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Assessment of Acute Motor Effects and Tolerance Following Self-Administration of Alcohol and Edible THC in Adolescent Male Mice.

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Abstract

Background: Cannabinoids and their principle psychoactive target, the cannabinoid type 1 receptor (CB1R), impact a number of alcohol-related properties, and although alcohol and cannabis are often co-used, particularly in adolescence, few animal models of this phenomenon exist. We modeled the co-use of alcohol and Δ^9 -tetrahydrocannabinol (THC) in adolescent mice using ingestive methods popular during this developmental period in humans, namely binge-drinking and edible THC. With this model, we assessed levels of use, acute effects, and tolerance to each substance.

Methods: Adolescent male C57BL/6J mice had daily, limited access to one of two edible doughs (THC or control), to one of two fluids (ethanol or water), and in one of two orders (dough-fluid or fluid-dough). Home cage locomotor activity was recorded both during and after access. On the day following the final access session, a subset of mice were assessed for functional and metabolic tolerance to alcohol using accelerating rotarod and blood ethanol concentrations, respectively. The remaining mice were assessed for tolerance to THC-induced hypothermia, and whole-brain CB1R expression was assessed in all mice.

Results: Ethanol intake was on par with levels previously reported in adolescent mice. Edible THC was well-consumed, but consumption decreased at the highest dose provided. Locomotor activity increased following ethanol intake and decreased following edible THC consumption, and edible THC increased fluid intake in general. Use of alcohol produced neither functional nor metabolic tolerance to an alcohol challenge. However, use of edible THC impaired subsequent drug-free rotarod performance and was associated with a reduction in THC's hypothermic effect.

Conclusions: Adolescent mice self-administered both alcohol and edible THC to a degree sufficient to acutely impact locomotor activity. However, only edible THC consumption had lasting effects during short-term abstinence. Thus, this adolescent co-use model could be

used to explore sex differences in self-administration as well as the impact substance co-use might have on other domains such as mood and cognition.

Keywords: Adolescent; Alcohol; Edible; Δ^9 -Tetrahydrocannabinol; Tolerance

1. Introduction

Adolescence is characterized by a relatively high prevalence of substance co-use, with alcohol and cannabis being two of the most prominent during this developmental period (Midanik et al., 2007; Schulenberg et al., 2017). Furthermore, in individuals with an alcohol use disorder, co-use of cannabis is highest among all illicit drugs (Falk et al., 2008). While there are many models of alcohol self-administration in animals (Bell et al., 2006; Rhodes et al., 2005; Samson, 1986), models of cannabinoid self-administration have only more recently been established (Barrus et al., 2018; Justinova et al., 2005; Kruse et al., 2019; Melis et al., 2017; Panagis et al., 2008; Smoker et al., 2019; Spencer et al., 2018; Wakeford et al., 2017), and models of alcohol-cannabinoid co-administration are sparse (Nelson et al., 2018).

Synthetic cannabinoids have been shown to facilitate alcohol-drinking in both mice and rats (Colombo et al., 2002; Linsenbardt and Boehm, 2009a); however, this is in contrast to Δ^9 -tetrahydrocannabinol (THC), which has no effect or reduces alcohol-drinking depending on route of administration (Nelson et al., 2018). This suggests a difference in effects on alcohol drinking between synthetic cannabinoids and the phytocannabinoid THC. Cannabinoid type 1 receptors (CB1Rs) have been shown to play a role in a number of properties of alcohol, including self-administration, reward, and neurochemical effects (Agoglia et al., 2016; Henderson-Redmond et al., 2016; Houchi et al., 2005; Hungund et al., 2003; Marcus et al., 2017; Thanos et al., 2005). Furthermore, decreases in CB1R expression and function have been found in high vs low alcohol-drinking mouse strains (Hungund and Basavarajappa, 2000) and in alcohol-dependent vs non-dependent humans (Henderson-

Redmond et al., 2016). Although this implies that cannabis or THC should impact properties related to alcohol use, more studies specifically examining THC in this domain are needed.

Legal access to cannabis and cannabis-based products is increasing, and so too is the variety of products available. Edible THC products are becoming increasingly popular (Giombi et al., 2018; Wang et al., 2019), and given their formulations (e.g. cookies, candies), are likely appealing to adolescents. Although viewed as safer by adolescents (Friese et al., 2017), edible THC administration in rodents induces CB1R-mediated effects similar to those produced by other routes of administration (Kruse et al., 2019; Nelson et al., 2018; Smoker et al., 2019), and high doses are known to produce adverse reactions in humans (Benjamin and Fossler, 2016; Bui, 2015; Favrat et al., 2005; Monte et al., 2015). It is still unclear to what degree use of edible THC might induce facets of substance use disorder, such as craving or tolerance, but it has been shown to impact CB1R expression in drug reward-related circuitry (Kruse et al., 2019).

