet, sedentary lifestyles, stress, and exposure to exogenous drugs and toxicants (ACOG 2017; Eckel et al. 2005; Mensah et al. 2004). Hence, a holistic understanding of the mechanisms linking lifestyle factors and the environment with an individuals' risk of developing metabolic diseases will be advantageous for public health promotion. Work in this dissertation will examine the effects of moderate alcohol, Δ^9-tetrahydrocannabinol (the primary psychoactive component of *Cannabis sativa*), and their combined use on energy balance, glucose homeostasis, cognitive functions, and neurobiological

changes in a rodent model. In the context of metabolic and cognitive fitness, we will also investigate the possible interactions between drug and high-fat diet exposure on the aforementioned health variables. Empirical findings on the association between moderate alcohol and cannabinoid use with the risk of developing metabolic diseases will inform future research efforts that will pave promising paths toward treatment and prevention of the associated health complications.

Alcohol

Alcohol drinking motives and patterns

Alcoholic beverages are typically consumed for their rewarding, mood-elevating, stress-relieving, and pain-reducing properties across numerous environmental/social contexts (Eckardt et al. 1998; Schrieks et al. 2014). Thus, alcohol consumption often promotes gregarious behaviors in humans. The effects of alcohol on physiology and behavior are strongly dictated by drinking patterns and quantity consumed per unit time (He et al. 2007; Nelson et al. 2016; Schrieks et al. 2014). Most of the positive health effects of alcohol are realized following moderate but not binge consumption. Because different amounts of alcohol can mean different things to different individuals, a unified definition of the two broad drinking patterns is appropriate.

According to the National Institute on Alcohol Abuse and Alcoholism (NIAAA), **binge** alcohol drinking is a pattern of drinking that increases blood alcohol concentration (BEC) to 80 mg/dl or greater. In a strict sense of the definition, a BEC $\geq$ 80 mg/dl is achieved after men consume five or more drinks or after women consume four or more drinks within two hours. Chronic binge drinking can injure the brain damage and produce detrimental health effects that I will expand upon later (George and Koob 2017; Monti et al. 2005; Siqueira et al. 2015; World et al. 1985).

Low-to-moderate alcohol drinking is the consumption of non-intoxicating amounts of alcohol. Health-conscious individuals who consume alcohol in moderation reap its rewarding psychoactive effects, as well as its purported beneficial health effects (Eckardt et al. 1998; Goldberg et al. 1999; Hendler et al. 2013; Kloner and Rezkalla 2007; Koppes et al. 2005). The

2015–2020 Dietary Guidelines for Americans recommend that women and men of the legal drinking age (21 years and over) who use alcohol should not consume more than one and two standard drinks per day, respectively (DeSalvo et al. 2016). In North America, one standard drink corresponds to a beverage that contains 14 g (or 0.6 fluid ounces) of pure alcohol or 12 fluid ounces of regular 5% beer. In other cultures, however, consumption of up to six standard drinks per day is considered moderate drinking (Harper et al. 1988; Wannamethee and Shaper 1996). A more apt definition for moderate drinking should be in terms of BEC kinetics or the BECs attained and maintained across time, which should be below 80 mg/dl (Hendler et al. 2013; Nelson et al. 2016). The bioavailability of a given dose of alcohol varies based on body composition (lean vs. fat mass) and the activity of alcohol metabolizing enzymes, which is further determined by age, sex, ethnicity/genetics, or drinking history, amongst other factors (Baraona et al. 2001; Gemma et al. 2006). Consequently, a "moderate" amount of alcohol may not be tolerated by everyone alike.

An individuals' pattern of alcohol use can be influenced by social expectation or the specific motivation for consuming alcoholic beverages (e.g., to relieve stress and pain or to get drunk). The manner of alcohol use can also be modified by biological sex, age, or an interaction between both factors. Sex differences in alcohol drinking are known to emerge during adolescence, when experimentation with exogenous drugs often begins (Crews et al. 2016; Gulley and Juraska 2013; Nixon and McClain 2010). In humans, males consume more alcohol compared with females (Gruchow et al. 1985; SAMHSA 2017; Schulte et al. 2009), though the rate of binge-drinking among females is steadily rising (reviewed in (Dir et al. 2017)). Sex differences in alcohol drinking behavior might be influenced by the disparate rate of alcohol metabolism among men and women or by the increased susceptibility of females to experience more severe unpleasant consequences of repeated alcohol use (Baraona et al. 2001; Dir et al. 2017; Mumenthaler et al. 1999). Adolescents are known to consume higher amounts of alcohol than adults do (Crabbe et al. 2011; Spear 2018). Among other factors, the age differences in alcohol drinking behavior can be partly explained by not only personality traits (sensation-seeking behavior) and environmental factors (peer, sibling, and parental influence) (Siqueira et al. 2015), but also by the heightened sensitivities of adolescents to the rewarding effects of alcohol or by their reduced sensitivities to alcohol's aversive properties (Crews et al. 2016; Nixon and McClain 2010). Evidence suggests that an early age at onset of alcohol use and binge

drinking during adolescence predicts problem with alcohol use in adulthood (Crews et al. 2007; Crews et al. 2016; Grant and Dawson 1997; Nixon and McClain 2010). Among young adults and the aged population, a greater proportion of men consume alcohol in moderation or excess compared with women (Colditz et al. 1991; Jones et al. 1982; Schulte et al. 2009). Yet it was also discovered that a slightly higher proportion of aged women (aged 70–89 years) were heavy drinkers compared with aged men (Jones et al. 1982).

Alcohol drinking behavior is not unique to humans. Fruit flies, zebrafish, rodents, and primates are some model organisms employed in the study of not only the neural systems that regulate alcohol drinking, but also the consequences of excessive drinking (Phillips 2002). Rodents are the commonly used preclinical animal models in the alcohol field (Phillips 2002). Alcohol researchers have devised several paradigms to induce moderate to high alcohol drinking in rats and mice (Crabbe et al. 2011; Ron and Barak 2016). When provided with an incentive to drink alcohol (e.g., use of sweetened alcohol solutions or intermittent access), rats can voluntarily regulate their alcohol intake to maintain BECs close to or below 80 mg/dl (Carnicella et al. 2014; Roberts et al. 1999; Vetter-O'Hagen et al. 2009). To simulate the voluntary nature of human alcohol consumption, we will employ intermittent drinking paradigms where rodents will be allowed to voluntarily consume saccharin-sweetened or unsweetened alcohol solutions in the experiments described in this dissertation.

As in humans, the pattern of alcohol consumption in rodents varies by sex and age, amongst other factors. In rats, sex differences in alcohol intake are predominantly influenced by age. Adolescent males can consume more grams of alcohol per body weight compared with adolescent females and adults of both sexes, although findings have been inconsistent (Doremus et al. 2005; Lancaster et al. 1996; Marco et al. 2017; Vetter-O'Hagen et al. 2009). Several groups, including ours, have shown that adolescent or adult female rats can consume higher doses of alcohol compared with adolescent or adult males, respectively (Cailhol and Mormede 2001; Doremus et al. 2005; Nelson et al. 2017; Sherrill et al. 2011a; Vetter-O'Hagen et al. 2009). Additionally, age differences in alcohol intake have been found to depend on the specific alcohol exposure context or protocol employed. Under the stress of single housing, adult but not adolescent male rats consume lower doses of alcohol compared with their group-housed counterparts (Doremus et al. 2005). There have also been reports of no sex or age differences in

adolescent rats' alcohol intake (Doremus et al. 2005; Lancaster et al. 1996; Marco et al. 2017; Vetter-O'Hagen et al. 2009) or in BECs attained in adult animals (Vetter-O'Hagen et al. 2009). Finally, genetic background and the concentration of alcohol solution presented can affect alcohol drinking behavior in rats (Cailhol and Mormede 2001; Vetter-O'Hagen et al. 2009). Above all, the fact that rodents can reliably consume alcohol to achieve behaviorally-relevant BECs renders them a useful experimental model.

Effects of alcohol on caloric intake and body weight

The caloric value of alcohol (7 kcal/g) is between that of carbohydrate (4 kcal/g) and fat (9 kcal/g) (Suter 2005; Yeomans 2010). Alcohol is commonly consumed alongside food during dinner and festive occasions, especially in Westernized societies where the beverage features prominently in diets. Among regular drinkers in the United States, calories derived from alcoholic beverages can comprise close to 30% of the recommended daily calorie intake in men and women (Bebb et al. 1971; Jones et al. 1982; Shelton and Knott 2014). The high global incidence of obesity and the fact that social drinkers are often not mindful of the caloric content of alcohol necessitates careful study of the relationship between alcohol use and energy balance (Suter 2005; Yeomans 2010).

The effects of alcohol on food intake and body weight are complex. For unclear reasons, men, but not women, who consume moderate amounts of alcohol with meals can adjust their daily caloric intake such that alcohol calories displace some calories from food (Cordain et al. 1997; Ferro-Luzzi et al. 1988). Some researchers found that there were no immediate or enduring changes in caloric intake, weight gain, and percent body fat in a group of healthy men who consumed wine for six weeks (Cordain et al. 1997). By contrast, women alcohol drinkers consumed excess daily calories and gained more weight compared with men (Ferro-Luzzi et al. 1988). Furthermore, alcohol can differently affect energy balance in humans since not everyone can efficiently utilize calories from alcohol. Compared with their lean counterparts, women with higher body mass index (BMI) who consumed 30 g of alcohol per day for three months required less calories from solid food in order to maintain their baseline body weight – a finding that suggest women with higher BMI can efficiently utilize calories from alcohol and carbohydrate compared with lean subjects (Clevidence et al. 1995). Conversely, substitution of 20–30% of

daily calories with alcohol in lean men resulted in no net gain in weight compared with obese subjects that gained weight (Crouse and Grundy 1984). The differential findings sometimes observed in men and women reinforce why it is necessary to include male and female subjects in physiology experiments.

Several large epidemiological studies reported that moderate-to-high alcohol intake is associated with decreases in dietary carbohydrate, protein, and fat consumption (Colditz et al. 1991; Gruchow et al. 1985; Jones et al. 1982; Tremblay et al. 1995). Such decreases in macronutrient intake, however, might not preclude an increase in total caloric intake. In those studies, alcohol drinkers did not gain extra weight and were not heavier than non-drinkers. (Colditz et al. 1991; Gruchow et al. 1985; Jones et al. 1982; Tremblay et al. 1995). In fact, women who drank moderate amounts of alcohol had lower obesity index relative to non-drinking controls; and there was an inverse relationship between BMI and the amount of alcohol consumed by men (Gruchow et al. 1985; Jones et al. 1982). A U-shaped relationship between alcohol intake and BMI was observed in women, where low and excessive alcohol intake are associated with weight gain (Colditz et al. 1991). By contrast, alcohol consumption had no effect on BMI in men (Colditz et al. 1991). The above findings speak to the fact that alcohol may prevent the efficient utilization of dietary macronutrients in a sometimes sexually-dimorphic fashion.

Alcohol is sometimes used as an aperitif or appetite stimulator prior to a meal. For example, experimental research has documented that alcohol consumption can favor excess caloric intake in men and women (Caton et al. 2004; Caton et al. 2007; Poppitt et al. 1996; Schrieks et al. 2015), possibly by increasing the rewarding value of savory foods (Schrieks et al. 2015). In one study, alcohol calories (red wine) contributed to excess caloric intake when it was consumed before or alongside a meal (Caton et al. 2007). Other researchers have shown that the appetite-stimulating effects of alcohol was dose-dependent, which indicates that there is a threshold for alcohol to serve as an aperitif (Caton et al. 2004; Hetherington et al. 2001).

The above studies in humans are, however, limited by their "artificial" nature. In most studies, data were generated from survey, questionnaires, or unnatural laboratory settings. The reliability of data generated through these means might not be guaranteed due to bias in self-reports or problem with retrospective recall. Further, the correlational nature of most of the

studies precludes the establishment of a cause-effect relationship between alcohol use and energy balance. More empirical evidence from animal studies may help clarify how alcohol use affects appetite and body weight control.

Rodent studies have also revealed mixed findings regarding the effects of alcohol on food intake and weight gain. Some studies show that rodents can compensate for the calories supplied by alcohol, while others reported no compensation. Rats forced to consume alcohol solution as the only liquid available to them reduced their food intake, but their daily caloric intake (sum of kcal from food and alcohol) and body weight remained unaffected (Cornier et al. 2002; Richardson et al. 1990; Richter 1926; Richter 1941). In two other studies, four weeks of forced alcohol consumption in rats was associated with reduced solid food intake and body weight but unchanged total caloric intake relative to a control group that consumed water (Benicky et al. 2000; Strbak et al. 1998). Interestingly, McCoy and colleagues observed that although alcohol-derived calories supplanted portions of the daily caloric intake in Syrian golden hamsters, involuntary consumption of high concentration of alcohol solution slowed the rate of weight gain (McCoy et al. 1981). Furthermore, rats fed ethanol-containing liquid diet consumed fewer daily calories and gained less body weight compared with those fed control diet (Reidelberger et al. 1996). Another study found that because rats that received intravenous or intragastric infusions of 3% alcohol solution did not sufficiently reduce their consumption of solid food to account for the energy supplied by alcohol, they maintained higher daily caloric intake but unchanged weight gain relative to controls (Giner and Meguid 1993). Our lab has previously shown that the effects of alcohol on caloric intake and body weight in rats rely on the BEC time course. While rats who consumed moderate doses of alcohol reduced their intake of solid food in proportion to the calories derived from alcohol, a prolonged binge-like BEC reduced food intake and body weight to a greater degree compared with the effect of a shorter binge-like BEC (Nelson et al. 2016). The sedative effects of binge alcohol had little to do with our results since the activity level of the alcohol- and saline-injected rats were indistinguishable 3 h after the single binge dose as well as on the subsequent days when alcohol was not administered.

Several neural and hormonal mechanisms may mediate the appetite altering effects of alcohol. For instance, human and rodent studies have shown that alcohol-related changes in the secretion of appetite-stimulating (orexigenic; e.g., neuropeptide Y, ghrelin) or appetite-

suppressing hormones (anorexigenic; e.g., cholecystokinin, leptin, glucagon-like peptide-1) can differently affect food intake as excellently reviewed in (Yeomans et al. 2003) and (Traversy and Chaput 2015). Alcohol can alter appetite by modulating GABAergic, dopaminergic, serotonergic, and opioidergic neurotransmissions (Traversy and Chaput 2015; Yeomans et al. 2003). Further, alcohol can suppress hepatic fatty acid oxidation and induce heat production and dissipation when alcohol is metabolized via the "energy-wasteful" microsomal ethanol-oxidizing system (MEOS) (Suter 2005; Suter et al. 1992). Consequently, frequent alcohol consumption can erroneously inform the brain that the body is in a state of energy deficit and promote caloric intake (Suter et al. 1992; Yeomans 2010; Yeomans et al. 2003). Paradoxically, while the alcohol-induced sparing of fat from oxidation may increase adiposity in the long run, metabolism of alcohol via the activation of MEOS can prevent weight gain. Moreover, a recent study that utilized an assortment of molecular techniques showed that binge doses of alcohol acutely increased food intake in singly-housed mice via stimulation of hypothalamic AgRP neurons (Cains et al. 2017). In that study, *in vitro* slice electrophysiological recording, calcium imaging, and chemogenetic experiments indicated that alcohol dose-dependently activates AgRP neurons in the arcuate nucleus of the hypothalamus to levels comparable to that in fasted animals (Cains et al. 2017). Whether binge and moderate alcohol doses differentially affect the neural-hormonal systems that regulate energy balance deserves attention.

The supposed appetite-stimulating property of alcohol cannot be attributed solely to the drink's biologic effects. Alcohol promotes gregarious behaviors, and social cues may interact with the psychoactive effects of alcohol to promote excess caloric intake (de Castro and de Castro 1989). For example, excess caloric intake was not observed when experimental human subjects consumed alcohol in isolation (Poppitt et al. 1998). Such observation is yet another testament to the complex interaction between alcohol and caloric intake in humans (Yeomans et al. 1999). Alcohol may also facilitate or forestall socially-driven feeding behavior and learned food preference in rodents. Alternatively, species differences (i.e., ethological characteristics) and other subtle experimental/housing characteristics may partly explain the sometimes-opposing findings. More carefully-controlled human and preclinical studies are needed to fully understand how alcohol consumption affects energy balance.

Effects of alcohol on cognitive outcomes

The detrimental neurobiological effects of binge doses of alcohol are prominent during prenatal and postnatal development (Gil-Mohapel et al. 2014; Ikonomidou et al. 2000). Human and animal research has repeatedly shown that low, moderate, and high doses of alcohol can elicit neuro-chemical, -immune, and -anatomical changes in several brain regions, with attendant cognitive deficits (Coleman et al. 2011; Cui and Koob 2017; Eckardt et al. 1998; Fadda and Rossetti 1998; Kuzmin et al. 2012; Obernier et al. 2002; Pascual et al. 2007; Vetreno et al. 2016). For example, alcohol consumption results in dose-dependent memory impairments in humans (Dry et al. 2012; Ryback 1971). Severe memory deficits that manifest at higher doses can culminate in blackout, or the temporary inability to recall details of events during the intoxication state (Goodwin et al. 1969; Nash and Takarangi 2011). At an extreme end of the spectrum of alcohol-related cognitive pathology is the Wernicke-Korsakoff syndrome – a brain disease characterized by loss of motor coordination, mental confusion, and the inability to form new declarative memories or to recall old memories following chronic heavy drinking to substantially high BECs (Fadda and Rossetti 1998). The syndrome is often observed in chronic alcohol abusers, and is associated with severe malnutrition, thiamine deficiency, and reduced life expectancy (World et al. 1985).

Multiple studies have shown that adolescents and females may be uniquely sensitive to the detrimental effects of alcohol on brain structure and functions when compared with adults and males, respectively (Maynard et al. 2018; Monti et al. 2005; White and Swartzwelder 2004). An earlier age of onset of alcohol use elicits neuroadaptations that can increase the risks for psychiatric disorders later in life (Broadwater et al. 2017; Crews et al. 2007; De Bellis et al. 2000; Spear 2011). Adolescents who begin using alcohol before 15 years of age have an increased risk of transitioning into heavy drinking adults who may become dependent on the drink as compared with those that begin drinking after 20 years of age (Grant and Dawson 1997). An age-dependent effect of alcohol on cognitive functions was reflected in the finding of greater semantic memory impairment in young adults aged 21–24 years compared with those aged 25–29 years (Acheson et al. 1998). Recent data from the National Consortium on Alcohol and NeuroDevelopment in Adolescence (NCANDA), a large cross-sectional study of 831 adolescents aged 12–21 years, show that adolescents with more alcohol drinking experience not only

performed poorer than the no/low drinkers on neuropsychological tasks that assessed autobiographical memory, cognitive ability, attention, emotional control, and motor functions, but were also more impulsive (Sullivan et al. 2016). Remarkably, the older no/low alcohol drinkers outperformed the younger no/low drinkers on measures of attention, cognitive ability, motor performance, and impulse control (Sullivan et al. 2016) – findings that mirror the age-dependent effects of alcohol shown by Acheson and co-workers (Acheson et al. 1998). Finally, sex differences in the effects of alcohol on cognitive functions are less clear, with boys outperforming girls on some but not all cognitive measures (Sullivan et al. 2016). The available human studies to date do not reveal whether alcohol consumption is a cause or consequence of cognitive dysfunctions. The ongoing Adolescent Brain Cognitive Development (ABCD) project, a large-scale prospective longitudinal study that tracks a cohort of over 11,000 drug-naïve children aged nine to ten years who reside in the United States, monitors adolescent drug use behavior or the lack of it (https://abcdstudy.org/). Findings from the ABCD study will shed light on how alcohol and other substance use can affect brain development, cognitive outcomes, and general health during the formative years (Volkow et al. 2018).

The detrimental effects of alcohol on cognitive functions are not restricted to the adolescent period or solely associated with the duration and pattern of drinking. A recent longitudinal study conducted in the United Kingdom reported that moderate and heavy alcohol consumption by adult men and women could pose a significant risk for cognitive decline as they aged (Topiwala et al. 2017). The researchers found that moderate alcohol drinking was associated with higher likelihood of hippocampal atrophy in regular drinkers compared with abstainers. They also documented that individuals with a history of heavy alcohol use had more pronounced hippocampal atrophy, disruption of white matter architecture, and cognitive decline when compared with moderate drinkers and abstainers (Topiwala et al. 2017).

Not all studies have documented alcohol-related memory impairments. Moderate alcohol use did not impair performance in driving and divided attention tasks in adolescents and young adults (Lenne et al. 2010; Macavoy and Marks 1975). In the study by Topiwala *et al.*, performance of aged men and women on some cognitive tasks was not significantly affected by a history of alcohol use (Topiwala et al. 2017). An inverted-U relationship between alcohol use and risk for cognitive decline has previously been proposed, where low-to-moderate drinking

protects against dementia in the elderly (Ruitenberg et al. 2002) and may be innocuous to the cognitive functioning of individuals with a history of heavy drinking (Goodwin et al. 1973). A recent cross-sectional study found that low-to-moderate alcohol consumption was associated with increased total brain volume in an elderly population without dementia (Gu et al. 2014). That study also reported that the positive association between alcohol use and total brain volume was mostly driven by red wine consumption, but not by beer or other types of alcoholic beverages (Gu et al. 2014). This observation raises the possibility that anti-inflammatory flavonoids and polyphenols abundant in red wine might be the crucial beneficial element of wine consumption on healthy brain aging.

Research with animal models has also provided valuable insight into the effects of alcohol on cognitive outcomes. As in humans, alcohol can dose-dependently impair cognitive functions in rodents (Acheson et al. 2001; Markwiese et al. 1998). For instance, adolescent rats demonstrated robust spatial memory deficits in a Morris water maze (MWM) task compared with adult rats (Markwiese et al. 1998), though this age-dependent effect was not noticeable after sufficiently low (0.5 g/kg) and high (2.5 g/kg) doses of alcohol were administered (Acheson et al. 2001). In the latter study, acute 0.5 g/kg dose of alcohol had no effect on spatial learning in adolescent and adult male rats, while the 2.5 g/kg dose impaired acquisition of the spatial MWM task in both age groups (Acheson et al. 2001). Spear and her colleagues showed that adolescent male rats exposed to 0.5 and 1.0 g/kg alcohol subsequently displayed worse odor discrimination learning compared with adults (Land and Spear 2004). Their results further suggest that an intact hippocampus may not be dispensable for odor discrimination learning in adolescent rats as it is for adult rats (Land and Spear 2004). Another study observed that intermittent binge alcohol exposure during adolescence impaired object recognition memory in both male and female rats, though sex-specific brain changes were noted (Marco et al. 2017). Collectively, the above studies show that alcohol can dose-dependently alter behaviors that rely on different memory systems.

As previously mentioned, the female brain is believed to be sensitive to the damaging effects of binge alcohol exposure. Sex differences in the effects of alcohol on neuroanatomy and cognitive functions in rats may be brain-region-specific and rely on the type of behavioral task employed, respectively (Koss et al. 2012; Maynard et al. 2018; West et al. 2018). Leasure and co-workers observed that the hippocampus, but not the medial prefrontal cortex (mPFC), of adult

female rats may be more sensitive than that of adult males to the detrimental effects of binge doses of alcohol (Maynard et al. 2018; West et al. 2018). This sex difference in the neural effects of alcohol (reduced neuron number in the dentate gyrus in adult females) was reflected in greater impairment in hippocampus-dependent task in the adult females compared with males (Maynard et al. 2018). The Juraska group, however, found that treatment of adolescent male and female rats with binge doses of alcohol had no effect on neuron number in the mPFC and basolateral amygdala during adulthood, but was associated with reduced glia number only in the mPFC of males (Koss et al. 2012). The functional significance of the reduced glia number was not investigated in that study, but it may be associated with abnormal changes in cellular homeostasis and functional connectivity in the mPFC. Thus, changes in the neural-hormonal milieu associated with age in rodents and possibly humans may mediate some of the sex-specific effects of alcohol on brain architecture and function.

The mechanisms responsible for the age and sex differences in the neurotoxic effects of alcohol are not well understood. Alcohol disrupts synaptic activity at hippocampal CA3-CA1 synapses, and the effects are more robust in slices from adolescent-exposed brains compared with those from adult-exposed brains (White and Swartzwelder 2004). In one report, the concentrations of alcohol that attenuated NMDA-mediated long-term potentiation (LTP) in hippocampal slices from adolescent rats were ineffective at disrupting LTP in slice preparations from adult rats (Pyapali et al. 1999). Fulton Crews and his crew have shown that acute and chronic alcohol administration dose-dependently alters hippocampal neurogenesis in adolescent and adult rats by inhibiting neural precursor cell proliferation and survival (Crews et al. 2006; Morris et al. 2010; Nixon and Crews 2002). Cellular and histological assays have been used to link acute and chronic alcohol administration to increased cell death in the dentate gyrus of young rats (Gil-Mohapel et al. 2014; Ikonomidou et al. 2000; Morris et al. 2010; Obernier et al. 2002; Pascual et al. 2007). In rats, chronic binge alcohol exposure during adolescence activates microglia in the dentate and Cornu Ammonis (CA) fields of the hippocampus, and such microglial response can persist into adulthood (McClain et al. 2011). Findings from preclinical animal studies suggest that, among many possibilities, binge doses of alcohol induce cell death, promote microgliosis, reduce trophic factors, hinder neurogenesis, and alter neurotransmission to a greater degree in the brains of females as compared with males (Marco et al. 2017; Maynard et al. 2018).

Some of the detrimental effects of alcohol on the brain and behavior can be reversed by healthy lifestyle changes, including abstinence, aerobic exercise, and adequate nutrition. Further, preclinical rodent studies have shown that immunotherapy (particularly non-steroidal anti-inflammatory drugs and antioxidants) may minimize some of the negative neuro-immune effects of chronic alcohol use (Pascual et al. 2007). However, a history of binge drinking during adolescence can leave indelible marks on brain development and executive functions that might not be erased by abstinence alone. Crews *et al.* opined that the negative impact of adolescent binge drinking on executive functions can persist into adulthood even following a period of sobriety (Crews et al. 2007). Combinations of lifestyle and therapeutic interventions may be effective at reversing some alcohol-related brain and behavioral changes.

Is alcohol consumption a risk factor for metabolic syndrome?

Scientists have long debated the relationship between alcohol consumption and cardio-metabolic health (Fernandez-Sola 2015; Molina et al. 2014; Yeomans 2010). The term "French Paradox" was coined in the early 1990s to describe the reduced prevalence of cardiovascular diseases among inhabitants of France, despite their substantial consumption of dietary fats and alcohol. This initial observation inspired other studies that also concluded that low-to-moderate alcohol consumption may have beneficial effects on metabolic and cardiovascular health in men and women (Dixon et al. 2002; Kao et al. 2001; Koppes et al. 2005; Phillips and Safrit 1971; Rasouli et al. 2014). A case-control study conducted in Sweden concluded that regular alcohol consumption (specifically wine) was associated with improved insulin sensitivity and reduced risk of type 2 diabetes in adult men and women (Rasouli et al. 2014). The researchers observed a U-shaped dose-dependent relationship, where abstainers had higher odds of developing type 2 diabetes compared with moderate drinkers. The investigators also found that every 5 g/day increase in alcohol consumption within the range of 0.6–23 g/day was associated with 5% lower odds of developing type 2 diabetes (Rasouli et al. 2014). The purported beneficial cardio-metabolic effects of light-to-moderate alcohol consumption can be mediated via increases in antioxidants from wine as well as favorable lipid profiles, specifically increases in high-density lipoprotein (HDL) cholesterol (Dixon et al. 2002; Goldberg et al. 1999). Conversely, others have shown that alcohol can be toxic to the liver and pancreas (Miyake et al. 2016; Phillips and Safrit

1971). A study conducted in the Japanese population found that any amount of alcohol consumption increases an individual's risks for developing non-alcoholic fatty liver disease and glucose intolerance (Miyake et al. 2016). Furthermore, a population-based study showed that alcohol consumption during adolescence increases the risk of type 2 diabetes during adulthood (Liang and Chikritzhs 2014), suggesting that adolescence is a period of heightened susceptibility to the effects of a metabolic disruptor like alcohol. The discrepancies on the effects of alcohol on diabetes risk may be explained by the type of alcohol consumed (beer, wine, liquor), ethnic differences in alcohol metabolism, the *sick quitter effect*, or bias in retrospective self-reporting, amongst other confounds.

Related to its association with weight gain and obesity is the scare that alcohol may be a significant risk factor for certain facets of metabolic syndrome, including insulin resistance and impaired glucose tolerance. Insulin resistance and glucose intolerance are some of the defining hallmarks of diabetes. Exposure to binge doses of ethanol not only injured the liver of genetically obese Zucker rats by upregulating markers of apoptosis, inflammation, and mitochondrial dysfunction, but also promoted hepatic steatosis, and lowered the activity of antioxidants in hepatocytes (Carmiel-Haggai et al. 2003). A study with inbred rats showed that chronic binge-like alcohol consumption damages the pancreas and liver and resulted in impaired glucose tolerance (Lee et al. 2015). Reports on the effects of alcohol on insulin receptor signaling are often at odds. Some researchers posit that moderate alcohol consumption has beneficial effects on hepatic insulin sensitivity (Furuya et al. 2005; He et al. 2007), while others show that chronic binge alcohol consumption impairs insulin signaling in liver, adipose, muscle, and brain tissues (de la Monte et al. 2012; de la Monte et al. 2009; He et al. 2007; He et al. 2006; Lindtner et al. 2013; Onishi et al. 2003). Collectively, it appears that the effects of alcohol on glucose homeostasis and insulin action are dose-dependent or biphasic and may depend on the specific tissues analyzed (He et al. 2007; Onishi et al. 2003). The inconsistent reports outlined above reflect the ongoing debate on whether regular alcohol consumption is healthful (Burton and Sheron 2018).

Cannabinoid

Cannabinoid neuropharmacology in brief

Humans have cultivated the marijuana plant (*Cannabis sativa*) for centuries (Earleywine 2002; Farrimond et al. 2011). Portions of the hemp plant have been consumed as food, or used to produce fiber, fabrics, paper, personal care products, and medicines. Concoctions prepared with the plant are used for certain religious rituals (due to the plant's mind-altering effects), and have proven useful for the treatment of an array of medical conditions ranging from menstrual cramps to poor appetite, asthma, convulsions, insomnia, earache, headache, glaucoma, and myriad other ailments (Earleywine 2002).

Despite some of its known therapeutic potential, marijuana is currently classified by the U.S. federal government as a schedule I controlled substance – a category that includes substances with high abuse potential, no accepted medical use, and lack of safe use (e.g., heroin, ecstasy, and hallucinogens). Yet, marijuana continues to be the most widely used illicit recreational drug in the United States and in several countries (National Academies of Sciences 2017). An estimated 2.7–4.9% or 129–238 million individuals around the world used marijuana in 2015 (UNODC 2017). The progressive decrease in the number of young people who believe marijuana use is risky stems in part from the rarity of overdose from marijuana when compared to the perils of using "more harmful" drugs like heroin and cocaine. Owing to advances in cannabis research and the growing public support for marijuana legalization, the political landscape governing recreation and medicinal marijuana continues to change. The possession of certain quantities of marijuana is no longer considered a criminal offense in several U.S. states and growing (National Academies of Sciences 2017).

Δ^9-tetahydrocannabinol (THC) is one of over 100 known phytocannabinoids derived from the marijuana plant. THC is typically administered via inhalation (smoking) of marijuana cigarettes or "joints" and by oral consumption of edibles prepared with marijuana extracts. The psychoactive effects of marijuana are brought about mainly by the actions of THC and its active metabolite (11-hydroxy-tetrahydrocannabinol) within the endocannabinoid system in the brain and select peripheral tissues (Grotenhermen 2003; Kirkham et al. 2002; Mackie 2005; Tseng et al. 2004; Volkow et al. 2017). In brief, the endocannabinoid system is composed of anandamide (AEA) and 2-diacyl glycerol (2-AG), their main receptors (cannabinoid receptors 1 and 2, CB_1R

and CB_2R), as well as their synthesizing and metabolizing enzymes. In the central nervous system (CNS), AEA and 2-AG are synthesized on-demand and retrogradely act on CB_1R, which is a $G_{i/o}$ subtype of G-protein coupled receptors. CB_1R activation by AEA, 2-AG, THC, and other agonists triggers a collection of cellular activities that result in the attenuation of presynaptic cell activity (see (Castillo et al. 2012; Lu and Mackie 2016; Silvestri and Di Marzo 2013; Volkow et al. 2017) for excellent reviews). Amongst other functions, the CNS endocannabinoid system is involved in the modulation of appetite, executive functions, mood, motivation, stress reactivity, and pain (Di Marzo and Matias 2005; Earleywine 2002; Volkow et al. 2017; Williams and Kirkham 2002a). CB_1R and CB_2R expressed in glial cells and in peripheral tissues also play important roles in the hedonic, homeostatic, or mnemonic mechanisms that regulate food intake and energy balance (Bowles et al. 2015; Silvestri and Di Marzo 2013; Thaler et al. 2012).

Effects of THC on caloric intake, alcohol intake, and body weight

Anecdotal reports of the appetite-stimulating property of marijuana (Tart 1970) have been corroborated by clinical reports of the efficacy of dronabinol (a schedule 3 medication containing synthetic THC) in promoting food intake and reversing weight loss in HIV/AIDS-infected subjects that present with anorexia (Beal et al. 1997; DeJesus et al. 2007). In a laboratory setting, smoked marijuana increased the consumption of palatable food in healthy volunteers (Foltin et al. 1988). Another study with casual marijuana users found that the hunger-stimulating effect of smoked marijuana wanes over time (Greenberg et al. 1976). It remains to be seen whether THC administered via other routes stimulates appetite in humans.

More empirical support for the endocannabinoid system's role in regulating energy balance comes from rodent studies where infusion of AEA and 2-AG into the ventromedial hypothalamus and nucleus accumbens stimulated food intake in rats (Jamshidi and Taylor 2001; Kirkham et al. 2002). Interestingly, compared with satiated rats, fasted rats had elevated levels of AEA and 2-AG in brain regions that modulate the homeostatic and hedonic aspects of food intake (Kirkham et al. 2002). AEA, THC, and other exogenous CB_1R agonists dose-dependently stimulated food intake in rats (Farrimond et al. 2010; Merroun et al. 2009; Williams and Kirkham 1999; Williams and Kirkham 2002b; Williams et al. 1998). Pretreatment with SR141716A (rimonabant; a selective CB_1R antagonist) or AM251 (a CB_1R inverse agonist)

averted the effects of CB_1R agonists on consumption of energy-dense foods in rodent (Colombo et al. 1998; Williams and Kirkham 2002b) and primate (Simiand et al. 1998) models, thus verifying that the orexigenic effects of cannabinoids are mainly transduced via CB_1R stimulation. Additionally, oral THC or subcutaneous AEA administration was found to increase the motivation for food in pre-fed rats by reducing the latency to feeding bouts and increasing feeding duration and frequency (Farrimond et al. 2010; Williams and Kirkham 2002a). The cellular and molecular bases for the "munchies" have been reviewed elsewhere (Koch 2017; Morozov et al. 2017; Patel and Cone 2015), as have the effects of many other cannabinoid drugs or marijuana constituents on eating behavior (Farrimond et al. 2010; Farrimond et al. 2011; Pagotto et al. 2006).

Cannabinoid receptor stimulation does not always elicit a hyperperphagic response. WIN 55,212-2, a full CB_1R and CB_2R agonist, dose-dependently reduced motivation for and consumption of a food reward (casein pellets) in an operant behavior task in rats (Drews et al. 2005). The reduced food intake observed at a higher dose of WIN was not driven by the locomotor-altering effects of CB_1R activation. Compared with controls, 0.6 mg/kg WIN stimulated locomotor activity but suppressed consumption of casein pellets, while 1.8 mg/kg WIN had no effect on locomotor activity but reduced casein consumption (Drews et al. 2005). Other rodent studies have also shown that THC can increase, reduce, or have no effect on food intake depending on the doses administered and other experimental specifics (Farrimond et al. 2011; Graceffo and Robinson 1998; Pagotto et al. 2006; Rubino et al. 2008). For instance, a previous research study demonstrated the biphasic effects of THC on feeding, where 1 mg/kg THC acutely reduced while 2 mg/kg THC increased food intake in fasted female Sprague-Dawley rats (Glick and Milloy 1972). Thus, it is logical to suspect that unforeseen biological factors or experimental subtleties may explain the often contradictory effects of cannabinoids on appetite and motivation.

The endocannabinoid system also regulates alcohol intake; and endocannabinoid dysregulation is thought to be implicated in alcohol use disorder (Ceccarini et al. 2014). Stimulation of CB_1R in neural circuits that control alcohol reinforcement increases rodents' motivation to obtain and consume alcoholic beverages – an effect that was averted by pretreatment with SR141716A (Colombo et al. 2002; Gallate et al. 1999). However, McGregor

and his team have called attention to the fact that the effect of THC in promoting alcohol consumption in rats was non-specific since intake of "near-beer" and a non-alcohol containing beverage (sucrose) can be enhanced by THC (McGregor et al. 2005). More research is needed to determine the factors that may explain the unclear effects of exogenous cannabinoids on alcohol consumption.

The effects of THC on body weight are less clear. Epidemiological data have shown that habitual marijuana users have lower BMI and waist circumference when compared with non-marijuana users (Penner et al. 2013). Chronic marijuana smoking have been found to increase weight gain in humans (Greenberg et al. 1976). The fact that THC use produces the "munchies" that can in turn engender weight gain and obesity partly motivated the development of rimonabant for the treatment of metabolic syndrome associated with obesity (Matias and Di Marzo 2007; Pagotto et al. 2006). However, this drug was pulled from the market due to the accompanying unpleasant side effects. A rodent study observed that twice a day intraperitoneal administration of 2.5–10 mg/kg THC (BID, 5–20 mg/kg/day) reduced caloric intake and weight gain in adolescent male and female rats (Rubino et al. 2008). In line with the study by Rubino *et al.*, another study reported that mutant mice with enhanced CB_1R activity neither consumed more high-fat and low-fat foods nor gained more weight compared with controls (Marcus et al. 2016).

The contrasting findings on the effects of THC on caloric intake and weight gain may also be explained by the fact that THC is a partial CB_1R and CB_2R agonist, can elicit opposing synaptic effects, and may activate a combination of other putative cannabinoid receptors like GPR55, GPR199, and TRPV1. Indeed, CB_1R activation can elicit hyperphagia or hypophagia depending on the cell population on which it is expressed. Selective activation of CB_1Rs located on presynaptic glutamatergic terminals in hypothalamic networks account for the orexigenic effects of THC, while selective activation of CB_1Rs expressed on presynaptic GABAergic terminals account for the anorexigenic effects of THC (Bellocchio et al. 2010; Matias and Di Marzo 2007). The balance between inhibitory and excitatory endocannabinoid transmission in the brain and endocannabinoid signaling in the gut differentially influences the action of orexigenic hormones (ghrelin, glucocorticoid, orexin, melanin-concentrating hormone), anorexigenic peptides (leptin, cholecystokinin, corticotropin-releasing hormone), and ventral

striatal dopamine transmission that all affect feeding behavior and energy balance (Bellocchio et al. 2010; Di Marzo et al. 2001; Di Marzo et al. 2009b; Matias and Di Marzo 2007; Silvestri and Di Marzo 2013). The inconsistent findings regarding the effects of THC on energy balance underscores the need for more empirical research aimed at uncovering additional factors that dictate the effects of THC on energy balance.

Effects of THC on cognitive outcomes

Existing reports on the effects of THC on several cognitive domains are inconsistent (Broyd et al. 2016). Studies in humans have shown that recreational THC doses (2.6, 5.2, and 20 mg/kg THC cigarette, 2.4% THC NIDA cigarette) impair performance on tasks that require sustained attention and quick reaction time (Leirer et al. 1991; Lenne et al. 2010; Macavoy and Marks 1975; Perez-Reyes et al. 1988). Others found that smoking of 1.75% and 3.33% THC cigarettes had no effect on driving performance (Liguori et al. 2002). A recently published longitudinal study concluded that children with low intelligence quotient (IQ) were more likely to initiate cannabis use during adolescence, though adolescent cannabis use was not associated with further cognitive decline (Meier et al. 2018). Conversely, preliminary findings from an ongoing longitudinal study demonstrate that medicinal marijuana use is associated with modest improvement in some aspects of executive function (Gruber et al. 2016).

It can be difficult to establish a definite cause-effect relationship between marijuana use and cognitive deficits in human subjects because evidence of reciprocal casualty exists. Cognitive dysfunctions can precede the initiation of marijuana use or can occur because of repeated use in vulnerable individuals and thwart successful substance abuse treatment outcomes. Other extraneous variables can also complicate result interpretation. One of such variables is the ratio of THC to cannabidiol (CBD) present in recreational and medicinal marijuana products. CBD, the primary non-psychoactive component of marijuana, antagonizes some of the effects of THC and is purported to confer the medicinal benefit of the marijuana plant. Marijuana cultivated for recreational use has higher THC to CBD ratio compared with those grown for medical purposes (Volkow et al. 2017; Wilson et al. 2019). Moreover, the THC and CBD contents of recreational marijuana can differ between geographical locales. In general, the use of high potency cannabis that is increasingly available is especially harmful to the global

cognitive wellbeing of adolescents (Wilson et al. 2019). An epidemiological study published in The Lancet estimated that frequent use of marijuana with high THC content is associated with increased risk of mental illness (Di Forti et al. 2019). The researchers, however, relied on self-reports of cannabis use that were not verified with biological measures (e.g., quantification of cannabinoids and their metabolites in urine, saliva, blood, or hair samples), and they did not adequately control for many lifestyle factors, including co-use of medications or other drugs of abuse.