Binge drinking is particularly prevalent among adolescents (Miller et al., 2007; Schulenberg et al., 2017), and adolescent rodents consume greater amounts of alcohol in binge-like fashion than adults (Holstein et al., 2011; Moore et al., 2010; Quoilin and Boehm, 2016). The Drinking-in-the-Dark (DID) model of binge-like drinking in C57BL/6J (B6) mice produces relatively high blood ethanol concentrations (> 80 mg/dl) and motor incoordination (Rhodes et al., 2005; Rhodes et al., 2007; Thiele and Navarro, 2014). A history of binge-like drinking leads to tolerance to alcohol-induced ataxia, alterations in alcohol metabolism, and a subsequent increase in alcohol consumption (Cox et al., 2013; Linsenhardt et al., 2011), but not withdrawal-related behaviors (Cox et al., 2013), in adult mice. However, assessment of tolerance to alcohol following binge-like drinking appears to be lacking for adolescent mice.

Given the need for models of adolescent substance co-use, the role of CB1Rs in various properties of alcohol, and the limited knowledge of the effects of edible THC, we assessed alcohol and edible THC use or co-use, its short-term consequences, and its impact on CB1R expression in adolescent mice. We hypothesized that adolescent mice would consume alcohol and THC to a degree sufficient to impact behavior acutely and to induce tolerance to an experimenter-administered challenge in a drug-specific manner. We were also interested to see if their combined use would have synergistic effects on these measures.

2. Materials and Methods

2.1. Animals

64 male C57BL/6J (B6) mice were obtained from Jackson Laboratory (Bar Harbor, ME) at postnatal day (PND) 21. Upon arrival, all mice were single-housed in standard mouse cages with wood chip bedding (Sani-Chips, PJ Murphy Forrest Products Corp., Montville, NJ) under a 12-hour, reverse light/dark schedule in temperature- and humidity-controlled rooms. All mice were PND 26 at the start of experimentation (Day 0). All mice had *ad libitum* access to food and water for the duration of the experiment, except for the dough access period, and were weighed daily. Procedures were approved by the IUPUI School of Science Institutional Animal Care and Use Committee and conformed to the Guide for the Care and Use of Laboratory Animals.

2.2. Drugs

THC was provided by the National Institute on Drug Abuse (Bethesda, MD) dissolved in 95% ethanol at a concentration of 100 mg/ml. 190-proof ethanol was purchased from Pharmco, Inc. (Brookfield, CT). Edible dough was created as previously described (Smoker et al., 2019). Briefly, the THC-ethanol solution, or 95% ethanol alone, was dissolved in glycerol and combined with flour, sugar, and salt to produce THC dough or

control dough, respectively. Control and THC dough contained a small, but equal, amount of ethanol (0.023-0.090 g/kg) depending on THC dose provided (Table 1). For injection, THC was dissolved in a vehicle of Tween-20, ethanol, and saline (1:1:18). Ethanol was dissolved at 20% (v/v) in tap water or saline for drinking or injection, respectively. All injections were given at 10 ml/kg.

2.3. Procedure

Mice were randomly assigned to conditions in a 2 x 2 x 2 (Dough x Fluid x Order) design (n = 8 per condition). Mice had access to one of two doughs (THC or control), to one of two fluids (ethanol or water), and in one of two orders (dough-fluid or fluid-dough). On day 0, all mice had access to control dough for 2 hours. Access to dough was given in a clean, empty cage with water, but not food, available. On days 1-5 and 8-11, mice had access to THC or control dough for 30 minutes and had access to 20% ethanol or water for 2 hours. Fluid access always began at 2 hours into the dark cycle (DID; adapted from Rhodes et al., 2005; Thiele and Navarro, 2014) and was immediately preceded (dough-fluid) or followed (fluid-dough) by dough access. THC dose and dough volume were adjusted across days, and values are provided in Table 1. For mice in the fluid-dough order, home cage locomotor activity was recorded at all times in 10-minute bins, except during dough access, as previously described (Linsenbardt and Boehm, 2012). Mice receiving dough-fluid and fluid-dough were housed in separate vivariums until day 12 to maximize the use of the home cage locomotor monitoring system and to permit independent control of light cycles for each order during self-administration. The two vivariums were adjacent and had similar temperature (22.8 ± 0.4 vs 22.7 ± 0.5 °C) and humidity (Mean 26.4% vs 23.5%) throughout the experiment.

2.4. Tolerance Testing

On day 12, approximately 24 hours after the last drug exposure, an equal number of mice from each condition were assigned to be evaluated for tolerance to ethanol ($n = 32$) or THC ($n = 32$). Prior to assignment, mice were matched for cumulative ethanol and/or THC exposure. Functional and metabolic tolerance to a fixed dose of ethanol were assessed using rotarod performance and blood ethanol concentration (BEC), respectively. Latency to fall from the rotarod was assessed on 3 consecutive trials 4-5 hours before and 30 minutes after a 2.5 g/kg (i.p.) injection of ethanol. The rotarod (Rotamex-5, Columbus Instruments, Inc., Columbus, OH) was 3 cm in diameter, was made of PVC with a knurled finish, had a fall height of 44.5 cm, and accelerated from 0 rpm to 100 rpm at 20 rpm/min on each trial with a 1-minute inter-trial interval. Retro-orbital sinus blood samples were taken 10 minutes after the final rotarod trial. Tolerance to THC-induced hypothermia was determined by assessing body temperature ($^{\circ}\text{C}$) 2.5 hours before and 1 hour after a 10 mg/kg (i.p.) injection of THC using a thermometer (Physitemp Instruments, Inc., Clifton, NJ) equipped with a mouse rectal probe (Braintree Scientific, Inc., Braintree, MA) inserted to a depth of 2 cm until a stable temperature was reached (~ 4 sec). On day 13, all mice were sacrificed via cervical dislocation, and brains were immediately removed, flash frozen in 2-methylbutane at -40°C , and stored at -80°C .

2.5. BEC and CB1R Expression

Blood samples were centrifuged, and plasma was separated and stored at -80°C until analyzed for BEC using gas chromatography as previously described (Lumeng et al., 1982). Whole-brain CB1R expression was determined via western blot as previously described (Kasten et al., 2017). Briefly, whole brain samples were homogenized in 2 ml of RIPA buffer, protein concentration was determined using a Bio-Rad protein assay kit (Bio-Rad, Hercules, CA), and 20 μg of protein in 20 μl of 4x loading buffer was loaded. For CB1R,

primary and secondary antibodies used were Anti-Cannabinoid Receptor 1, Rabbit polyclonal to Cannabinoid Receptor 1 (1:1000 dilution, Abcam, Cambridge, MA) and IRDye 800CW Goat anti-Rabbit IgG (H+L) (1:5000 dilution, LI-COR, Lincoln, NE), respectively. For β -actin, primary and secondary antibodies used were β -actin mouse monoclonal (1:1000 dilution, LI-COR) and IRDye 680RD Donkey anti-Mouse IgG (H+L) (1:5000 dilution, LI-COR), respectively. The image was scanned using Odyssey CLx (Image Studio Lite 5.2), and CB1R expression was normalized using β -actin as the reference.

2.6. Statistical Analysis

Statistical analysis was conducted using SPSS or GraphPad Prism, with significance set at $p < .05$. For self-administration, daily mean consumption of control and THC dough (% consumed) was compared using t-tests. As the edible THC dose provided was systematically increased across the experiment, and as there was a main effect of Day on THC dose consumed and ethanol intake, F 's(8, 224) > 7.25, p 's < .001, THC dose consumed (mg/kg), ethanol intake (g/kg), and water intake (ml/kg) were analyzed independently for each combination of THC dose and volume using repeated-measures, 3-way ANOVA's with Day, Order, and either Fluid (for THC dose) or Dough (for intake) as factors. Analyses were followed by 2-way ANOVA, 1-way ANOVA, t-test, and/or Tukey's HSD post-hoc. Baseline locomotor activity was calculated as the average activity for 1 hour preceding dough access. Relative locomotor activity (% of baseline) was calculated for each 10-minute bin during the 2 hours of DID or 2 hours immediately post-DID. Average relative locomotor activity during DID and post-DID was analyzed separately for each combination of THC dose and volume using 2-way ANOVA's with Dough and Fluid as factors, followed by Tukey's HSD post-hoc.

For tolerance testing, both baseline and post-ethanol rotarod performance were analyzed using repeated-measures, 3-way ANOVA's with Trial, Dough, and Fluid as factors, followed by 2-way ANOVA where appropriate. Both baseline and post-THC body temperature were analyzed using 3-way ANOVA with Vivarium, Dough, and Fluid as factors. As there was an effect of vivarium on baseline temperature $F(1, 24) = 57.18, p < .001$, but not post-THC temperature, $p = .173$, change in temperature (post-THC – baseline) was normalized as Z-scores within each vivarium. Thus, a positive Z-score indicates a smaller decrease in temperature, while a negative Z-score indicates a greater decrease in temperature, relative to the mean change in temperature of mice in a given vivarium. Normalized temperature change was analyzed using 2-way ANOVA with Dough and Fluid as factors and was also analyzed based on day 11 edible THC dose consumed using a t-test. BEC was analyzed using 2-way ANOVA with Dough and Fluid as factors, and whole-brain CB1R expression was analyzed using 4-way ANOVA with Dough, Fluid, Order, and Tolerance Test (EtOH vs THC) as factors.