Because the results from studies of human marijuana use are confounded by several extraneous factors that are beyond the control of researchers, preclinical studies are necessary. Rodent studies have documented cognitive impairment (Cha et al. 2007; Han et al. 2012; Murphy et al. 2017; Varvel et al. 2005; Zamberletti et al. 2015) and lack of impairment (Bilkei-Gorzo et al. 2017; Cha et al. 2007) due to acute or chronic THC administration. The detrimental effects of THC on spatial and non-spatial learning can differ based on age and sex. Persistent working memory deficits and repetitive behaviors were observed in rodents chronically dosed with 3 mg/kg THC (i.p.) during adolescence (P28–P48) but not during adulthood (P69–P89) (Murphy et al. 2017). Interestingly, the detrimental effects in adolescents were reversed when THC and CBD (3 mg/kg each) were co-administered (Murphy et al. 2017). That finding agrees with the view that CBD can reverse some detrimental effects of THC. Another study that included adolescent and adult male and female rats found that 5 and 10 mg/kg THC (i.p.) acutely disrupted both spatial and non-spatial learning to a greater extent in adolescent and female rats compared with adult and male rats, respectively (Cha et al. 2007; Cha et al. 2006). The pronounced vulnerability of adolescents was not observed when testing occurred twenty-eight days after the end of a 21 consecutive-day exposure to 5 mg/kg THC (Cha et al. 2006), suggesting that the timing of drug treatment relative to behavioral testing is crucial. Conversely, researchers found that old mice chronically dosed with 3 mg/kg/day delivered via subcutaneously-implanted osmotic minipumps for 28 days demonstrated improved spatial memory along with enhanced synaptic marker proteins, enhanced hippocampal spine density, and favorable epigenetic changes (Bilkei-Gorzo et al. 2017). The neurobiological mechanisms that mediate the detrimental effects of acute or chronic THC exposure on cognitive functions are similar to those that mediate the effects of alcohol, and have been extensively reviewed elsewhere (Han et al. 2012; Keeley et al. 2015b; Mizrahi et al. 2017; Renard et al. 2017; Rubino

et al. 2015; Rubino et al. 2009; Rubino et al. 2008; Zamberletti et al. 2015). A better understanding of these mechanisms may help explain the relatively high susceptibility of adolescents and females to drug-induced cognitive deficits.

Is the use of THC a risk factor for metabolic syndrome?

CB_1 and CB_2 receptors are ubiquitously expressed in the nervous system and peripheral tissues (liver, skeletal muscle, pancreas, and fat) involved in energy homeostasis (Mackie 2005; Onaivi et al. 2006). It is little wonder that THC plays an important role in the regulation of energy balance and metabolism (Gatta-Cherifi and Cota 2016; Mazier et al. 2015; Pagotto et al. 2006; Silvestri and Di Marzo 2013). Chronic activation of peripheral and central cannabinoid receptors by THC (especially CB_1R) induces glucose intolerance, adipose tissue insulin resistance, and visceral adiposity in relatively healthy humans (Hollister and Reaven 1974; Maccarrone et al. 2015; Matias and Di Marzo 2007; Muniyappa et al. 2013; Podolsky et al. 1971). In parallel with this observation, pharmacological antagonism of CB_1Rs reduces dyslipidemia and glucose intolerance in overweight and obese individuals (Matias and Di Marzo 2007; Triay et al. 2012). In rats, CB_1R antagonism with AM251 can reverse the glucose intolerance caused by acute 10 mg/kg AEA (Bermudez-Siva et al. 2006). Additionally, endogenous and exogenous cannabinoids acting through CB_1R activation stimulate appetite for dietary fat (Di Marzo and Matias 2005; DiPatrizio et al. 2013), slow gastric emptying (McCallum et al. 1999), and reduce energy expenditure (Mazier et al. 2015), thereby permitting greater lipid absorption and adipose tissue expansion (especially the expansion of white adipocytes). Prolonged high-fat diet consumption has also been associated with neuroinflammation and injury to the mediobasal hypothalamus (MBH) that can perpetuate metabolic syndrome in humans and rodents (Thaler et al. 2012; Valdearcos et al. 2018). The observation that elevated circulating levels of AEA and 2-AG are related to abdominal adiposity led some to propose that marijuana use engenders metabolic dysregulation in obese but not in normal weight subjects (Cote et al. 2007; Di Marzo et al. 2009a; Gatta-Cherifi and Cota 2016). The reasons for such findings are unclear and may be related to hypothalamic pituitary adrenal (HPA) axis dysregulation in obese individuals. Preclinical research shows that expression of glucocorticoid-induced metabolic dysregulation required peripheral and central CB_1R activation (Bowles et al. 2015). Moreover, chronic

corticosterone treatment increased hepatic and circulating levels of AEA, suggesting a cross-talk between peripheral endocannabinoid signaling and the hypothalamic pituitary adrenal axis in regulation of energy homeostasis (Bowles et al. 2015).

Not all human and rodent studies support the notion that cannabis use is detrimental to cardio-metabolic health. Administration of THC via inhalation (but not via intravenous injection) had no effect on glucose tolerance and plasma insulin levels in healthy non-obese males (Hollister and Reaven 1974; Permutt et al. 1976). Another study observed no effect of cannabis smoking on pancreatic β-cell function and lipid profiles in men and women (Muniyappa et al. 2013). In fact, others have shown that chronic cannabis smoking was associated with low BMI, low abdominal fat, favorable lipid profiles, and improved insulin sensitivity (Penner et al. 2013; Warren et al. 2005). Several prominent cross-sectional and longitudinal studies (NHANES, NSDUH, and CARDIA) consistently found null or negative association between marijuana use and indices of metabolic syndrome [though a cross-sectional analysis of data from CARDIA uncovered a modest positive association between marijuana use and incidence of prediabetes (reviewed in (Sidney 2016)]. Furthermore, administration of THC and JZL195 (a potent inhibitor of the enzymes that break down AEA and 2-AG) to transgenic mice expressing desensitization-resistant CB_1Rs did not result in positive energy balance or impaired glucose homeostasis when compared with wild-type controls (Marcus et al. 2016). Collectively, the effects of marijuana use on diabetes risk in humans may be 1) dose-dependent, where large doses may favor glucose intolerance, 2) route-of-administration-dependent, and 3) minimized by the development of tolerance to recreational doses in regular users. Given the inadequate control of genetic and lifestyle factors that often confound human research, more empirical evidence is needed before a definite association between THC exposure and metabolic syndrome can be established.

Alcohol and THC co-use

Regardless of the imposed restrictions on their accessibility, alcohol and marijuana remain the most widely used legal and illegal substance in most countries, respectively. In the year 2016, an estimated 137 or 24 million Americans over the age of 12 reported current use of alcohol or marijuana, respectively (SAMHSA 2017). The high prevalence of alcohol and marijuana use increases the occasions of their co-use, especially by adolescents and young adults (Briere et al.

2011; Pape et al. 2009; Subbaraman and Kerr 2015). Co-use of both drugs tends to be favored under specific social contexts (Lipperman-Kreda et al. 2018), and for different reasons, including use for self-medication, for relaxation, to be "cross-faded," or to be social and conform to peers (Lukas and Orozco 2001; O'Hara et al. 2016; Patrick et al. 2018; Skalisky et al. 2019). Consequently, and in parallel with the animal literature, adolescents who regularly consume alcohol are likely to use marijuana and vice versa (Grella et al. 2014; O'Hara et al. 2016).

Effects on energy balance and glucose homeostasis

To my knowledge, no study has documented the effects of alcohol and THC co-use on food intake, energy balance, and glucose homeostasis. Considering the vast literature that links alcohol or cannabis use with the incidence of metabolic diseases (reviewed above), it will be important to understand how the combined use of both drugs may predispose an individual to metabolic syndrome.

Effects on cognitive outcomes

Investigations of the effects of alcohol and marijuana co-use on memory and performance have arrived at dissimilar conclusions. In some studies, alcohol and THC co-use impaired psychomotor performance of males and females in a driving simulator (Downey et al. 2013; Perez-Reyes et al. 1988; Sutton 1983). Researchers have also investigated the additive or synergistic pharmacodynamics interaction between alcohol and THC. Additive interaction of two drugs on a particular variable is approximately equivalent to the arithmetic sum of the individual effect of each drug; while synergistic interaction is when the combined effect of the two drugs is supra-additive. In one study, the additive or synergistic interaction between alcohol and THC varied as a function of BEC, where higher peak BECs (e.g., approximately 110 mg/dl) were associated with worse performance outcomes compared with lower peak BECs (e.g., < 60 mg/dl) (Perez-Reyes et al. 1988). Others have found no additive effects of alcohol and THC combination on performance in specific measures of driving ability (Lenne et al. 2010; Liguori et al. 2002). In a within-subject cross-over study, low doses of alcohol (0.1 g/kg) or THC (2.5 mg/kg) administered to healthy male and female volunteers moderately impaired cognitive

performance but no additive or synergistic effects were uncovered when both drugs were combined (Ballard and de Wit 2011). Nevertheless, the presence of additive effects but lack of synergistic effects of combined alcohol and THC treatment on cognitive performance have been found by other researchers (Chait and Perry 1994; Chesher et al. 1976).

The lack of agreement between studies may be explained by the fact that volunteers in most of those studies were casual alcohol and marijuana users. Regular drugs users may not only metabolize a given amount of drug faster than infrequent users would, but also may experience lower degrees of the subjective effects. In a divided attention task, individuals with a lifetime of THC use showed no impairment under a specific alcohol + THC dose combination compared with never-users who demonstrated greater impairment (Macavoy and Marks 1975). Also, because most of the studies evaluated the effects of the drugs in a driving simulator, a more experienced driver may outperform a less experienced one (Lenne et al. 2010). The ability of an experienced drug user to adapt their behavior to perform optimally under shifting task demands (behavioral tolerance) may partly explain the paradoxical observations.

Past preclinical studies have examined the interactive effects of alcohol and THC on cognitive functions. Two rodent studies observed impairments in object recognition/working memory caused by additive or synergistic interactions between alcohol and THC (Ciccocioppo et al. 2002; Swartzwelder et al. 2012). In adult Marchigian Sardinian alcohol-preferring rats, neither moderate alcohol consumption nor 2 or 5 mg/kg THC (i.p.) impaired working memory, but their combination effectively impaired working memory (Ciccocioppo et al. 2002). The use of the genetically selected alcohol-preferring rats limits the extent to which the result can be generalized to other subjects. Furthermore, adolescent and adult Sprague-Dawley rats that received instant 1.0 mg/kg THC + 1.5 g/kg alcohol (i.p.) displayed memory impairment compared with when they received either drug alone (Swartzwelder et al. 2012). In that study, however, the acute effects of alcohol, THC, and their combination were studied in the same subjects, thus not controlling for drug tolerability or practice effect. Additionally, it was recently demonstrated that a potential mechanism by which combined exposure to alcohol and CB_1R stimulation may affect cognitive function is via reduced neurogenesis and increased cell death in the hippocampus (Khatri et al. 2018). More preclinical research on the cognitive effects of chronic moderate alcohol and THC co-use is needed.

Adolescent model of alcohol and THC co-use

In both humans and animal models, adolescence marks the end of childhood and the beginning of adulthood. It is a developmental stage characterized by robust synaptic pruning, change in hormone and neurotransmitter receptor densities, as well as the maturation and remodeling of brain circuits that orchestrate cognitive, emotional, reproductive, and reward-related behaviors (Crews et al. 2007; Sisk and Foster 2004; Spear 2000). It is hypothesized that adolescents may be more sensitive to the rewarding effects, but less sensitive to the aversive effects of alcohol and exogenous drugs compared with adults (Crews et al. 2016; Nixon and McClain 2010; Spear 2011). This combination can facilitate the transition from experimentation with drugs to excessive use by vulnerable individuals.

Alcohol and cannabis co-use is typically initiated during adolescence (Briere et al. 2011; Grella et al. 2014; Pape et al. 2009). According to the 2017 Monitoring the Future survey, over 60% and 45% of high school seniors have experimented with alcohol and marijuana/hashish in their lifetime, respectively (Johnston et al. 2018). Adolescent exposure to either drug can result in unwanted changes in brain areas that regulate cognitive, appetitive, affective, and reward-related behaviors (Ellgren et al. 2008; Gulley and Juraska 2013; Koss et al. 2012; Philpot et al. 2009; Renard et al. 2017; Rubino et al. 2015; Rubino et al. 2008). For example, exposure to alcohol or THC during adolescence can alter the maturation of the prefrontal cortex (PFC) – the principal brain region important for executive function control (Koss et al. 2012; Rubino et al. 2015). Compared with their separate use, the co-use of alcohol and THC during adolescence predicts poor academic achievements and is believed to be more detrimental to general health and safety (Subbaraman and Kerr 2015; Yurasek et al. 2017). In fact, when alcohol or cannabinoid use begins during adolescence, there is a greater chance that problem drug use and other psychopathology will manifest in adulthood (Crews et al. 2007; Crews et al. 2016; Renard et al. 2014; Spear 2016; Spear 2018; Wilson et al. 2019). Finally, given that adolescents are sensitive to lasting metabolic alterations caused by alcohol and cannabinoid use (Liang and Chikritzhs 2014; Renard et al. 2014), studies that systematically examine the mechanisms that mediate the effects of alcohol and THC co-use on metabolic and behavioral outcomes during adulthood are needed.

CHAPTER 2: DISSERTATION OBJECTIVES

Food intake and preference are governed by a myriad of neurohormonal and mnemonic mechanisms (Schneider et al. 2013; Schwartz et al. 2000). In addition, exogenous substances like alcohol and cannabinoids alter appetite, energy homeostasis, and cognitive functions. Unhealthy ingestive behavior is responsible, at least in part, for the global economic burden of metabolic syndrome (a collection of health conditions that include obesity, dementia, type 2 diabetes, and cardiovascular diseases) and several psychiatric disorders. A clear understanding of the mechanisms by which alcohol and cannabinoid use are culpable for the metabolic syndrome interests the general public and the entire medical community (Muniyappa et al. 2013; Sidney 2016; Suter 2005; Yeomans 2010).

Prevailing research on the link between alcohol use and metabolic syndrome, especially type 2 diabetes mellitus, often arrives at contrasting conclusions (Goldberg et al. 1999; Phillips and Safrit 1971; Pietraszek et al. 2010). Individuals who adhere to the Dietary Guidelines for Americans drink alcohol in moderation for its mood-elating and stress-reducing effects (DeSalvo et al. 2016; Eckardt et al. 1998). Similarly, most rodents do not voluntarily consume alcohol to the point of intoxication (Crabbe et al. 2011; Nelson et al. 2016). Because low-to-moderate alcohol consumption alters neurophysiology and peripheral organ functions (Cui and Koob 2017; Eckardt et al. 1998; Molina et al. 2014), it is likely that moderate alcohol consumption will alter ingestive behaviors, glucose metabolism, and cognitive functions.

Δ^9-tetrahydrocannabinol (THC) is recognized as the main psychoactive cannabinoid derived from the marijuana plant. THC primarily acts as a partial CB_1 receptor agonist. THC can act in the central nervous system to stimulate appetite for palatable/energy-dense foods in most (Foltin et al. 1988) but not all studies (Graceffo and Robinson 1998). THC can also stimulate CB_1 and CB_2 receptors in peripheral tissues to trigger metabolic dysregulation (Matias and Di Marzo 2007; Silvestri and Di Marzo 2013). Anecdotal and epidemiological reports show that marijuana use impairs cognitive functions (Bolla et al. 2002; Tart 1970), but such observations are not universal across a variety of experimental conditions (Broyd et al. 2016; Pattij et al. 2008). More empirical data are needed to clarify how THC affects feeding and cognitive behaviors.

Despite the popularity of alcohol and the increasing public acceptance of marijuana, research on the effects of chronic moderate alcohol and THC co-use on energy balance and cognitive functions are scarce. Empirical knowledge of the mechanistic links between their co-use and development of metabolic syndrome will facilitate therapy development to mitigate the detrimental outcomes associated with their use. We predict that, compared to their separate use, alcohol and THC co-use will predispose an individual to overweight, type 2 diabetes, and cognitive deficits via triggering pronounced and lasting neurobiological dysregulations in vulnerable subjects like adolescents who are predisposed to co-use both drugs (Lipperman-Kreda et al. 2018; Yurasek et al. 2017).

Adolescence, the developmental stage between childhood and adulthood, is characterized by thrill-seeking behaviors, including experimentation with alcohol and drugs of abuse. Because of the systematic neurobiological reorganizations that occur during adolescence, the brains of adolescents are particularly sensitive to the harmful influence of exogenous substances like alcohol and cannabinoids (Gulley and Juraska 2013; Spear 2000; Spear 2015). The endocannabinoid system plays a crucial role in the neurobiological remodeling that occurs during adolescence (Rubino et al. 2015; Spear 2016). Chronic exposures to alcohol or cannabinoids can alter the expression and function of components of the endocannabinoid system to produce abnormal neuroplastic changes (Ceccarini et al. 2013; Ellgren et al. 2008; Farquhar et al. 2019; Rubino et al. 2008) in brain circuits that regulate feeding, cognitive behavior, and energy metabolism.

The **objective** of my dissertation is to determine how separate or combined alcohol and THC use affects energy balance, metabolic fitness, and cognitive outcome using an adolescent rat model. My *central hypothesis* was that repeated alcohol or THC use would change food intake and negatively alter glucose homeostasis and cognitive functions. I further predicted that alcohol or THC use during adolescence would alter the expression of key protein/enzyme in the brain (CB_1R and GSK-3β) during abstinence, and would prime the subject for heightened metabolic dysregulation upon subsequent physiological challenge (high-fat diet consumption) in adulthood. Importantly, I expected that, compared to separate drug use, combined alcohol and THC use would uniquely affect energy balance and be associated with more detrimental effects

on glucose homeostasis, cognitive outcomes, and neurobiology. I tested my hypotheses in two specific aims outlined below:

Aim 1: Examine the effects of alcohol on energy balance, memory, and emotion-like behaviors.

My *working hypothesis* was that adolescent-onset chronic intermittent alcohol consumption would alter memory, emotion-like behaviors, and glucose tolerance during adulthood. Experiment one (Chapter 3) tested this hypothesis by employing conventional rodent behavioral tasks and an oral glucose tolerance test. This experiment also explored potential sex differences.

Aim 2: Investigate the acute or long-term effects of adolescent alcohol and THC exposure, alone or in combination, on energy balance, glucose homeostasis, and cognitive behaviors.

My *working hypotheses* were that, compared with the effects of their separate use, chronic moderate alcohol and THC co-use during adolescence would 1) uniquely alter caloric intake, cognitive behaviors, glucose homeostasis, and neurobiology, and 2) elicit worse metabolic outcomes following a high-fat diet (HFD) challenge in adulthood. Experiment two (Chapter 4) established a rodent model of alcohol and THC co-use and used it to examine the effects of adolescent drug exposure on food intake, body weight, and cognitive behaviors during treatment and abstinence. Experiment two also assessed the effects of drug exposure on behavioral anhedonia, glucose homeostasis, and changes in brain CB_1R protein expression and GSK-3β activity during abstinence. Experiment three (Chapter 5) assessed whether adolescent drug exposure would alter ingestive behaviors and metabolic response following a period of HFD consumption during young adulthood. HFD accurately models the typical diet of human drug users.

CHAPTER 3: CHRONIC MODERATE ALCOHOL DRINKING ALTERS PLASMA INSULIN CONCENTRATION WITHOUT AFFECTING COGNITIVE AND EMOTION-LIKE BEHAVIORS IN RATS[1]

Abstract

Because the consumption of alcoholic beverages prevails in society, its effects on diabetes risk is a subject of interest. Extant literature on this topic often disagrees. Here, we probed the effects of chronic moderate alcohol consumption on glucose metabolism in rats. The effect of chronic moderate alcohol drinking on depression- and anxiety-like behaviors and memory was also explored. Adolescent male and female Long-Evans rats consumed saccharin-sweetened 5% (one week) and 10% ethanol (seven weeks) under a 7.5-h/day (Monday–Friday) access schedule. This exposure was followed by sucrose preference and elevated plus maze (EPM) tests during an intervening week before a 6-week intermittent access (Monday, Wednesday, Friday) to 20% unsweetened ethanol in a 2-bottle choice drinking paradigm was implemented (EtOH). A free-feeding control group received water (Water). Our prior work revealed that voluntary ethanol consumption decreases food intake in rats. Hence, a second control group that received water was mildly food-restricted (FR) and their average body weight was matched to that of the EtOH group. During the week following week 6 of intermittent access to 20% ethanol, rats were submitted to sucrose preference, EPM, and novel object recognition (NOR) tests. Insulin response to a glucose load was subsequently assessed via an oral glucose tolerance test (OGTT). Rats attained and maintained blood ethanol concentrations of ~55 mg/dl that correlated with the dose of sweetened 10% ethanol ingested within 1–3 h. Relative to intake by Water controls, EtOH rats consumed less chow. There was no body weight difference between both groups. Neither sex of EtOH rats showed increased depression- and anxiety-like behaviors, as respectively measured by sucrose preference and EPM, nor did they show deficit in object recognition memory during abstinence. Male EtOH rats, however, showed signs of reduced general activity on the EPM. During OGTT, male EtOH rats showed a time-dependent

[1] This chapter is an updated version of a manuscript previously published as: Nelson NG, Suhaidi FA, Law WX, Liang NC (2017) Chronic moderate alcohol drinking alters insulin release without affecting cognitive and emotion-like behaviors in rats Alcohol 70:11-22 doi:10.1016/j.alcohol.2017.12.001. The copyright owner has granted permission to reprint.

potentiation of insulin concentration for proper glucose clearance. Such an effect was not observed in females. This preliminary study shows that chronic moderate alcohol consumption can have negative metabolic consequences in the absence of overt behavioral deficits, especially in males.

Introduction

Alcohol consumption is a behavior that prevails in society. Most drinkers consume alcohol in moderation (14–30 g or 1–2 drinks/day), especially with meals and during special occasions. Excessive drinking, however, is common among adolescents and young adults (Crews et al. 2007; SAMHSA 2015). Problematic drinking behavior is strongly associated with risk of developing alcohol use disorder, bodily injury and death, poor academic performance, delinquent behaviors, and many health consequences (Burton and Sheron 2018; Crews et al. 2007; Klarich et al. 2015; Saitz 2005; Siqueira et al. 2015; White and Hingson 2013). Habitual alcohol use can be deleterious to multiple organ systems (Eckardt et al. 1998; Molina et al. 2014). Habitual alcohol use has also been associated with ill health outcomes, including hyperglycemia and insulin resistance, which are core features of diabetes mellitus (Fernandez-Sola 2015; Metz et al. 1969).

Diabetes mellitus is a debilitating metabolic disease that affects over 400 million people worldwide, and this number is projected to exceed 600 million by 2040 (Ogurtsova et al. 2017). The American Diabetes Association declares that 1.5 million Americans are diagnosed with diabetes yearly, and diabetes ranks as the seventh leading cause of death in America (American Diabetes Association 2018). Management of diabetes and its associated health complications cost American tax payers a staggering $327 billion in 2017 (American Diabetes Association 2018). Clinicians and other health-care experts emphasize the necessity for avoidance of obesogenic diets in favor of exercise and pharmacotherapy or cell-based therapy in diabetes care (Knowler et al. 2002; Kobayashi 2006). Unfortunately, insufficient attention has been directed to the contribution of a modifiable lifestyle risk factor such as alcohol consumption to the diabetes epidemic.

Reports on the association between alcohol consumption and diabetes risk in men and women are equivocal. Several investigators posit that regular moderate (not excessive) alcohol consumption has no effect on insulin sensitivity and cardiovascular health (Cordain et al. 2000;

Goldberg et al. 1999; Zilkens et al. 2003), or could improve indices of insulin sensitivity and efficient glucose utilization in both men and women (Baliunas et al. 2009; Carlsson et al. 2005; Kao et al. 2001; Kiechl et al. 1996; Koppes et al. 2005; Pietraszek et al. 2010). Others, however, caution against alcohol use by pre-diabetic individuals or those predisposed to diabetes due to their genetic or environmental endowments (Babor et al. 2012; Baliunas et al. 2009; Greenhouse and Lardinois 1996; Pietraszek et al. 2010; Vaeth et al. 2014). The supposed benefits of moderate alcohol use by healthy individuals may not generalize to others due to the possibility of interaction with medications or detriment to general health (Emanuele et al. 1998; Fernandez-Sola 2015; Greenhouse and Lardinois 1996; Molina et al. 2014). The few studies that investigated the potential for sex differences in the link between moderate alcohol consumption and diabetes risk often disagree (Kao et al. 2001; Koppes et al. 2005). Hence, more empirical studies on the topic are warranted.

Sex differences in ethanol intake often appear during human adolescent years, with men consuming more alcohol than women did (SAMHSA 2015; Schulte et al. 2009). This trend also persists into adulthood (Schulte et al. 2009). Findings from rodent studies are inconsistent. Around adolescence (P35–P48), male rats can consume higher ethanol doses relative to females (Lancaster et al. 1996; Vetter-O'Hagen et al. 2009). Others reported no sex difference in rodent drinking behavior during adolescence (Doremus et al. 2005). We and others, however, have shown that adult female rats consistently consume more ethanol than males do (Broadwater et al. 2013; Lancaster et al. 1996; Nelson et al. 2016; Sherrill et al. 2011b). Hence, it would be important to investigate how sex differences in ethanol intake will translate into contrasting vulnerabilities to developing type 2 diabetes in male and female rats.

Disagreements between findings from clinical studies and meta-analysis conducted to date illustrate the complexity of this topic. In human studies, subtle variations in how data were collected, handled and interpreted limit comparison between findings from different labs (Goldberg et al. 1999; Koppes et al. 2005; Waki et al. 2005). Most human studies utilize questionnaires that rely on the ability of subjects to recall details of their drinking episodes. In situations where comprehensive medical records are available (Babor et al. 2012; Chen and Garcia-Webb 2014), the chronicle of alcohol consumption is often lacking or inaccurate at best (Saitz 2005). Preclinical animal studies on the topic are sparse. It was recently shown that

chronic alcohol consumption impaired glucose metabolism and increased diabetes risk in outbred rats (He et al. 2006), experimental diabetic Otsuka Long-Evans Tokushima Fatty (OLETF) rats (Lee et al. 2015), and aldehyde 1B1 knockout mice (Singh et al. 2015). This was indicated either by hyperglycemia during an intraperitoneal glucose challenge test or altered insulin signaling. It is noteworthy that, in addition to the use of modified rodent strains as subjects in two of those studies, when rodents were exposed to high ethanol doses (5–13 g/kg/day) involuntarily or via ethanol-containing diet as the only calorie source, blood ethanol concentrations (BECs) were seldom reported, and potential sex differences were not investigated.

Several human and rodent studies have documented impaired cognitive functions due to alcohol administration that was associated with a myriad of detrimental brain changes (Coleman et al. 2011; Obernier et al. 2002; Pascual et al. 2007; Vetreno et al. 2016). Studies that found learning and memory impairments in rodents employed binge-like ethanol doses via involuntary ethanol administration (Obernier et al. 2002; Pascual et al. 2007). Binge-like ethanol doses result in intoxicating BECs (e.g., $\geq$ 80 mg/dl) that is often accompanied by noticeable motor-incoordination (Carnicella et al. 2014; Sherrill et al. 2011b). Since outbred rats tend not to voluntarily consume ethanol to achieve intoxicating BECs (Furuya et al. 2003; Nelson et al. 2016), the extent of cognitive decline, if any, remains to be seen with voluntary ethanol intake.

The aim of the present study was to investigate the consequences of chronic moderate ethanol consumption on emotion-like and cognitive behaviors and glucose metabolism. We utilized two different rodent voluntary alcohol consumption paradigms that promote moderate-to-high ethanol drinking and produce BECs of approximately 40–100 mg/dl during the initial 30–120 min of access in Long-Evans rats (Barson et al. 2015; Carnicella et al. 2014; Nelson et al. 2016; Simms et al. 2008). Behavioral assays such as sucrose preference, elevated plus maze (EPM), and novel object recognition (NOR) tests were employed to respectively evaluate depressive-like, anxiety-like, and cognitive behaviors after weeks of moderate alcohol drinking. Glucose tolerance and insulin release were then assessed via oral glucose tolerance test (OGTT). We hypothesized that chronic moderate alcohol consumption will alter performance on the above behavioral tasks during abstinence. We further expected that long-term moderate alcohol consumption would lead to impaired glucose homeostasis.

Materials and methods

Subjects and housing

Male and female Long-Evans rats (n = 38/sex) that weighed 80–97 g (postnatal day 33, P33) upon arrival from Envigo (Indianapolis, Indiana) were the subjects. The colony was maintained in a climate-controlled vivarium where rats were individually housed in conventional laboratory cages on a 12-h/12-h light/dark photoperiod (lights on at 11:30 AM). Rats had *ad libitum* access to water and standard rodent chow (3.1 kcal/g; 24% protein, 58% carbohydrate, 18% fat from soybean oil; Harlan 2018, Madison, Wisconsin) during habituation. Chow and fluid accesses during experimental manipulations are described in the "Procedures" below. All experimental procedures were approved by IACUC at the University of Illinois, Urbana-Champaign and were in accordance with the guidelines of the National Institutes of Health.

Test fluids

0.1% saccharin solutions were prepared by mixing sodium saccharin (Sigma) with tap water. Sweetened ethanol solutions (5% and 10% v/v) were prepared by diluting 200 proof (100%) ethanol (Decon™ Labs, Inc., King of Prussia, Pennsylvania) with 0.1% saccharin. The 20% ethanol and 1% sucrose solutions were respectively prepared by diluting ethanol and sucrose (Sigma) with tap water. Fluids were presented in 250 ml glass bottles fitted with stainless steel sipper tubes that minimized spillage and evaporation. Bottles were weighed (to the nearest 0.1 g) to determine fluid intake. During the experiment, ethanol from a previous drinking session was discarded and replaced with freshly-prepared solutions before presentation to the rats. Water was replaced every 3–5 days.

Procedures

The experiment was performed with two separate cohorts of rats (cohort 1: n = 17/sex; cohort 2: n = 13/sex). Another set of 16 rats (n = 8/sex) was received from the same vendor during week 7 of experimental procedures on cohort 2. They were used as a second control group as described in the "Procedure for food restricted (FR) group" below. After habituation, rats in each of the two cohorts were divided into EtOH and Water groups. Both groups had *ad libitum* access to chow except during food deprivation that preceded the OGTT. Experimental manipulations began when rats were juveniles (P39). Two paradigms of intermittent access to ethanol were

used, i.e., rats did not receive ethanol every day. This intermittent nature of ethanol consumption is to better parallel the intermittent drinking patterns in people who drink moderately (Eckardt et al. 1998).

The first ethanol administration paradigm was a five consecutive day (Monday–Friday/week) drinking schedule. All rats in the EtOH groups were introduced to the sweet taste of 0.1% saccharin solution from 8:00 AM to 3:30 PM (i.e., the last 3.5 h of the dark cycle and the first 4 h of the light cycle) to acquire voluntary drinking during this period. We chose this duration and timing of alcohol presentation based on the facts that 1) rats are nocturnal animals and can consume substantial amounts of food and fluid during the final hours of the dark cycle and 2) it approximates human alcohol consumption around meal or rest time (Clevidence et al. 1995; Dixon et al. 2002). The following week, these rats had access to sweetened 5% ethanol, which was used to acclimate them to the ethanol plus 0.1% saccharin taste combination (Roberts et al. 1999). Subsequently, the 5% ethanol was replaced with 10% sweetened ethanol for the next seven weeks. The Water groups had *ad libitum* access to water throughout. Body weight and chow consumption were recorded daily at 8:00 AM. During Monday–Friday, 7.5-h/day fluid intakes by the Water and EtOH rats were recorded at 3:30 PM after which ethanol bottles were replaced with water bottles. Hence, all rats had access to one water bottle during the succeeding 16.5 h. During weekends, no ethanol was provided and thus food and water intakes were measured only at 8:00 AM (24-h intake). All rats underwent the same behavioral tests during week 9 (Fig. 3.1).

After the week of behavioral evaluations, ethanol access for the EtOH groups was switched to the second ethanol administration schedule: a weekly Monday, Wednesday, Friday (24-h/day) intermittent access to 20% ethanol in a 2-bottle choice drinking paradigm. This paradigm was established by other alcohol researchers as a protocol that induces moderate-to-high ethanol drinking that often results in average blood alcohol levels of approximately 60 mg/dl within a short time (Barson et al. 2015; Carnicella et al. 2014; Simms et al. 2008). We applied it to encourage drinking and to determine how rats would respond to an ethanol solution devoid of sweetener (Roberts et al. 1999) after they have had prior exposure to sweetened ethanol (Broadwater et al. 2013). During regular 8:00 AM measurements on alcohol access days, EtOH rats were presented with simultaneous access to one bottle each of 20% ethanol and tap water for 24 h. Rats in the Water groups were presented with two bottles of tap water. The

placement of the ethanol (right or left) was alternated on each drinking session to avoid development of side preference. During the intervening days (e.g., Tuesday, Thursday, and weekend days), the ethanol bottle from the EtOH rats and one water bottle from the Water rats were removed so that all rats had unlimited access to one water bottle. On these intervening days, food and water intakes were measured at 8:00 AM (24-h intake). This schedule of three ethanol intake sessions per week occurred for six weeks. Subsequently, another series of behavioral tests were performed with all rats during weeks 15 and 16 (Fig. 3.1).

Procedure for food-restricted (FR) group

Studies in our lab show that voluntary ethanol consumption suppresses food intake and has no effect on weight gain in Long-Evans rats (Nelson et al. 2016). The additional rats received during cohort 2 experiment (n = 8/sex) served as an additional group employed to control for the food intake suppression effect of alcohol that might confound result interpretation. The procedures for these rats began during week 7 of cohort 2 experiment. These rats were mildly food-restricted while their average body weight was closely monitored and made equivalent to that of the EtOH group of the respective sex (cohort 1 and 2 combined). The FR rats had free access to water, and the daily chow ration was provided during 8:00 AM measurements. All experimental procedures (daily measurements and behavioral tests) with the FR group were the same with those for rats in cohort 1 and 2.

Blood ethanol concentration (BEC) analysis

To determine BEC reached under the 20% ethanol administration paradigm, tail blood was collected from all rats. During week 15 (the last week of intermittent-access drinking), rats were presented with 20% ethanol or water per the experimental procedure. Intakes were recorded and tail blood (80 μl) was collected into a heparinized microcapillary tube 3 h after ethanol presentation and 5 h after ethanol removal on an intervening day of the 2-bottle choice drinking procedure. Accordingly, blood collection from all FR rats occurred during week 15.

Tail blood for BEC measurement was not collected during the sweetened 10% ethanol drinking paradigm. To evaluate the resulting BEC at different time points under this drinking paradigm, we conducted a separate experiment with 18 alcohol-naïve Long-Evans rats (6 males, 12 females), and the age at which these rats drank overlapped with rats in this study. These rats

were the progeny of those initially obtained from Envigo and were housed in the same animal facility. Daily handling of these rats was consistent with what was performed in this study. Alcohol access began when the rats were at P59–P61, and it was presented according to a 3-h/day (8:00–11:30 AM) Monday–Friday access schedule. The ethanol access time was reduced to 3 h from the 7.5 h because results from the original experiment showed that rats consume most of their ethanol dose within the first 3 h of the 10% ethanol access. Prior to tail blood collection, the rats had voluntarily consumed sweetened 10% ethanol for one week. On separate days during weeks 2 and 3 of drinking, ethanol intakes were measured and tail blood was collected immediately after 1- or 3-h access. Ethanol access under this schedule continued until Monday of week 5.

Blood samples were centrifuged at 1000 × g for 10 min at 4 °C after which plasma was collected and immediately stored at –80 °C. BEC was quantified using a colorimetric assay kit, which measures ethanol by an enzyme-driven oxidation reaction (STA-620; Cell Biolabs, Inc., San Diego, California) with a protocol supplied by the manufacturer. Ethanol concentrations were estimated using a standard curve per manufacturer's recommendations.

Behavioral tests

We employed a series of behavioral tests to examine the effects of chronic moderate ethanol consumption on cognitive and emotion-like behaviors. Elevated plus maze (EPM) and novel object recognition (NOR) tests were performed in the middle of the light cycle after 1 h habituation in a room adjacent to the sound-attenuated testing room. Both rooms were illuminated to approximately 800 lux by white fluorescent lights. A concealed ceiling-mounted camera connected to a computer in an adjacent room was applied to allow real-time behavioral analysis and video recording for offline analysis by individuals blind to group assignments. Between each animal, the apparatuses were cleaned with a non-alcohol based cleaning agent (Coverage Spray; STERIS Corp., St. Louis, Missouri). Behavioral data presented are the average values from three trained scorers.

Sucrose preference

Among the constellation of symptoms that qualify for the diagnosis of clinical depression is anhedonia – the avoidance of an otherwise pleasurable stimulus (Anisman and Matheson 2005). Behavioral anhedonia was assessed by sucrose preference test (Monteggia et al. 2007). The tests were performed within three to seven days after the end of each ethanol drinking paradigm (P102, P103, P106, and P153; Fig. 3.1). Using the classic 2-bottle choice procedure (Monteggia et al. 2007), the rats were presented with two bottles, 1% sucrose vs. tap water, immediately after 8:00 AM measurements. The positions of the bottles were alternated 12 or 24 h later to prevent the development of location preference for drinking. Sucrose preference ratio is equal to $\frac{\text{sucrose intake (g)}}{\text{sucrose + water intake (g)}} \times 100$.

EPM

The EPM procedure we employed was adapted from that described elsewhere (Barson et al. 2015; Walf and Frye 2007). The black wooden EPM had two open arms (OA), 50 cm × 10 cm, and two enclosed arms (CA), 50 cm × 10 cm with 40 cm high walls. All arms had an open roof. The OA and CA were elevated from the ground to a height of 93.5 cm. The tests were performed on day 6 of abstinence from ethanol (P105 and P154; Fig. 3.1). At the start of each trial, rats were placed at the intersection of the four arms of the plus-maze facing an OA and were allowed to explore for 5 min. Videos were scored for time spent on the OA, and for total arm entries. An arm (OA or CA) entry occurred when a rat had at least the fore paws on the arm (Barson et al. 2015). Time spent on the OA was used as an index for anxiety-like behavior, while total arm entry (OA + CA entries) was used as a measure of general activity level in the maze (Carobrez and Bertoglio 2005; Monteggia et al. 2007; Walf and Frye 2007).

NOR

The NOR test exploits a rodents tendency to gravitate toward an unfamiliar object (Leger et al. 2013). Performance on this task is believed to be an assessment of hippocampus and perirhinal cortex learning and memory functions (Ennaceur et al. 1996; Gaskin et al. 2010). Prominent alcohol researchers have utilized this task to assess rodents' cognitive function (Pascual et al. 2007; Vetreno et al. 2016). The test was performed from day one to four of abstinence from

ethanol (P149–P152; Fig. 3.1). On day 1, rats were habituated to an open-field arena for 5 min. The arena was composed of a black-colored high-density polyethylene panel (100 cm × 100 cm) with raised sides (35.5 cm high) constructed of black Plexiglas®. The central arena (60 cm × 60 cm) was demarcated with white correction fluid. The open-field apparatus was elevated to 70 cm above the floor. Videos were scored for the number of crossings into and time spent in the central arena. Number of crossings was considered a metric of general activity level (Hale et al. 2008; Hamezah et al. 2017). On days 2 and 3, rats explored two identical objects positioned in opposite corners along the border of the central arena. On day 4, one of the sample objects was replaced by a novel object. The position of the novel object (right or left) during the object recognition trial was alternated between rats to avoid the possible influence of side preference. Videos were scored for time spent exploring each object. Exploratory behaviors included direct contacts with the objects: touching while gazing at or sniffing within 3 cm of the objects. Non-directed contacts or climbing an object without sniffing was not counted. Data from an EtOH male rat that did not approach either object on test day 4 were excluded from analysis. The percent time spent exploring the novel object relative to the total time spent exploring both objects was computed. A score above 50% signifies novel object recognition.

Oral glucose tolerance test (OGTT) and plasma insulin ELISA

From P149 after alcohol availability was discontinued, all rats had unlimited access to tap water. Rats in cohorts 1 and 2 were evenly divided into two groups for OGTT performed on the eighth (P156) or ninth (P157) day after ethanol access cessation. Accordingly, OGTT with the FR groups was conducted on P156. After an overnight 12-h fast with only water available, OGTT began at 12:30 PM, during which fasting blood glucose was measured (baseline) from tail blood using a handheld glucometer (AlphaTRAK2 blood glucose test strips, Abbott Labs). Subsequently, blood (80 μl) was collected into heparinized capillary tubes for fasting plasma insulin determination. Rats were then challenged with glucose (2.0 g/kg body weight, 20% glucose prepared with distilled water) administered by oral gavage. Blood glucose concentrations were measured at 15, 30, 60, and 120 min post-glucose challenge. Blood was drawn at those time points for plasma insulin measurements.

Blood samples were kept on ice before immediate centrifuge at 1000 × g and 4 °C for 15 min. Plasma was collected and stored at –80 °C until used for insulin measurements. Plasma

insulin was quantified using a commercially available rat insulin ELISA kit (ALPCO; Salem, New Hampshire), according to the manufacturer's instructions.

Data analysis

Total caloric intake (kcal) comprised the sum of calories from chow (3.1 kcal/g) and ethanol (7 kcal/g) for EtOH rats, or calories from only chow for Water and FR rats. BEC data were analyzed by one-way ANOVA. Sucrose preference, EPM, NOR, fasting glucose, fasting insulin, and area under curve (AUC) of the glucose and insulin data were analyzed by two-way ANOVA with group and sex as factors. EPM and NOR data of each sex were also independently analyzed by one-way ANOVA. Ethanol dose was analyzed by two-way repeated measures ANOVA, with time (in weeks) as a repeated measure. Caloric intake, body weight, glucose, and insulin data were analyzed by repeated measures factorial ANOVA with group, sex, and time as factors. Significant main effects and interactions ($p < 0.05$) were accompanied with Fisher LSD post hoc tests. Pearson correlation was used to assess the linear relationship between ethanol dose and the ensuing BEC. All data are presented as mean ± standard error of the mean (SEM), and analyses were conducted in Statistica 13.1 (Dell Inc.; Round Rock, Texas). Parts of data from the cohort 1 experiment (n = 17/sex) were reported in our previous publication (Nelson et al. 2016).

Results

Ethanol intake and BEC

Ethanol intake dose (g of pure ethanol per kg of body weight) is presented as the daily average across 5 and 3 measurement days per week during the weekday- and intermittent-accesses, respectively. Throughout the experiment, females consumed higher ethanol doses than males did [sex effect, $F(1,32) = 29.86$, $p < 0.0001$; Fig. 3.2]. As we previously reported (Nelson et al. 2016), ethanol intake increased from week 1 to week 2 of drinking when the concentration of sweetened ethanol was switched from 5% to 10% [post hoc, male: 2.03 ± 0.14 g/kg to 2.49 ± 0.09 g/kg, female: 2.99 ± 0.10 g/kg to 3.50 ± 0.13 g/kg, both $p < 0.05$]. When the ethanol administration paradigm was changed from 10% during weekdays to intermittent 20%, ethanol, intake decreased significantly [time effect, $F(13,416) = 7.59$, $p < 0.0001$]. Ethanol intake reduced to a similar extent in male and female rats during the transition from 10% to 20% ethanol access, i.e., intake during the last week of 10% ethanol drinking relative to intake during

the first week of 20% ethanol drinking [post hoc, male: 2.19 ± 0.11 g/kg to 1.58 ± 0.20 g/kg (28% reduction), $p < 0.01$; female: 3.23 ± 0.14 g/kg to 2.21 ± 0.33 g/kg (33% reduction), $p < 0.0001$]. Despite the decrease, females maintained higher ethanol consumption relative to males.

On the day tail blood was sampled during the weekly Monday, Wednesday, and Friday intermittent access to 20% ethanol, females consumed more ethanol during the first 3 h than males did [1.06 ± 0.11 g/kg vs. 0.68 ± 0.10 g/kg, $F(1,32) = 7.10$, $p < 0.02$]. Our efforts to detect BEC at 3-h after ethanol presentation and 5 h after next-day ethanol removal were unsuccessful, as BECs at these time points were mostly below the detection threshold of our kit.

In the separate group of 6 male and 12 female rats given sweetened 10% ethanol, females consumed more daily ethanol dose than males did during the entire 21-day access [time and sex effects: $F(20,320) = 2.66$, $p < 0.0002$ and $F(1,16) = 14.63$, $p < 0.002$]. On blood sampling days during week 2 or 3 of drinking, the doses consumed after 1-h and 3-h accesses were measured and blood samples were collected for BEC measurement. Pearson correlations taking both male and female data into account indicated that BECs significantly correlated with ethanol intake after both 1-h [$r = 0.77$, $p < 0.0002$] and 3-h [$r = 0.68$, $p < 0.002$] access sessions (Fig. 3.3). The rats consumed 1.10 ± 0.08 g/kg and 2.29 ± 0.11 g/kg ethanol at the 1-h and 3-h sampling time points, respectively. BECs detected at these two time points were 52 ± 10 mg/dl and 59 ± 8 mg/dl, respectively. On the day tail blood was sampled, females consumed an average ethanol dose that was comparable to that consumed by males [1-h: $F(1,16) = 0.07$; 3-h: $F(1,16) = 0.10$, $p > 0.05$]. Likewise, there was no sex difference in the BEC attained at the aforementioned sampling points [1-h: $F(1,16) = 0.80$; 3-h: $F(1,16) = 0.45$, $p > 0.05$].

Caloric intake and body weight

Alcohol drinking resulted in reduced chow intake in male and female rats [group, sex, time, and group × sex × time effects: $F(2,35) = 19.62$, $F(1,35) = 405.93$, $F(14,490) = 42.01$, and $F(28,490) = 3.54$, respectively, all $p < 0.0001$]. EtOH males (post hoc, $p < 0.0002$) and females ($p < 0.02$) consumed less chow than their Water counterparts did (Fig. 3.4A and 3.4B). The significant main effects held true when data of the FR groups were excluded in another analysis [group, sex, and time effects: $F(1,28) = 18.87$, $F(1,28) = 316.36$, and $F(14,392) = 37.09$, respectively, all $p < 0.0002$]. During food restriction, the FR groups consumed their entire daily chow ration. Their

chow intake was significantly lower than that of the Water groups (post hoc for both sexes, $p < 0.002$). The average daily chow of the male and female FR groups was 87.4 ± 0.8% and 88.2 ± 1.7%, respectively, of intake by their corresponding free-feeding Water groups. The respective average daily chow (kcal) consumed by male and female FR groups was 95.2 ± 0.9% and 95.3 ± 1.8% of chow intake by the corresponding EtOH groups. Fluctuations in the amount of chow provided to the FR rats were pronounced early during the food-restriction procedure, especially in males. Despite these fluctuations, there were insignificant differences in amounts of chow consumed by EtOH and FR rats of both sexes (post hoc, $p > 0.05$).

Whereas the chow diet was the only energy source for the Water and FR groups, calorie from ethanol was another energy source for the EtOH rats. Alcohol consumed by the male and female EtOH rats contributed approximately 5% of their daily total caloric intake. Analysis of total caloric intake indicated significant group, sex, time, and group × sex × time effects [$F(2,35) = 17.58$, $F(1,35) = 395.20$, $F(14,490) = 47.74$, and $F(28,490) = 3.78$, respectively; all $p < 0.0001$]. Thus, total caloric intakes by Water and EtOH groups in both sexes did not differ (post hoc, $p > 0.05$). Furthermore, the respective average daily chow (kcal) consumed by male and female FR groups was 90.6 ± 0.8% and 89.9 ± 1.7% of total caloric intake by their corresponding EtOH groups (total caloric intake post hoc for both sexes $p < 0.004$). That is, the significant overall group effect of total caloric intake was presumably driven by fluctuations of the chow diet provided to the FR rats. With the exclusion of FR rats in another analysis, the group and group × sex × time effects disappeared [$F(1,28) = 2.24$ and $F(14,392) = 0.61$, $p > 0.05$]. Our analyses clearly indicate that 14 week moderate alcohol drinking did not alter total caloric intake in rats.

Male rats gained weight at a faster pace and weighed significantly more than females (Fig. 3.4C and 3.4D). Analysis of all data revealed significant group [$F(2,35) = 5.95$, $p < 0.006$], sex [$F(1,35) = 1131.91$, $p < 0.0001$], time [$F(14,490) = 3068.79$, $p < 0.0001$], and group × sex × time effects [$F(28,490) = 1.58$, $p < 0.05$]. Body weight of male Water and EtOH rats did not differ, but Water males were heavier than FR males (post hoc, $p < 0.005$). Body weight of female Water and EtOH rats did not differ, and Water females were marginally heavier than FR females ($p = 0.06$). In addition, body weights of male and female EtOH rats were not different from that of their respective FR counterparts (both $p > 0.05$). Because the significant overall group effect was driven by the FR groups, their exclusion in a separate analysis clearly indicated

that 14 week voluntary ethanol consumption had no effect on weight gain [group and group × week × time effects: $F(1,28) = 1.84$ and $F(14,392) = 1.28$, $p > 0.05$].

Sucrose preference

Sucrose preference tests performed on P102, P103, and P106 were combined into a 72-h preference test, while that performed on P153 was analyzed as a 24-h preference test. Analysis of the 72-h sucrose preference test indicated a near-significant group effect [$F(2,35) = 2.91$, $p = 0.068$; Fig. 3.5A]. This was driven by the tendency of EtOH male and female rats toward higher sucrose preference compared with the FR rats. Analysis of the 24-h sucrose preference test revealed insignificant group effect [$F(2,34) = 0.11$, $p > 0.05$; Fig. 3.5B].

EPM

EPM conducted on day 6 of abstinence from sweetened ethanol (P105) indicated a near-significant group effect in time spent on the open arms (OA) [$F(2,33) = 2.76$, $p = 0.078$; Fig. 3.6A]. An independent analysis of male rats' data revealed a significant group effect [$F(2,35) = 7.43$, $p < 0.003$]. Relative to Water and EtOH males, FR males spent more time on the OA (post hoc, both $p < 0.002$). In contrast, time on the OA was similar in all three groups of female rats. Analysis of total arm entries, a measure of general activity level (Walf and Frye 2007), revealed significant group and group × sex effects [$F(2,33) = 3.43$ and 3.78, respectively; both $p < 0.05$]. EtOH males exhibited reduced activity compared with Water and FR males (post hoc, $p < 0.002$ and $p < 0.03$, respectively; Fig. 3.6C). EtOH males made fewer arm entries than Water and EtOH females did (post hoc, both $p < 0.006$).

EPM conducted on day 6 of abstinence from 20% unsweetened ethanol (P154) indicated a near-significant group effect in OA time [$F(2,35) = 2.76$, $p = 0.077$]. Overall, females spent more time in the OA compared with males [sex effect, $F(1,35) = 18.59$, $p < 0.0002$; Fig. 3.6B]. Analysis of total arm entries indicated no significant group and group × sex effects [$F(2,35) = 1.02$ and 2.03, respectively; both $p > 0.05$]. Albeit, females exhibited greater activity level compared with males [sex effect, $F(1,35) = 20.08$, $p < 0.0001$; Fig. 3.6D].

Open field activity and NOR

Relative to males, females spent more time in the center of the open-field arena [sex effect, $F(1,33) = 28.96$, $p < 0.0001$; Fig. 3.7A]. Females also demonstrated higher activity level compared with males, as indicated by more frequent crossings into the central arena [sex effect, $F(1,33) = 25.03$, $p < 0.0002$; Fig. 3.7B]. Group and group × sex effects were not significant [$F(2,33) = 1.28$ and 0.73, respectively; both $p > 0.05$]. These findings corroborate results from the EPM conducted on P154.

There was a significant group effect [$F(2,34) = 6.36$, $p < 0.005$] and insignificant sex and group × sex effects [$F(1,34) = 0.52$ and $F(2,34) = 0.34$, respectively; both $p > 0.05$] in novel object exploration time. In males, FR rats explored the novel object for a longer duration than the Water and EtOH groups did [group effect, $F(2,34) = 3.56$, $p < 0.04$ in male one-way ANOVA; post hoc, both $p < 0.03$; Fig. 3.7C]. There were insignificant subgroup differences in total object exploration time among females [group effect, $F(2,35) = 2.54$, $p = 0.093$ in female one-way ANOVA]. Analysis of total object exploration time during the object recognition trial indicated significant group effect [$F(2,34) = 4.85$, $p < 0.02$], and insignificant sex and group × sex effects [$F(1,34) = 1.84$ and $F(2,34) = 1.12$, respectively; both $p > 0.05$]. In males, FR rats explored both objects for a longer duration than the Water and EtOH groups did, but the difference was marginally significant [group effect, $F(2,34) = 3.10$, $p = 0.058$ in male one-way ANOVA]. There were insignificant subgroup differences in total object exploration time among females [group effect, $F(2,35) = 2.29$, $p > 0.05$ in female one-way ANOVA]. All groups preferentially explored the novel object during the test session [percent novel object exploration time: group $F(2,34) = 2.34$ and sex $F(1,34) = 0.41$ effects, both $p > 0.05$; Fig. 3.7D]. An independent analysis of female rats' data revealed a significant group effect [$F(2,35) = 3.48$, $p < 0.05$]. Relative to EtOH females, FR females spent more percent time exploring the novel object (post hoc, both $p < 0.02$).

Glucose tolerance and insulin levels

Fasting glucose levels were similar between male and female rats, and among subgroups of males and females (Fig. 3.8A and 3.8C). Chronic moderate ethanol consumption did not impair glucose clearance during OGTT [group effect, $F(2,34) = 0.28$, $p > 0.05$]. Blood glucose was cleared at comparable rates in males and females [time and sex effects: $F(4,136) = 161.33$, $p <$

0.0001 and $F(1,34) = 3.69$, $p = 0.063$]. Males demonstrated elevated overall glucose response relative to females [glucose AUC: sex effect, $F(1,33) = 25.10$, $p < 0.0001$; Fig. 3.8E].

Male and female rats had similar fasting insulin levels, and there were no subgroup differences in both sexes of rats (Fig. 3.8B and 3.8D). Chronic moderate ethanol consumption was associated with elevated plasma insulin concentration in males during OGTT [group, sex, time, and group × sex × time effects: $F(2,33) = 3.56$, $p < 0.04$, $F(1,33) = 37.34$, $p < 0.0001$, $F(4,132) = 85.14$, $p < 0.0001$, and $F(8,132) = 3.12$, $p < 0.003$, respectively]. EtOH males had significantly more insulin in plasma at the 15-min time point compared with the Water and FR males (post hoc, both $p < 0.0001$). FR males had lower plasma insulin at the 15-min time point relative to Water males ($p < 0.02$). Post hoc tests revealed no significant subgroup differences in insulin levels among females. Additionally, male rats displayed elevated overall insulin response relative to females [insulin AUC: group $F(2,33) = 3.09$, $p = 0.06$, sex $F(1,33) = 35.93$, $p < 0.0001$, and group × sex $F(2,33) = 1.48$, $p > 0.05$ effects; Fig. 3.8F]. An independent analysis of male rat's insulin AUC revealed a significant group effect [$F(2,35) = 3.49$, $p < 0.05$]. EtOH males had a significantly higher AUC compared with FR males (post hoc, $p < 0.03$), and a marginally-higher AUC compared with Water males ($p = 0.062$). There were no group differences in insulin AUC in female rats.

Discussion

This study aimed to clarify the associations between chronic moderate alcohol consumption, type 2 diabetes, and cognitive functions in rats. In support of our and others' earlier work (Barson et al. 2015; Furuya et al. 2003; Nelson et al. 2016), rats that voluntarily ingested ethanol reduced their chow intake while their total daily caloric intake and body weight remained unaffected. Although female rats consumed higher ethanol doses as compared with males, most rats did not attain BEC above the defined intoxication level of 80 mg/dl. Such a kind of chronic voluntary moderate ethanol consumption in males led to a transiently potentiated plasma insulin level for proper blood glucose clearance during OGTT. It is important to note that the changes in plasma insulin concentration that we observed could represent an imbalance between insulin secretion from the pancreas and its degradation in the liver and kidneys. Further, neither male nor female rats showed depression- and anxiety-like behaviors and cognitive deficits, which indicates that the 14-week moderate drinking did not lead to alcohol dependence. Altogether, these findings

indicate that moderate alcohol consumption can pose a sex-dependent risk for pre-diabetes in normal-weight subjects who consume a relatively healthy diet.

Contrary to some reports, rats in this study did not escalate their ethanol intake during the 6-week period of intermittent 20% ethanol drinking. Nonetheless, escalation of ethanol intake under this paradigm was not universal to all rats (Carnicella et al. 2014). Our finding of reduced ethanol intake when the ethanol-access paradigm was changed may be due to absence of sweetener in the 20% ethanol after rats had previously consumed lower concentrations of sweetened ethanol as adolescents. In fact, other alcohol researchers have observed that male rodents with prior exposure to sweetened 10% ethanol solutions during adolescence do not increase their intake of unsweetened 20% ethanol during young adulthood (Broadwater et al. 2013). Our results extend those findings and show that familiarity with the taste of sweetened ethanol during adolescence reduced intake of unsweetened ethanol by male and female adult rats.

The sweetened ethanol-drinking paradigm used in this study produces average BECs close to 60 mg/dl in outbred rats within 30–120 min of drinking (Barson et al. 2015; Broadwater et al. 2013; Carnicella et al. 2014; Nelson et al. 2016; Simms et al. 2008). Similar BECs, measured 40–100 min after initiation of drinking, have been detected in humans that consumed moderate amounts of alcohol (Weissenborn and Duka 2003). Our inability to detect BEC when measured 3–5 h after intermittent 20% ethanol access suggests a near-complete metabolism of blood ethanol within the delay between ethanol accesses to blood sampling. The non-detectable BEC indicates that EtOH rats went from moderate drinking that can result in BECs of ~55 mg/dl during sweetened 10% ethanol exposure to mild drinking during the 20% ethanol exposure. Furthermore, in the locally bred rats given sweetened 10% ethanol, we observed an insignificant sex difference in ethanol intake and BEC attained on the day tail blood was sampled. This lack of sex difference in ethanol intake might be due to interruption to their drinking during the blood sampling procedure. Importantly, the results from this 14-week moderate-to-mild voluntary ethanol drinking study offered new insights into the association between alcohol consumption and the risk of prediabetes and type 2 diabetes.

Our finding that chronic moderate alcohol consumption was associated with an increase in the amount of plasma insulin needed to maintain normal glucose clearance, i.e., an early sign of impairment in insulin sensitivity, concurs with that from studies with healthy humans, dogs, and standard rats (Cullmann et al. 2012; Dornhorst and Ouyang 1971; Lochner et al. 1967; Metz

et al. 1969; Phillips and Safrit 1971; Singh and Patel 1976). Most of these studies were designed to observe the acute effects of ethanol or ethanol plus glucose loads on glucose metabolism. Here, we show that early insulin insensitivity during an OGTT procedure occurred in male but not female rats that chronically consumed moderate amounts of ethanol. Similarly, increased risk of diabetes manifestation in male drinkers compared with females was observed in Swedish (Cullmann et al. 2012) and Japanese (Waki et al. 2005) populations. Our result is also consistent with a recent meta-analysis that concluded that decreased risk of type 2 diabetes among moderate alcohol drinkers is confined to women (Knott et al. 2015). Our finding is more remarkable considering that female rats consumed a higher cumulative ethanol dose. This observation reinforces the idea that females may be spared from some negative metabolic consequences of ethanol (Mumenthaler et al. 1999). This sex difference in the diabetogenic effect of ethanol alludes to the protective effect of ovarian hormones in alcohol-related toxicity (Kishimoto et al. 2002; Mumenthaler et al. 1999). The mechanism still needs to be worked out, and future studies can explore this possibility. Additionally, since all animals consumed the same alcohol, our results imply that the differing types of alcohol cherished by men and women might not fully explain the contrasting vulnerabilities to diabetes observed in humans.

Proponents of the notion that regular moderate alcohol consumption confers protection from cardiovascular diseases and type 2 diabetes in men and women often arrive at such conclusions based on data generated from surveys and questionnaires (Baliunas et al. 2009; Carlsson et al. 2005; Cullmann et al. 2012; Kao et al. 2001; Kiechl et al. 1996; Koppes et al. 2005; Rasouli et al. 2014). While their conclusions have merits, methodological constraints limit their significance. Our findings suggest that moderate alcohol use may not be beneficial to everyone. In fact, the genetic make-up of subjects might interact with the metabolic effects of alcohol to modify diabetes outcome. This has been observed in humans (Vaeth et al. 2014) and demonstrated in rodents (Singh et al. 2015).

Our results contrast with those of studies where a 4-week low-to-moderate ethanol consumption improved hepatic insulin sensitivity in standard laboratory rats via enhancement of the insulin signaling pathway (Furuya et al. 2003; Furuya et al. 2005). In one study, 3% ethanol was the only fluid source available, and rats were under anesthesia during the intravenous insulin tolerance tests employed (Furuya et al. 2003). Additionally, a recent study found that 10% ethanol intake improved tolerance to an intraperitoneal glucose injection in C57BL6 mice with

prolonged high-fat diet exposure (Gelineau et al. 2017). In both studies, insulin sensitivity and glucose tolerance were assessed during continuous ethanol access. By contrast, the oral glucose tolerance test in the present study was performed eight or nine days after alcohol access ended. In addition to differences in the timing of glucose tolerance assessment in relation to ethanol availability, other methodological disparities between the above studies hamper direct comparisons. Nonetheless, our findings underscore the need for closer examination of factors such as diet, genetics, and lifestyle that may contribute to potential sex and individual differences in the risk of type 2 diabetes among moderate alcohol drinkers.

The lowered plasma insulin concentration observed in the FR males at 15 min post-glucose challenge supports the hypothesis that calorie restriction is beneficial. Calorie restriction is known to improve insulin sensitivity and other indices of efficient glucose metabolism (reviewed in (Trepanowski et al. 2011)). Our results further show that improved insulin sensitivity is possible with minor weight loss. This finding should be interpreted cautiously since 1) insulin AUC of the FR males was only reduced relative to that of the EtOH group but not the Water controls, and 2) we did not observe similar beneficial effects in females. Mild calorie restriction in male FR rats was associated with reduced anxiety-like behavior, evidenced by increased open-arm time during the EPM conducted soon after 8-week voluntary alcohol consumption. Because total arm entries during both EPM tests in FR and Water groups did not differ, it is unlikely that the significantly increased open-arm time displayed by the FR male rats was due to a higher activity level. We speculate that chronic mild calorie restriction might have made these rats immune to approach-avoidance stress in early adulthood. Furthermore, as indicated by the number of crossings into the central arena during the open-field test, FR male and female rats were as active as their respective Water and EtOH counterparts. By contrast, during the object recognition trial, male FR rats spent more time exploring both familiar and novel objects as compared with the Water and EtOH groups. Altogether, results of the behavioral tests suggest that the FR rats could be more exploratory in the presence of objects.

Moderate alcohol use can impair cognition in humans (Crews et al. 2007; Weissenborn and Duka 2003), and gender differences in alcohol-related cognitive impairment have been documented (reviewed in (Mumenthaler et al. 1999)). Abstinence from alcohol can also induce craving and mood disturbances (Becker 2000). We employed the sucrose preference, EPM, and NOR tests to respectively assess depression-like, anxiety-like, and cognitive behaviors in the rats

during abstinence from alcohol. To the extent that behavior on these tasks reveals the cognitive and emotional status of rodents, the 14-week voluntary, intermittent moderate ethanol consumption in this study did not elicit dependence and cognitive deficits in rats. Nonetheless, EtOH males displayed reduced general activity levels after 8-week ethanol drinking (on P105), which disappeared at the second EPM test (on P154). Such disappearance may be the consequence of reduced activity level of male rats with age (Hamezah et al. 2017). The time-dependent appearance or disappearance of sex and group differences on behavior highlight the influence of several factors on rodents' EPM performance (Carobrez and Bertoglio 2005).

To reiterate, we showed that regular moderate alcohol consumption might not be innocuous as previously suggested. Male rats that chronically consumed ethanol expressed an early sign of insulin insensitivity (a marker of prediabetes) during an OGTT. This occurred without impaired glucose clearance or body weight disparity between ethanol consumers relative to controls. Additionally, abstinence from moderate ethanol consumption did not lead to emotion-like and cognitive behavioral deficits. Our findings reveal that regular moderate alcohol use can have negative metabolic consequences independent of weight gain. Despite the purported benefits of moderate drinking reported by other researchers, our data bolster the thesis that consumers should do a cost-benefit analysis in their decision to consume or abstain from alcohol (Fernandez-Sola 2015; Klarich et al. 2015; Pietraszek et al. 2010; Saitz 2005; Vaeth et al. 2014).

Acknowledgements

Startup funds from the Psychology Department of the University of Illinois at Urbana-Champaign supported this study. The authors are grateful to Firmino Pinto, Weijie Xu, Haley York, and Tiffany Yang for their technical assistance. We also acknowledge Dr. Timothy Moran for his scholarly input early in the study, and Dr. Justin Rhodes for providing access to a plate reader for BEC quantification.

Figures

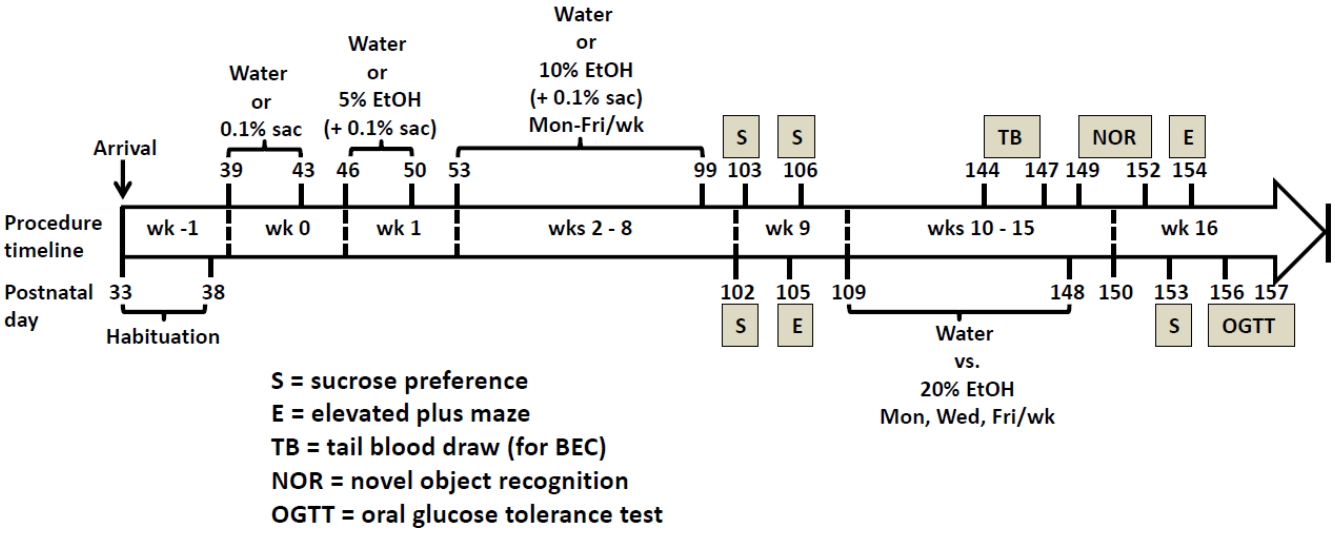


Figure 3.1: Timeline of ethanol consumption and behavioral tests. Long-Evans rats voluntarily consumed ethanol for 14 weeks under Monday–Friday, 7.5-h/day access to saccharin-sweetened 5% and then 10% ethanol, and intermittent access (Monday, Wednesday, Friday, 24-h/day) to unsweetened 20% ethanol. After both drinking paradigms, rats underwent some behavioral assessments and an oral glucose tolerance test (OGTT).

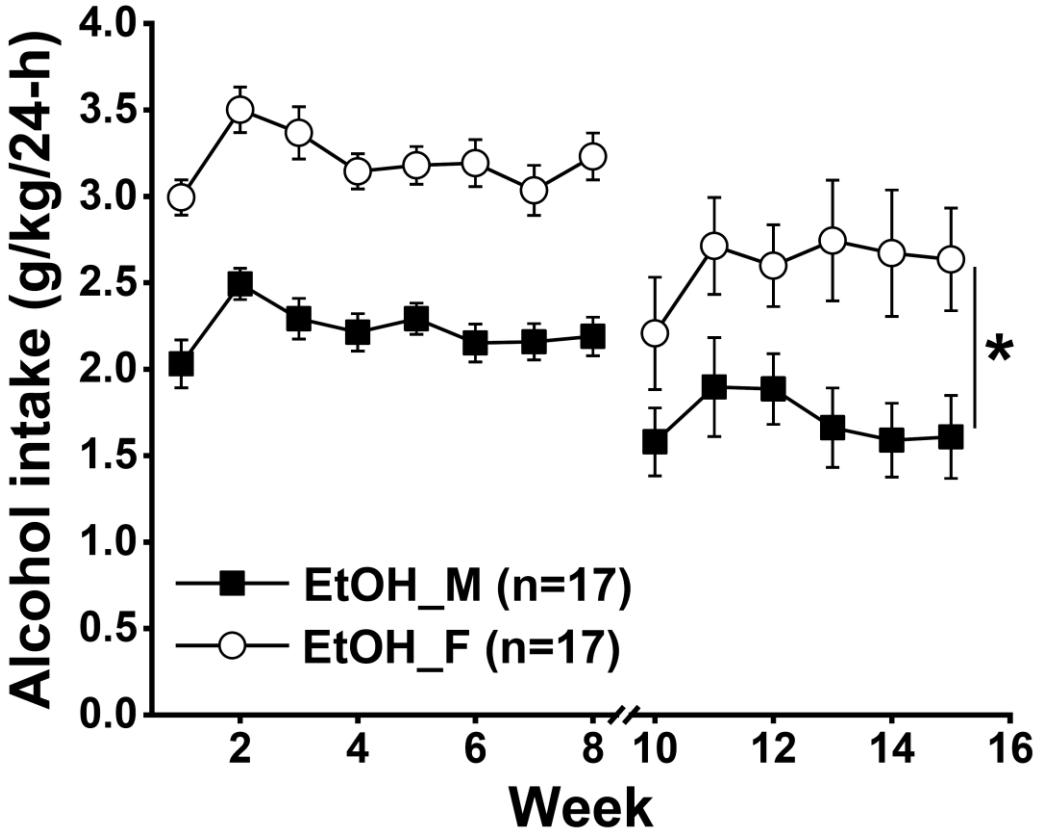


Figure 3.2: Female rats consistently consumed higher doses of alcohol than males did. The break signifies transition from the alcohol access schedule of Monday–Friday (7.5-h/day) to Monday, Wednesday, Friday (24-h/day). *$p < 0.05$

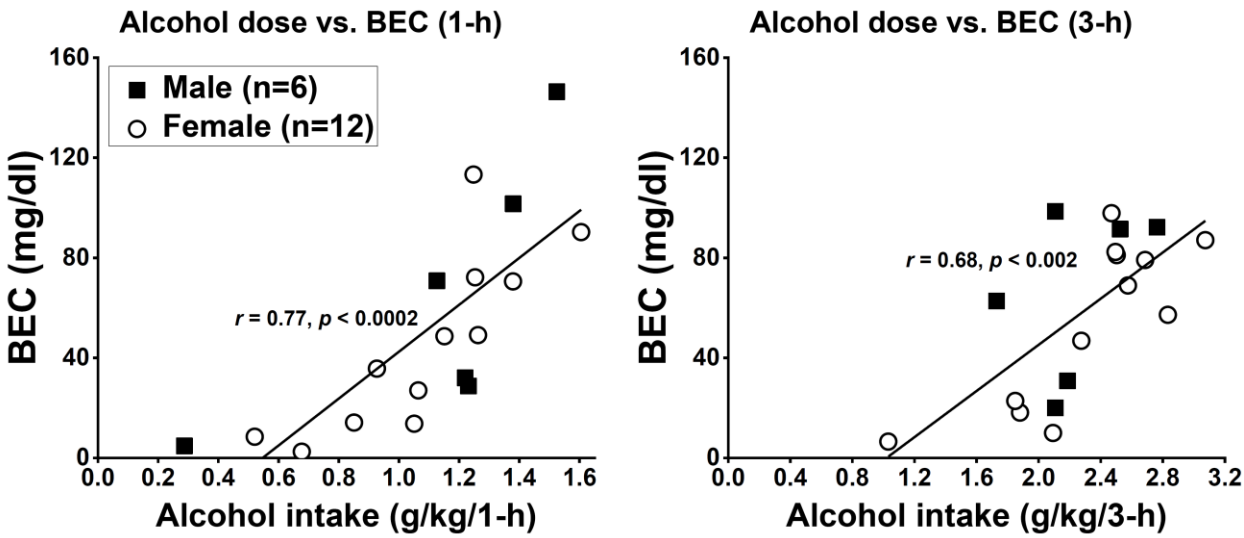


Figure 3.3: Pearson correlation shows blood ethanol concentrations (BEC) significantly correlated with alcohol intake dose after both 1-h ($r = 0.77$, $p < 0.0002$) and 3-h ($r = 0.68$, $p < 0.002$) access to saccharin-sweetened 10% alcohol.

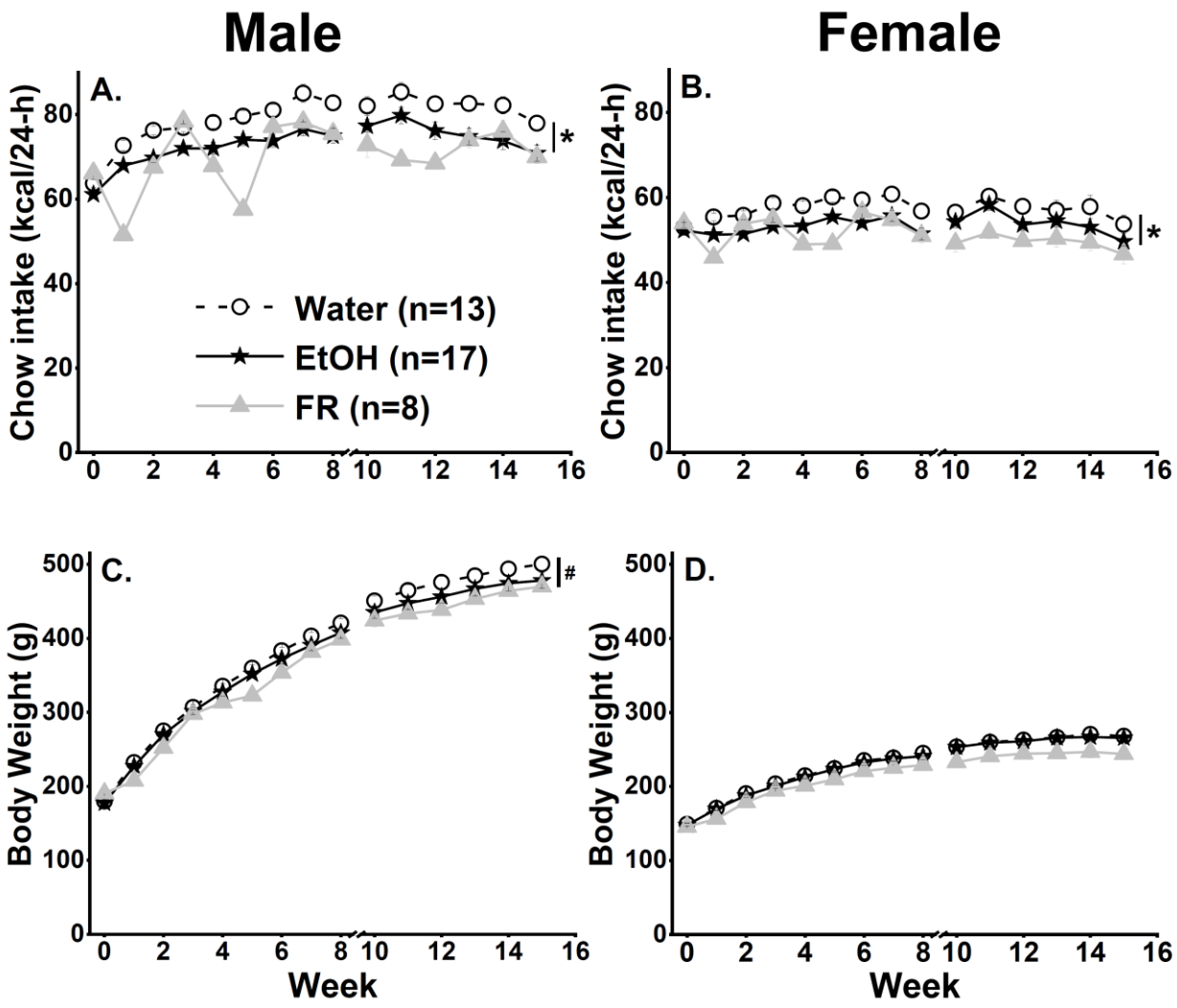


Figure 3.4: Chronic voluntary ethanol consumption was associated with reduced daily chow intake and unchanged body weight in both male and female rats. (**A** and **B**) Average weekday chow intakes during ethanol access period. (**C** and **D**) Weekly body weight throughout the experiment. The breaks signify transition from the alcohol access schedule of Monday–Friday (7.5-h/day) to Monday, Wednesday, Friday (24-h/day). *Water vs. EtOH and FR; #Water vs. FR, $p < 0.05$.

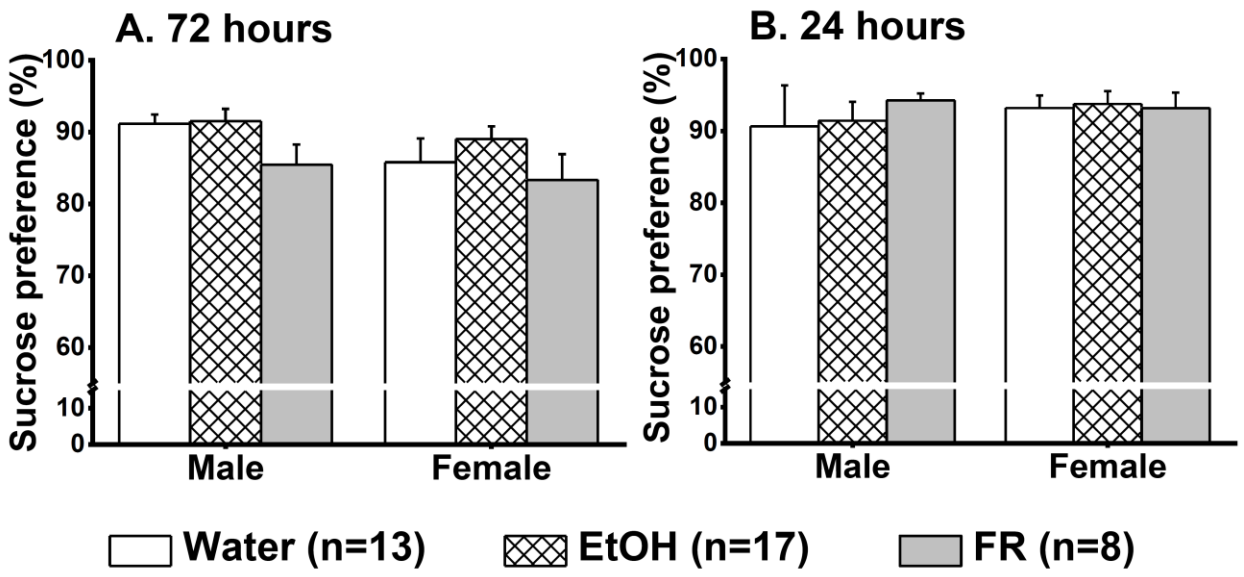


Figure 3.5: Abstinence from chronic moderate alcohol consumption did not elicit depression-like behavior as indicated by lack of effect on sucrose preference. (**A**) 72-h sucrose preference test conducted around day 3 to 7 (P102, P103, and P106) of abstinence from sweetened 10% ethanol. (**B**) 24-h sucrose preference test conducted on day 5 (P153) of abstinence from intermittent access to 20% ethanol. Bars represent percent of total fluid consumed that was sucrose. All rats, regardless of sex, equally preferred 1% sucrose over plain water.