As a complement to group-level effects, and in consideration of within-group variability, Pearson correlations were run on a number of variables, including THC dose consumed, ethanol intake, water intake, relative locomotor activity, rotarod performance, body temperature, BEC, and CB1R expression.

3. Results

3.1. Adolescent mice self-administer ethanol and edible THC alone and in combination, and THC increases fluid intake.

Mean daily consumption of dough for each condition is shown in Table 2. Control dough was consumed at 100% on all days for mice receiving water DID, and was slightly less than 100% on one and three days, respectively, for mice receiving ethanol after or before

dough. For mice in the dough-fluid order, less THC dough than control dough was consumed on days 4, 5, 10, and 11 when followed by water, and on days 1, 4, 10, and 11 when followed by ethanol. For mice in the fluid-dough order, less THC dough than control dough was consumed on day 10 only when preceded by water and on days 9, 10, and 11 when preceded by ethanol.

Figure 1 shows edible THC dose consumed across days for each combination of THC dose and volume. There were no significant effects on days 1-3 (max dose 3 mg/kg), p 's > .257. On days 4-5 (max dose 6 mg/kg), there was a main effect of Order, $F(1, 28) = 6.96$, $p = .013$, with mice consuming more THC when dough was given after DID. There were no significant effects on days 8-9 (max dose 6 mg/kg), p 's > .159. On days 10-11 (max dose 12 mg/kg), there was a main effect of Day, $F(1, 28) = 7.65$, $p = .010$, with dose consumed decreasing significantly on day 11, as well as a main effect of Order, $F(1, 28) = 4.62$, $p = .040$, and a Fluid x Order interaction, $F(1, 28) = 4.75$, $p = .038$. Post-hoc t-tests comparing Order for each Fluid separately indicated greater THC dose consumed in mice in the fluid-dough vs dough-fluid order when drinking water, $t(30) = 3.75$, $p < .001$, but no effect of Order in mice drinking ethanol, $p = .982$.

Figure 2 shows ethanol intake across days for each combination of THC dose and volume. There were no significant effects on days 1-3 or on days 4-5, p 's > .080. However, on days 8-9, there was a main effect of Day, $F(1, 28) = 10.10$, $p = .004$, main effect of Dough, $F(1, 28) = 6.39$, $p = .017$, and a Day x Dough x Order interaction, $F(1, 28) = 4.63$, $p = .040$. Follow-up 2-way ANOVA's on each day indicated no significant effects on day 8, but a main effect of Dough, $F(1, 28) = 7.28$, $p = .012$, on day 9, with Tukey's HSD indicating lower intake in mice receiving THC dough after ethanol than in mice receiving control dough in either order, p 's < .05. Finally, on days 10-11, there was a main effect of Day, $F(1, 28) =$

6.42, $p = .017$, with intake increasing on day 11, and a trend towards a Dough x Order interaction, $F(1, 28) = 4.03$, $p = .055$.

Figure 3 shows water intake across days for each combination of THC dose and volume. On days 1-3, there was a main effect of Order, $F(1, 28) = 4.35$, $p = .046$, with higher water intake when DID was preceded by dough. On days 4-5, there was a main effect of Dough, $F(1, 28) = 8.58$, $p = .007$, a main effect of Order, $F(1, 28) = 9.20$, $p = .005$, and a Dough x Order interaction, $F(1, 28) = 5.40$, $p = .028$, with Tukey's HSD indicating higher intake in mice receiving THC dough before water than in all other conditions, p 's $< .01$. On days 8-9, there was a Dough x Order interaction, $F(1, 28) = 4.97$, $p = .034$. Post-hoc t-tests comparing Order for each Dough separately indicated greater water intake in mice in the dough-fluid vs fluid-dough order when consuming THC dough, $t(30) = 2.32$, $p = .028$, but no effect of Order in mice drinking ethanol, $p = .343$. Finally, on days 10-11, there was a trend towards a main effect of Order, $F(1, 28) = 4.06$, $p = .054$.