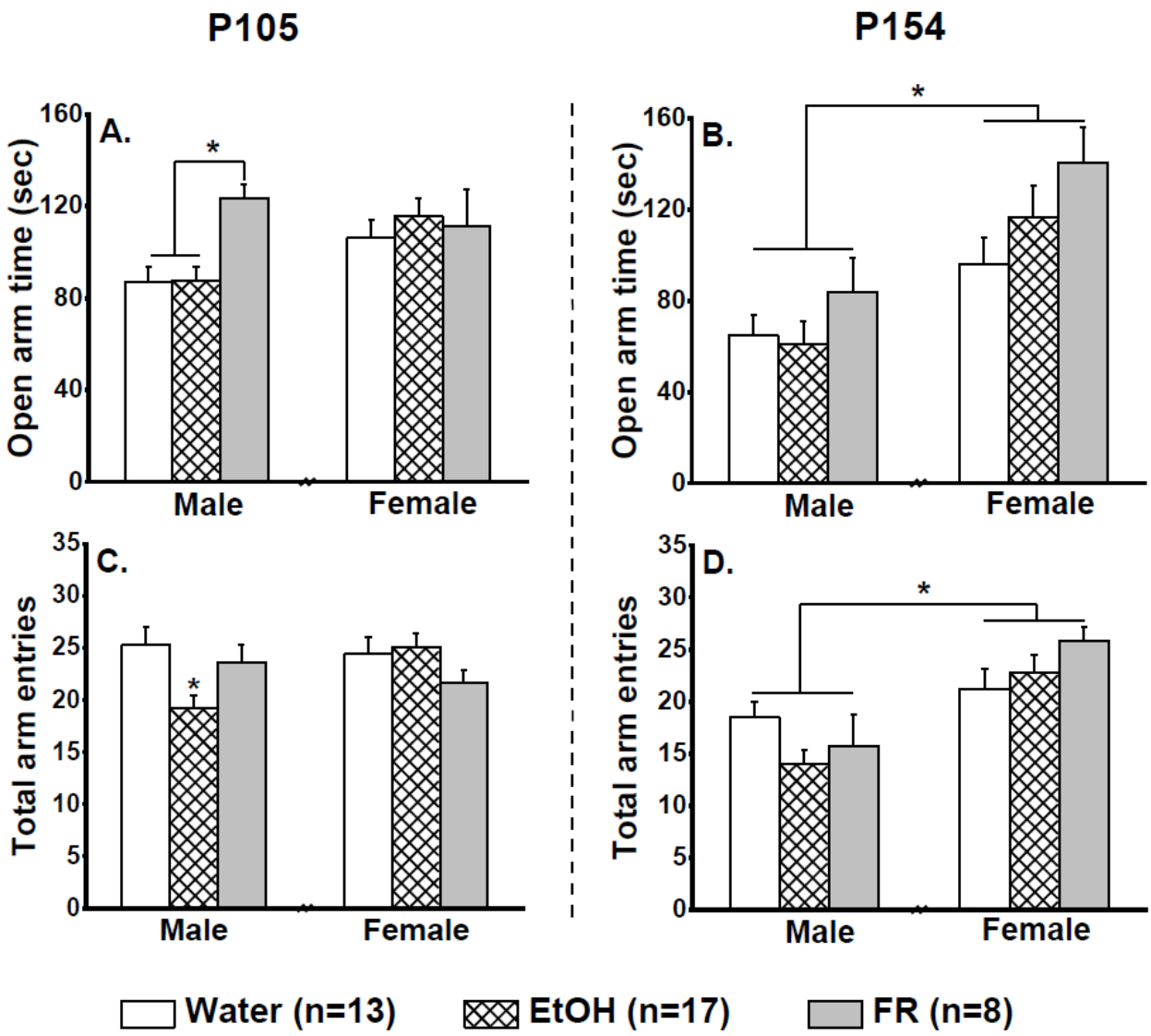


Figure 3.6: Abstinence from chronic moderate ethanol consumption did not increase anxiety-like behaviors in male and female rats on the elevated plus maze (EPM) conducted on day 6 of abstinence from 10% sweetened (P105) and intermittent access to 20% ethanol (P154). Bars represent time spent in the open arms (OA, **A** and **B**) and total number of arm entries (**C** and **D**). FR males spent more time on the OA than Water and EtOH males did on P105, but not on P154. Likewise, the reduced total arm entries displayed by EtOH males on P105 disappeared by P154. Females collectively spent more time on the OA and made more arm entries than males did on P154. *$p < 0.05$

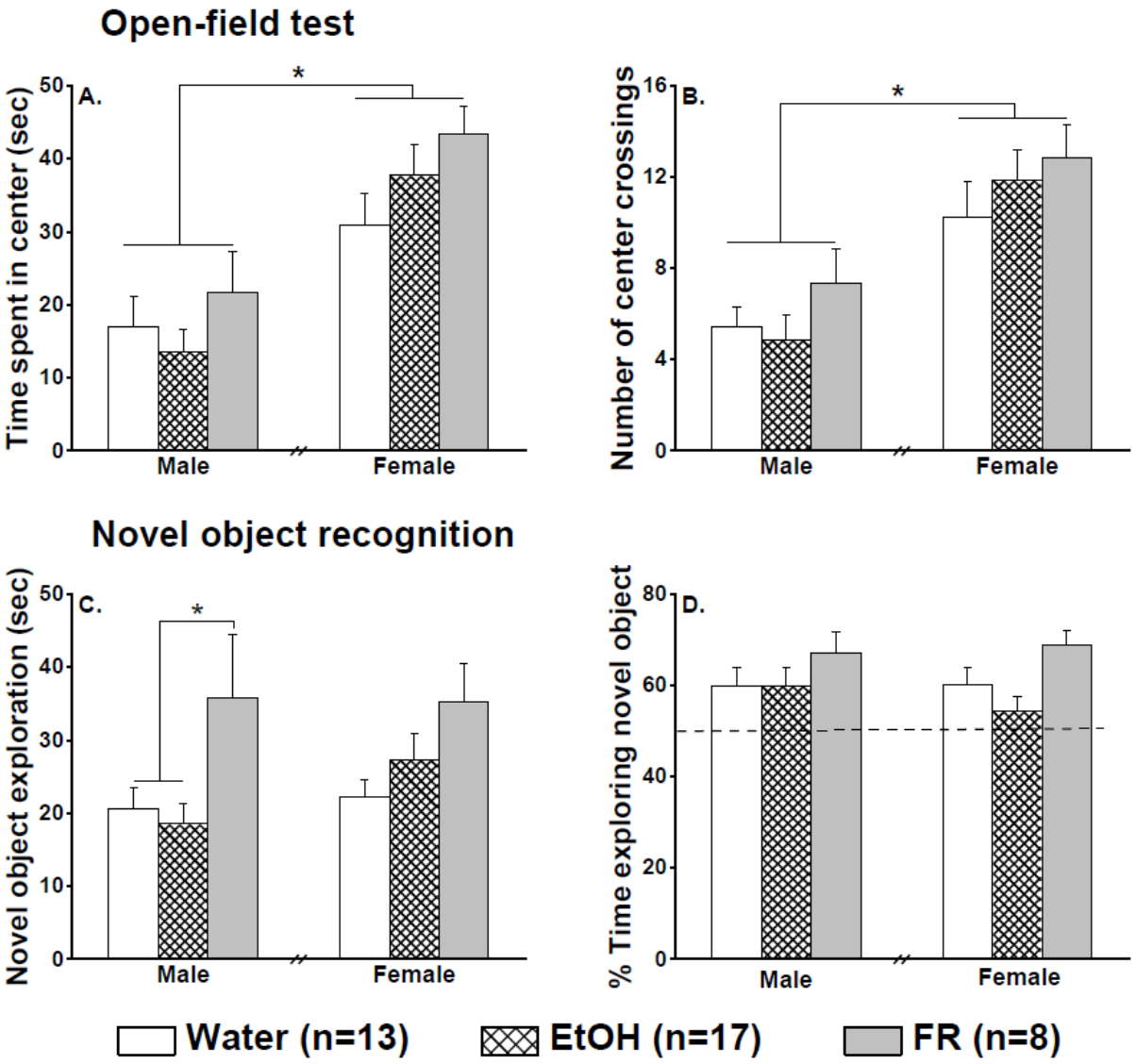


Figure 3.7: Abstinence from chronic moderate ethanol consumption did not alter behavior on open-field and novel object recognition tests (P149–P152). (**A**) Time spent in the center of the open-field arena. Females spent more time in the central arena than males did. (**B**) Number of crossings into the center of the open arena. Females made more center crossings compared with males. (**C**) Novel object exploration time. FR males and females spent more time exploring the novel object than the respective Water groups did. Male FR rats also spent more time exploring the novel object relative to EtOH males. (**D**) Percent novel object interaction time. All male and female groups spent more than 50% of exploration time on the novel object. Relative to EtOH females, FR females spent more percent time exploring the novel object. The dashed line demarcates the 50% recognition threshold. *$p < 0.05$

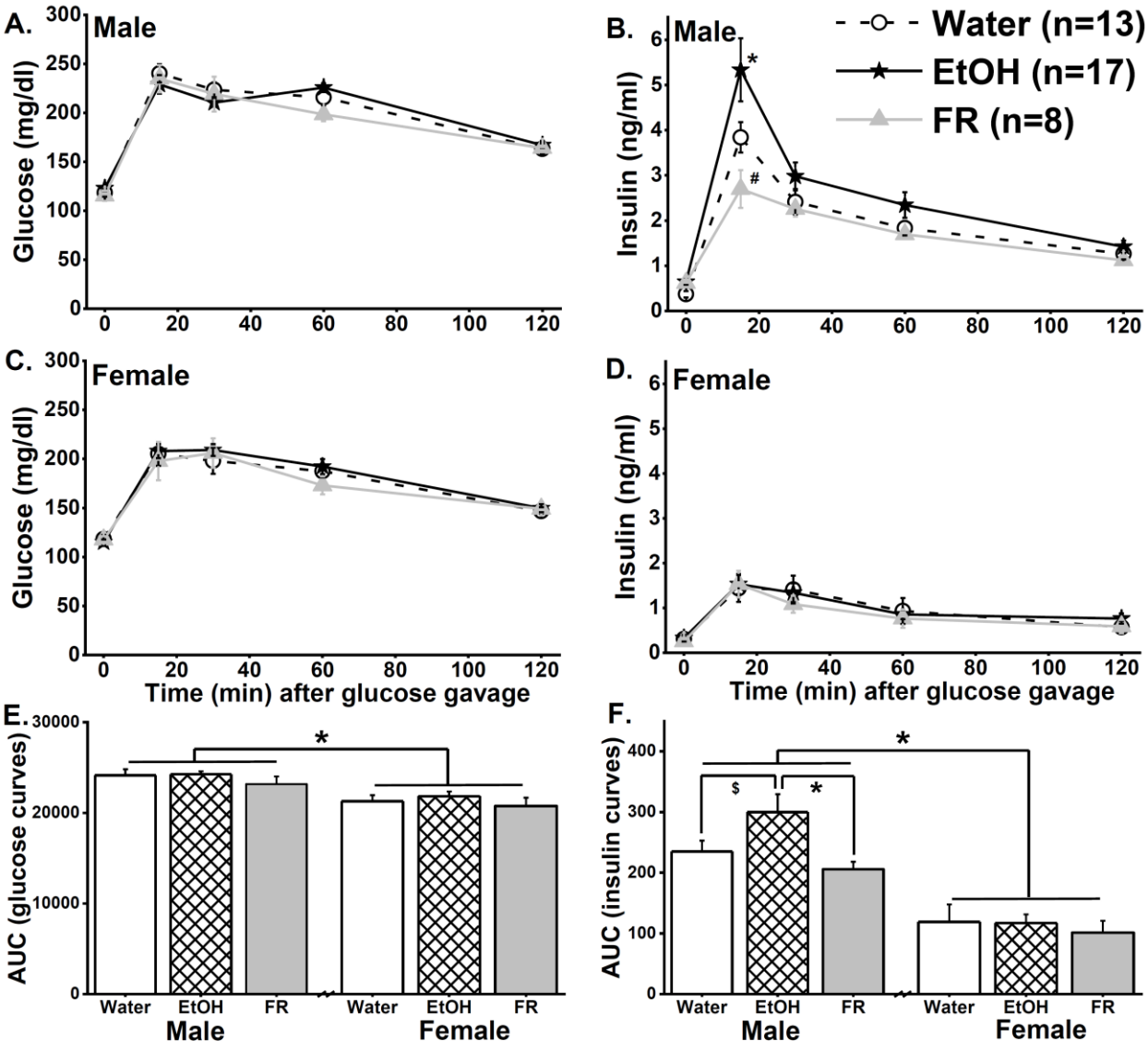


Figure 3.8: Blood glucose levels and plasma insulin responses in male (**A** and **B**) and female (**C** and **D**) rats after oral gavage of 2.0 g/kg glucose solution. *EtOH vs. Water and FR; #Water vs. FR, $p < 0.05$. Areas under glucose (**E**) and insulin (**F**) curves (AUC) were calculated by the trapezoid method. Males showed greater glucose and insulin responses compared with females. EtOH males showed greater insulin response compared with FR males, and a marginally greater insulin response compared with Water males. *$p < 0.05$; \$$p = 0.062$

CHAPTER 4: COMBINED Δ^9-TETRAHYDROCANNABINOL AND MODERATE ALCOHOL ADMINISTRATION: EFFECTS ON INGESTIVE BEHAVIORS AND GLUCOSE HOMEOSTASIS IN ADOLESCENT MALE RATS[2]

Abstract

Whereas co-use of alcohol and marijuana is prevalent in adolescents, the effects of such drug co-exposure on ingestive behaviors, cognition, and glucose homeostasis remain largely unexplored. We hypothesized that co-exposure to alcohol and Δ^9-tetrahydrocannabinol (THC), the main psychoactive constitute of marijuana, alters feeding behavior, cognition, and glucose homeostasis differently from either drug alone. Male rats received daily THC (3–20 mg/kg/day) or oil vehicle through subcutaneous injection or consumption of a cookie with access to saccharin or saccharin-sweetened alcohol during adolescence (P30–P45). Barnes maze and sucrose preference tests were applied to assess spatial memory and behavioral flexibility and abstinence-related anhedonia, respectively. Subsequently, an oral glucose tolerance test (OGTT) was used to assess glucose homeostasis nine days following the last subcutaneous THC and oral alcohol administration. Cannabinoid-1 receptor (CB_1R) and basal glycogen synthase kinase-3 beta (GSK-3β) activity were measured in the medial prefrontal cortex, mediobasal hypothalamus, and hippocampus via immunoblot at 1–5 weeks after last drug exposure. Subcutaneous THC did not affect alcohol intake, but dose-dependently increased acute (3-h) chow intake and reduced weight gain. Moderate alcohol consumption reduced the acute hyperphagic effect of subcutaneous THC. By contrast, oral THC at a dose > 5 mg/kg robustly reduced alcohol intake without affecting 3-h chow intake. At this dose, some rats stopped consuming the THC-laced cookies. Furthermore, oral THC reduced weight gain, and co-exposure to alcohol alleviated this effect. Chronic subcutaneous, but not oral, THC reduced sucrose intake during abstinence. Neither treatment impaired cognitive behaviors in the Barnes maze. Finally, alcohol and THC co-exposure differently changed insulin response to a glucose load during the OGTT. We observed insignificant effects of separate or combined drug use on

[2] This chapter is an updated version of a manuscript previously published as: Nelson NG, Law WX, Weingarten MJ, Carnevale LN, Das A, Liang NC (2019) Combined Δ^9-tetrahydrocannabinol and moderate alcohol administration: effects on ingestive behaviors in adolescent male rats Psychopharmacology (Berl) doi:10.1007/s00213-018-5093-3. The copyright owner has granted permission to reprint.

CB_1R and GSK-3β protein expressions. Moderate alcohol and THC consumption can interact to elicit unique outcomes on ingestive behaviors, energy balance, and glucose homeostasis. Importantly, this study established a novel model of voluntary alcohol and THC consumption for studying mechanisms underlying the consequences of adolescent onset co-use of the two drugs.

Introduction

Alcohol and marijuana/hashish are among the most widely used drugs. The 2016 National Survey on Drug Use and Health estimates that 81% and 44% of individuals aged 12 or older in the United States have used alcohol or marijuana at least once in their lifetime, respectively (SAMHSA 2017). The ease of access to alcohol and the growing popularity and decriminalization of marijuana increase the likelihood for both drugs to be used in combination (Mechoulam and Parker 2003). Despite this increasing trend, how alcohol and marijuana co-use uniquely affects ingestive and cognitive behaviors and glucose homeostasis in adolescents and young adults remain unclear.

In the human and animal literature, reports on the effects of alcohol on appetite, cognition, and glucose homeostasis are mixed (Dry et al. 2012; Spear 2018; Steiner et al. 2015; Yeomans 2010). We previously showed that the route of alcohol administration (oral vs. parenteral) and the time course of blood ethanol concentration (BEC) dictate the effects of alcohol on food intake in rats (Nelson et al. 2016). Rats that drank moderate amounts of alcohol aptly reduced their food intake to maintain their daily caloric intake and weight gain at the level of controls. Furthermore, most rodent studies that reported memory impairments administered a dose of alcohol that rapidly elevates BEC above the defined intoxication threshold of 80 mg/dl (Coleman et al. 2014; Kuzmin et al. 2012; Pascual et al. 2007). By contrast, we recently showed that chronic moderate alcohol consumption from adolescence to young adulthood did not alter object recognition memory in rats (Nelson et al. 2017). In that study, glucose tolerance was also altered by the long-term alcohol exposure (Nelson et al. 2017). How the effects of moderate alcohol consumption on food intake, cognitive behavior, and glucose homeostasis will be modified by simultaneous marijuana use is yet to be explored.

Δ^9-tetahydrocannabinol (THC) is one of over 100 different phytocannabinoids derived from the marijuana plant (*Cannabis sativa*). The psychoactive effects of marijuana are mediated

mainly by the actions of THC within the brain endocannabinoid system (Kirkham et al. 2002; Manwell et al. 2014b). THC activates cannabinoid receptors 1 and 2 (CB_1R and CB_2R) in the central nervous system and in select peripheral tissues to modulate appetite, cognition, mood, glucose homeostasis, and other physiological functions (Di Marzo and Matias 2005; Earleywine 2002; Merroun et al. 2009; Muniyappa et al. 2013; Pertwee 2008). In healthy human volunteers, smoked marijuana increased caloric intake (Foltin et al. 1988; Greenberg et al. 1976) and acutely altered glucose homeostasis (Podolsky et al. 1971). Notwithstanding, an epidemiological study documented some beneficial metabolic effects of chronic marijuana use (Penner et al. 2013). In rats, exogenous cannabinoids (THC, WIN 55,212-2, and CP 55,940) dose-dependently increased not only food intake (Merroun et al. 2009; Williams and Kirkham 2002b; Williams et al. 1998), but also operant responding for sucrose and alcoholic beverages (Gallate et al. 1999; Malinen and Hyytia 2008). Other rodent studies, however, concluded that CB_1R agonists either decreases or have no effect on food intake (Drews et al. 2005; Graceffo and Robinson 1998; Rubino et al. 2008). The effects of THC on glucose homeostasis can differ depending on the cannabinoid receptor subtype it mostly activates, where stimulation of CB_1R or CB_2R impaired or improved systemic glucose homeostasis, respectively (Bermudez-Siva et al. 2006; Maccarrone et al. 2015). These observations underscore the need for more research aimed at dissecting the factors that predict the effects of THC on ingestive behaviors and glucose homeostasis. To our knowledge, the potential for the route of THC administration to dictate the outcome of its interaction with popular appetite-altering compounds (e.g., alcohol) has not been explored.

The effects of THC on cognitive functions are equivocal. The smoking of a 20 mg/kg THC cigarette impaired performance in tests of attention and reaction time (Leirer et al. 1991) whereas medicinal marijuana use is associated with modest improvement in some aspects of executive function (Gruber et al. 2016). Notwithstanding, establishing cause-effect relationship between marijuana use and cognitive deficits in humans can be difficult (Earleywine 2002). Experiments with rodents have also documented cognitive impairment (Han et al. 2012; Murphy et al. 2017), lack of impairment (Cha et al. 2007), and even improved spatial memory (Bilkei-Gorzo et al. 2017) following THC exposure. These reports reinforce the idea that, amongst others, the effects of THC may depend on subtle experimental paradigms like the THC dosage, duration and age of exposure, sensitivity of cognitive tasks employed, and timing of drug exposure relative to behavioral testing.

Research to understand the neurobiological underpinnings of the unique effects of alcohol and THC co-use on brain and behavior are warranted. Among several possibilities, alterations to glycogen synthase kinase-3 beta (GSK-3β; the widely expressed GSK-3 isoform in the central nervous system) and CB_1R activity deserve attention. Normal GSK-3β and CB_1R activity in many brain regions are crucial for maintaining synaptic plasticity and cellular homeostasis (Beurel et al. 2015; Han et al. 2012). Chronic exposures to cannabinoids or alcohol can transiently downregulate or desensitize CB_1R in many brain regions (Breivogel et al. 1999; Ceccarini et al. 2013; Ceccarini et al. 2014; Rubino et al. 2008; Zamberletti et al. 2015). Heavy alcohol use is also associated with impaired GSK-3β activity in many brain regions (Neznanova et al. 2009; van der Vaart et al. 2018). Alterations in central CB_1R and GSK-3β expression or activity are implicated in many metabolic, neurological, and psychiatric diseases (Beurel et al. 2015; Di Marzo et al. 2009b). How chronic moderate alcohol and THC co-use will affect CB_1R protein expression and GSK-3β activity is currently unknown.

The purpose of this study is to establish a rodent model of alcohol and cannabinoid co-administration to examine how the route of THC administration (parenteral vs. oral) interacts with the effects of moderate alcohol to modify ingestive and cognitive behaviors. We hypothesized that moderate alcohol consumption would suppress acute chow intake with no effect on daily total caloric intake. Regardless of administration route, THC would dose-dependently increase acute and daily caloric intake, and alcohol would lessen the hyperphagic effect of THC. Alcohol and THC co-administration will alter glucose homeostasis differently than either drug alone would. We further predicted that memory impairments, abstinence-related anhedonia, and alteration to CB_1R expression and GSK-3β activity would be severe in rats exposed to combinations of alcohol and THC compared with those only exposed to either drug alone.

Materials and methods

Animal husbandry

Preadolescent male Long-Evans rats weighing 35–50 g (Envigo, Indianapolis, IN, USA) were semi-pair housed in large polyethylene tubs with transparent Plexiglas cage dividers that contained six spaced-out holes (~4 mm radius) that permitted visual, tactile, and olfactory interactions between cage pairs. This arrangement enabled us to track individual rats' food and

fluid intake. All animals arrived at postnatal day 22 (P22) except those used for Experiment 2a that arrived at P25. A total of 148 rats were used – 40 each in Experiments 1a and 1b, and 32 and 36 in Experiments 2a and 2b, respectively. Colony was maintained in a temperature and humidity-controlled vivarium on a 12-h:12-h light/dark cycle (lights on at 11:30 AM). Rats had *ad libitum* access to tap water and a standard rodent chow (3.1 kcal/g; 58% carbohydrate, 24% protein, and 18% fat from soybean oil; 2018 Teklad global rodent diet, Indianapolis, IN, USA) and varying accesses to fluids as described in the "Experimental designs" below. Daily animal handling and care occurred at 8:00 AM when body weight, food, and water intakes were recorded. All experiments were approved by IACUC at the University of Illinois, Urbana-Champaign, and they conformed to the guidelines stipulated in the *Guide for the care and use of laboratory animals* by the National Research Council, 2011.

Drug

The contents of 10 mg dronabinol capsules, a commercially-available synthetic Δ^9-tetrahydrocannabinol (Actavis Pharm, Inc.; Parsippany, NJ, USA), were suspended in sesame oil vehicle (Fisher Scientific) for the 3, 5, and 10 mg/kg subcutaneous (s.c.) injections. In Experiment 1, the 3 mg/kg THC doses were administered in a volume of 3 ml/kg body weight, while both the 5 and 10 mg/kg doses were administered at a volume of 1.5 ml/kg body weight. In Experiment 2, dronabinol to be overlain on cookies (Goldfish Grahams Fudge Brownie, Pepperidge Farm; Norwalk, CT, USA) was diluted with sesame oil for the 1.5, 3, 5, and 10 mg/kg doses. The 1.5 and 3 mg/kg THC doses were applied onto cookie at a volume of 0.383 ml/kg body weight; the 5 and 10 mg/kg doses were respectively applied at 0.639 and 0.444 ml/kg body weight. Oil vehicle was administered isovolumetrically to the different THC doses.

Test fluids: saccharin and alcohol

Saccharin (0.1%) and sweetened ethanol (10% and 5% v/v) solutions were prepared as we have previously described (Nelson et al. 2016). Test fluids were presented at home cages in 50 ml graduated plastic bottles fitted with stainless steel sipper tubes that minimize spillage and evaporation. Bottles were weighed (to the nearest 0.1 g) to determine fluid intake. We recorded spillage of < 0.2 g from empty drip cages and thus, statistical analyses were based on raw intake

measurements (i.e., not corrected from the empty drip cages). Ethanol solution from a previous drinking session was discarded, and freshly-prepared solution was presented to the rats daily.

Experimental designs

Experiment 1: Chronic moderate alcohol drinking and subcutaneous THC administration, alone or in combination

Experiment 1a: Spatial learning after chronic alcohol and THC exposure

Forty pair-housed rats were habituated to the vivarium for four days. At 8:00 AM on the fourth habituation day, a cage divider was inserted in the center of each cage to enable separate tracking of individual animals' intake. Over the next four days (P26–P29), the animals were entrained to consume 0.1% saccharin along with chow during the last 3 h of the dark cycle (8:30–11:30 AM). After 11:30 AM measurements, saccharin bottles were replaced with water bottles. Rats were assigned to one of four groups (n = 10/group) by matching average body weight and saccharin intake during entrainment: control (CTL) given saccharin and oil injection, ethanol (EtOH) given 10% ethanol and oil injection, THC given saccharin and THC injection, and combination (COM) given 10% ethanol and THC injection. At 8:30 AM from P30–P45, animals received daily subcutaneous THC or oil injections immediately before test fluid access in their home cages. Chow and test fluid intakes were measured at 11:30 AM (3-h intakes) when test fluids were supplanted by tap water. Drug and test fluid exposures spanned 16 days (P30–P45) that overlapped with the onset of adolescence. The 3 mg/kg/day dose lasted for eight days, while 5 and 10 mg/kg/day doses each lasted for four days (Rubino et al. 2009; Rubino et al. 2008). The doses were increased across time to control for drug tolerability and to model the escalation of drug intake commonly observed in humans. From P32, preputial separation – the complete manual retraction of the prepuce from the glans penis of male rats – was assessed during 11:30 AM measurements to determine how drug exposure would affect puberty onset (Korenbrot et al. 1977). One rat in the EtOH group was discovered dead at 8:00 AM on P42. Another EtOH rat was accidentally injected with 10 mg/kg THC on P43. The subsequent data from this rat were excluded from analysis. Except during the behavioral tests, chow and water were freely available for one week after the last drug exposure day. The experimental timeline is summarized in Fig. 4.1A.

Blood ethanol concentration (BEC) analysis

To determine how low-to-moderate THC doses would affect BEC in rats exposed to both drugs concurrently, tail blood was sampled on the eighth (P37) and second (P43) day of 3 and 10 mg/kg/day THC doses, respectively. On those days, rats were presented with 10% alcohol or saccharin per the experimental procedure. After a 1-h (3 mg/kg THC) or 3-h (10 mg/kg THC) free access, intakes were recorded and tail blood (80 µl) collected into heparinized microcapillary tubes. The blood samples were centrifuged at 1000 × g for 10 min at 4 °C after which plasma was collected and immediately stored at –80 °C. BEC was quantified in plasma gotten from EtOH and COM rats using a colorimetric assay kit (STA-620; Cell Biolabs, Inc., San Diego, CA) with a protocol supplied by the manufacturer. Ethanol concentrations were estimated using a standard curve per manufacturer's recommendations (Nelson et al. 2016).

Behavioral tests

Sucrose preference

During abstinence from THC and alcohol, sucrose preference test was used to assess intake of a mildly rewarding sucrose solution (Carlin et al. 2016; Nelson et al. 2017). On the test days (Fig. 4.1A), rats were presented with two bottles, 1% sucrose vs. tap water, immediately after 8:00 AM measurements. The positions of the bottles were alternated on each day to prevent the development of location preference for drinking. After the sucrose preference tests, all rats were returned to *ad libitum* water access. Sucrose preference (%) was calculated as $\frac{\text{sucrose consumption}}{\text{sucrose + water consumption}} \times 100$.

Barnes maze

Rats were submitted to the Barnes maze (BM) test to examine the effects of periadolescent alcohol and THC exposure on spatial learning, memory, and cognitive flexibility. The BM has been used by other researchers to assess such behaviors following different experimental manipulations (Coleman et al. 2014; Kuzmin et al. 2012). The maze consisted of a circular dark grey high-density polyethylene panel (122 cm diameter) with 20 holes (10 cm diameter) that are 2 cm away from the edge and evenly distributed around the circumference of the platform. An escape chamber (39 cm × 12.5 cm × 14.5 cm) constructed of black Plexiglas® was mounted on a

rotatable wooden support that permitted a 360° rotation on the horizontal plane to align the box underneath any hole of the maze. The escape chamber had a 20° incline base that allowed the rats to carefully descend into the box. Only one hole led to the escape chamber. The entire set-up was supported on a 99 cm high movable pedestal with adjustable wheels that remained locked during testing.

BM testing began four days after the last drug exposure (Fig. 4.1A) and was performed in the middle of the light cycle after 1 h habituation in a room adjacent to the sound-attenuated testing room. Both rooms were illuminated to approximately 900 lux white diffuse fluorescent lights. The testing room contained five apparent visuo-spatial cues. In the BM, rats must use the spatial cues to locate the escape chamber. A concealed ceiling-mounted camera connected to a computer in an adjacent room enabled real-time behavioral analysis and video recording for offline analysis by individuals blind to the group assignments. The time it took a rat to locate and enter the escape chamber (escape latency) was manually scored by trained scorers. Where reported, average distance travelled in the maze was tracked by TopScan (CleverSystems Inc.; Reston, VA, USA). Between each animal, the apparatus was cleaned with a non-alcohol-based cleaning agent (Coverage Spray; STERIS Corp., St. Louis, MO, USA) to eliminate odor cues.

Spatial learning and memory

Spatial learning occurred on two consecutive days (P49–P50), and each daily session consisted of four trials with an inter-trial interval of at least 1 h. At the start of a trial, a rat is placed in the center of the maze and allowed to explore for 3 min. A trial ended when the rat entered the escape chamber or when the 3 min elapsed, whichever occurred first. In the situation when a rat did not enter the chamber by the end of a trial, it was gently guided into the chamber where it remained for 90 sec before being returned to its cage.

Twenty-four hours after the spatial learning task, rats underwent spatial memory evaluation (P51). The procedure was identical to that used for spatial learning, except that the starting orientation of a rat during the four trials alternated between the North, East, South, and West cardinal directions.

Behavioral flexibility

Four behavioral flexibility trials were conducted on the day after the spatial memory test (P52). The arrangement of the first trial was identical to that of the spatial learning trials. In the second, third, and fourth trials, the escape chamber was rotated 180° or 90° relative to its position during the first trial. Hence, the escape chamber alternated among the four quadrants corresponding with its cardinal location during each of the four trials in this order: South, North, West, and East. The rotations of the escape chamber ensured that rats were unable to escape the maze using acquired spatial cues but had to identify the new locations – a measure of cognitive/behavior flexibility (Coleman et al. 2014; Kuzmin et al. 2012; Pattij et al. 2008).

Oral glucose tolerance test (OGTT)

Rats were returned to their home cages at the conclusion of the Barnes maze testing. Following an overnight 16-h fast, we performed the OGTT on P54 (Fig. 4.1A) according to our established protocol (Nelson et al. 2017). After we measured fasting blood glucose from a drop of tail blood using an AlphaTRAK glucometer (Abbott Labs) and collected tail blood using 80 µl heparinized microcapillary tubes, we administered an oral gavage of 2.0 g/kg body weight glucose with a 20% glucose solution. We then collected glucose readings and tail blood at 15, 30, 60, and 120 min post glucose dosing. The blood samples were transported on ice and centrifuged at 1000 × g for 15 min at 4 °C. Plasma was collected and stored at –80 °C for later insulin measurement as we have previously done (Nelson et al. 2017).

Brain extraction and western blot

Within 1 h after the OGTT, the animals were rapidly decapitated and their brains immediately extracted and snap-frozen in powdered dry ice. Brains were stored at –80 °C until they were processed for western blotting. The medial prefrontal cortex (mPFC, +4.20 to +2.52 mm relative to bregma), mediobasal hypothalamus (MBH, -1.72 to -3.36 mm), and hippocampus (HIP, -2.40 to -4.56 mm) were punched from 400–500 µm thick frozen coronal sections using a 2.0 mm diameter Miltex biopsy punch (Electron Microscopy Sciences; Hartfield, PA, USA) based on published coordinates for the rat brain (Paxinos and Watson 2007).

Tissues for western blot were homogenized in cold RIPA buffer containing protease (Sigma; Cat. # P8340) and phosphatase (Sigma; Cat. # P5726) inhibitor cocktails and allowed to incubate on ice for 40 min. The homogenates were then centrifuged at 12,000 rpm, 4 ℃ for 15 min, after which the supernatant was carefully collected. Total protein concentration was quantified via bicinchoninic acid method (Pierce BCA Protein Assay Kit; Thermo Fisher Scientific, Cat. # 23227). Equal amounts of protein per sample (10 µg) was mixed in a 3:1 ratio with 4x laemmli buffer containing 2-mercaptoethanol and denatured at 95 °C for 5 min. Proteins (10 µg/20 µl) were fractionated by SDS/PAGE in 4–15% pre-cast polyacrylamide gels (Mini-Protean TGX Gels; Bio-Rad, Cat. # 456-1084) at 100 V before transfer onto polyvinylidene difluoride membrane (Bio-Rad). Portions of the membrane containing CB_1R and vinculin molecular weights were cut and blocked for 40 min at room temperature with 5% non-fat dry milk in TBS-T (Tris Buffered Saline with 0.05% Tween 20). The pre-incubations were followed by overnight incubation at 4 °C with gentle shaking in anti-cannabinoid 1 receptor or vinculin antibody in 2.5% milk solutions (see Table 4.1 for information on the specific primary antibodies and dilutions used in this chapter). Primary incubations were followed by four 10 min washes with TBS-T before the membranes were incubated in HRP-linked anti-rabbit IgG (1:2000; Cell Signaling Technology, Cat. # 7074) with gentle shaking for 2 h at room temperature and protected from light. Blots were washed and incubated for 4 min in Clarity Western ECL Substrate (Bio-Rad, Cat. # 170-5060). Chemiluminescent protein bands were visualized and imaged with a ChemiDoc Touch Imaging System (Bio-Rad). Using Image J software (NIH; Bethesda, MA, USA), relative CB_1R protein quantity was determined by normalized intensity to loading control vinculin. The average density of the EtOH, THC, and COM groups were normalized to that of the CTL.

Experiment 1b: Spatial learning during alcohol and THC exposure

During the BM procedure in Experiment 1a, we noticed that drug exposure during adolescence had no effect on performance. We reasoned that the drug might interfere with BM performance if 1) we increased the cumulative THC dose administered to the rats, 2) drug exposure overlapped with training in the maze, and 3) spatial memory and cognitive flexibility were assessed a few days after BM training. The 40 rats (n = 10/group) were housed under identical conditions as in Experiment 1a. Habituation and drug administration procedure were also consistent with that of

Experiment 1a, except that the THC and COM groups in Experiment 1b received 3, 5, 10, and 20 mg/kg/day THC injections (s.c.), each dose lasting for four days for a total of 16 drug exposure days (Fig. 4.1B). Importantly, to minimize THC-induced locomotor depression (Manwell et al. 2014b; McGregor et al. 2005; Williams and Kirkham 2002a), the 20 mg/kg/day dose was administered as two separate 10 mg/kg doses. The first was administered at 8:30 AM as per the usual protocol, while the second was given at 9:30 PM (i.e., 2 h before dark onset). Two 48-h sucrose preference tests were performed (Fig. 4.1B).

The Barnes maze set-up and procedure employed here was consistent with that of Experiment 1a. The 12-day BM procedure spanned a 15-day period that was punctuated by three days within which the first 48-h sucrose preference test was performed (Fig. 4.1B). Rats underwent eight days of training (1 trial/day) that overlapped with the last eight days of alcohol and THC exposures. Spatial memory (2 trials/day) was assessed on days 4 and 5 after the last drug exposure. Behavioral flexibility test (2 trials/day) occurred on abstinence days 6 and 7 (P51 and P52). Except for days when sucrose preference tests were performed, rats were maintained on free access to chow and water during a 2-week abstinent period.

Between four (P73) to five (P81) weeks after the last drug exposure day (P45), a subset of the rats (n = 6/group) were decapitated and their brains were rapidly removed and frozen in powdered dry ice. [The other subset of rats was processed for pilot electrophysiological recordings in collaboration with the Gulley Lab (the preliminary data are not presented in this dissertation)]. The mPFC, MBH, and HIP were micropunched and processed for quantification of protein levels of CB_1R, phosphorylated GSK-3β, total GSK-3β, and vinculin via western blot as described above. The GSK-3β membranes were first probed with the phosphorylated protein that was stripped before membranes were probed for total protein. Relative protein densities were determined by normalized intensity to house-keeping protein vinculin (CB_1R) or to total protein (GSK-3β) in the same lane. Average density of the EtOH, THC, and COM groups were expressed as a percentage of the CTLs.

Experiment 2: Chronic moderate alcohol drinking and oral THC consumption, alone or in combination

Experiment 2a: Spatial learning during voluntary alcohol and THC consumption

Although smoking or vaping is the preferred route of human THC administration, consumption of edibles laced with cannabis extracts is becoming popular (Grella et al. 2014; National Academies of Sciences 2017). THC-containing edible products like beverages (tea, coffee, cannabis cocktails), confectionaries (cookie, brownies, candy, chocolate), and cooking condiments are available in states where recreational and medicinal marijuana are legal (Grella et al. 2014; Vandrey et al. 2015). To enhance the relevance of our model by mimicking this growing pattern of THC ingestion, we sought to administer it to the rats via cookies. The 32 rats (n = 8/group) were aged P25 at arrival. The drug treatment timeline employed here was consistent with that of Experiment 1b, except that drink training began on P26. The initial plan was to present rats with cookie overlaid with 3, 5, 10, and 20 mg/kg/day THC in their home cages. Each dose was to last for four days, and the 20 mg/kg/day THC was to be given as two 10 mg/kg doses. After the first 5 mg/kg/day THC consumption, THC and COM rats consumed less test fluids which became pronounced after the first two 10 mg/kg/day doses when food intake also reduced (see *Results*). Consequently, we modified our protocol to where lower THC doses were provided to the same rats twice a day: 1 h after the end of test fluid consumption, i.e., 1 h after light onset (12:30 PM), and 3 h before dark onset (9:00 PM). We also reduced the concentration of sweetened alcohol solution from 10% to 5% to mitigate any potential aversive effects caused by the interaction of THC (and its metabolites) and the higher alcohol concentration (Fig. 4.2A). The 5% alcohol solution was first introduced to the EtOH and COM rats on P40 when oil cookie was provided to all groups. Under this modified protocol (P41–P45), rats were first presented with 3-h access to saccharin or 5% alcohol solution (8:30–11:30 AM). After chow and test fluid intakes were recorded, the first and second THC-laden cookies of the day were respectively presented at 12:30 and 9:00 PM. Barnes maze set-up and timeline were consistent with that of Experiment 1b. Except for days of sucrose preference tests (Fig. 4.2A), chow and water were freely available during a 2-week abstinent period.

Experiment 2b: Testing the modified voluntary alcohol and THC consumption paradigm

Experiment 2b tested the modified drug administration paradigm developed in Experiment 2a in a new cohort of rats. After habituation to the animal facility, the 36 rats (CTL and COM, n = 10/group; EtOH and THC, n = 8/group) were entrained to the new drug exposure schedule. Immediately after daily care during the 4-day training period, rats had 3-h access to 0.1% saccharin solution and chow (last 3 h of the dark cycle, 8:30–11:30 AM). Following measurement of chow and saccharin consumption, the first and second oil-laden cookies were respectively provided at 12:30 PM and 9:00 PM. During the 16-day treatment period, rats first had 3-h access to saccharin or sweetened 5% alcohol solution followed by a cookie laced with oil or THC (1.5, 3, or 5 mg/kg) presented twice per day at 12:30 PM and 9:00 PM (Fig. 4.2B). Each cumulative daily dose of 3, 6, 8, and 10 mg/kg THC lasted for four days. To ascertain how oral THC consumption would affect BEC kinetics, we measured BEC at different time points (see Supplemental Information). Barnes maze test was not performed in Experiment 2b. Except on the days when sucrose preference tests were conducted (Fig. 4.2B), rats had *ab libitum* access to chow and water for three weeks following the last drug exposure day.

Plasma levels of THC and its metabolites

In Experiments 1b and 2b, tail blood was collected shortly after light onset on the day after the last THC exposure (i.e., 14 h after). This timing for blood collection was chosen mainly because it coincided with a period when daily measurements were performed. The blood samples were centrifuged, and plasma was immediately stored at –80 °C. Lipid components of the plasma samples were extracted, and levels of THC, 11-OH-THC, and THC-COOH were quantified by liquid chromatography-tandem mass spectrometry (LC-MS/MS) as we have previously done (Arnold et al. 2018; Tiscione et al. 2016).