3.2. Locomotor activity is bi-directionally impacted by ethanol and edible THC self-administration.

Relative locomotor activity was analyzed for a 2-hour period during DID and immediately post-DID as an average of days for each combination of THC dose and volume (Figure 4). Mice with edible THC consumption < 1 mg/kg or ethanol intake = 0 g/kg on more than one day in a given day range were excluded. On days 1-3, there were no significant effects either during DID or post-DID, p 's $> .186$. On days 4-5, during DID there was a main effect of Dough, $F(1, 27) = 5.71$, $p = .024$, and a Dough x Fluid interaction, $F(1, 27) = 5.29$, $p = .029$, with mice receiving THC dough and water being less active than all other conditions, p 's $< .05$, and post-DID there was a trend towards a main effect of Fluid, $F(1, 27) = 3.89$, $p = .059$. On days 8-9, during DID there was a main effect of both Dough, $F(1, 27) = 4.37$, $p = .046$, and Fluid, $F(1, 27) = 6.86$, $p = .014$, with less activity in mice

receiving THC dough and water than those receiving control dough and ethanol, $p < .05$, and the main effect of Dough persisted post-DID, $F(1, 27) = 6.07$, $p = .020$, with reduced activity in mice receiving THC dough. Finally, on days 10-11, there was a trend towards a main effect of Fluid during DID, $F(1, 26) = 3.78$, $p = .063$, but no significant effects either during DID or post-DID, p 's $> .125$.

3.3. Edible THC consumption, but not ethanol, has effects during short-term abstinence, but BEC and CB1R expression are unaffected.

On day 12, tolerance to ethanol, using rotarod and BEC, and tolerance to THC, using temperature, was assessed. With respect to baseline rotarod performance, there was a main effect of both Trial, $F(2, 84) = 3.35$, $p = .040$, and Dough, $F(1, 84) = 6.47$, $p = .013$, as well as a trend towards a main effect of Fluid, $F(1, 84) = 3.57$, $p = .062$ (Figure 5). Overall, performance increased over trials and was poorer in mice having previously consumed THC dough. Given that performance differences based on Dough and Fluid (trend) were present at baseline, post-ethanol rotarod performance was analyzed using latencies to fall expressed as percent of baseline. With respect to post-EtOH rotarod performance, there were no significant effects, p 's $> .127$ (Figure 5), as ethanol greatly reduced performance in all groups, and there were also no significant effects on BEC, p 's $> .432$ (Figure S1). A subset of mice (8 of 32) was mistakenly given ethanol injections based on a different mouse's body weight, resulting in slightly higher ethanol doses (Mean = 2.67 g/kg). Importantly, neither post-ethanol rotarod performance nor BEC differed between these mice and those receiving 2.5 g/kg ethanol, p 's $> .363$. Compared to baseline temperature, THC was effective in inducing hypothermia 1 hour after injection, $t(31) = 12.51$, $p < .001$; however, there were no significant effects of Dough or Fluid on normalized temperature change, p 's $> .161$ (Figure 6). Two mice receiving control dough and water showed almost no change in temperature in response to THC (-0.6 °C each) and had the two highest normalized temperature change

scores (2.50 and 2.63). Removal of these two mice did not alter the lack of effect of condition on THC-induced hypothermia. Looking at the most recent exposure to THC (day 11) in mice consuming THC dough only, those consuming the entire dose (12 mg/kg) showed less change in temperature than those consuming a partial dose (< 12 mg/kg), $t(14) = 3.13$, $p = .007$ (Figure 4). Finally, there were no significant effects on whole-brain CB1R expression, p 's $> .216$ (Figure S1).

3.4. Both ethanol and THC dose consumed are associated with degree of change in locomotor activity, and THC dose consumed is associated with a reduction in THC's hypothermic effect.

Correlations were run using relevant variables for particular behaviors of interest, including self-administration, locomotor activity, ethanol tolerance, and THC tolerance.

With respect to self-administration, the relationship between THC dose consumed and either ethanol or water intake was examined within each condition independently for each combination of THC dose and volume. THC dose consumed was negatively associated with ethanol intake on days 1-3 for mice receiving dough after DID only, $r(24) = -.44$, $p = .033$.

All other correlations failed to reach significance, p 's $> .135$ (Table S1). In addition to self-administration, the relationship between relative locomotor activity (DID and post-DID) and either THC dose consumed, ethanol intake, or water intake was examined within each condition, and collapsed, across all days. For a subset of data points ($n = 4$ for each time period (DID and post-DID)), mice had extremely high relative locomotor values as compared to mice within their condition on a given day (≥ 3 SD), and the data points in question were excluded from these analyses. THC dose consumed was negatively associated with activity for all conditions during DID, r 's $< -.24$, p 's $< .032$ (Figure 4, Table S2), but not post-DID, p 's $> .110$ (Table S2). Ethanol intake was positively associated, $r(142) = .17$, $p = .043$, while water intake was negatively associated, $r(142) = -.18$, $p = .035$, with activity during DID

(Figure 4, Table S2). Intake of each fluid was negatively associated with activity post-DID for mice consuming THC only, r 's $< -.36$, p 's $< .003$ (Table S2).