Statistical analysis

In Experiment 1, daily caloric intake in CTL and THC rats consisted of calories from chow (3.1 kcal/g), while EtOH and COM rats consumed calories from chow and alcohol (7 kcal/g). The loss of one rat and the mishap during THC injection in Experiment 1a reduced the number of EtOH rats to 8. Furthermore, some volume of glucose was spilled during the oral gavage procedure in Experiment 1a. The OGTT data from the correspondent rat were eliminated from

analysis (the eventual sample sizes are provided in the legend of Fig. 4.6). In Experiment 2, daily caloric intake in CTL and THC rats included calories from chow and cookie (4.7 kcal/g), while EtOH and COM rats consumed calories from chow, cookie, and alcohol. The inconsistency with which rats consumed THC cookies prompted us to apply separation or exclusion criteria (see Experiment 2 results below). In Experiment 2a, the respective final sample sizes of the THC and COM cookie-consuming groups (THC_C and COM_C) were 5 and 4, and that of the cookie-avoiding groups (THC_A and COM_A) were 3 and 4. In Experiment 2b, the final sample size of the COM group was seven. The dose-response effects of THC on alcohol, saccharin, and caloric intakes were analyzed using the average value from the respective doses. These intake analyses were repeated with body weight-adjusted intakes, which were computed as intake per 100 g body weight of individual rat.

Alcohol, saccharin, and caloric intake during treatments were analyzed by one-way ANOVA (paradigm 2 of Experiment 2a) or by two-way repeated-measures ANOVA with treatment as a between-subject factor and THC dose or day as the repeated measure. Likewise, Barnes maze data were analyzed by two-way repeated-measures ANOVA with treatment as a between-subject factor and trial session as the repeated measure. The blood glucose and insulin concentrations from the OGTT were analyzed by two-way repeated-measures ANOVA with time (in min) after oral glucose gavage as the repeated measure. The effects of specific THC dose on caloric intake, alcohol intake, and BEC were determined by one-way ANOVA, as was analysis of weight gain during treatment, caloric intake and weight gain during abstinence, protein expression, fasting blood glucose and insulin concentrations during the OGTT, areas under the glucose and insulin curves (AUC), sucrose preference, and fluid intakes during the preference tests. Significant main effects and interactions ($p < 0.05$) from the ANOVAs were accompanied with Tukey's HSD post hoc tests. Plasma levels of THC and its major metabolites were analyzed via Student's *t*-test. Pearson correlation was used to assess the linear relationship between ethanol dose and the ensuing BEC. Data are presented as mean ± standard error of the mean (SEM). Analyses were conducted in Statistica 13.3 (TIBCO Software Inc.; Palo Alto, California).

Results

Experiment 1: Chronic moderate alcohol drinking and subcutaneous THC administration, alone or in combination

Subcutaneous THC reduced saccharin intake, but had no effect on alcohol intake and BEC

In Experiments 1a and 1b, THC and CTL groups increased their saccharin intake across treatment days [$F(2,36) = 40.33$ and $F(3,54) = 21.24$, respectively; both $p < 0.0001$]. Compared with the CTLs, subcutaneous THC reduced saccharin intake in Experiment 1b [$F(1,18) = 7.83$, $p < 0.02$], but not in 1a. The above result held true for body weight-adjusted saccharin intakes. Ethanol consumed by EtOH and COM rats did not differ at any of the administered THC dose (Fig. 4.3A and 4.8A).

The effects of subcutaneous THC on blood ethanol concentration (BEC) were assessed in Experiment 1a. After a 3 mg/kg THC injection, EtOH (1.54 ± 0.13 g/kg) and COM (1.4 ± 0.18 g/kg) rats consumed similar alcohol dose within 1 h. Both groups attained comparable BECs of 4.60 ± 1.41 mg/dl – a value near the lower detection limit of our kit. BEC attained after 1 h of alcohol access significantly correlated with the ingested dose in only the EtOH group (Fig. 4.3B). After a 10 mg/kg THC injection, EtOH (1.93 ± 0.13 g/kg) and COM (2.11 ± 0.19 g/kg) rats consumed similar alcohol dose within 3 h to achieve BECs of 18.07 ± 8.39 and 22.81 ± 9.68 mg/dl, respectively. BEC achieved after 3-h alcohol drinking significantly correlated with the ingested dose in both EtOH and COM groups (Fig. 4.3C).

Subcutaneous THC stimulated short-term chow intake, and reduced weight gain and caloric intake during abstinence

Subcutaneous THC dose-dependently increased while moderate alcohol consumption reduced 3-h chow intake in Experiments 1a [group and group × time effects: $F(3,35) = 14.16$ and $F(6,70) = 5.47$, respectively; both $p < 0.0002$; Fig. 4.3D] and 1b [group and group × time effects: $F(3,36) = 11.46$ and $F(9,108) = 3.55$, respectively; both $p < 0.0007$; Fig. 4.8B]. In both experiments, the reduced caloric intake by the EtOH group relative to the CTL group (post hoc, both $p < 0.02$) did not persist when alcohol calorie was included. The respective hyperphagic and hypophagic effect of THC and alcohol appeared to oppose each other as chow intake of the COM group did not differ from that of the CTLs in both experiments. The significant main effects on 3-h chow

intake outlined above persisted when intake was adjusted to individual body weight (see Supplementary table 4.1).

Regardless of body weight adjustment, daily caloric intake did not differ between groups. In parallel with the total daily caloric intake results of Experiment 1a, there were no group differences in weight gain during drug exposure. In Experiment 1b, however, THC and COM rats gained less weight than did CTL and EtOH rats [$F(3,36) = 7.84$, $p < 0.0004$; post hoc, all $p < 0.02$; Fig. 4.8C].

In Experiment 1a, prior drug exposure did not significantly affect average caloric intake during the week of abstinence, though COM rats consumed fewer calories per body weight compared with the CTLs [$F(3,34) = 3.27$, $p < 0.04$; post hoc, $p < 0.05$]. During this period, COM rats gained less weight than did EtOH rats and tended to weigh less than THC rats [$F(3,34) = 4.30$, $p < 0.02$; post hoc, $p < 0.02$ and $= 0.057$, respectively; Fig. 4.3E].

During the 2-week abstinent period in Experiment 1b, THC rats consumed fewer daily calories than did CTL and EtOH rats [$F(3,36) = 6.87$, $p < 0.0009$; post hoc, both $p < 0.02$], but analysis of weight-adjusted energy intake revealed no treatment effect. Finally, THC rats gained less weight than EtOH rats did during abstinence [$F(3,36) = 3.32$, $p < 0.04$; post hoc, $p < 0.02$; Fig. 4.8D].

Subcutaneous THC reduced sucrose intake during abstinence

Abstinence from chronic subcutaneous THC and voluntary alcohol drinking had no effect on sucrose preference as all the groups preferred 1% sucrose solution to plain water during the 48-h sucrose preference tests. In Experiment 1a, subcutaneous THC, but not moderate alcohol drinking, reduced sucrose consumption in the THC and COM groups compared with the CTL group [$F(3,33) = 4.46$, $p < 0.01$; post hoc, $p = 0.058$ and < 0.05, respectively; Fig. 4.4A]. THC and COM groups consumed less total fluid compared with the CTL group [$F(3,33) = 4.25$, $p < 0.02$; post hoc, $p = 0.086$ and < 0.04, respectively]. These effects persisted with body weight-adjusted analyses.

In the first sucrose preference test of Experiment 1b (P47–P48), THC rats consumed less sucrose and total fluid than did the CTL and EtOH rats [sucrose: $F(3,36) = 8.37$, $p < 0.0003$; post hoc, $p < 0.02$ and 0.0004, respectively; total fluid: $F(3,36) = 10.21$, $p < 0.0001$; post hoc, $p < 0.02$ and 0.0002, respectively; Fig. 4.9A]. In the second sucrose preference test (P54–P55),

THC rats also consumed less sucrose and total fluid compared with the CTL and EtOH groups [sucrose: $F(3,36) = 5.37$, $p < 0.004$; post hoc, $p < 0.04$ and 0.004, respectively; total fluid: $F(3,36) = 5.18$, $p < 0.005$; post hoc, $p < 0.05$ and 0.004, respectively; Fig. 4.9B]. All the significant main findings outlined above held true for body weight-adjusted sucrose and total fluid intakes.

Subcutaneous THC, moderate alcohol, and their combination had no effect on Barnes maze performance

In Experiments 1a and 1b, escape latency decreased equivalently in all groups across learning sessions [Experiment 1a, $F(7,238) = 135.11$ and Experiment 1b, $F(7,252) = 148.10$; both $p < 0.0001$; Fig. 4.5 and 4.10A, respectively]. There were no group differences in this parameter during spatial memory tests, and all groups displayed equal facility in locating the new escape chambers during behavioral flexibility sessions. In Experiment 1b, all groups of rat travelled progressively reduced distance across learning sessions [$F(7,245) = 90.36$, $p < 0.0001$; Fig. 4.10B]. There were no enduring effects of treatment on spatial memory and behavioral flexibility.

Combined subcutaneous THC and moderate alcohol consumption differently altered glucose tolerance

The 16 days of subcutaneous THC and moderate alcohol treatment did not alter fasting blood glucose levels (Fig. 4.6A). All the groups of rats comparably cleared blood glucose across time during the OGTT [time effect, $F(4,136) = 94.95$, $p < 0.0001$; Fig. 4.6B]. The fasting plasma insulin concentration of the COM group was mildly lower than that of the THC group [group effect, $F(3,27) = 3.00$, $p < 0.05$; post hoc, $p = 0.051$; Fig. 4.6C]. Rats in each group had a slightly different profile of plasma insulin concentration during the 2-h following the intragastric 2.0 g/kg glucose load [group, time, and group × time effects: $F(3,27) = 2.58$, $p = 0.075$, $F(4,108) = 73.90$, $p < 0.0001$, and $F(12,108) = 2.01$, $p < 0.03$, respectively]. At the 15-min time point, plasma insulin level of the COM group was lower than that of both the EtOH (post hoc, $p < 0.009$) and THC ($p = 0.068$) groups. Finally, the COM group demonstrated a trend for an overall reduced insulin response compared with the CTLs [insulin AUC: group effect, $F(3,27) = 2.45$, $p = 0.085$; post hoc, $p = 0.087$; Fig. 4.6D]. In support of our *a priori* hypothesis that drug

combination will elicit unique effects, independent samples *t*-test revealed that the area under the insulin curve of the COM group was significantly lower than that of the CTLs [$t(15) = 2.19$, $p < 0.05$].

Subcutaneous THC, moderate alcohol, and their combination had no effect on brain CB_1R expression and basal GSK-3β activity

Animals in Experiment 1a were sacrificed nine days after the last drug treatment. Western blot analysis of the brain revealed no effect of 16-day separate or combined THC and alcohol exposure on CB_1R protein expression in the medial prefrontal cortex (mPFC), mediobasal hypothalamus (MBH), and hippocampus (HIP) [main effects: $F(3,34) = 0.33$, 1.38, and 0.41, respectively; all $p > 0.05$; Fig. 4.7]. In the MBH, *a priori t*-test revealed that the COM group had lower CB_1R protein expression compared with the CTL group [$t(18) = 2.40$, $p < 0.03$].

In Experiment 1b, animals were sacrificed 4–5 weeks after the last day of drug treatment. There were nonsignificant changes to CB_1R expression and GSK-3β activity in the three brain regions we analyzed (Fig. 4.11A and 4.11B). However, in the mPFC, *a priori t*-test revealed that the EtOH group had higher GSK-3β activity (or lower pGSK-3β to tGSK-3β ratio) compared with the CTL group [$t(10) = 3.13$, $p < 0.02$]; while in the MBH, *a priori t*-test revealed that the THC group had lower GSK-3β activity compared with the CTL group [$t(10) = -2.80$, $p < 0.02$].

Experiment 2: Chronic moderate alcohol drinking and oral THC consumption, alone or in combination

Individual differences in voluntary THC consumption

Simultaneous cookie and test fluid exposure (paradigm 1): during the 16-day treatment period, all CTL and EtOH rats that received oil cookie consumed it within 1 h. Six of the eight THC rats and five of the eight COM rats that received cookie overlaid with 3 mg/kg, 5 mg/kg, and the first 10 mg/kg THC consumed it within 3 h. One THC and three COM rats utterly avoided the 10 mg/kg THC cookie.

Cookie exposure following test fluid access (paradigm 2 and Experiment 2b): when this schedule of drug exposure was implemented during the last five days of Experiment 2a (Fig. 4.2A), three THC and four COM rats sporadically had bits of leftover THC-laden cookies. Thus, we

separated both groups into cookie consumers (THC_C, n = 5; COM_C, n = 4) and cookie avoiders (THC_A, n = 3; COM_A, n = 4). The "cookie avoiders" were rats with left-over cookies on two or more days. Throughout Experiment 2b (Fig. 4.2B), except for three COM rats that intermittently had bits of leftover cookies, all rats that received THC cookie consumed it before the next cookie was provided.

Oral THC dose-dependently reduced alcohol intake when alcohol was simultaneously consumed with THC-laden cookie

In Experiments 2a and 2b, THC and CTL rats increased their saccharin intake across treatment days [$F(2,24) = 9.52$ and $F(3,48) = 42.51$, respectively; both $p < 0.001$]. Weight-adjusted analyses of saccharin intake revealed no significant group differences, but THC rats tended to consume less saccharin in Experiment 2a [$F(1,12) = 3.60$, $p = 0.08$]. The effect of oral THC on alcohol consumption depended on the temporal order of their consumption. Under paradigm 1 of Experiment 2a when sweetened 10% alcohol solution and THC-laden cookies of increasing doses were simultaneously available, COM_C rats progressively reduced their alcohol consumption compared with the EtOH rats [group and group × time, $F(1,11) = 8.33$ and $F(2,22) = 5.37$, $p < 0.02$, respectively; both $p < 0.02$; Fig. 4.12A]. Throughout paradigm 2 of Experiment 2a when rats consumed sweetened 5% alcohol solution before THC-laden cookies, both COM_C and COM_A rats continued to consume less alcohol compared with the EtOH rats [$F(2,103 = 8.00$, $p < 0.006$; Fig 4.12C]. Although EtOH rats appeared to reduce their alcohol intake when the concentration of alcohol solution was reduced from 10% (cookie simultaneously available) to 5% (cookie not simultaneously available), the COM rats still consumed significantly less alcohol. When alcohol was presented prior to THC-laden cookies of increasing doses in Experiment 2b, the EtOH and COM rats consumed similar alcohol doses i.e., intake was not THC dose-dependent (Fig. 4.13A).

Tail blood was sampled during drug exposure in Experiment 2b to ascertain how oral THC consumption would affect BEC kinetics. EtOH (1.53 ± 0.15 g/kg) and COM (1.24 ± 0.10 g/kg) rats consumed similar alcohol doses within 1 h following 8 mg/kg/day THC consumption. BECs attained by the EtOH and COM groups were 24.8 ± 8.2 mg/dl and 25.4 ± 11.6 mg/dl, respectively. The BECs significantly correlated with the consumed dose only in the EtOH group (Fig. 4.13B). After 3 h of alcohol access following 10 mg/kg/day THC consumption, EtOH (1.64

± 0.18 g/kg) and COM (1.36 ± 0.18 g/kg) rats consumed comparable alcohol doses and BECs were barely-detectable (< 1.0 mg/dl) at this time point.

Oral THC, moderate alcohol, and their interaction reduced short-term chow intake

The temporal order of THC and alcohol consumption differently affected 3-h chow intake. In paradigm 1 of Experiment 2a, the EtOH group consumed less 3-h chow compared with the CTL and THC groups [$F(3,23) = 8.93$, $p < 0.0005$; post hoc, both $p < 0.004$; Fig. 4.12B]. But in paradigm 2, EtOH, THC, and COM groups consumed less 3-h chow relative to the CTLs [$F(5,26) = 9.72$, $p < 0.0001$; post hoc, all $p < 0.008$; Fig. 4.12D]. In Experiment 2b, although THC-laden cookies were not available during the last 3 h of the dark cycle, COM and EtOH rats consumed less 3-h chow compared with the CTLs [$F(3,29) = 3.64$, $p < 0.03$; post hoc, $p < 0.03$ and = 0.086, respectively; Fig. 4.13C]. However, this treatment effect was only marginal (p = 0.089) with body weight-adjusted analyses (see Supplementary table 4.1).

In Experiment 2a, daily caloric intake and weight gain were unaffected during drug exposure and the 2-week abstinent period. In Experiment 2b, THC rats consumed fewer daily calories compared with the CTLs [$F(3,29) = 3.71$, $p < 0.03$; post hoc, $p < 0.03$], but this effect became insignificant when the intake data were adjusted by body weight. However, the THC rats gained less weight compared with the CTLs [$F(3,29) = 3.77$, $p < 0.03$; post hoc, $p < 0.02$; Fig. 4.13D]. Such group difference in weight gain did not persist into the third week of abstinence.

Oral THC, moderate alcohol, and their combination had no effect on sucrose intake during abstinence

In Experiment 2a, abstinence from oral THC and voluntary alcohol drinking had no effect on sucrose preference as all the groups preferred 1% sucrose solution to tap water during the 48-h (P47–P48) and 72-h (P54–P56) preference tests. There were no group differences in sucrose and total fluid intakes. Experiment 2b showed identical findings (Fig. 4.14A and 4.14B).

Oral THC, moderate alcohol, and their combination had no effect on Barnes maze performance

In Experiment 2a, escape latency and distance travelled decreased across learning sessions [latency, ($F(7,175) = 65.62$ and distance travelled, $F(7,140) = 57.23$; both $p < 0.0001$]. There

were no enduring effects of treatment on spatial memory and behavioral flexibility (Supplementary Fig. 4.1).

THC, alcohol, or their combination did not alter age of puberty onset

Pre-pubertal drug treatment had no effect on the age of puberty onset in Experiments 1 and 2. The average age of puberty onset was 37 days (P34–P41 among rats in all four groups) in Experiment 1, and 38 days (P33–P42) in Experiment 2.

Subcutaneous and oral THC resulted in significant plasma cannabinoid levels

Significant plasma levels of THC and THC-COOH were detected 14 h after injection of 10 mg/kg (Experiment 1b) or access to a 5 mg/kg THC-laced cookie (Experiment 2b) in both THC and COM rats (Table 4.2). However, significant levels of 11-OH-THC were detected only in rats that received THC injection. Finally, 11-OH-THC levels were significantly lower in the COM group compared with the THC group [$t(9) = 3.96$, $p < 0.01$] when THC was injected subcutaneously.

Discussion

The present study is the first to examine the effects of adolescent alcohol and THC exposure, alone or when combined, on ingestion, cognition, glucose homeostasis, and brain CB_1R and GSK-3β protein expressions in male rats. It revealed that the effects of co-exposure to the two drugs on ingestive behaviors depend on the route of THC administration. Consistent with our hypothesis, moderate alcohol suppressed acute chow intake and subcutaneous THC dose-dependently induced acute hyperphagia without affecting alcohol intake. By contrast, oral THC can alleviate the hypophagic effect of moderate alcohol, but doses higher than 5 mg/kg blunted alcohol intake. These drug administrations did not significantly change total daily caloric intake. However, higher cumulative doses of subcutaneous and oral THC suppressed weight gain. Inconsistent with our hypothesis, co-exposure to alcohol and subcutaneous or oral THC did not impair spatial learning and memory in a Barnes maze. Also inconsistent was our observation that 16 days of alcohol and subcutaneous THC co-exposure mildly improved glucose homeostasis without altering CB_1R protein expression and basal GSK-3β activity in the cortex, hippocampus,

and mediobasal hypothalamus. Finally, chronic subcutaneous but not oral THC reduced intake of 1% sucrose solution during abstinence, with no significant interaction with alcohol.

Caloric intake and body weight

The route and schedule of THC administration dictated its dose-dependent effects on caloric intake in rats. Results of Experiment 1 indicate that rats compensated for the immediate hyperphagic effect of THC by reducing chow intake during the subsequent period of the day. The acute hyperphagic effects of subcutaneous THC injection concur with early studies with low dose (< 1 mg/kg) intraperitoneal THC injection in rats (Jarbe and DiPatrizio 2005). Other studies with THC doses > 1 mg/kg, however, reported no change or reduced food intake immediately following intraperitoneal THC injection (Drewnowski and Grinker 1978; Graceffo and Robinson 1998). Thus, compared with intraperitoneal injection, higher doses (> 5 mg/kg) of subcutaneous THC are required to produce immediate increase in appetite.

Previous studies have shown that oral gavage of THC increases short-term (within 2 h) food intake at a dose as low as 0.50 mg/kg in adult rats (Farrimond et al. 2010; Williams et al. 1998). By contrast, in Experiment 2a, voluntary oral THC consumption did not produce immediate (3-h) hyperphagia. The progressive increase in food intake during adolescence may mask the moderate hyperphagic effects of oral THC that may appear given a larger sample size. Furthermore, the use of calorie-rich cookie (approximately 4 kcal/cookie) to deliver THC might have precluded a hyperphagic response by making the rats consume less chow. But it is unlikely that the calorie from cookie masked the hyperphagic effect of THC because the rats could consume greater than 20 kcal as shown in Experiment 1 or greater than half of their daily energy intake within 15 min when they are hyperphagic (Liang et al. 2013). Alternatively, the decrease in 3-h chow intake observed in the THC-exposed groups in Experiment 2a (paradigm 2) and 2b is possibly a compensatory response to the acute effects of the previous oral THC dose since daily total caloric intake was unaltered.

The divergent effects of subcutaneous versus oral THC on caloric intake may relate to the bioavailability and pharmacokinetic profiles of THC. Compared with injected or inhaled THC, orally-consumed THC peaks slowly in plasma (Grotenhermen 2003; Manwell et al. 2014a). The lipophilic nature of THC permits its easy permeation across the blood brain barrier. Thus, the timeline of plasma THC and its major active metabolite (11-OH-THC) reflects changes to brain

levels (Tseng et al. 2004; Wiley and Burston 2014) where they act on select brain regions that regulate appetite and motivation (Di Marzo and Matias 2005; Gallate et al. 1999; Kirkham et al. 2002; Williams and Kirkham 2002a). THC can either stimulate or suppress appetite depending on the relative proportion of presynaptic glutamatergic or GABAergic terminals on which it acts, and the ensuing effects on hormone signaling (Bellocchio et al. 2010; Gatta-Cherifi and Cota 2015; Mazier et al. 2015). Moreover, cannabinoid activity in mitochondria and glia, hepatocytes, adipocytes, myocytes, and other tissue types are involved in energy balance regulation as reviewed in (Gatta-Cherifi and Cota 2015) and (Patel and Cone 2015). Paradoxically, low-concentration THC can antagonize the canonical synaptic effects of the endocannabinoid – 2-arachidonyl glycerol (2-AG), and of the full agonist – WIN 55,212-2 (Kelley and Thayer 2004) especially in brain areas containing lower CB_1R density with varying coupling efficiencies (Pertwee 2008). The effects of THC on food intake in rodents are complex and can be dictated by an array of biological and methodological factors. Compared with existing involuntary alcohol and cannabinoid co-administration models (Khatri et al. 2018; Swartzwelder et al. 2012), our novel voluntary alcohol and THC co-use model will be useful for studies aimed to explore the underlying biological mechanisms that mediate the effects of oral drug consumption. As an added advantage, our model can be adapted to examine the dose-dependent effects of alcohol and THC co-use on behavior and energy balance.

Rats exposed to alcohol alone adequately regulated their caloric intake, and alcohol interacted differently with subcutaneous and oral THC to modify caloric intake. In Experiments 1 and 2, EtOH rats reduced their chow intake during the 3-h alcohol access period to compensate for the calories from alcohol. That is, chow intake during the other period of the day did not differ between EtOH rats and the controls. These results are consistent with others and our previous reports (Cornier et al. 2002; Nelson et al. 2016; Nelson et al. 2017). Furthermore, subcutaneous THC minimized the immediate hypophagic effect of alcohol in the COM group (Fig. 4.3D and 4.8B). Eating of < 5 mg/kg THC-laced cookie also reversed the hypophagic effect of alcohol (Fig. 4.12B). But when the oral THC dose reached 10 mg/kg, alcohol intake was robustly reduced (Fig. 4.12A) and some rats stopped consuming the THC-laced cookie. Individual differences in voluntary THC consumption has been observed among humans where some experience euphoria and others experience dysphoria or paranoia upon initial

experimentation with cannabis (Earleywine 2002). Future studies should explore individual differences in alcohol and THC co-use in-depth.

The unique effects of combined alcohol and THC reported in this study might relate to some pharmacokinetic interactions between both drugs. Exposure to THC after alcohol consumption can modestly increase circulating levels of THC and 11-OH-THC (Hartman et al. 2015a; Lukas and Orozco 2001). Concurrent alcohol consumption also increases and prolongs the pleasurable effects of smoking or inhaling vaporized cannabis (Hartman et al. 2016; Lukas and Orozco 2001). Our study shows that moderate alcohol consumption had insignificant effect on plasma levels of THC and THC-COOH in rats that received THC subcutaneously or orally. This observation concurs with prior studies that documented no significant effect of alcohol on blood or salivary THC concentrations, as well as on the subjective effects of smoked cannabis due to wide individual variations in these variables (Hartman et al. 2015b; Perez-Reyes et al. 1988). Interestingly, whereas 11-OH-THC was undetected 14 h following oral consumption of 5 mg/kg THC (via cookie), 11-OH-THC was significantly lower in alcohol-consuming rats injected with 10 mg/kg THC (Table 4.2). Future studies should elucidate the mechanisms by which moderate alcohol drinking alters metabolism of THC administered via different routes.

Subcutaneous and oral THC modestly influenced body weight. The high cumulative THC dose administered in Experiments 1b and 2b (152 and 108 mg/kg, respectively), compared with Experiments 1a and 2a (84 and 73.5 mg/kg, respectively), was associated with suppressed weight gain. Our results, in conjunction with findings from other groups (Drewnowski and Grinker 1978; Keeley et al. 2015b; Rubino et al. 2008), suggest that the effects of THC on weight gain may have more to do with the cumulative dose and frequency of daily administrations than with the route of administration. Intriguingly, whereas moderate alcohol drinking alone did not influence body weight, it interacted differently with subcutaneous versus oral THC to affect weight gain as observed in the results of the COM groups. In Experiment 1a, the COM group had the lowest weight gain during abstinence (Fig. 4.3E). When the THC dose increased in Experiment 1b, similar daily moderate alcohol drinking mildly alleviated the enduring suppressed weight gain associated with injected THC (Fig. 4.8D). Moderate alcohol drinking also modestly alleviated the suppressed weight gain caused by voluntary THC consumption during treatment in Experiment 2b (Fig. 4.13D).

Saccharin and alcohol intake and BEC

The results that THC administered subcutaneously or orally could reduce saccharin intake contradict previous reports that systemic or central administration of endocannabinoid or THC increases intake and palatability of sweet solutions (Gallate et al. 1999; O'Brien et al. 2013; Shinohara et al. 2009). Likewise, that oral THC can robustly reduce alcohol intake contrasts with previous findings where systemic injection of CB_1R agonists increases voluntary ethanol drinking (Colombo et al. 2002; Linsenbardt and Boehm 2009). These intake suppression effects, however, are in line with the notion that THC can be aversive for rodents. Intraperitoneal injection of THC can support conditioned taste as well as place aversion in both rats and mice (Han et al. 2017; Manwell et al. 2014b; Wakeford and Riley 2014). Although the procedures in our experiments were not specifically designed to examine the aversive property of THC, the results of alcohol intake support that rats are more tolerant to the aversive effects of subcutaneous than of oral THC. Conversely, alcohol may enhance the aversive effects of THC because more COM than THC rats stopped eating THC-laced cookies in Experiments 2a (4 vs. 3 rats) and 2b (3 vs. 0 rats). This phenomenon, colloquially termed being "crossfaded", can manifest in individuals who simultaneously use alcohol and cannabis to achieve an enhanced *high* (Patrick et al. 2018). It may result from high-potency or -dose THC interacting with alcohol to reverse the rewarding effects or to potentiate the aversive effects of their combined use. The dose-dependent effect of oral THC in reducing alcohol intake suggests that the phenomenon is not likely due to cookie availability. Additionally, the unchanged alcohol intake following subcutaneous 20 mg/kg/day THC administration that resulted in higher plasma levels of THC and its active metabolite than oral 10 mg/kg/day THC did suggests that the sedative effect of THC played a minor role in the robust alcohol intake reduction following oral THC administration.

We found no significant differences in BEC between the EtOH and COM groups. This finding is in line with the observation that smoking THC has no effect on BEC in humans (Chait and Perry 1994; Downey et al. 2013; Perez-Reyes et al. 1988). Yet, our results was unexpected considering evidence showing that alcohol dehydrogenase also metabolizes THC (Wall et al. 1983), and that THC inhibits alcohol metabolizing enzymes (Marselos et al. 1991). Thus, we expected BEC to be elevated in the COM group compared with the EtOH group, as has been observed in humans (Chesher et al. 1976). A previous study, however, demonstrated that

smoking cannabis can attenuate BEC and the psychoactive effects of alcohol in humans (Lukas et al. 1992). Overall, these results indicate that the unique effects of alcohol and THC co-use on the pharmacokinetics of each drug and on behavioral processes is an important avenue for further research.

Sucrose preference

As we previously observed (Nelson et al. 2017), abstinence from chronic moderate alcohol consumption did not elicit behavioral anhedonia measured by sucrose preference (Kang et al. 2016). Here we did not observe an effect on sucrose preference following abstinence from chronic THC, alone or when combined with moderate alcohol intake. Our findings are consistent with the report that chronic combined moderate alcohol consumption and marijuana smoking had no influence on mood in humans (Chait and Perry 1994). We also found that rats previously exposed to subcutaneous, but not oral, THC consumed less sucrose during abstinence compared with controls. Such behavioral manifestation may resemble an endophenotype of psychophathology and a sequela of drug abstinence (e.g., avolition) (Renard et al. 2014; Renard et al. 2017). However, other factors such as changes in taste detection and perception could have contributed to the reduced sucrose intake. This is the first study to demonstrate changes in sucrose intake during abstinence from THC and its combined use with alcohol. The differential effects of subcutaneous and oral THC and their interaction with moderate alcohol to affect sucrose intake during abstinence warrant further investigation.

Learning, memory, and behavioral flexibility

Cognitive dysfunction is a predisposing factor for drug use disorder, and it contributes to poor addiction treatment outcomes (Spear 2018). Our study is the first to investigate the combined effects of chronic THC and moderate alcohol exposure on cognitive functions assessed in the Barnes maze during adolescence. Surprisingly, exposure to neither drugs impaired spatial learning, memory, and behavioral flexibility (Fig. 4.5 and 4.10). Several factors may explain our inability to detect behavioral impairments in the rats, including the ease of the Barnes maze tasks we employed and the fact that THC is a partial CB_1R agonist. Congruent with both points, cognitive deficits have been observed when 1) sensitive behavioral tasks like novel object recognition (Murphy et al. 2017; O'Shea et al. 2004; Swartzwelder et al. 2012), eight-arm radial

maze (Rubino et al. 2009), Morris water maze (Cha et al. 2007; Tomas-Roig et al. 2017), and fear conditioning (Tomas-Roig et al. 2017) are employed, and 2) more potent CB_1R agonist like WIN 55,212-2 and CP 55,940 are used (O'Shea et al. 2004; Tomas-Roig et al. 2017). Finally, the coincident peak blood THC and alcohol concentrations attained in this study might have been subthreshold to that required to uncover interactive effects between THC and moderate alcohol (Ramaekers et al. 2006).

Glucose homeostasis

The OGTT performed on the ninth day following the last drug exposure revealed modest changes to systemic glucose tolerance. The plasma insulin content of the COM group was lower than that of the CTL group at the 15-min time point following an oral glucose load. The plasma insulin AUC showed that, compared with the CTLs, rats co-exposed to alcohol and THC for 16 days required slightly lower plasma insulin for proper glucose clearance. Ours is the first preclinical animal study to document mild beneficial effects of moderate alcohol and THC co-use on glucose homeostasis in adolescent male rats. Our finding corroborates the observation of null or favorable effects of moderate alcohol or THC use on glucose homeostasis in humans and rodents (Baliunas et al. 2009; Furuya et al. 2003; Penner et al. 2013; Permutt et al. 1976; Pietraszek et al. 2010; Steiner et al. 2015). To my knowledge, there exist no clinical or epidemiological research that has systematically investigated the metabolic effects of joint alcohol and marijuana use. The published epidemiological studies that investigated the metabolic effects of alcohol or marijuana use often statistically controlled for use of other drugs or screened for and excluded participants with a history of poly-substance use. Further study of the effects of alcohol and marijuana co-use on glucose homeostasis is warranted, as is investigations of the cellular mechanisms that mediate the outcomes.

Neurobiological changes

Studies have shown that adolescent alcohol or cannabinoid use can alter the developmental of diverse brain systems, and some of the changes can persist into adulthood (Crews et al. 2007; Ellgren et al. 2008; Gulley and Juraska 2013; Philpot et al. 2009; Spear 2016). CB_1R, an essential component of the endocannabinoid system, is the most widely expressed metabotropic receptor in the mammalian brain and plays an essential role in adolescent brain development and

functioning (Fernandez-Ruiz et al. 2000; Long et al. 2012). The kinase GSK-3β is an important player in the signaling cascades stimulated by diverse ligands in the brain (Sutherland 2011). Phosphorylation of GSK-3β at the N-terminal serine 9 residue disrupts its interaction with diverse cellular substrates (Beurel et al. 2015; Sutherland 2011). Thus, the ratio of phosphorylated to total GSK-3β protein expression serves as a proxy of its enzymatic activity. Reduced enzyme activity (via higher pGSK-3β to tGSK-3β ratio) correlates with many beneficial neurobiological and metabolic effects.

In Experiments 1a and 1b, we assayed how separate or combined alcohol and THC exposure would alter CB_1R and GSK-3β expression and activity in brain regions essential for regulating executive function (mPFC), energy balance (MBH), and memory (HIP). When measured 1–5 weeks after drug exposure, we did not detect significant changes in the expression of both proteins. Our inability to detect significant changes in CB_1R expression can be explained by its dynamic homeostatic regulation in the brain, but it does not preclude the fact that the receptors could have been desensitized by the repeated exposure to THC during adolescence (Farquhar et al. 2019; Rubino et al. 2008). Desensitized receptors sub-optimally respond to stimulation by ligands. Importantly, a previous study observed nonsignificant changes to CB_1R protein by western blot when measured five weeks after chronic THC exposure in adolescent male mice (Kasten et al. 2017). Another study observed nonsignificant changes in CB_1R expression in the hippocampus and frontal cortex of rats measured two weeks after chronic intermittent alcohol consumption (Marco et al. 2017). *In vivo* PET imaging studies have also generated inconsistent findings regarding the effects of alcohol on CB_1R dynamics. Human and rat PET studies have observed increases and decreases in CB_1R availability in several brain regions after acute and chronic alcohol administration, respectively (Ceccarini et al. 2013; Ceccarini et al. 2014). Further, the nonsignificant changes to basal GSK-3β activity in the brains of drug-abstinent rats could reflect compensation by the diverse signaling pathways reliant on the enzyme. Our *a priori* pairwise comparisons revealed opposite changes in GSK-3β activity in the mPFC and MBH. The implications of the modestly increased GSK-3β activity in the mPFC of EtOH rats relative to CTLs, and the modestly reduced GSK-3β activity in the MBH of THC rats relative to CTLs (Fig. 4.11B) are unclear in this preliminary study. The fact that rats in our study consumed moderate doses of alcohol, were administered relatively moderate doses of THC, and were abstinent for several weeks before their brains were harvested could explain the null or

modest results. Perhaps, the evolutionary importance of GSK-3β activity makes it resilient when perturbed by minor environmental insults. Other researchers have observed that moderate to high doses of alcohol acutely inhibits GSK-3β activity in rodent medial prefrontal cortex and nucleus accumbens (Neasta et al. 2011; Neznanova et al. 2009; van der Vaart et al. 2018).

Puberty timing

Prior studies found that prepubertal alcohol and cannabinoid exposure can alter puberty onset and estrous cycling in female rats (Dees and Skelley 1990; Marusich et al. 2014; Wenger et al. 1988). No much is known about the separate or combined effects of alcohol and THC exposure on puberty onset in male rats. Using preputial separation as a marker for puberty onset (Korenbrot et al. 1977), we found that THC and alcohol, alone or when combined, had no effect on puberty onset relative to controls. The male rats in this study, however, attained puberty at around P38, which is a few days earlier than what the literature suggests (P40–P48) (Juraska and Willing 2017).

Because our animals were received from the vendor around weaning (P22–P25), the hastened puberty onset may be the outcome of early life stresses (maternal/sibling separation and environmental changes associated with shipping or transportation) or interactions between early-life stresses and the injection exposure. The stress of the injection procedure in Experiment 1 might have masked any THC-specific effect on preputial separation. The enduring effects of injection procedure have been suspected to account for neuroanatomical and behavioral differences in adult rats that were injected during adolescence (Keeley et al. 2015a). Notwithstanding, the early age of puberty onset we observed may not be attributable to subcutaneous injection experience since rats not injected also attained puberty early (Experiment 2). Moreover, a previous study observed no effect of injection procedure on puberty onset in male Long-Evan rats (Kang et al. 2016).

Conclusion

Co-use of alcohol and cannabis is in vogue despite the lack of empirical data on how it affects brain and behavior. In establishing novel rodent models of alcohol and THC co-use, we found that injected or orally administered THC can dose-dependently interact with alcohol to differently influence feeding behaviors and glucose homeostasis. Importantly, our established

method of voluntary THC and alcohol ingestion is an improvement over the existing involuntary alcohol and cannabinoid co-administration models (Ciccocioppo et al. 2002; Khatri et al. 2018; Swartzwelder et al. 2012), and approximates the manner of human consumption. Our methods can be easily adapted for investigations of the behavioral, metabolic, and neurobiological mechanisms of joint alcohol and cannabinoid use in adolescent and adult subjects.

Acknowledgements

Startup funds from the Psychology Department of the University of Illinois at Urbana-Champaign (to NCL), and NIH grants R03DA043701 (to NCL), and R01GM115584-01A1 and R03DA042365-01A1 (both to AD) supported this study. We thank Dr. Lucas Li of the Roy J. Carver Metabolomics Center (UIUC) for performing the LC-MS/MS analyses. We acknowledge the technical assistance of Firmino Pinto, Charles Shoemaker, Jacqueline Sanchez, Tiffany Yang, and Sheel Vasavada, and are grateful to Dr. Justin Rhodes who provided access to the TopScan software. Portions of this work were presented at the 41st annual scientific meeting of the Research Society on Alcoholism in San Diego, CA, USA.

Tables and Figures

Table 4.1: Tabulation of the primary antibodies we used for Western blotting

Antibody	**Source and Cat. #**	**Isotype**	**Molecular weight (kDa)**	**Dilution used**
CB_1R	ThermoFisher Scientific PA1-745	Rabbit polyclonal	60	1:1000
pGSK-3β (S^9)	Cell Signaling 9336	Rabbit polyclonal	46	1:1000
tGSK-3β	Cell Signaling 9315	Rabbit monoclonal	46	1:10000
Vinculin	Cell Signaling 13901	Rabbit monoclonal	124	1:5000

The subscripts “p” and “t” denote “phosphorylated” and “total” protein, respectively.

Table 4.2: Plasma levels of THC and its major metabolites in Experiments 1b and 2b

Route / Group	**Subcutaneous THC injection**			**Oral THC consumption**		
	THC	11-OH-THC	THC-COOH	THC	11-OH-THC	THC-COOH
THC	16.9 (2.89)	5.0 (0.66)*	7.1 (1.02)	1.3 (0.28)	*nd*	1.8 (0.44)
COM	15.1 (1.29)	2.1 (0.36)*	5.5 (1.79)	8.6 (5.11)	*nd*	3.1 (0.81)

Data are mean (± SEM) concentrations (ng/ml) in each treatment group (n = 5–6). *: significantly different from each other according to Student's *t*-test ($p < 0.01$). *nd*: non-detectable.