A number of variables were examined for their relationship to ethanol tolerance measures, baseline and post-ethanol rotarod performance and BEC. BEC was negatively associated with post-ethanol rotarod performance (avg % of baseline), $r(32) = -.63$, $p < .001$, a relationship that held with the removal of one extreme data point, $r(31) = -.46$, $p = .010$ (Figure 5, Table S3), but all other correlations failed to reach significance, p 's $> .095$.

Similarly, a number of variables were examined for their relationship to THC tolerance. All correlations failed to reach significance, p 's $> .055$ (Table S4). However, with the removal of two extreme temperature values (≥ 2.5 SD), a positive association between cumulative week 2 THC dose consumed and normalized temperature change was revealed, $r(30) = .41$, $p = .027$ (Figure 6, Table S4). Finally, neither ethanol intake nor THC dose consumed was associated with CB1R expression, p 's $> .247$ (Table S5).

4. Discussion

This study used adolescent mice to model the use or co-use of alcohol and THC and did so using a formulation (edible THC) and pattern (binge drinking) popular in humans during this developmental period. Adolescent mice consumed edible THC, alcohol, or their combination to a degree sufficient to induce acute effects on locomotor activity. While alcohol use did not appear to produce either functional or metabolic tolerance, use of edible THC produced an impairment in motor coordination and was associated with tolerance to THC-induced hypothermia.

Edible control dough was well consumed, as was edible THC at lower doses. However, consumption of edible THC became more variable at the highest dose offered (12 mg/kg) and fell below levels of control dough consumption in all conditions on day 11.

Nevertheless, multiple mice consumed 12 mg/kg of THC, and one mouse consumed all the THC it was provided. Reduction in consumption of THC dough was likely not due to taste factors, as the highest THC concentration provided was consumed at levels similar to control dough (days 8-9) until the dose was increased by doubling the volume provided (days 10-11). Rather, this reduction is likely due to the association of THC's post-ingestive psychoactive effects with dough consumption (Smoker et al., 2019). Of note is that when provided double the volume of edible THC, consumption was higher in mice receiving dough after DID. This effect cannot be attributed to the influence of alcohol, as it was present across fluids (days 4-5) and in water-drinking mice only (days 10-11). One explanation is that, as the timing of DID was held consistent with respect to the light cycle for each order, while the timing of dough access was varied for each order, mice might be more willing to consume higher doses of THC a few hours into the dark cycle, consistent with effects on alcohol intake during limited access (Rhodes et al., 2005).

In this modified DID procedure, ethanol intake escalated across days and was on par with levels previously reported in adolescent B6 mice (Moore et al., 2010; Quoilin and Boehm, 2016), indicating that the addition of an edible dough component doesn't necessarily impact DID behavior. During the second week, when ethanol intake was highest, consumption of THC dough reduced ethanol intake. This effect was completely driven by mice receiving THC dough after DID and is consistent with decreased ethanol intake when combined with oral THC in rats (Nelson et al., 2018). What appears to be an increase in ethanol intake in mice receiving THC dough before DID (vs THC after) is likely explained by a general increase in fluid intake following consumption of THC, as mice had elevated water intake following THC consumption on multiple days. Given that the DID procedure only provides access to a single fluid, a 2-bottle choice procedure might better tease apart the effect of edible THC on fluid intake versus ethanol intake specifically.

For adolescents in the dough-fluid order, ethanol intake and consumption of edible THC had divergent effects on locomotor activity. Interestingly, the activity levels of mice with access to both substances fell between these two extremes, suggesting an additive rather than a synergistic effect on locomotion. Increased activity following repeated periods of voluntary ethanol intake in adolescent mice has been previously reported (Quoilin and Boehm, 2016) and appears to be an age-related phenomenon, as adult mice show a decrease in activity to ethanol intake over days (Linsenhardt et al., 2011; Quoilin and Boehm, 2016). Administration of THC typically decreases activity (Lichtman et al., 2001; McMahon and Koek, 2007), except at low doses in novel environments (Sanudo-Pena et al., 2000; Kruse et al., 2019), and although there is evidence for age-related differences in the locomotor response to THC (Shramm-Sapota et al., 2007; Kasten et al., 2017) the decreases in activity seen here in adolescent mice are similar to those seen in adult mice following edible THC consumption (Smoker et al., 2019). While these ethanol- and THC-induced locomotor effects were most pronounced during the 2 hours of DID, the effect of THC persisted post-DID on some days, consistent with the extended duration of action of THC following oral administration (Grotenhermen, 2003; Hlozek et al., 2017). Interestingly, water intake was negatively associated with locomotor activity during DID. This could be directly explained non-pharmacologically, as mice actively drinking water aren't moving, or indirectly explained pharmacologically, as mice consuming edible THC had both higher water intake and lower activity levels.