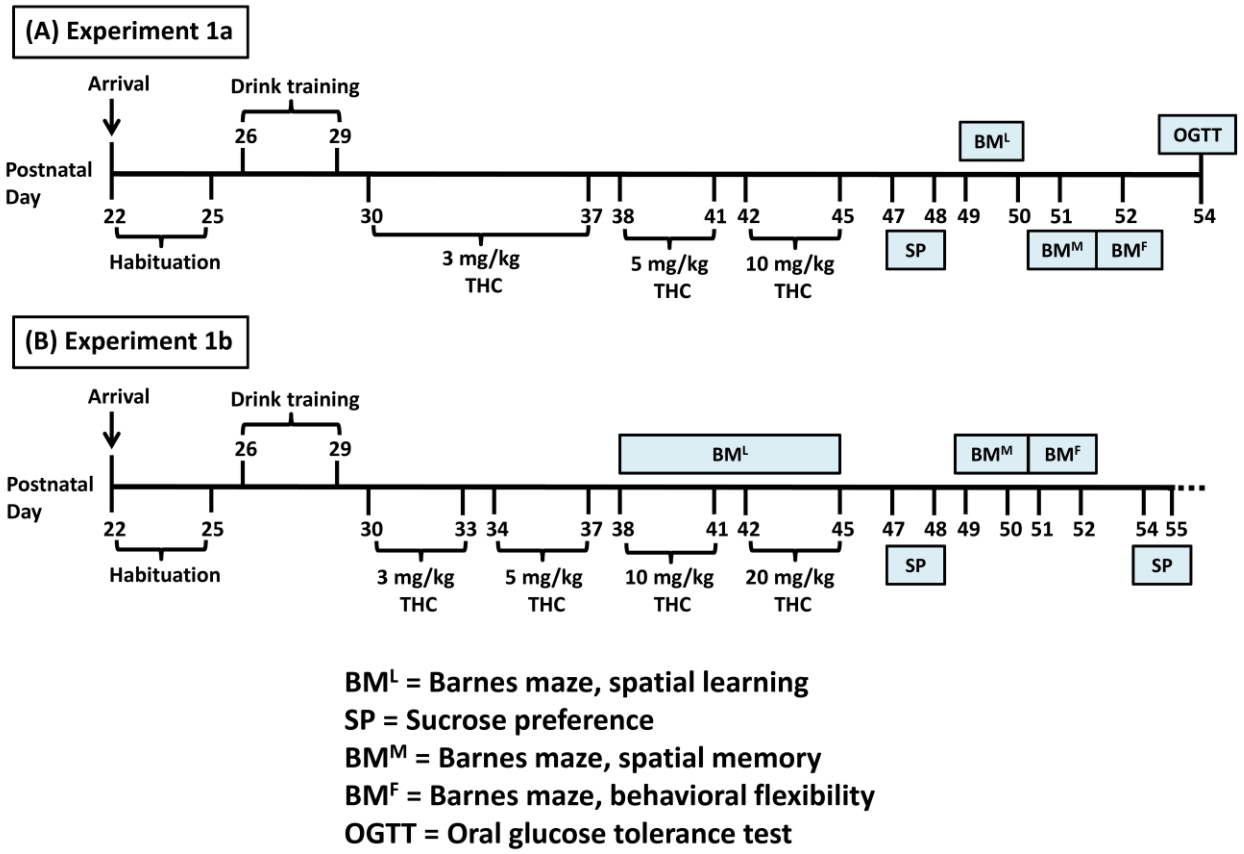
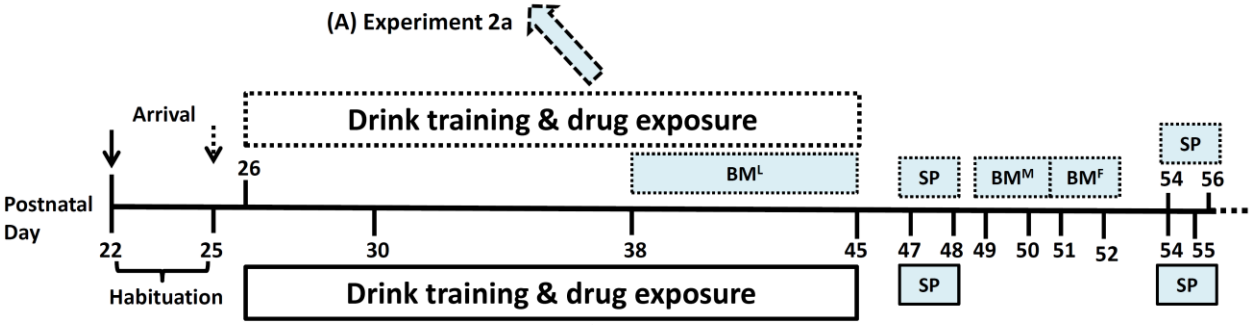


Figure 4.1: Timeline of chronic subcutaneous THC injection and voluntary alcohol drinking. (**A**) In Experiment 1a (EtOH, n = 8; CTL, THC, and COM, n = 10/group), 3 mg/kg/day THC lasted for eight days, while the 5 and 10 mg/kg/day THC each lasted for four days. Sucrose preference (SP), Barnes maze (BM), and oral glucose tolerance test (OGTT) were performed as indicated. (**B**) In Experiment 1b (n = 10/group), the four THC doses (3, 5, 10, and 20 mg/kg/day) each spanned four days. BM and SP tests were performed as indicated. Spatial learning (BM^L) occurred during the last 8 days of drug treatment.

Time/Age	P26–29	P30–33	P34–37	P38 & 39	P40	P41	P42	P43	P44	P45
8:30 – 11:30 AM Cookie Test fluid	oil	3 mg/kg or oil	5 mg/kg or oil	10 mg/kg or oil	5% EtOH or Sac	5% EtOH or Sac	5% EtOH or Sac	5% EtOH or Sac	5% EtOH or Sac	5% EtOH or Sac
	0.1% Sac	10% EtOH or Sac	10% EtOH or Sac	10% EtOH or Sac						
12:30 PM (Cookie)					oil	1.5 mg/kg or oil	1.5 mg/kg or oil	3 mg/kg or oil	3 mg/kg or oil	5 mg/kg or oil
9:00 PM (Cookie)					oil	1.5 mg/kg or oil	3 mg/kg or oil	3 mg/kg or oil	5 mg/kg or oil	5 mg/kg or oil



(B) Experiment 2b

Time/Age	P26–29	P30–33	P34–37	P38–41	P42–45
8:30 AM (Test fluid)	0.1% Sac	5% EtOH or Sac	5% EtOH or Sac	5% EtOH or Sac	5% EtOH or Sac
12:30 PM (Cookie)	oil	1.5 mg/kg or oil	3 mg/kg or oil	3 mg/kg or oil	5 mg/kg or oil
9:00 PM (Cookie)	oil	1.5 mg/kg or oil	3 mg/kg or oil	5 mg/kg or oil	5 mg/kg or oil

BM^L = Barnes maze, spatial learning
SP = Sucrose preference
BM^M = Barnes maze, spatial memory
BM^F = Barnes maze, behavioral flexibility

Figure 4.2: Timeline of chronic oral THC consumption and voluntary alcohol drinking. Treatments indicated in the shaded part of the tables occurred during the last 3 h of the dark cycle. (**A**) Manipulations in Experiment 2a (CTL and EtOH, n = 8/group; THC_C, n = 5; THC_A, n = 3; COM_C and COM_A, n = 4) are indicated in the dotted boxes and top table. The "A" or "C" following the underscores denote "Avoiders" and "Consumers" of THC-laden cookie, respectively. The transition from paradigm 1 to 2 occurred on P40. (**B**) Manipulations in Experiment 2b (CTL, n = 10; EtOH and THC, n = 8/group; COM, n = 7) are indicated in the solid boxes and bottom table. Barnes maze testing was not performed.

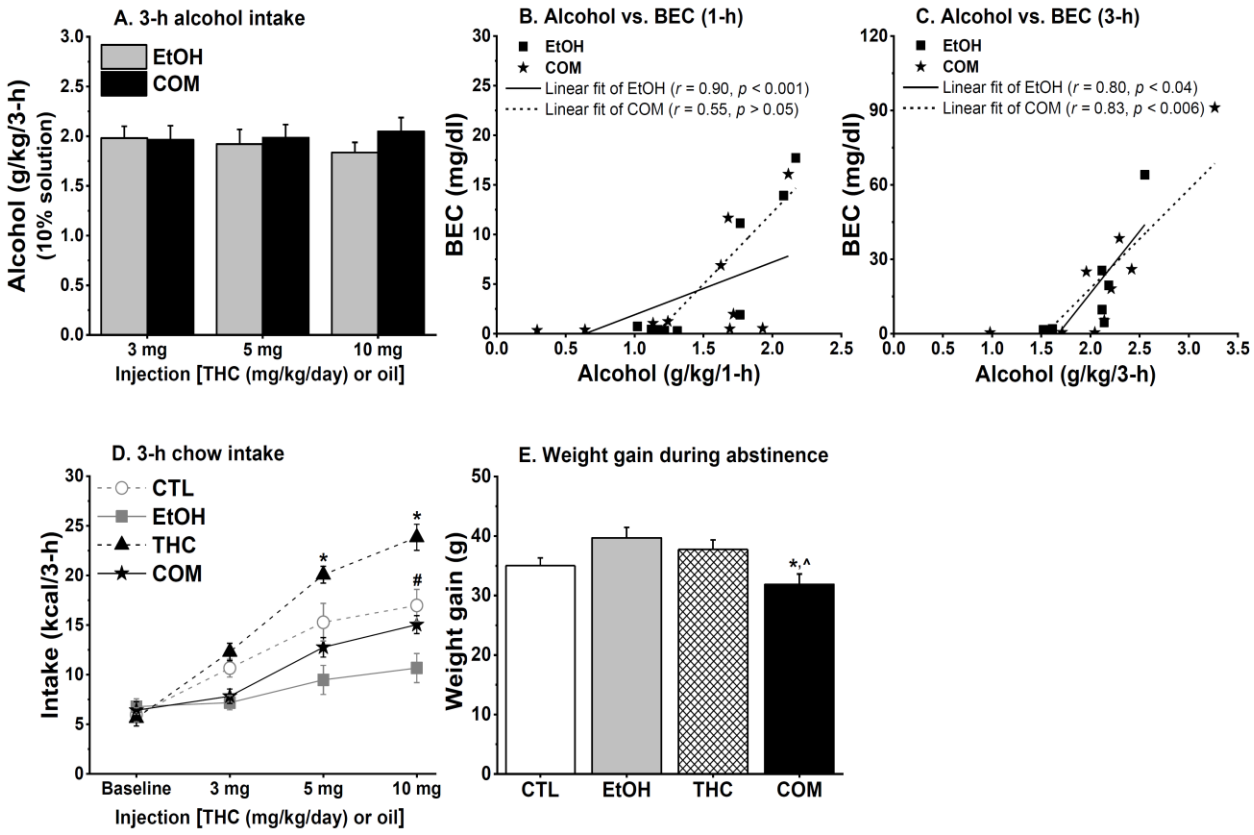


Figure 4.3: Chronic subcutaneous THC and voluntary alcohol consumption, alone or when combined, differently affected caloric intake and weight gain (Experiment 1a: EtOH, n = 8; CTL, THC, and COM, n = 10/group). (**A**) EtOH and COM rats consumed similar doses of sweetened 10% alcohol. (**B**) Blood ethanol concentration (BEC) correlated with 1-h alcohol intake in the EtOH group (r = 0.90, p < 0.001). (**C**) BEC correlated with 3-h alcohol intake in the EtOH group (r = 0.80, p < 0.04) and with 3-h alcohol intake in the COM group (r = 0.83, p < 0.006) after subcutaneous 10 mg/kg THC. (**D**) THC injection dose-dependently stimulated 3-h chow intake. Alcohol consumption reduced 3-h chow intake and blunted the hyperphagic effect of THC. THC vs. EtOH and COM: *p < 0.004; CTL vs. EtOH and THC: #p < 0.03. (**E**) COM rats gained the least weight during drug abstinence. COM vs. EtOH: *p < 0.02; COM vs. THC: ^p = 0.057.

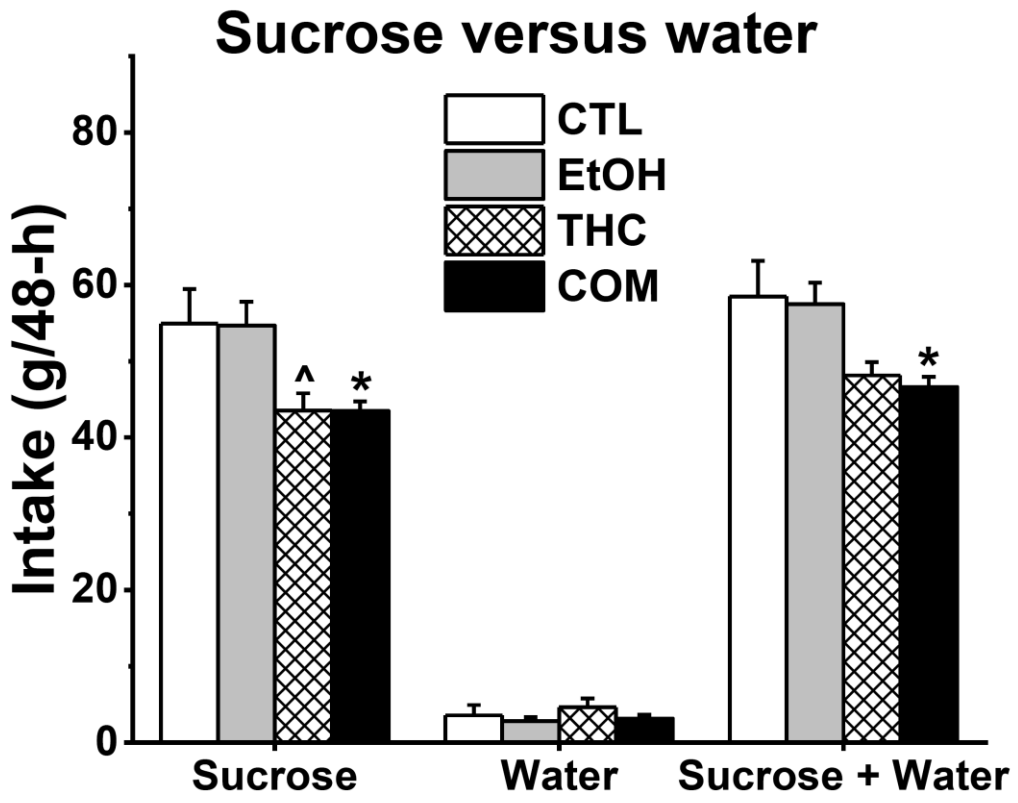


Figure 4.4: Chronic subcutaneous THC alone or when combined with voluntary alcohol consumption reduced sucrose and total fluid intake during a 48-h sucrose preference test conducted 2–3 days (P47–P48) after drug treatment ended (Experiment 1a: EtOH, n = 8; CTL, THC, and COM, n = 10/group). THC vs. CTL: ^p = 0.058; COM vs. CTL: *p < 0.05.

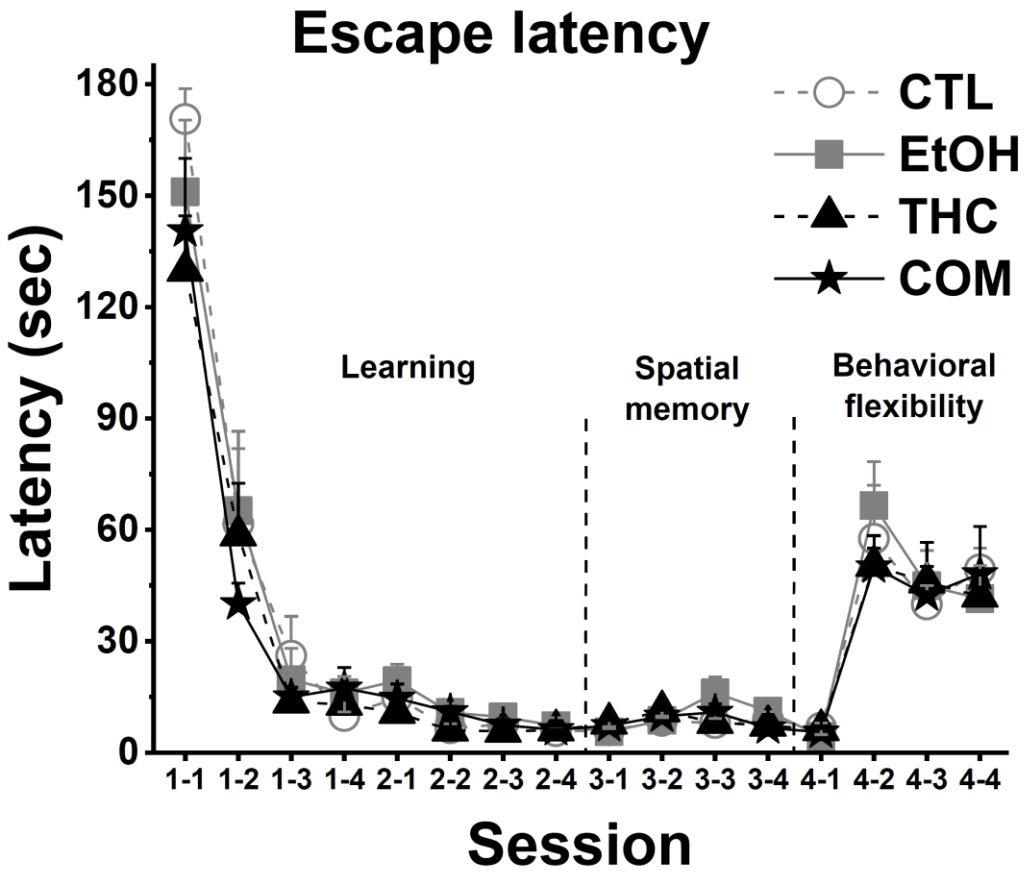


Figure 4.5: Chronic subcutaneous THC injection and voluntary alcohol consumption, alone or when combined, had no effect on cognitive behaviors assessed in the Barnes maze (Experiment 1a: EtOH, n = 8; CTL, THC, and COM, n = 10/group). There were no group differences in escape latency throughout the procedure.

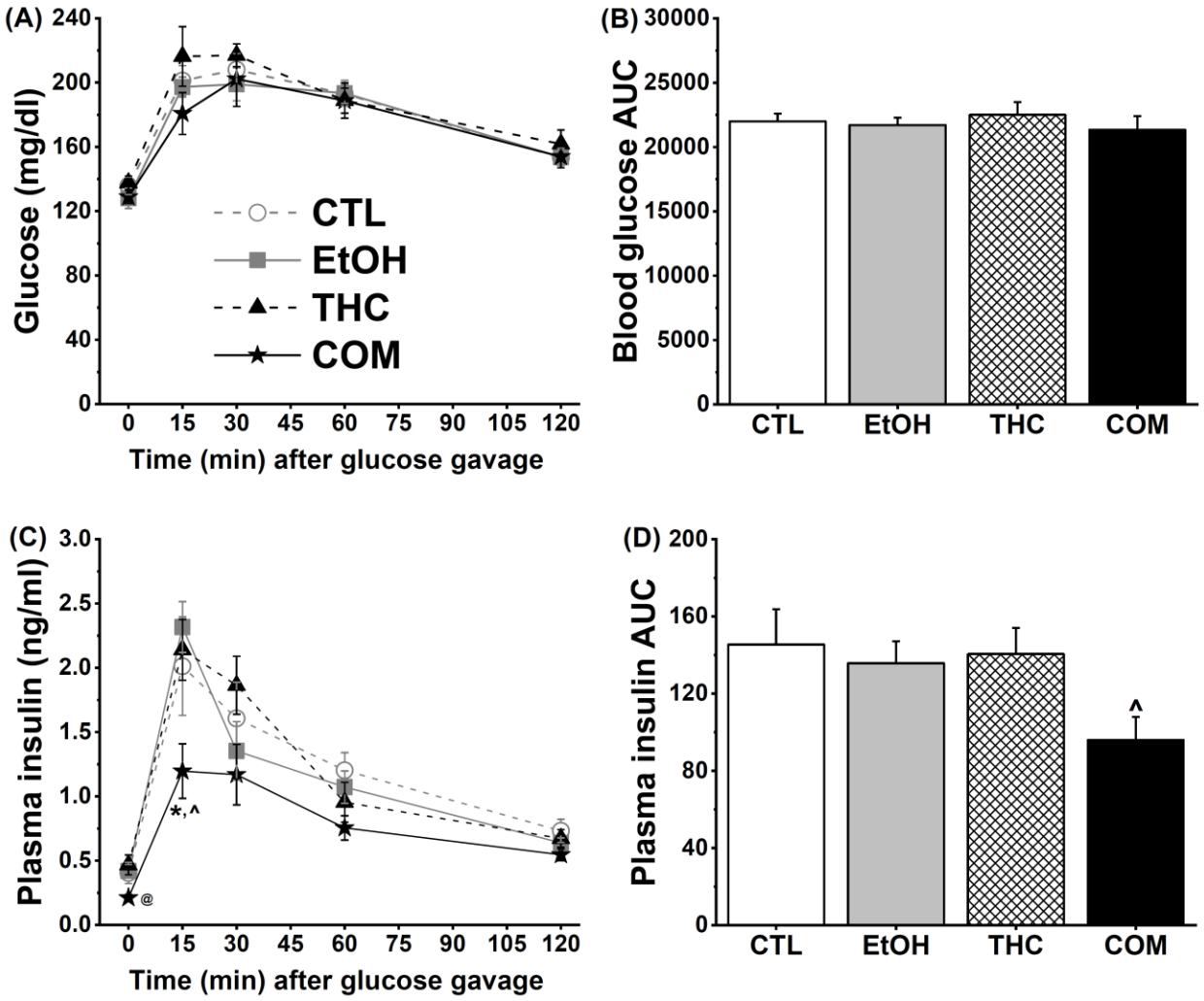


Figure 4.6: Chronic subcutaneous THC injection and voluntary alcohol consumption differently altered glucose tolerance. The OGTT was conducted nine days following the last drug exposure in Experiment 1a (CTL, n = 9; EtOH and THC, n = 7; COM, n = 8). (**A** and **B**) alcohol and THC alone or in combination had no effect on glucose clearance. (**C**) Combined chronic alcohol and THC exposure was associated with reduced plasma insulin content at 15 min following the oral glucose load. COM vs. EtOH: *$p < 0.009$; COM vs. THC: ^$p = 0.068$ and @$p = 0.051$. (**D**) The COM group had a moderately reduced AUC of the insulin curve compared with the CTLs. CTL vs. COM: ^$p = 0.087$.

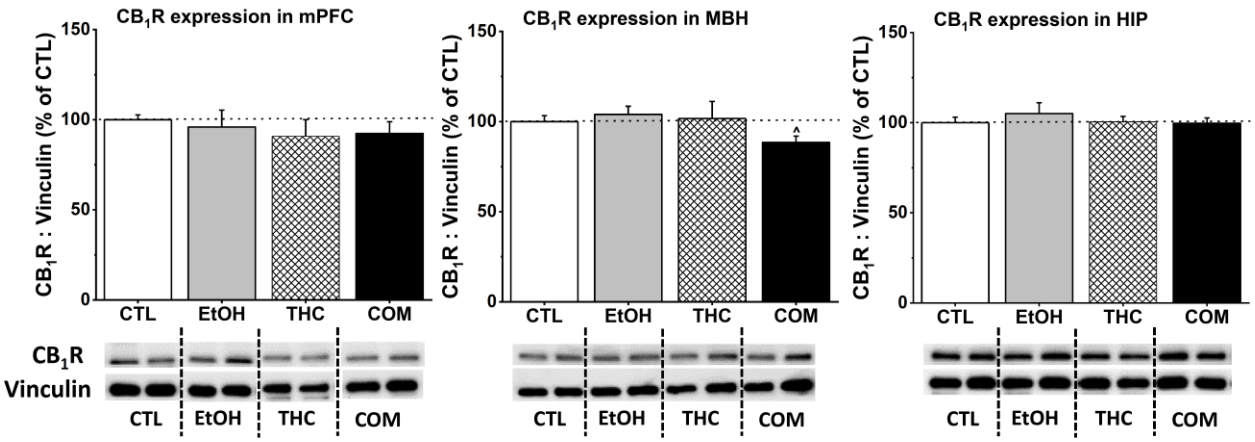


Figure 4.7: Chronic subcutaneous THC injection and voluntary alcohol consumption, alone or when combined, had negligible effect on CB_1R protein expression in the medial prefrontal cortex (mPFC), mediobasal hypothalamus (MBH), and hippocampus (HIP) of the male rats (Experiment 1a: EtOH, n = 8; CTL, THC, and COM, n = 10/group). In the MBH, the COM group had reduced CB_1R protein expression compared with the CTLs: $\hat{}p = 0.028$ by *t*-test. Rats were sacrificed nine days after the last drug treatment. Representative CB_1R (60 kDa) and Vinculin (124 kDa) Western blot images are depicted below the graphs.

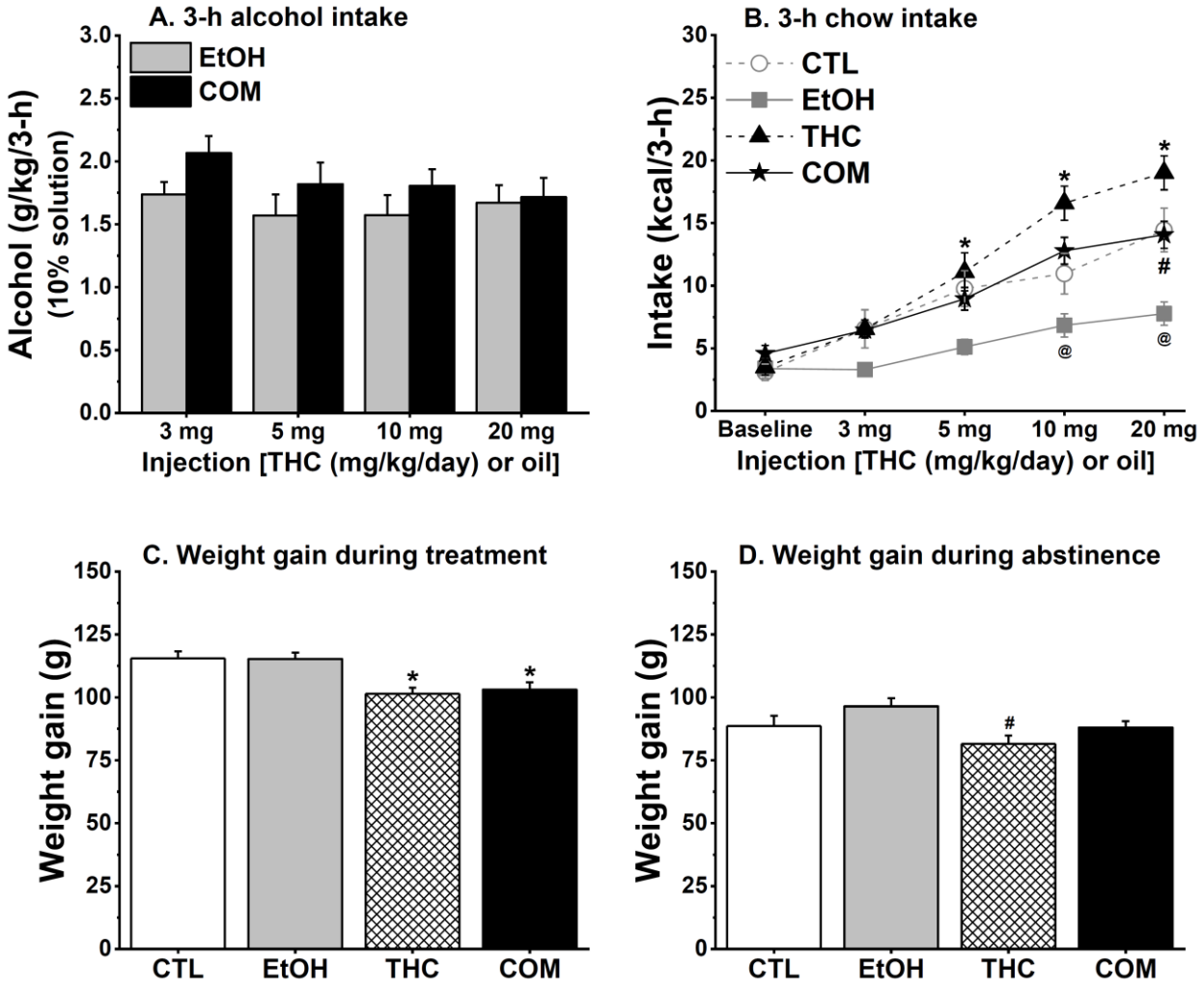


Figure 4.8: Chronic subcutaneous THC and voluntary alcohol consumption, alone or when combined, differently affected caloric intake and weight gain (Experiment 1b: n = 10/group). (**A**) EtOH and COM rats consumed similar doses of sweetened 10% alcohol solution. (**B**) THC injection dose-dependently increased 3-h chow intake only in the COM relative to the EtOH group. Alcohol consumption reduced 3-h chow intake, but THC averted the hypophagic effect of alcohol. THC vs. EtOH: *$p < 0.05$; EtOH vs. COM: @$p < 0.05$; CTL vs. EtOH: #$p < 0.02$. (**C**) Subcutaneous THC suppressed weight gain in the THC and COM groups. THC or COM vs. CTL or EtOH: *$p < 0.02$. (**D**) Reduced weight gain of the THC group persisted during the 2-week abstinence. THC vs. EtOH: #$p < 0.02$.

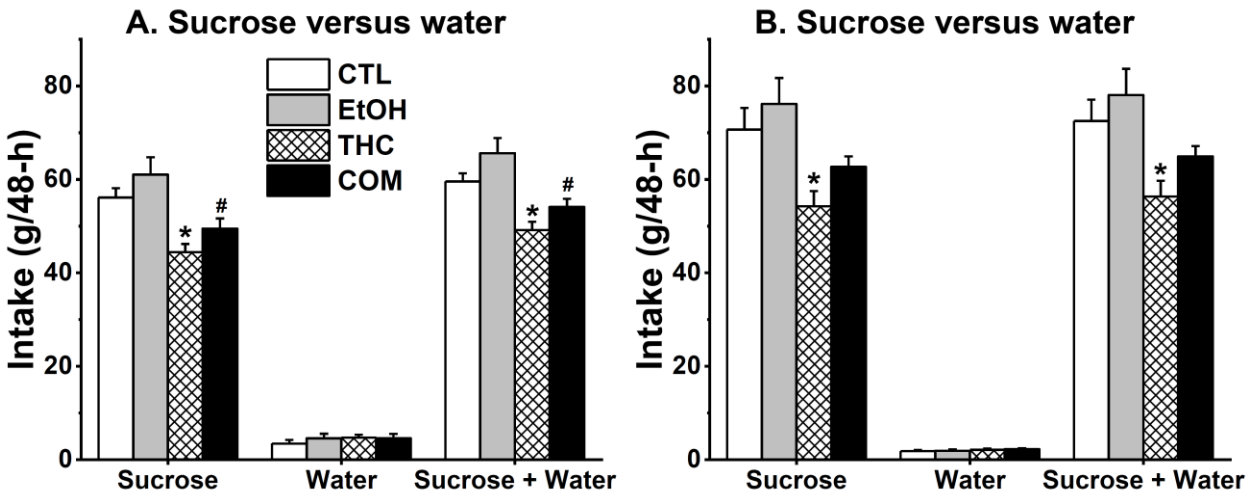


Figure 4.9: Chronic subcutaneous THC alone or when combined with voluntary alcohol consumption reduced sucrose and total fluid intake during sucrose preference tests (Experiment 1b, n = 10/group). (**A**) Sucrose and total fluid intake during the first 48-h sucrose preference test conducted 2–3 days (P47–P48) after drug treatment ended. THC vs. CTL and EtOH: *$p < 0.02$; COM vs. EtOH: #$p < 0.02$. (**B**) Sucrose and total fluid intake during the second 48-h sucrose preference test conducted 9–10 days (P54–P55) after drug treatment ended. THC vs. CTL and EtOH: *$p < 0.05$.

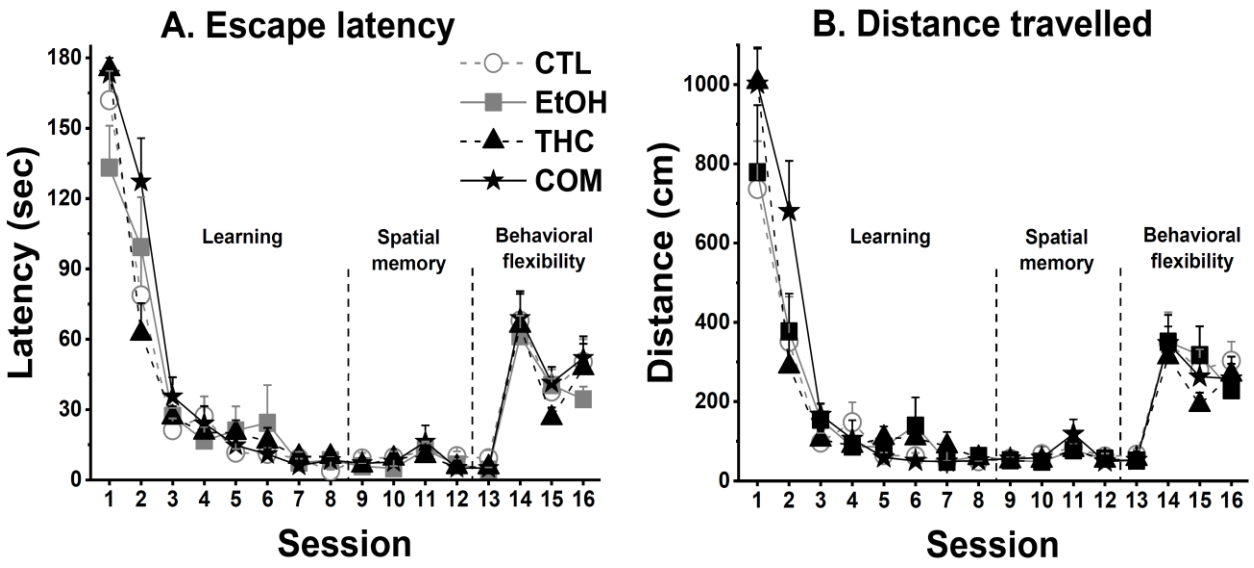


Figure 4.10: Chronic subcutaneous THC injection and voluntary alcohol consumption, alone or when combined, had no effect on cognitive behaviors assessed in the Barnes maze (Experiment 1b, n = 10/group). There were no group differences in the (**A**) escape latency, and (**B**) distance travelled throughout the procedure.

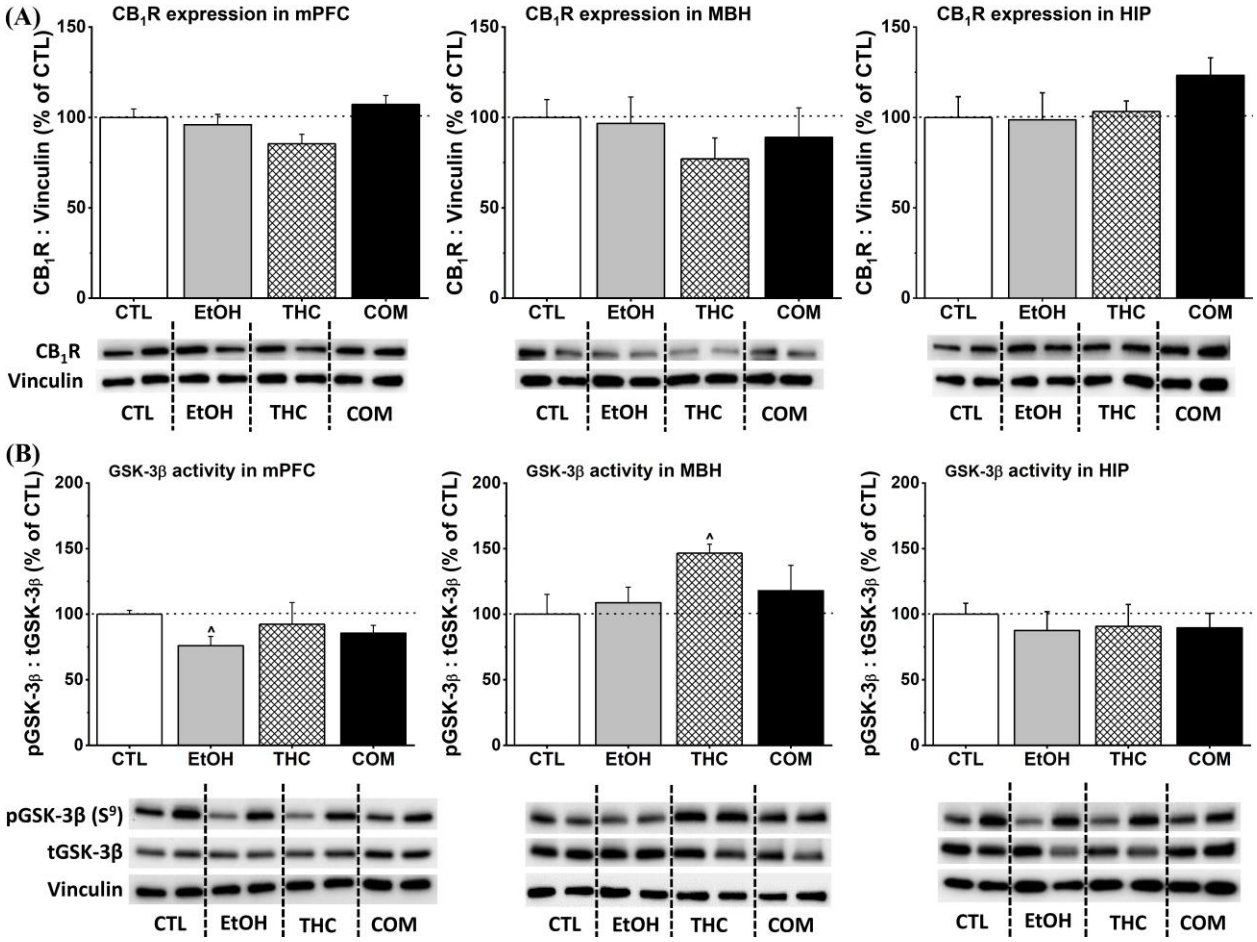


Figure 4.11: Chronic subcutaneous THC injection and voluntary alcohol consumption, alone or when combined, had negligible effect on (**A**) CB_1R protein expression and (**B**) GSK-3β activity in the mPFC, MBH, and HIP of the male rats (Experiment 1b, n = 6/group). Independent samples *t*-tests revealed that GSK-3β activity was greater in the mPFC of EtOH rats compared with CTLs: $\hat{}p = 0.011$, and that GSK-3β activity was lower in the MBH of THC rats compared with CTLs: $\hat{}p = 0.019$. Rats were sacrificed 4–5 weeks after the last drug treatment. Representative CB_1R (60 kDa), phosphorylated and total GSK-3β (46 kDa), and Vinculin (124 kDa) Western blot images are depicted below the graphs.

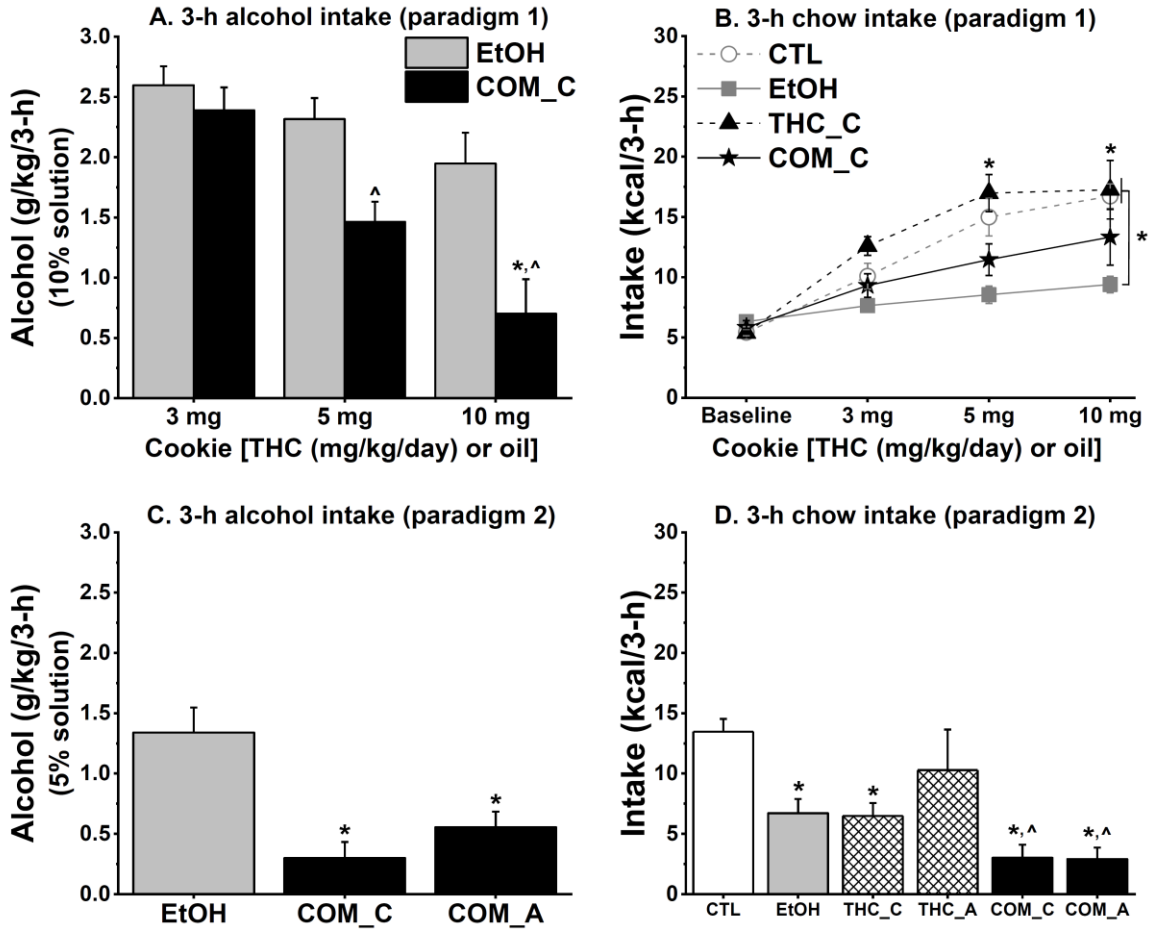


Figure 4.12: Chronic oral THC consumption reduced alcohol intake, while alcohol alone or when combined with oral THC reduced 3-h chow intake (Experiment 2a: CTL and EtOH, n = 8/group; THC_C, n = 5; THC_A, n = 3; COM_C and COM_A, n = 4). (**A**) In paradigm 1 (P30–P39), COM_C rats consumed lower doses of sweetened 10% alcohol solution than did EtOH rats: *$p < 0.009$. COM_C rats also consumed reduced alcohol doses during 5 and 10 mg/kg/day THC consumption than they did during 3 mg/kg/day THC consumption: ^$p < 0.03$. (**B**) Moderate 10% alcohol consumption suppressed 3-h chow intake in EtOH rats. EtOH vs. CTL and THC_C: *$p < 0.03$. (**C**) When THC or oil cookie was consumed after sweetened 5% alcohol access in paradigm 2 (P40–P45), COM_C and COM_A rats continued to consume less alcohol than the EtOH rats did. EtOH vs. COM_C and COM_A: *$p < 0.05$. (**D**) The EtOH, THC_C, COM_C, and COM_A groups consumed significantly lower 3-h chow compared with the CTLs. CTL vs. EtOH, THC_C, COM_C, and COM_A: *$p < 0.008$; THC_A vs. COM_C and COM_A: ^$p = 0.055$ and 0.050, respectively.