To determine if adolescent self-administration of ethanol and/or THC was sufficient to induce tolerance, mice were given either an ethanol or THC challenge on day 12. Mice having consumed ethanol performed marginally better on the rotarod at baseline, possibly resulting from experience moving with an altered sense of balance during previous ethanol intoxication. However, having consumed ethanol resulted in neither an enhancement of

performance on the rotarod nor an alteration in BEC following ethanol injection. Of note is that the 2.5 g/kg ethanol dose drastically impaired rotarod performance, with some mice falling prior to the initiation of rod rotation (< 3 sec). The rotarod parameters used were generally within the range previously reported for testing effects of ethanol on motor coordination in adult (Boehm et al., 2000; Offenhauser et al., 2006; Rustay et al., 2003; Rustay and Crabbe, 2004; Stinchcomb et al., 1989) and adolescent (Fish et al., 2016; Hefner and Holmes, 2007; Krahe et al., 2017) mice. In addition, the 2.5 g/kg dose used has successfully produced group differences on the rotarod in adult and adolescent mice (Hefner and Holmes, 2007; Offenhauser et al., 2006; Rustay et al., 2003; Rustay and Crabbe, 2004). In this study, the rod used was at the low end of reported diameters (3 cm) and may have contributed to the relatively poor rotarod performance post-ethanol; although, mice performed relatively well at baseline. Furthermore, the BEC's produced at 40 min post-injection appear to be reasonable for a 2.5 g/kg injection in adolescent B6 mice (Hefner and Holmes, 2007; Linsenbardt et al., 2009b). Overall, these results suggest that the 9 (of 11) days of binge-like ethanol intake (~3-5 g/kg) in this experiment were insufficient to induce functional or metabolic tolerance.

In contrast to alcohol, self-administration of edible THC produced notable short-term effects one day after the final exposure, including an impairment in baseline rotarod performance and a reduction in THC-induced hypothermia; however, whole-brain CB1R expression was unaltered. Repeated exposure to THC leads to both spontaneous and antagonist-precipitated withdrawal (Cook et al., 1998; Cutando et al., 2013; Trexler et al., 2018; Tzavara et al., 2000). Given that baseline rotarod performance was measured beyond the time of expected acute effects of orally-administered THC (Anderson et al., 1975; Hlozek et al., 2017; Kruse et al., 2019; Smoker et al., 2019), the impairment in motor coordination seen in mice having consumed edible THC is possibly due to its induction of withdrawal.

THC has been shown to impair cerebellar-based performance, including motor coordination, both acutely (Dar, 2000) and through various durations of abstinence (Cutando et al., 2013; Skosnik et al., 2008). Although consumption of edible THC in this study did not reduce CB1R expression at the whole-brain level, impairment in motor coordination could have been due to its effects on CB1R expression or function at the regional level (e.g. cerebellum) (McKinney et al., 2008), in accord with region-specific CB1R downregulation following oral THC consumption in rats (Kruse et al., 2019). In addition to its effect on motor coordination, individual variability in edible THC consumption in week 2, but not at the group level, was associated with a reduction in THC-induced hypothermia. Furthermore, when considering just the most recent exposure to THC, mice consuming a full 12 mg/kg dose showed a blunted hypothermic response to THC compared to those consuming a partial dose.

Tolerance to the hypothermic effect of THC can occur rapidly in mice (Pertwee et al., 1993), even following a single oral administration (Anderson et al., 1975). Therefore, it is reasonable to conclude that recent elevated consumption of edible THC, but not just exposure per se, might have been sufficient to induce tolerance to experimenter-administered THC. Overall, self-administration of edible THC in adolescents had short-term effects consistent with withdrawal and tolerance.

There are a few limitations to be considered, including the sex and strain of mice used, a few anomalies with injections during tolerance testing, and the assessment of global CB1R expression. With respect to sex, male mice were used for both practical and theoretical reasons. Practically, given the novelty of many aspects of the procedure, including adolescent mouse edible THC self-administration, edible THC in combination with alcohol, and varying substance access order, the use of one sex greatly limited the number of animals used in this initial assessment. Theoretically, being male is associated with a higher prevalence of alcohol use, cannabis use, and cannabis dependence (Le Strat et al., 2009;

Lopez-Quintero et al., 2011), and in young adults or adolescents, a higher prevalence of daily alcohol use, binge-drinking alcohol, daily cannabis use, and use of edible THC (Johnson et al., 2016; Schulenberg et al., 2017). In addition, we have previously shown that adult male mice have less variable control dough consumption and a more consistent response to THC dough than adult female mice (Smoker et al., 2019). The results of the current study suggest that investigation of sex differences in an adolescent alcohol-THC co-use model might be best served by providing edible THC a few hours into the dark cycle and using a choice procedure, limited access or 24-hour, with access to both alcohol and an alternate fluid.