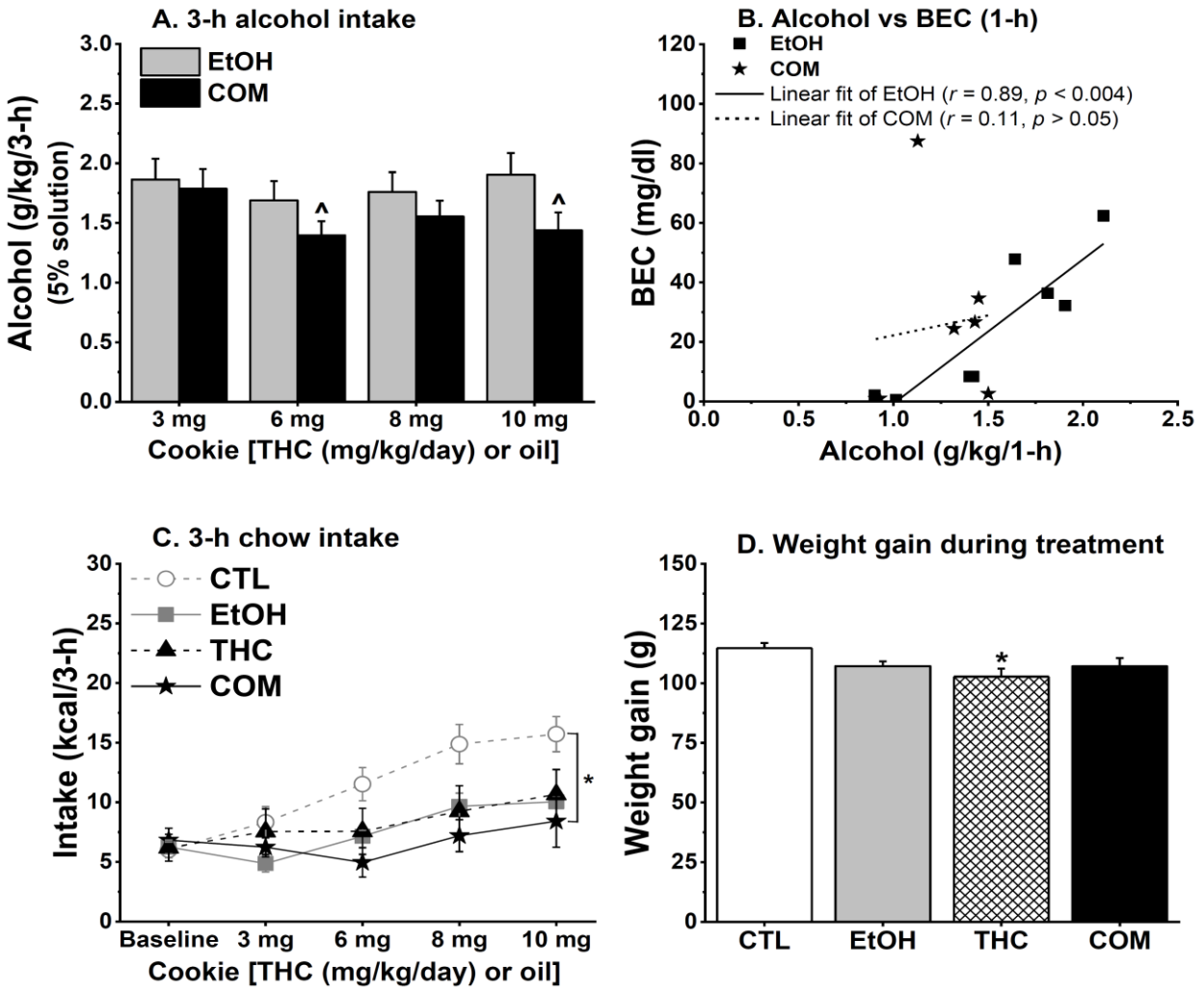


Figure 4.13: Chronic oral THC consumption mildly reduced alcohol intake and suppressed caloric intake and weight gain (Experiment 2b: CTL, n = 10; EtOH and THC, n = 8/group; COM, n = 7). (**A**) EtOH and COM rats consumed similar doses of 5% alcohol solution. COM rats consumed lower alcohol doses during 6 and 10 mg/kg/day THC consumption than during 3 mg/kg/day THC consumption: ^$p < 0.008$. (**B**) BEC correlated with 1-h alcohol intake in the EtOH group ($r = 0.89$, $p < 0.004$). (**C**) Drinking 5% alcohol reduced 3-h chow intake and augmented the somewhat hypophagic effect of oral THC. CTL vs. EtOH: $p = 0.086$; CTL vs. COM: *$p < 0.03$. (D) THC consumption suppressed weight gain in the THC group compared with the CTLs: *$p < 0.02$.

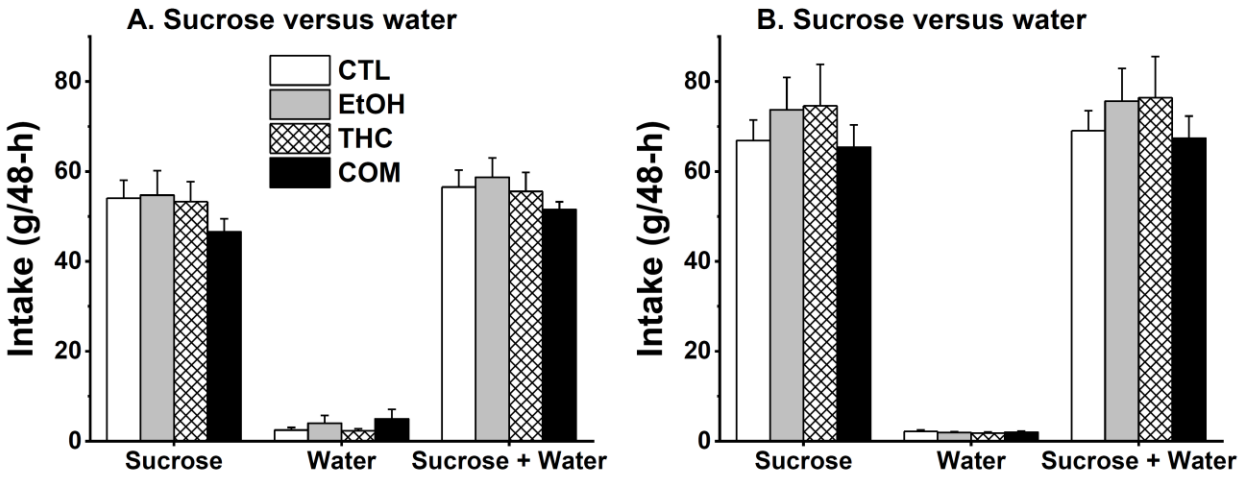
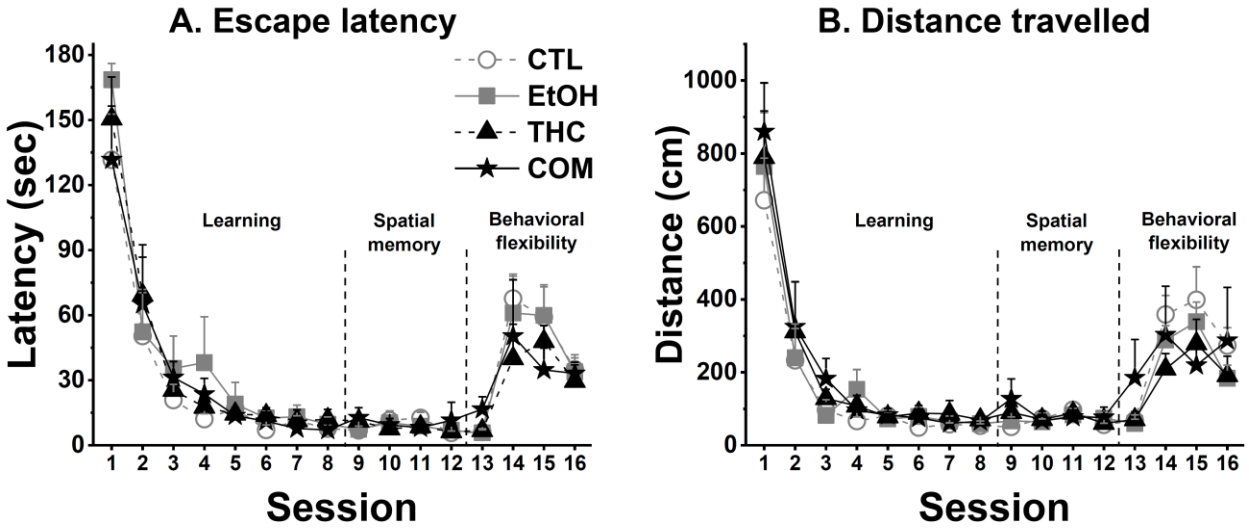


Figure 4.14: Chronic oral THC and voluntary alcohol consumption, alone or when combined, had no effect on sucrose and total fluid intake during the (**A**) first and (**B**) second 48-h sucrose preference tests in Experiment 2b (CTL, n = 10; EtOH and THC, n = 8/group; COM, n = 7).

Table: *p*-values of main group effects from raw and body weight-adjusted data analysis

Exp.	Parameter	Main group effect Raw data	Main group effect Weight-adjusted data
1a	Saccharin	*ns* ($p = 0.27$)	*ns* ($p = 0.32$)
	3-h chow	$p < 0.0001$	$p < 0.0001$
	24-h calorie	*ns* ($p = 0.94$)	*ns* ($p = 0.96$)
	24-h calorie (abstinence)	*ns* ($p = 0.07$)	$p < 0.04$
	Sucrose	$p < 0.01$	$p < 0.03$
	Sucrose + Water	$p < 0.02$	$p < 0.04$
1b	Saccharin	$p < 0.02$	$p < 0.02$
	3-h chow	$p < 0.0001$	$p < 0.0003$
	24-h calorie	*ns* ($p = 0.25$)	*ns* ($p = 0.60$)
	24-h calorie (abstinence)	$p < 0.0009$	*ns* ($p = 0.54$)
	Sucrose (SP 1)	$p < 0.0003$	$p < 0.009$
	Sucrose + Water (SP 1)	$p < 0.0001$	$p < 0.004$
	Sucrose (SP 2)	$p < 0.004$	$p < 0.03$
	Sucrose + Water (SP 2)	$p < 0.005$	$p < 0.04$
2a	Saccharin	$p < 0.05$	*ns* ($p = 0.08$)
	3-h chow (P 1)	$p < 0.0005$	$p < 0.0002$
	3-h chow (P 2)	$p < 0.0001$	$p < 0.0001$
	24-h calorie (P 1)	*ns* ($p = 0.23$)	*ns* ($p = 0.13$)
	24-h calorie (P 2)	*ns* ($p = 0.13$)	*ns* ($p = 0.42$)
	24-h calorie (abstinence)	*ns* ($p = 0.07$)	*ns* ($p = 0.47$)
	Sucrose (SP 1)	*ns* ($p = 0.18$)	*ns* ($p = 0.12$)
	Sucrose + Water (SP 1)	*ns* ($p = 0.49$)	*ns* ($p = 0.45$)
	Sucrose (SP 2)	*ns* ($p = 0.78$)	*ns* ($p = 0.64$)
	Sucrose + Water (SP 2)	*ns* ($p = 0.74$)	*ns* ($p = 0.54$)
2b	Saccharin	*ns* ($p = 0.99$)	*ns* ($p = 0.67$)
	3-h chow	$p < 0.03$	*ns* ($p = 0.09$)
	24-h calorie	$p < 0.03$	*ns* ($p = 0.48$)
	24-h calorie (abstinence)	*ns* ($p = 0.26$)	*ns* ($p = 0.15$)
	Sucrose (SP 1)	*ns* ($p = 0.58$)	*ns* ($p = 0.51$)
	Sucrose + Water (SP 1)	*ns* ($p = 0.64$)	*ns* ($p = 0.48$)
	Sucrose (SP 2)	*ns* ($p = 0.70$)	*ns* ($p = 0.39$)
	Sucrose + Water (SP 2)	*ns* ($p = 0.71$)	*ns* ($p = 0.39$)

Supplementary Table 4.1 compares the *p*-values of main group effects for raw and body weight-adjusted saccharin, calorie, and sucrose intake data. Almost all significant findings are consistent under both analyses; *ns* = non-significant ($p > 0.05$), SP = sucrose preference test, P = paradigm (in Experiment 2a).



Supplementary Figure 4.1: Chronic oral THC and voluntary alcohol consumption, alone or when combined, had no effect on cognitive behaviors assessed in the Barnes maze (Experiment 2a: n = 8/group). There were no group differences in the (**A**) escape latency, and (**B**) distance travelled throughout the procedure.

CHAPTER 5: ADOLESCENT MODERATE ALCOHOL AND Δ^9-TETRAHYDROCANNABINOL CONSUMPTION: BEHAVIORAL AND METABOLIC EFFECTS IN ADULTHOOD

Abstract

Obesity and the consequent metabolic syndrome are common, serious, and costly. Poor early-life environment, including exposures to drugs of abuse and obesogenic diets can predispose an individual to metabolic diseases later in life. Repeated alcohol or Δ^9-tetrahydrocannabinol (THC) use during adolescence can alter the maturation and function of brain systems that regulate energy balance. Little is known about how combined use of both drugs affects energy balance and glucose homeostasis in adolescents and young adults. Here, we investigated how co-use of both drugs during adolescence will affect feeding behavior and some metabolic parameters during early adulthood. Male Long-Evans rats voluntarily consumed saccharin or alcohol (3-h/day) before cookie laced with oil or increasing THC doses (1.5–5 mg/kg, twice per day) for 16 consecutive days during adolescence (P30–P45). Sucrose preference tests were used to measure abstinence-induced anhedonia. Following a three-week drug abstinent period, some rats in each group began consuming 45% high-fat diet (HFD). An oral glucose tolerance test (OGTT) and an intraperitoneal insulin tolerance test (ITT) were performed after four and nine days on the new diet regimen, respectively. Animals were sacrificed soon after the ITT for fat and plasma tissue harvest. Alcohol-consuming rats consumed fewer chow when alcohol was simultaneously available. THC-only-consuming rats ingested less saccharin solution and gained the least weight. Drug treatment had no effect on intakes of either 1% sucrose or daily calorie during abstinence. HFD-fed rats demonstrated hyperphagia and gained more weight, with no influence of prior drug exposure. Rats exposed to HFD for four days during drug abstinence presented with modest hyperglycemia and hyperinsulinemia at baseline and during the OGTT compared with those that continuously consumed chow. Nine days of HFD consumption resulted in elevated percent visceral fat and plasma leptin concentration. Surprisingly, HF diet feeding had no effect on insulin sensitivity during the ITT. Prior drug exposure had no effect on all the metabolic parameters. In this study, 16 days of moderate alcohol and THC co-use in adolescent rats had null effects on glucose homeostasis and insulin sensitivity under both chow and short-term HFD

feeding conditions during early adulthood. More studies are needed to clearly define the effects of combined alcohol and THC use on metabolic outcomes.

Introduction

The wide prevalence and burdensome nature of obesity and metabolic syndrome echoes the need for more studies to shed light on the predisposing and protective factors at play. Previous studies have established that early-life nutritional deficits or surfeits can influence neurological and metabolic health throughout the lifespan (Bouret et al. 2015; Paradis et al. 2017), as do active or passive exposure to exogenous drugs like alcohol and cannabinoids (Grant et al. 2018; Lopez-Gallardo et al. 2012; Thompson et al. 2009). Protracted consumption of diets rich in calorie, salt, saturated fatty acids, and refined sugars but low in fiber ("Western diet") coupled with sedentary lifestyle are notable contributors to the overweight/obese phenotype (Eckel et al. 2005; Kanoski and Davidson 2011; Moody et al. 2015). The allure of fatty and sweetened foods are partly responsible for the high incidence of adolescent obesity and type 2 diabetes (ACOG 2017), especially because adolescents are susceptible to the negative metabolic effects of Western diets (Hsu et al. 2015).

Use and co-use of alcohol and marijuana often begin during adolescence (Briere et al. 2011; Subbaraman and Kerr 2015). This drug use behavior is worrisome considering the cornucopia of empirical evidences suggesting that the adolescent brain is susceptible to the influence of exogenous compounds (Ernst et al. 2009; Nixon and McClain 2010; Tomas-Roig et al. 2017). Notably, alcohol or cannabis use during adolescence can alter the ontogeny of neural systems that regulate cognitive and reward-related behaviors (Ellgren et al. 2008; Gulley and Juraska 2013; Philpot et al. 2009; Rubino et al. 2015), to provoke aberrant brain functioning later in life (Broadwater et al. 2017; Crews et al. 2016; Higuera-Matas et al. 2015; Renard et al. 2014). Such neural alterations can translate into increased motivation to consume palatable/energy-dense diets during adulthood (Carlin et al. 2016; Figlewicz et al. 2013). Adolescents are also susceptible to the diabetogenic effects of alcohol (Liang and Chikritzhs 2014). Additionally, removal of access to alcohol or cannabis following protracted use can engage the stress system (Bruijnzeel and Gold 2005; Zorrilla et al. 2014), which, depending on severity, can in-turn lead to overconsumption of calorie-dense diets as a compensatory/coping mechanism (Liang et al. 2013; Torres and Nowson 2007). Despite the popularity of alcohol and cannabis co-use, there is

paucity of studies on how such co-use affects energy balance and metabolic outcomes during abstinence.

Epidemiological data have implicated chronic alcohol use with central adiposity and some facets of metabolic syndrome, including leptin resistance, insulin resistance, and type-2 diabetes (Fernandez-Sola 2015; Pietraszek et al. 2010; World et al. 1985). Other researchers conclude that moderate alcohol consumption may either be beneficial to cardio-metabolic health or can have null effects [(Baliunas et al. 2009); reviewed in (Nelson et al. 2017)]. Several central and peripheral mechanisms can directly mediate the detrimental metabolic effects of alcohol. For example, chronic exposure to excessive amounts of alcohol has been found to not only impair hepatocyte and pancreatic β-cell functions, but to also alter brain glucose utilization and insulin action (Carmiel-Haggai et al. 2003; de la Monte et al. 2012; Lee et al. 2015; Lindtner et al. 2013). The metabolic effects of marijuana use are also less clear (Penner et al. 2013; Sidney 2016). Human laboratory studies have documented that marijuana smoking can acutely increase plasma insulin concentration – a marker of prediabetes (Podolsky et al. 1971), impair glucose tolerance (Hollister and Reaven 1974), or may have no effect on glucose homeostasis (Permutt et al. 1976) in adults subjects. A cross-sectional, case-control study reported that chronic marijuana smoking can reduce circulating HDL-cholesterol and increase adipose tissue hypertrophy and insulin resistance without significant detriment to pancreatic β-cell function (Muniyappa et al. 2013). The negative metabolic effects of cannabinoids are assumed to be mediated by the action of THC on cannabinoid sybtype-1 receptor in the brain and peripheral tissues (Bermudez-Siva et al. 2006; Jourdan et al. 2016; Liu et al. 2012; Maccarrone et al. 2015; Matias and Di Marzo 2007; Nogueiras et al. 2008; Silvestri and Di Marzo 2013). In addition to potential methodological limitations, the inconsistent reports on the metabolic effects of marijuana in humans may relate to the action of other potent compounds in the plant on diverse receptor targets (Maccarrone et al. 2015; Muniyappa et al. 2013). Hence, animal studies are needed to understand the metabolic effects of THC.

In Chapter 3 of this dissertation, we demonstrated that male rats that chronically consumed moderate alcohol doses showed early signs of glucose intolerance (Nelson et al. 2017). In Chapter 4, we further showed that adolescent alcohol and THC co-use altered glucose-mediated insulin release early during abstinence. The lack of systematic investigation of the lasting behavioral and metabolic effects of alcohol and THC co-use prompted the undertaking of

this study using an adolescent rodent model of voluntary consumption of the two drugs. Given the negative metabolic outcomes of short-term Western diet consumption in young subjects (Brons et al. 2009; Kraegen et al. 1991), we sought to investigate whether high-fat diet (HFD) exposure would exacerbate the impact of prior alcohol and THC use. Importantly, to our knowledge, this will also be the first study on the metabolic effects of THC consumed via an edible product. We hypothesized that, compared to separate use of alcohol and THC, their co-use during adolescence will lead to lasting alterations in feeding behavior that will prime the subject for heightened reactivity to metabolic challenge in adulthood. Hence, we predicted that rats previously co-exposed to alcohol and THC will demonstrate hyperphagia and increased adiposity when presented with HFD compared with the drug naïve rats. Combined drug co-use and HFD overconsumption would greatly impair glucose tolerance and insulin sensitivity during adulthood.

Materials and methods

Animal husbandry

A total of 100 male Long-Evan rats (Envigo, Indianapolis, IN, USA) that arrived at postnatal day 22 (P22) were used. They were semi-pair housed in large polyethylene tubs with transparent Plexiglas cage dividers that enabled measurement of food and fluid intake for each rat. The colony was maintained in a temperature and humidity-controlled vivarium on a 12-h light/dark cycle (lights on at 11:30 AM). During habituation and drug exposure, rats had *ad libitum* access to a standard rodent chow (3.1 kcal/g; 58% carbohydrate, 24% protein, and 18% fat from soybean oil; 2018 Teklad global rodent diets 2018, Indianapolis, IN, USA). See the "Experimental procedures" below for diet manipulation during drug abstinence. Tap water was provided in glass bottles fitted with stainless steel sippers. Daily animal handling and care occurred at 8:00 AM when body weight, food, and water intakes were recorded. All study protocols were approved by the Institutional Animal Care and Use Committee (IACUC) at the University of Illinois at Urbana-Champaign, and they conformed to the guidelines stipulated in the *Guide for the care and use of laboratory animals* by the National Research Council, 2011.

Test fluids (saccharin, alcohol) and Δ^9-tetrahydrocannabinol (THC)

Saccharin (0.1%) and sweetened 5% ethanol solutions were prepared as previously described (Nelson et al. 2019). Test fluids were presented at home cages in 50 ml graduated plastic bottles fitted with stainless steel sipper tubes that minimize spillage and evaporation. Bottles were weighed (to the nearest 0.1 g) to determine fluid intake. Alcohol and solution from a previous drinking session was discarded, and freshly-prepared solutions were presented to the rats daily.

The contents of 10 mg dronabinol capsules, a commercially-available synthetic THC (Actavis Pharm, Inc.; Parsippany, NJ, USA) to be overlain on cookies (Goldfish Grahams Fudge Brownie, Pepperidge Farm; Norwalk, CT, USA) was diluted with sesame oil vehicle (Fisher Scientific). The 1.5 and 3 mg/kg THC doses were applied onto cookie at a volume of 0.383 ml/kg body weight; the 5 mg/kg dose was applied at 0.639 ml/kg body weight (Nelson et al. 2019). Oil vehicle was applied onto cookie isovolumetrically to the different THC doses.

Experimental procedures

Drug exposure

Drug training and exposure procedures were performed as we have previously described (Nelson et al. 2019). Rats were first habituated to the vivarium for four days. During the initial three habituation days, they were pair-housed without cage dividers. Body weight of individual rat and food and water intakes of each pair were recorded daily. At 8:00 AM on the fourth habituation day, a cage divider was inserted in the center of each cage to enable tracking intakes of individual animal. Over the next four days (P26–P29), the animals were entrained to consume 0.1% saccharin along with chow during the last 3 h of the dark cycle (8:30–11:30 AM). Following measurement of chow and saccharin consumption, saccharin bottles were replaced with water bottles and the first and second oil-laden cookies were respectively provided at 12:30 PM and 9:00 PM. At the end of training, rats were assigned to one of four groups by matching average body weight and saccharin intake during entrainment: control (CTL, n = 26) given saccharin and oil-laden cookie, ethanol (EtOH, n = 24) given 5% ethanol and oil-laden cookie, THC (n = 24) given saccharin and THC-laden cookie, and combination (COM, n = 26) given 5% ethanol and THC-laden cookie. During the 16-day treatment period, rats first had 3-h access to saccharin or 5% alcohol solution followed by a cookie laced with oil or THC (1.5, 3, or 5 mg/kg) presented twice per day at 12:30 PM and 9:00 PM (Fig. 5.1). Each cumulative daily dose

of 3, 6, 8, and 10 mg/kg THC lasted for four days. One of the CTL rats, which had a deformed incisor that prevented it from consuming solid food, was maintained on wet pulverized chow and excluded from further experimentation. During 11:30 AM measurements starting on P32, we assessed how drug exposure would affect puberty onset in each rat by checking for preputial separation or the complete manual retraction of the prepuce from the glans penis (Korenbrot et al. 1977). To ascertain how oral THC would affect blood ethanol concentration (BEC) in these rats, tail blood (80 µl) was sampled at 1-h during the second/third day of 8 mg/kg/day THC consumption, and at 3-h during the first/second day of 10 mg/kg/day THC consumption. BEC was analyzed in those samples as we have previously described (Nelson et al. 2019).

Sucrose preference test

The first (P47–P48) and second (P54–P55) tests respectively began two and nine days after the last drug and test fluid exposure day. On the test days, rats were presented with two bottles, 1% sucrose versus tap water, immediately after 8:00 AM measurements. The positions of the bottles were alternated on each day to prevent the development of location preference for drinking. After the sucrose preference tests, all rats were returned to *ad libitum* water access. Sucrose preference (%) was calculated as $\frac{\text{sucrose consumption (g)}}{\text{sucrose + water consumption (g)}} \times 100$.

High-fat diet exposure and oral glucose tolerance test (OGTT)

For the next three weeks after the last drug exposure day (P46–P66), except during the sucrose preference tests, rats were maintained on *ad libitum* access to chow and water. Starting on P67, rats in all four groups were subdivided into a chow group that continued consuming regular chow diet (CTL and COM, n = 14/group; EtOH and THC, n = 12/group) or a high-fat diet (HFD) group whose diet was switched to a 45% high-fat diet (4.73 kcal/g; 45% fat from lard and soybean oil, 35% carbohydrate, and 20% protein; D12451, Research Diets, New Brunswick, NJ, USA; CTL, n = 11; EtOH, THC, and COM, n = 12/group). On the fourth day on this diet regimen (P70), rats were overnight food-restricted such that their intake was 70% of their respective intake on P69. OGTT was performed in the middle of the light cycle (between 2:00 and 7:00 PM) on P71 according to our established protocol (Nelson et al. 2017). Fasting blood glucose was measured with an AlphaTRAK2 blood glucose monitoring system (Abbots Laboratories) before 80 µl of tail blood was drawn with heparin-coated capillary tubes for fasting

plasma insulin determination. Rats received 2.0 g/kg body weight glucose with a 20% glucose solution delivered via an oral gavage. Blood glucose was measured, and tail blood was drawn for plasma insulin measurement at 15, 30, 60, and 120 min after delivery of the glucose load. Blood samples were centrifuged at 1000 × g, 4 °C for 15 min, and the plasma was collected and stored at –80 °C for later insulin measurement using a rat enzyme-linked immunosorbent assay (ELISA) kit (Cat. # 80-INSTRU-E01, ALPCO, Salem, NH, USA) according to the manufacturer's instructions (Nelson et al. 2017).

Insulin tolerance test (ITT) and tissue harvest

After the OGTT, rats were returned to their home cages to resume *ad libitum* access to their respective new diets for four more days (P71–P74). On the fifth day (P75), they were mildly food-restricted to 90% of their average free-feeding intakes on previous two consecutive days (P72 and P73) following the OGTT. Between 2:00 and 7:00 PM on P76, fasting blood glucose measurements were taken before rats were intraperitoneally injected with 1.0 U/kg body weight of insulin (Humulin R, U-100; Lilly USA, LLC, Indianapolis, IN, USA). The 1.0 U/kg insulin dose was chosen based on its use by other researchers to assess systemic insulin sensitivity (Underwood and Thompson 2016). Seventy-five min later, blood glucose was measured before rats were rapidly decapitated. The trunk blood was collected into EDTA-coated tubes and the brains rapidly extracted and immediately frozen in powdered dry ice before storage at –80 °C. The blood samples were centrifuged and plasma collected to measure plasma levels of insulin (ALPCO) and leptin (Cat. # 90040; Crystal Chem, Elk Grove Village, IL, USA). Animal carcasses were stored at 4 °C overnight. Epididymal and retroperitoneal fat pads were dissected and weighed by researchers blinded to the group assignment. Percent visceral fat composition was calculated with reference to the body weight on the day of sacrifice (Nelson et al. 2016).

Statistical analysis

Subsequent data from rats with bits of leftover cookie on two or more days were excluded from analyses. Hence, the eventual sample sizes of the THC_chow, COM_chow, and COM_HF groups were 10, 11, and 11, respectively. Caloric intake, saccharin intake, alcohol intake dose during drug exposure, and blood glucose concentrations from the ITT were analyzed by two-way repeated-measures ANOVA. Blood glucose and insulin concentrations from the OGTT were

analyzed by three-way repeated-measures ANOVA with drug, diet, and time as factors. Caloric intake and weight gain during diet manipulation (i.e., during HFD or chow exposure), mild fasting blood glucose and insulin concentrations during the OGTT, areas under glucose and insulin concentration curves, percent visceral fat, and trunk plasma insulin and leptin concentrations were analyzed by two-way ANOVA. Weight gain during drug treatment, alcohol intake 1–3 h before blood draw, BEC, sucrose preference, and fluid intake during the preference tests were analyzed by one-way ANOVA. Comparison between two groups within a diet condition was conducted with independent samples *t*-test. The linear relationship between ingested alcohol dose and BEC, as well as between plasma leptin and percent visceral fat were analyzed by Pearson's correlation. Statistically significant main effects were accompanied by post hoc Tukey's honestly significant difference tests. Data are presented as mean ± SEM. Analyses were performed in Statistica 13.3 (TIBCO Software Inc.; Palo Alto, California), and differences with $p < 0.05$ were considered significant.

Results

Voluntary THC consumption

Most rats consumed the THC-laced cookie within 3 h. Two and four rats from the THC and COM groups, respectively, had bits of left-over cookies. The subsequent data of these rats were excluded from analyses.

Effects of oral THC on saccharin intake, alcohol intake, and BEC

The CTL and THC rats consumed increasing amounts of saccharin across treatment days [$F(3, 135) = 78.37$, $p < 0.0001$]. But the THC rats consumed less saccharin compared with the CTLs [$F(1, 45) = 4.93$, $p = 0.031$; Fig. 5.2A]. This group difference did not remain after saccharin intake was normalized to body weight [$F(1, 45) = 3.32$, $p = 0.075$]. The THC and COM groups consumed similar doses of alcohol across treatment days (Fig. 5.2B). Despite no group effect on alcohol intake, COM rats appeared to reduce alcohol intake during consumption of higher doses of THC (6 and 10 mg/kg/day). On the days BECs were measured, EtOH (1.36 ± 0.07 g/kg) and COM (1.20 ± 0.08 g/kg) rats consumed similar dose of alcohol within 1 h following 8 mg/kg/day THC consumption. Both groups of rats attained comparable BECs of 25.50 ± 4.08 mg/dl and 25.95 ± 5.67 mg/dl, respectively. After 3 h of alcohol access following 10 mg/kg/day THC

consumption, EtOH (1.65 ± 0.10 g/kg) and COM (1.45 ± 0.13 g/kg) rats consumed comparable alcohol doses and attained BECs of 13.57 ± 3.63 mg/dl and 18.35 ± 5.30 mg/dl, respectively. The BECs attained after both 1- and 3-h alcohol drinking significantly correlated with the ingested dose in both the EtOH [$r = 0.74$, $p < 0.0002$ and $r = 0.64$, $p < 0.008$, respectively] and COM [$r = 0.65$, $p < 0.004$ and $r = 0.91$, $p < 0.0001$, respectively] groups (Fig. 5.2C and 5.2D).

Effects of THC and alcohol consumption on food intake, sucrose consumption, and weight gain

Consumption of THC cookie on the previous day blunted chow intake during the last 3 h of the dark cycle on the next day in the THC and COM groups relative to the CTLs [$F(3, 89) = 8.46$, $p < 0.0001$; post hoc, both $p < 0.007$; Fig. 5.2E]. The EtOH group consumed less 3-h chow compared with the CTL group ($p < 0.04$). The lower 3-h chow intake by THC and COM rats compared with the CTLs was retained after intake was normalized to body weight. There were no group differences in daily caloric intake during the 16-day treatment period, but the THC rats gained less weight than did the EtOH and CTL rats [$F(3, 89) = 5.39$, $p < 0.002$; post hoc, both $p < 0.02$; Fig. 5.2F].

One-way ANOVA revealed no effect of prior oral THC and alcohol consumption on sucrose and water intakes during the sucrose preference tests (Fig. 5.3A and 5.3B). Based on our *a priori* hypothesis that combined alcohol and THC consumption will elicit unique effects, an independent samples *t*-test revealed that the COM group consumed less sucrose compared with the CTLs [$t(46) = 2.00$, $p = 0.051$] during the first preference test. The COM group also tended to consume less total fluid compared with the CTLs during the first preference test [$t(46) = 1.78$, $p = 0.082$].

Rats that consumed 45% HFD for four days (P67–P70) demonstrated hyperphagia [$F_{(1, 38)} = 412.58$, $p < 0.0001$] and modest weight gain [$F(1, 38) = 3.64$, $p = 0.064$]. There were no drug effects on daily chow or HFD intake during the 9-day test period (P67–P75). As expected, the HFD-fed rats consumed approximately 30% more daily calories [$F(1, 38) = 252.48$, $p < 0.0001$; Fig. 5.4A] and gained slightly more weight [$F(1, 38) = 25.23$, $p < 0.0001$; Fig. 5.4B] compared with the chow-fed rats. Under the HFD condition, the COM group gained more weight than the CTLs did [$t(20) = -2.21$, $p < 0.04$]. Importantly, the significant diet effect remained when the intake data were normalized to body weight.

Effects of THC and alcohol consumption on glucose and insulin tolerance during abstinence

The HFD consumers had elevated basal blood glucose and insulin concentrations relative to the chow consumers following a mild fast [$F(1, 24) = 42.25$ and 28.92, respectively; both $p < 0.0001$; Fig. 5.5A, 5.5B, 5.5D, and 5.5E]. During the OGTT, three-way repeated-measures ANOVA uncovered no effect of drug treatment and diet on blood glucose clearance and plasma insulin concentrations across time. The HFD groups demonstrated greater area under curve (AUC) of blood glucose [$F(1, 24) = 78.52$, $p < 0.0001$; Fig. 5.5C] and plasma insulin [$F(1, 24) = 32.78$, $p < 0.0001$; Fig. 5.5F] concentrations compared with the chow groups. Within each diet condition, there were no effects of prior alcohol and THC exposure on the metabolic parameters. Because there was no effect of prior drug exposure on blood glucose levels during the ITT, we collapsed the groups by diet condition. The HFD-fed groups had higher fasting blood glucose concentration compared with the chow-fed groups [diet × time effect: $F(1, 88) = 4.61$, $p < 0.04$; post hoc, $p < 0.002$; Fig. 5.6A]. There was, however, no diet effect on blood glucose level at the 75-min time point – indicating that the nine-day HFD consumption did not alter insulin sensitivity in the rats. Furthermore, the HFD-fed rats had higher trunk plasma leptin concentration [$F(1, 23) = 10.48$, $p < 0.004$; Fig. 5.6B] and percent visceral fat [$F(1, 38) = 84.56$, $p < 0.0001$; Fig. 5.6C], with no effect of prior alcohol or THC exposure. Under the HFD condition, and mirroring the body weight results, the COM group had slightly higher percent visceral fat content compared with the CTLs [$t(20) = -2.02$, $p = 0.057$]. Neither drug treatment nor diet condition influenced trunk plasma insulin concentration (Fig. 5.6D). Finally, percent visceral fat significantly correlated with plasma leptin in both the chow both the chow- [$r = 0.39$, $p < 0.009$] and HFD-fed [$r = 0.58$, $p < 0.0007$] groups (Fig. 5.6E).

Effects of oral THC and moderate alcohol, alone or when combined on age of puberty onset

Neither drug, alone or when combined, altered age of puberty onset. The average age of puberty onset was P37 (range: P34–P42).

Discussion

We designed this study to explore the lasting behavioral and metabolic outcomes of adolescent alcohol and THC co-use in male rats. Our principal aim was to observe how prior alcohol and THC exposures will interact with the metabolic effects of a high-fat diet (HFD) to affect glucose

homeostasis and insulin sensitivity in adulthood. In consonant with our previous findings (Nelson et al. 2019), rats that consumed THC-laced cookie subsequently consumed less saccharin solution during the last 3 h of the dark cycle. Although oral THC consumption had no effect on daily calorie intake, it modestly suppressed weight gain during treatment. THC consumption immediately after alcohol access also had no effect on blood alcohol concentration and intake of a mildly-rewarding 1% sucrose solution during abstinence. Novel findings from this experiment were that separate or combined alcohol and THC use by adolescent male rats did not alter intake of a preferred HFD and had no effect on glucose tolerance and insulin sensitivity when assessed during young adulthood following a three-week abstinent period. The lack of metabolic effects of the drugs was observed under both chow and short-term HFD feeding conditions. Notably, HFD feeding altered glucose homeostasis. Four days of HFD feeding increased mild fasting glucose and insulin concentrations following a brief fast, and mildly altered glucose tolerance during the OGTT. But nine days of HFD feeding had no effect on insulin sensitivity measured via the ITT.

Voluntary alcohol consumption during the last 3 h of the dark cycle resulted in reduced chow intake. The calorie deficit was compensated for during the subsequent hours of the day as demonstrated by unchanged daily caloric intake and weight gain by the EtOH group relative to the CTL group. Such finding has been previously demonstrated by us and others (Cornier et al. 2002; Furuya et al. 2003; Nelson et al. 2019; Nelson et al. 2016; Nelson et al. 2017). Our finding that voluntary consumption of low to moderate cumulative THC doses did not alter daily caloric intake also concur with our previous results (Nelson et al. 2019) and supports that of other researchers (Williams et al. 1998). Notwithstanding, our observed null effect of oral THC on daily caloric intake goes against the acclaimed hyperphagic effects of THC (Cota et al. 2003; Pagotto et al. 2006; Williams et al. 1998). Our findings, instead, reinforce the conclusion that the effects of THC on appetite is influenced by several experimental variables (Pagotto et al. 2006). The complexities of cannabinoid signaling in the brain feeding circuits, sensory systems, and peripheral tissues involved in energy balance regulation are yet to be untangled, and research efforts in these domains have blossomed in recent years (Bellocchio et al. 2010; Gatta-Cherifi and Cota 2016; Koch et al. 2015; Maccarrone et al. 2015; Mazier et al. 2015). For example, activation of known (CB_1R and CB_2R) and putative cannabinoid receptors in not only hindbrain circuits, but also in hypothalamic circuits that include POMC and AgRP neurons and glial cells

prominently modulate the neuroendocrine control of feeding behavior and energy balance (Koch 2017; Koch et al. 2015; Mazier et al. 2015; Morozov et al. 2017).

We expected that the impact of alcohol and THC on the adolescent rat brains will translate into the overconsumption of calories in the absence of metabolic need that is reminiscent of overeating in humans. Instead, we observed that separate or combined alcohol and THC exposure did not exacerbate hyperphagia on a palatable 45% HFD during abstinence. This contrary observation can be due to several factors. First, although researchers who have used similar adolescent THC exposure timeline (parenteral administration) observed persistent neural and behavioral abnormalities during abstinence (Renard et al. 2017; Rubino et al. 2008), our 16-day oral drug exposure duration may have been insufficient to elicit brain changes that can engender abnormal feeding behavior during abstinence. The voluntary nature of our alcohol and THC exposure may have permitted the rats to titrate their drug intake to avert aversive consequences. The circulating levels of THC and its major active metabolite, 11-OH-THC, can vary based on the route of administration, with oral THC leading to slow elevation in serum drug levels in rodents and humans (Grotenhermen 2003; Nelson et al. 2019). Second, any brain abnormalities caused by drug treatments might have been mitigated during the three-week abstinent/washout period. Studies have observed brain-region-specific downregulation and desensitization of CB_1R following chronic alcohol or THC exposure that resolved to varying extent during abstinence (Breivogel et al. 1999; Ceccarini et al. 2014; Hirvonen et al. 2012; Rubino et al. 2008). Hence, a shorter washout period or even an earlier onset and simultaneous consumption of HFD with drug treatment may uncover robust drug/diet interaction. Third, the short duration (nine days) of high-fat diet exposure and the type of HFD used (45%) may have precluded observation of over eating behavior. In typical diet-induced obese models, the rodents are maintained on diets containing $\geq$ 50% calories from fat for several weeks to months (Almeida-Suhett et al. 2019; Douglass et al. 2017; Pistell et al. 2010; Thaler et al. 2012). Prolonged exposure to HFD alters the organization and function of neurons and glia in the hypothalamus to promote obesity and metabolic dysfunctions (McNay et al. 2012). Moreover, several classic studies have shown that long-term HFD feeding stimulates astrocyte and microglia (the resident immune cell of the central nervous system) transition from resting to reactive/pro-inflammatory with concomitant obesity and metabolic disturbances (Douglass et al. 2017; Thaler et al. 2012; Valdearcos et al. 2018). In support of this third scenario, chronic (over

16 weeks) consumption of 60% HFD elicited reactive microgliosis and impaired cognition whereas 41% HFD had no such effect, though both diets promoted obesity and astrocyte activation (Pistell et al. 2010). Thus, it is also likely that an extended access to the 45% HFD might be necessary to observe an interaction between HFD and the lingering effects of prior drug exposure.

We found that short-term HFD consumption led to increased caloric intake and modest weight gain and altered glucose homeostasis but not insulin sensitivity in young male rats. Specifically, the OGTT performed on P71 (Fig. 5.1) immediately following a mild overnight fast revealed that the HFD-fed rats had elevated basal glucose and insulin concentrations, and areas under the glucose and insulin curves (AUC) compared with the chow-fed rats. There were no effects of drug treatment or drug by diet interaction on these findings. After nine days on the distinct diets, the HFD-fed rats had elevated basal blood glucose and plasma leptin concentrations and visceral adiposity compared with chow-fed rats. An elevated fasting glucose concentration within normal physiological ranges is an independent risk factor for the later onset of type 2 diabetes in otherwise healthy male subjects (Park et al. 2006). In support of our *a priori* hypothesis, pairwise comparison revealed that the COM rats that consumed HFD gained more weight and had higher visceral adiposity compared with the CTLs on the same diet. Although the HFD-consuming rats in our study were not obese *per se*, it is plausible that the increased visceral adiposity may be associated with alterations to endocannabinoid tone in peripheral tissues like pancreas and visceral fat. Accordingly, previous studies have observed increases in the endocannabinoid content and cannabinoid receptor activity in sensory systems (taste buds, olfactory neurons) during food deprivation or in hepatic, pancreatic, visceral adipose, blood, muscle, and gastrointestinal tissues following diet-induced obesity in mice (Argueta and DiPatrizio 2017; Mazier et al. 2015; Osei-Hyiaman et al. 2005; Pagotto et al. 2006; Starowicz et al. 2008). Similar changes to the peripheral endocannabinoid tone have been observed in obese as compared with non-obese humans (Matias and Di Marzo 2007; Matias et al. 2006). What's more, the increased adiposity in our HFD-exposed rats could have translated into increased expression of cytokine markers of inflammation and oxidative stress (TNF-α, IL-1β, IL-6, and other adipocytokines) that have been linked with altered energy balance and metabolic fitness (Lavin et al. 2011).

The lack of significant group differences in plasma insulin concentration 75 min after 1 U/kg insulin administration suggest that prior drug and diet manipulations did not alter insulin break-down by insulin degrading enzymes (IDEs) in the liver and kidneys. Our observation is significant considering that alcohol, cannabinoid, and HFD exposure can compromise hepatic enzyme function to promote hyperinsulinemia and impair insulin signaling (Bermudez-Siva et al. 2006; Carr et al. 2013; de la Monte et al. 2009; Liu et al. 2012). Thus, it appears that our limited drug and HFD exposure regimen precluded us from observing the full spectrum of drug- and diet-induced metabolic alterations. Further, we observed no effect of drug or diet on insulin tolerance. Besides the fact that we should have measured blood glucose concentration at least once before the 75-min time point post insulin injection during the ITT, the 1 U/kg insulin dose we administered to the rats may have been too high to reveal subtle drug- or diet-induced changes in insulin sensitivity. An insulin clamp experiment would have provided a more reliable assessment of insulin sensitivity compared with the ITT (Bowe et al. 2014; Carr and Correnti 2015). Utilizing the insulin clamp, others have shown that 1) three days of HFD (59% fat) feeding induced hepatic and adipose insulin resistance in rats (Kraegen et al. 1991), and 2) a five-day overconsumption of a diet containing 60% fat increased fasting blood glucose and induced hepatic insulin resistance and other metabolic aberrations in young healthy men (Brons et al. 2009). Future studies should also measure plasma corticosterone levels because HFD-induced elevation of corticosterone alters glucose homeostasis (Underwood and Thompson 2016) via an endocannabinoid mechanism (Bowles et al. 2015).

Proper insulin signaling in the brain and peripheral tissues mediate a broad range of biological functions, including reward, cognition, appetite, and energy balance (Heni et al. 2015; Kullmann et al. 2015; Xu et al. 2010). The detrimental metabolic and behavioral effects of alcohol, cannabinoids, and HFD can be transduced via alteration of brain insulin signaling (de la Monte et al. 2009; Maccarrone et al. 2015; Nogueiras et al. 2008; Silvestri and Di Marzo 2013). The HFD-induced alteration of glucose homeostasis in our study did not reveal how insulin signaling might have been impacted. Impaired insulin-induced phosphoinositide 3-kinase/protein kinase B (PI3K/AKT) signaling is a likely mechanism. A mouse genetic study demonstrated that dysregulated PI3K/AKT signaling in steroidogenic factor-1 expressing neurons of the ventromedial hypothalamus predisposes rodents to diet-induced obesity due to reduced energy expenditure (Xu et al. 2010). In keeping with that finding, alterations to brain insulin signaling

via PI3K/AKT/GSK pathway in rodents promoted “addiction-like” behaviors that manifested as overconsumption of energy dense diets and the eventual development of obesity (Dadalko et al. 2015). An fMRI study showed that, following intranasal insulin treatment, subjects with higher visceral adiposity had impaired insulin action in the prefrontal cortex and hypothalamus and expressed increased wanting for sweet foods compared with lean subjects (Kullmann et al. 2015). Intriguingly, reduced monoamine turnover in the mesolimbic system and striatum of rodents exposed to HFD can be mediated by aberrant insulin signaling via the AKT pathway (Dadalko et al. 2015; Speed et al. 2011). Data presented in Chapter 4 of this dissertation show that low-to-moderate doses of alcohol and THC may have no effect on basal GSK-3β enzyme activity in the mediobasal hypothalamus, medial prefrontal cortex, and hippocampus. Accordingly, we did not observe significant drug-induced hyperphagia on HFD during abstinence (Fig. 5.4A). Follow-up work in our lab investigates the effects of drug and diet treatment on insulin-stimulated AKT

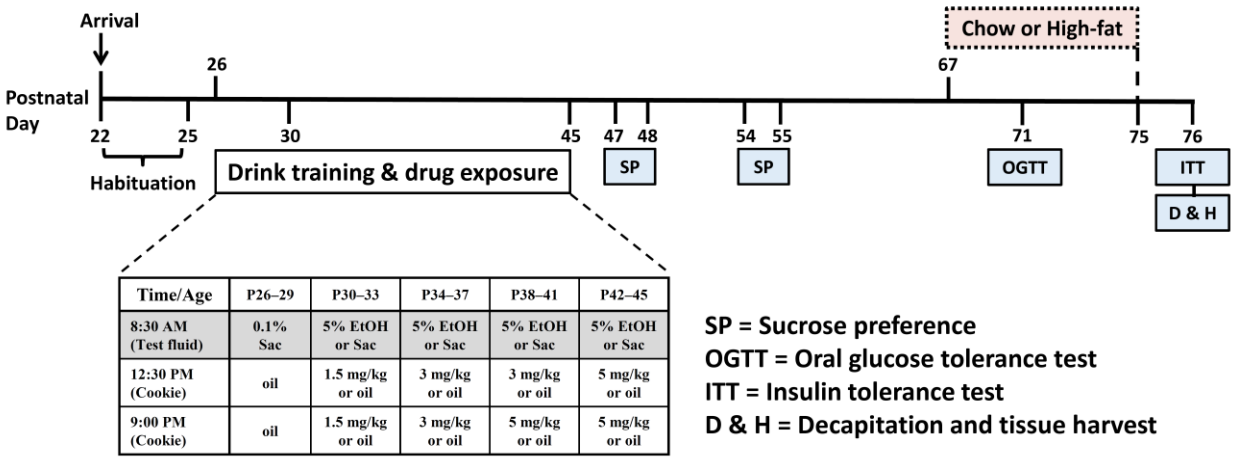
/GSK-3β activity in the brain, with a focus on sex differences.

In summary, this is the first study to our knowledge that examines the long-term effects of adolescent alcohol and THC co-use on feeding behavior and metabolic outcome during young adulthood. We also examined the effects of prior drug exposure on glucose and insulin homeostasis in the context of HFD feeding. We observed no persistent metabolic effects of 16-day moderate alcohol and oral THC consumption in the male rats under both chow and HFD feeding conditions. We did, however, found that short-term (4–9 days) HFD feeding resulted in modest signs of glucose intolerance, and elevated mild fasting blood glucose concentration, visceral adiposity, and plasma leptin concentration. It is worth noting that we compared the behavioral and metabolic effects of 45% HFD to that of standard chow diet. Use of control low-fat diet (LFD) is a preferable option for metabolic studies (Warden and Fisler 2008), but standard chow diet (SD) can be a bona fide substitute when low-fat diet is not available. This point is buttressed by the recent observation that an 18-week consumption of SD or LFD elicited similar phenotypic, behavioral, and metabolic effects in rodents (Almeida-Suhett et al. 2019). Further studies on the effects of moderate alcohol and THC use on energy balance and glucose homeostasis are warranted.

Acknowledgements

Startup funds from the Psychology Department of the University of Illinois and NIH grant R21DA045175-01A1 (both to NCL) supported this study. We thank Sheel Vasavada, Jacquelin Sanchez, and the psychology department animal care staff for their superb technical assistance. This chapter will be revised and submitted for publication with Michael Weingarten, Wen Xuan Law, Daniel Sangiamo, and Nu-Chu Liang as co-authors.

Figures



Time/Age	P26–29	P30–33	P34–37	P38–41	P42–45
8:30 AM (Test fluid)	0.1% Sac	5% EtOH or Sac	5% EtOH or Sac	5% EtOH or Sac	5% EtOH or Sac
12:30 PM (Cookie)	oil	1.5 mg/kg or oil	3 mg/kg or oil	3 mg/kg or oil	5 mg/kg or oil
9:00 PM (Cookie)	oil	1.5 mg/kg or oil	3 mg/kg or oil	5 mg/kg or oil	5 mg/kg or oil

Figure 5.1: Experimental timeline. Drug exposure spanned 16 days (P30–P33). Treatments indicated in the shaded part of the table occurred during the last 3 h of the dark cycle. From P67, some rats continued consuming regular chow diet while the others received 45% high-fat diet. Glucose and insulin tolerance tests were performed on P71 and P76, respectively [Chow (CTL, n = 14; EtOH, n = 12; THC, n = 10; COM, n = 11) and HF (CTL and COM, n = 11/group; EtOH and THC, n = 12/group)].

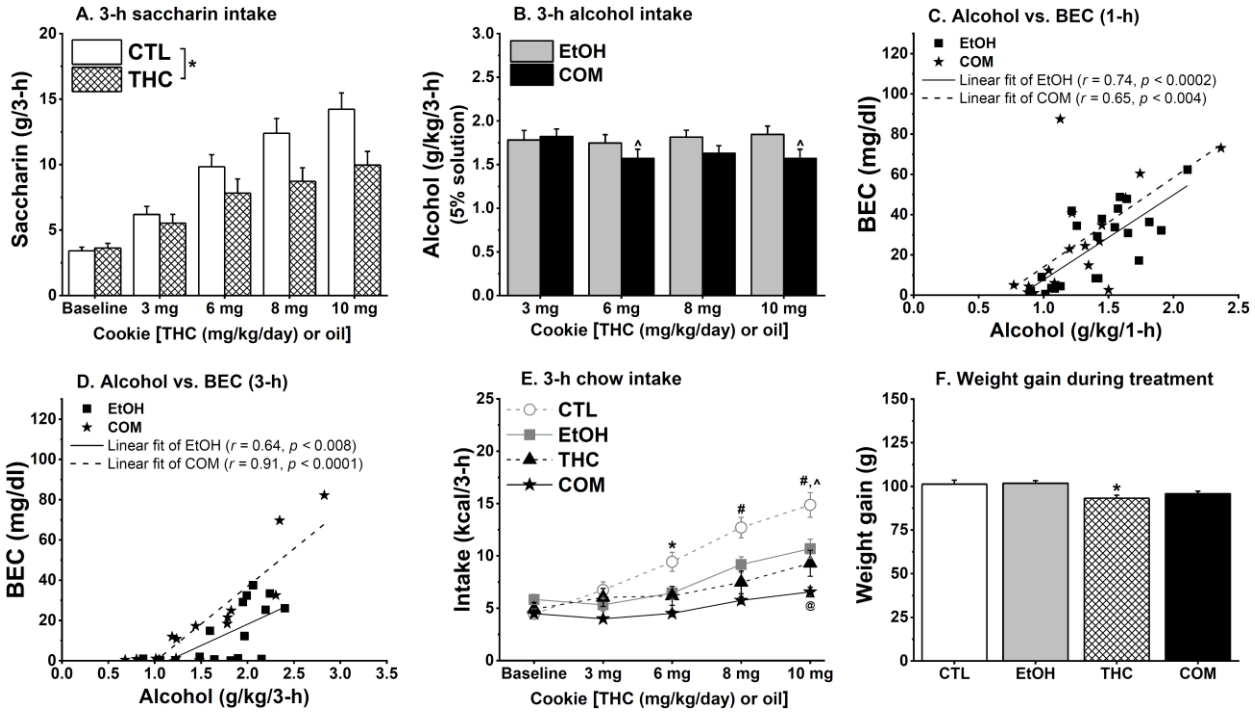


Figure 5.2: Chronic oral THC consumption reduced saccharin intake, chow intake during the last 3 h of the dark cycle and weight gain, whereas moderate alcohol alone or when combined with oral THC reduced 3-h chow intake (CTL, n = 25; EtOH, n = 24; THC and COM, n = 22/group). (**A**) THC rats consumed less saccharin compared with the CTL rats. *$p < 0.04$. (**B**) EtOH and COM rats consumed similar doses of alcohol during each THC dose. COM rats' alcohol intake during 6 and 10 mg/kg/day THC consumption was lower than their intake during 3 mg/kg/day THC consumption: ^$p < 0.03$. (**C**) Blood ethanol concentration (BEC) correlated with 1-h alcohol intake in the EtOH group ($r = 0.74$, $p < 0.0002$) and with intake in the COM group ($r = 0.65$, $p < 0.004$) after oral 8 mg/kg/day THC. (**D**) BEC correlated with 3-h alcohol intake in the EtOH group ($r = 0.64$, $p < 0.008$) and with intake in the COM group ($r = 0.91$, $p < 0.0001$) after oral 10 mg/kg/day THC. (**E**) Drinking 5% alcohol reduced 3-h chow intake and augmented the somewhat hypophagic effect of oral THC. CTL vs. COM: *$p < 0.005$; CTL vs. THC and COM: #$p < 0.004$; CTL vs. EtOH: ^$p = 0.051$; EtOH vs. COM: @$p < 0.03$. (**F**) Oral THC suppressed weight gain in the THC group compared with both the CTL and EtOH groups: *$p < 0.02$.

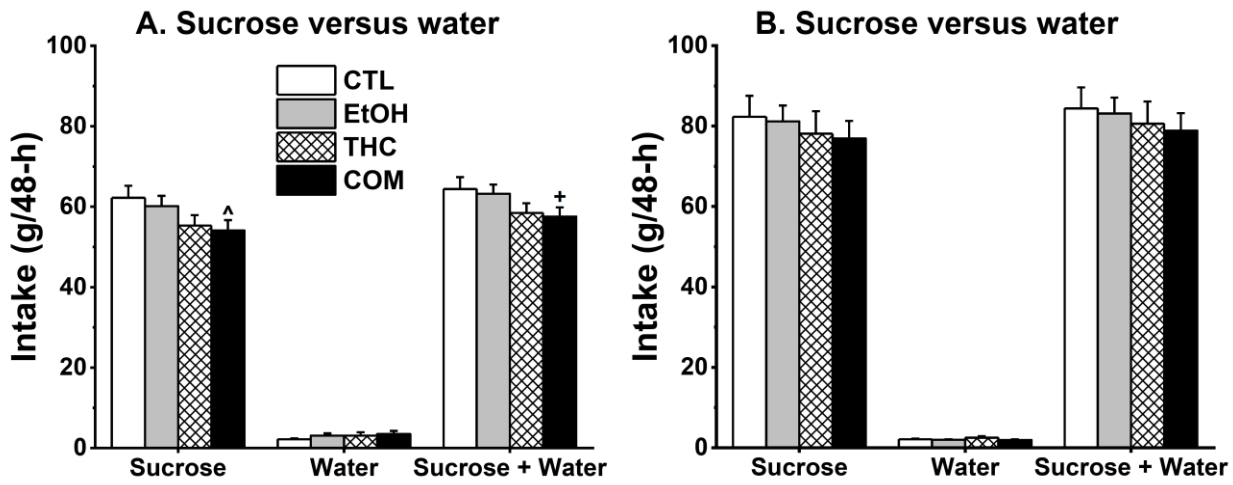


Figure 5.3: Chronic combined oral THC and voluntary alcohol consumption reduced sucrose and total fluid intake during the (**A**) first but not during the (**B**) second 48-h sucrose preference test. CTL vs. COM: ^*p* and +*p* = 0.051 and 0.082, respectively, by *t*-test. Alcohol and THC alone had no effect on sucrose and total fluid intake during both sucrose preference tests (CTL, n = 25; EtOH, n = 24; THC and COM, n = 22/group).

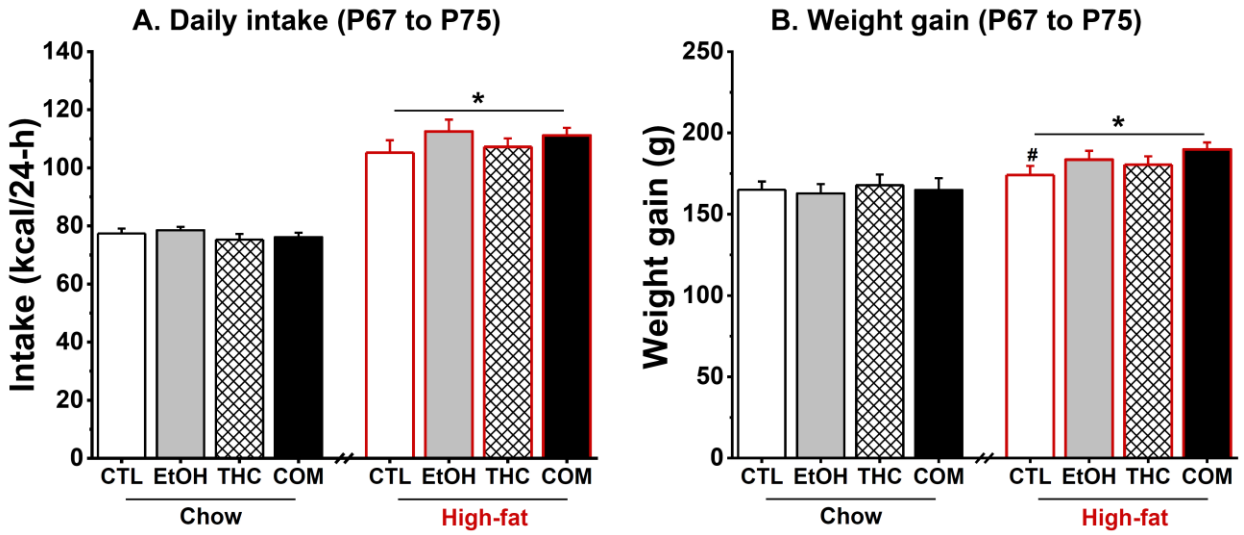


Figure 5.4: Chronic oral THC and voluntary alcohol consumption, alone or when combined, had no effect on daily caloric intake and weight gain during abstinence (from P67–P75) [Chow (CTL, n = 14; EtOH, n = 12; THC, n = 10; COM, n = 11) and HFD (CTL and COM, n = 11/group; EtOH and THC, n = 12/group)]. (**A**) Rats maintained on 45% high-fat diet consumed more average daily calories compared with intake by those maintained on regular chow diet: **p* < 0.0001. (**B**) HFD-fed rats gained more weight compared with the chow-fed rats: **p* < 0.0001 by ANOVA. Under the HFD condition, the COM rats gained more weight compared with the CTLs: #*p* < 0.04 by *t*-test.

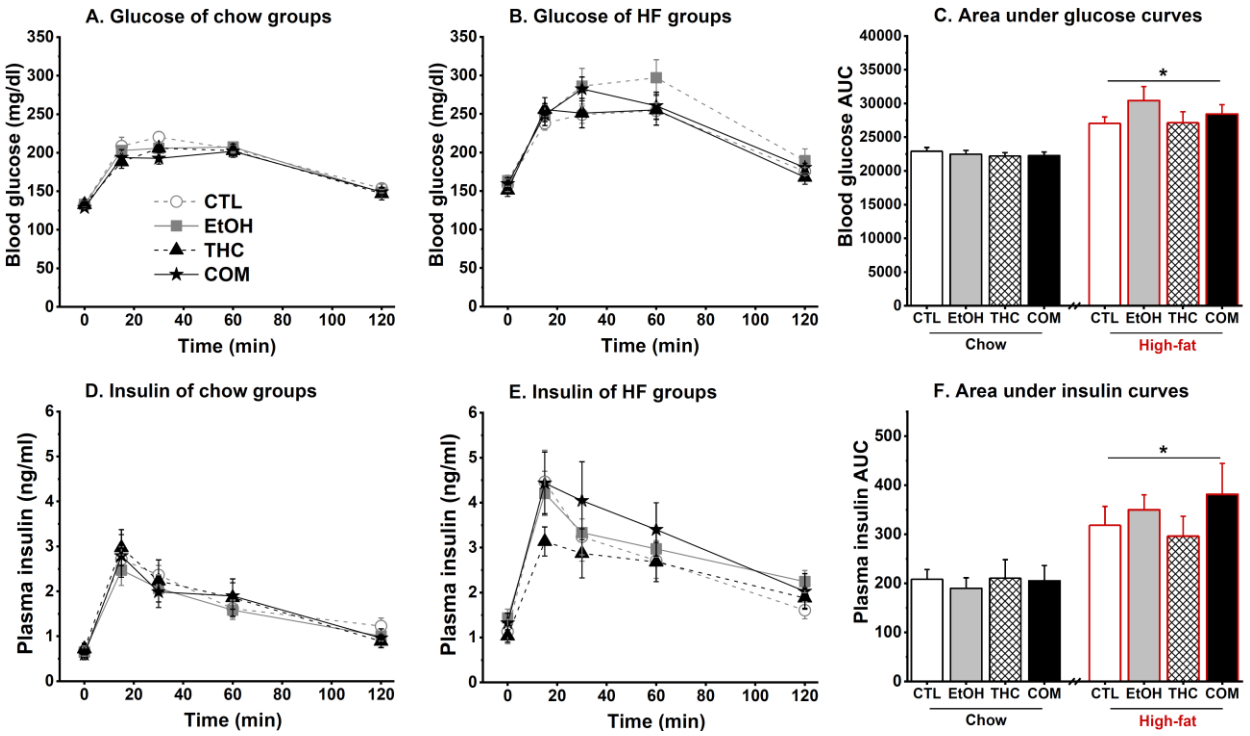


Figure 5.5: Effects of oral THC, moderate alcohol, and diet on glucose tolerance [Chow (CTL, EtOH, THC, and COM, n = 10–12/group) and HFD (CTL, EtOH, THC, and COM, n = 7–8/group)]. Prior drug exposure had no effect on glucose clearance in both the groups of rats (**A**) perpetually maintained on chow diet, and those (**B**) that consumed HFD for 4 days (P67–P70) after the diet switch (see Fig. 5.1). (**C**) Area under curve revealed that 4-day HFD consumption altered glucose tolerance with no significant effect of prior drug exposure: *$p < 0.0001$. Prior drug exposure had no effect on insulin release in response to the oral glucose loads in both groups of rats that were (**D**) perpetually maintained on chow diet, and those (**E**) that consumed HFD for 4 days (P67–P70) after the diet switch. (**F**) Area under curve revealed that 4-day HFD consumption potentiated insulin release with no significant effect of prior drug exposure: *$p < 0.0001$.

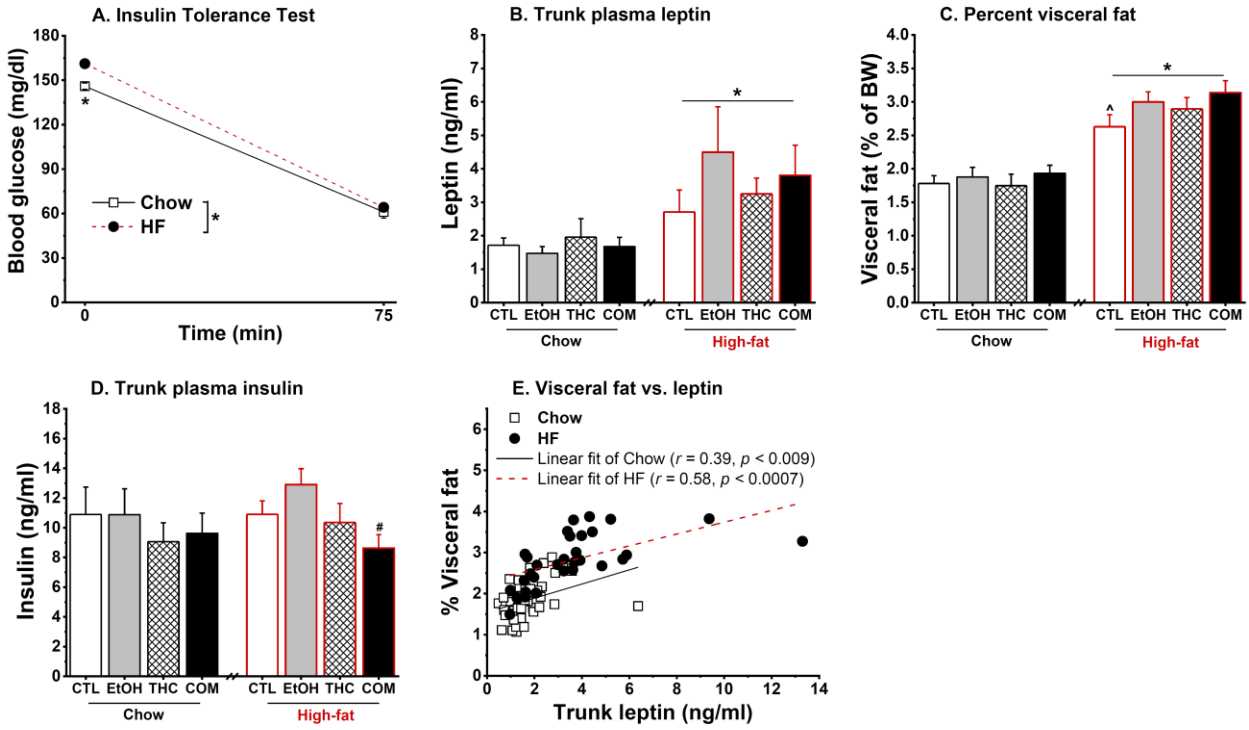


Figure 5.6: Effects of oral THC, moderate alcohol, and diet on some metabolic parameters [Chow (CTL, EtOH, THC, and COM, n = 10–12/group) and HFD (CTL, EtOH, THC, and COM, n = 7–12/group)]. Nine days of HF diet feeding (**A**) increased fasting blood glucose with no effect on insulin sensitivity during the insulin tolerance test: *$p < 0.002$, (**B**) increased plasma leptin concentration: *$p < 0.004$, (**C**) increased percent visceral fat: *$p < 0.0001$, and (**D**) had no effect on plasma insulin concentration. Prior alcohol or oral THC consumption did not significantly influence the above measures. However, under the HFD condition, *t*-test revealed that the COM rats tended to have higher percent visceral adiposity compared with the CTLs: ^$p = 0.057$ and lower trunk insulin compared with the EtOH group #$p < 0.007$. (**E**) Percent visceral fat significantly correlated with leptin measured in trunk plasma in both the chow- ($r = 0.39$, $p < 0.009$) and HFD-fed ($r = 0.58$, $p < 0.0007$) groups.

CHAPTER 6: SUMMARY AND FUTURE DIRECTIONS

The objective of this dissertation is to examine the effects of separate and combined chronic alcohol and Δ^9-tetrahydrocannabinol (THC) use on energy balance, glucose homeostasis, and cognitive behavior. Our work shows that chronic THC or moderate alcohol exposure differently influences energy balance and glucose homeostasis and reveals unique effects of the co-use of both drugs. Importantly, this doctoral research established a novel rodent model of alcohol and THC co-use that can be adapted to study the behavioral and biological effects of polydrug use in other experimental models. This work advanced our understanding of the metabolic effects of alcohol and cannabinoid use.

As reviewed in Chapter 1, the caloric value of alcohol (7 kcal/g) is nestled between that of carbohydrate (4 kcal/g) and fat (9 kcal/g). Alcohol drinkers are often not cognizant of the calories supplied by alcoholic beverage when they consume it alone or alongside meals; and the extent to which alcohol-derived calories contribute to the high incidence of obesity and metabolic syndrome begs for further clarification (Suter 2005; Yeomans 2010). We previously demonstrated that the appetite-reducing effects of binge doses of alcohol were related to the time course of blood alcohol concentration (Nelson et al. 2016). Chapter 3 of this dissertation further reported that rats who consumed moderate alcohol doses compensated for the extra calories as demonstrated by the reduced solid food intake and unchanged daily caloric intake and body weight relative to controls (Nelson et al. 2017). We also observed that chronic moderate alcohol consumption could predispose males to prediabetes and that mild caloric restriction produced beneficial effects on glucose homeostasis. We did not explore the mechanisms that mediate the reduced food intake during alcohol consumption. Future work in this domain should investigate how alcohol affects the activity of cells and signaling molecules within the hypothalamus and other feeding centers in a dose- or time-dependent manner. Moreover, metabolic studies that employ a Comprehensive Lab Animal Monitoring System (CLAMS) will reveal whether the briefly reduced food intake in rats who consume alcohol is related to an acute imbalance between caloric intake and energy expenditure or whether it is mainly mediated by reduced appetite. Additionally, islet function assay and further metabolic phenotyping using the euglycemic-hyperinsulinemic clamp and insulin tolerance tests will provide more insight into the mechanisms that mediate altered glucose tolerance and insulin sensitivity (Bowe et al. 2014). To

understand the molecular underpinnings of the altered glucose homeostasis in male rats that chronically consume alcohol, future studies should explore any correspondent change to insulin signaling in the brain and peripheral tissues (e.g., muscle, fat, liver, heart, and pancreas). Sex differences in the diabetogenic effects of alcohol may be explored by surgically or pharmacologically manipulating sex hormones in rodents and observing the correspondent effects on glucose homeostasis. If sex differences are observed, further analysis of the molecular mechanisms that mediate the effects of sex steroids on energy balance and metabolism should be pursued. The involvement of other hormones involved in metabolism (e.g., growth hormone, thyroid hormone, epinephrine, orexin, and glucocorticoid) also deserves attention.

Alcohol and marijuana are the most commonly used legal and illegal drugs, respectively (Johnston et al. 2018). It is concerning that both substances are commonly used in combination by adolescents (Patrick et al. 2018), despite the paucity of studies on the health implications of their co-use (Briere et al. 2011; Yurasek et al. 2017) including the effects on energy balance and glucose homeostasis. There is also a lack of preclinical models of voluntary alcohol and THC co-use that reliably approximates human use pattern. To fill these gaps, we established a rodent model of alcohol and THC co-use in Chapter 4 and investigated its effects on energy balance, glucose homeostasis, cognitive behaviors, and some neurobiological changes. We showed that subcutaneous or oral THC administration combined with moderate alcohol consumption differently alters feeding behaviors in adolescent male rats. THC elicited a hyperphagic effect within 3 h after subcutaneous administration and moderate doses of orally consumed alcohol reduced this effect. Alternatively, oral THC consumption via cookie had null effect on food intake during the initial 3 h after administration, and both subcutaneous and oral THC had no effect on daily caloric intake (Nelson et al. 2019). Similar time- and route-of-administration-dependent effects of THC on food intake have been noted by other researchers. For example, in rodents, high doses of THC produce anorectic-like effects, while low doses produce hyperphagic effects often within the initial 1–6 h following drug administration (Pagotto et al. 2006; Williams et al. 1998). The different outcomes we observed when THC was administered subcutaneously or orally may be explained by the fact that orally consumed THC undergoes first-pass hepatic metabolism that can reduce the effective concentration of the drug in circulation or favor more sequestration of the lipophilic drug into fat depots in the body (Grotenhermen 2003). In consonant with the above possibilities, 11-hydroxy-THC (a potent CB_1R ligand) was

undetectable in plasma obtained from rats that consumed THC cookie (Nelson et al. 2019). Furthermore, compared with their separate use, we observed that co-administered alcohol and THC differently altered glucose homeostasis. Nine days after the 16 days of subcutaneous THC and moderate alcohol consumption (P30–P45), the COM rats had reduced plasma insulin content in response to a glucose load that was indicative of improved systemic insulin sensitivity. To clearly dissect the effects of separate or combined THC and alcohol use on feeding behavior and metabolism, meal pattern analyses (meal size, meal frequency, latency to a feeding bout, and feeding rate) will be useful (Argueta and DiPatrizio 2017; Drewnowski and Grinker 1978; Higgs et al. 2003), as would further metabolic phenotyping as previously mentioned (Bowe et al. 2014).

In both Chapters 3 and 4 summarized above, we were unable to detect detrimental effects of separate or combined THC and alcohol administration on short-term object recognition memory and on spatial learning, memory, and behavioral flexibility measured in the Barnes maze. An essential next step would be to adopt more sensitive measures of behavioral flexibility, especially attentional set-shifting or operant strategy shifting tasks. Additionally, modifications of our novel object recognition (reducing the inter-trial intervals) and Barnes maze (slightly increasing its difficulty) tasks may make them sensitive to subtle drug-induced changes in cognition. Preliminary Barnes maze data from a follow-up study in our lab lend credence to the latter scenario. In Chapter 4, we also expected that exposure to alcohol and THC will affect the expression of the chief metabotropic receptor in the central nervous system (CB_1R) and dysregulate the activity of the vital signaling molecule glycogen synthase kinase-3 beta (GSK-3β) in the medial prefrontal cortex (mPFC), hippocampus (HIP), and mediobasal hypothalamus (MBH). We observed insignificant changes in CB_1R expression and basal GSK-3β activity in the mPFC, HIP, and MBH of rats when measured 4–5 weeks following drug treatment. Future studies should analyze more brains soon after the termination of chronic drug exposure.

The long-term effects of marijuana use on energy balance and glucose homeostasis are unclear (Muniyappa et al. 2013; Penner et al. 2013; Permutt et al. 1976). There is also a scarcity of studies on the interactive effects of alcohol and THC combination in conjunction with western diet consumption on metabolic fitness. We investigated whether any lingering effects of prior alcohol and THC exposure will interact with that of a high-fat diet (HFD) to generate heightened metabolic dysregulation in Chapter 5. Utilizing the voluntary oral drug co-use model that we

established in Chapter 4, male rats consumed alcohol and THC-infused cookies during adolescence (P30–P45) with *ad libitum* access to chow diet and subsequently consumed a 45% HFD *ad libitum* for a few days during young adulthood (P67–P75). Surprisingly, adolescent onset alcohol and THC exposure under our paradigm had no lingering effects on the phenotypic and metabolic parameters we assessed during young adulthood. Moreover, previous drug consumption and subsequent HFD exposure did not interact. This is the first study to systematically investigate whether adolescent alcohol and/or THC use can affect energy balance and metabolism upon HFD challenge in adulthood.

The seemingly beneficial effects of moderate alcohol and THC co-use on glucose homeostasis observed in Chapter 4 but not in Chapter 5 may be explained by notable differences in experimental design, including the disparate route of THC administration (subcutaneous in Chapter 4 vs. oral in Chapter 5), the cumulative dose of THC administered (84 mg/kg/rat in Chapter 4 vs. 108 mg/kg/rat in Chapter 5), the frequency of THC administration (once per day in Chapter 4 vs. twice per day in Chapter 5), and the interval between drug treatment relative to execution of the glucose tolerance test (8 days in Chapter 4 vs. 25 days in Chapter 5). Thus, it appears that there is a J-shaped relationship, where the beneficial effects of moderate alcohol and THC co-use manifest soon after brief exposure to lower cumulative doses that were less frequently administered and no benefit is obtained as the cumulative dose and length of exposure increase. Accordingly, a previous study found that the effects of THC on glucose homeostasis may be dose-dependent, route-of-administration-dependent, or modified by a history of marijuana use (Hollister and Reaven 1974). In that study, intravenous 6 mg of THC impaired glucose tolerance in healthy men who casually smoked marijuana, while smoking of marijuana that contained 16 mg of THC had no effect on glucose tolerance possibly due to the development of tolerance to the effects of smoked THC (Hollister and Reaven 1974). It is important to note that not all the THC contained in a marijuana cigarette is bioavailable following smoking. Because of loss associated with combustion, the systemic bioavailability of smoked THC typically ranges from 5 to 30% depending on the users' smoking history (Grotenhermen 2003). Consequently, between 0.8–4.8 mg THC is expected to be present in plasma following smoking of the 16 mg THC cigarette in the study by Hollister and Reaven (1974).

Although 16 days of exposure to moderate alcohol and THC had null effects on glucose and insulin tolerance in Chapter 5, our finding in the COM group in Chapter 4 is partly in line with reports that brief low alcohol consumption can improve insulin sensitivity in rats (Furuya et al. 2003), and THC-mediated CB_2R stimulation in peripheral tissues enhances insulin sensitivity (reviewed in (Maccarrone et al. 2015)). Moving forward, in addition to calculating the amount of glucose and insulin to administer during OGTT and ITT using lean body mass rather than total body mass, utilizing metabolic cages to assess energy balance, and performing further metabolic phenotyping experiments as previously suggested (also see Chapter 5), analyses of brain, muscle, adipose, liver, and other tissues involved in energy metabolism may unveil whether drug-induced alterations that might not have manifested at the behavioral level can portend future metabolic dysregulations. Planned future studies in our lab will investigate the effects of alcohol and THC co-use on IRS-1/AKT/GSK-3β activity in the aforementioned tissues following application of exogenous insulin. The lab will also explore the interactive effects of HFD.

As can be expected of any new field of scientific endeavor, the avenues for advancement of research on alcohol and cannabinoid co-use are innumerable. In addition to those previously mentioned, there is more knowledge to be gleaned from studies on the impact of the drug co-use on synaptic plasticity, immune function, neuroanatomy, gastrointestinal physiology, and behavior, including possible sex-dependent and transgenerational or epigenetic effects. Our lab is currently studying the effects of alcohol and THC co-use on energy balance and cognitive functions in female rats and has plans to investigate the sex-dependent effects of drug co-use and HFD exposure on homeostatic synaptic plasticity. It will also be important for future studies to investigate whether and how the effects of moderate alcohol and THC co-use on physiology are modified by genetics (individual differences). It is likely that they would because there is evidence of individual differences on the neurobiological or behavioral effects of alcohol (Singh et al. 2015; Vaeth et al. 2014) and THC (Earleywine 2002; Nelson et al. 2019; Polissidis et al. 2010). Although the preclinical rat model may not perfectly reproduce the complexities of human drug use (e.g., the erratic schedule of alcohol and/or THC use), it nonetheless grants us the flexibility to pursue research that is otherwise unethical or cumbersome to conduct in humans. Emergent data from this field of work could hopefully open doors to management of metabolic and behavioral disorders caused by chronic alcohol and cannabinoid use. There is more work to be done.

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