B6 mice were chosen for their propensity to self-administer psychoactive drugs (Belknap et al., 1993; Rhodes et al., 2007), including edible THC (Smoker et al., 2019).

However, at least two other strains of mice have demonstrated reliable edible THC consumption in our lab (unpublished data), suggesting this co-use model might be adaptable for use in strains other than B6. With respect to injections during tolerance, a subset of mice received ethanol injections at a dose slightly higher than 2.5 g/kg due to experimenter error. However, these mice did not differ in BEC or post-ethanol rotarod performance (Figure S1). In addition, two mice receiving THC injection showed an unreasonably blunted hypothermic response for having had no prior alcohol or THC exposure (Figure 6A/B). Inclusion of these two mice completely obscures the relationship between degree of edible THC consumption and subsequent THC-induced hypothermia. Out of a total of 64 mice, it appears that two mice tested for THC tolerance and one mouse tested for ethanol tolerance (Figure 5C) likely received incomplete injections. Finally, given the potential for a relationship between CB1R expression and the many behaviors assessed in this study, including the self-administration of alcohol and/or THC, locomotor activity, motor coordination, and hypothermia, analysis was conducted at the whole-brain level rather than being targeted towards all the CB1R-expressing regions involved in these behaviors. While the results of this analysis were null,

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this in no way precludes regional differences in CB1R expression, G-protein-coupled signaling, or other endocannabinoid signaling components, often seen following exposure to alcohol or THC (Grotenhermen, 2003; Henderson-Redmond et al., 2016; Mechoulam and Parker, 2003). Instead, this study indicates that repeated self-administration of alcohol and/or THC in adolescent male mice is not associated with alteration of global CB1R expression, and suggests at least one region (cerebellum) for more targeted investigation following repeated edible THC consumption.

In conclusion, the current study presents a mouse model of adolescent alcohol and THC co-use producing acute effects, and in the case of THC, effects during short-term abstinence. This model could be adapted to investigate sex differences in adolescent substance use or co-use, and to investigate the potentially unique impact of adolescent substance co-use in a number of relevant domains, including cognition, mood, and addiction liability.

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Fig. 1. Edible THC dose consumed across days (Mean $\pm$ SEM, n's = 8) during A) week 1 and B) week 2. Labels above the x-axis indicate maximum THC dose provided (2x = double dough volume). $^c p < .05$ vs dough-fluid, $^{ccc} p < .001$ water-THC vs THC-water, $^@ p < .05$ vs day 11.

Fig. 2. Ethanol intake across days (Mean $\pm$ SEM, n's = 8) during A) week 1 and B) week 2. Labels above the x-axis indicate maximum THC dose provided (2x = double dough volume). $^* p < .05$ EtOH-THC vs control dough in either order, $^{\#} p = .055$ dough x order interaction, $^{@@} p < .01$ vs day 8, $^@ p < .05$ vs day 10.

Fig. 3. Water intake across days (Mean $\pm$ SEM, n's = 8) during A) week 1 and B) week 2.

Labels above the x-axis indicate maximum THC dose provided (2x = double dough volume). $^c p < .05$ vs fluid-dough, $^{**} p < .01$ vs all other conditions, $^* p < .05$ THC-water vs water-THC, $^{\#} p = .054$ vs fluid-dough.

Fig. 4. Relative home cage locomotor activity (% of baseline, Mean $\pm$ SEM, n's = 7-8) of mice in the dough-fluid order for 2 hrs A) during DID and B) immediately post-DID, $^* p < .05$ vs all other conditions, $^{\wedge} p < .05$ vs control-EtOH, $^a p < .05$ vs control, $^b p < .05$ vs water, $^{\#} p < .064$ vs water. Relationship between relative home cage locomotor activity for 2 hrs during DID and C) THC dose consumed or D) fluid intake. Mice with activity $\geq 3SD$ were excluded. All THC mice (dashed line in C), $r = -.24$, $p = .004$. All EtOH mice (solid line in D), $r = .17$, $p = .043$. All Water mice (dashed line in D), $r = -.18$, $p = .035$.

Fig. 5. Assessment of tolerance to ethanol. A) Baseline rotarod performance (Mean $\pm$ SEM, n's = 8), ^a p = .013 vs control, [#] p = .062 vs water, [@] p = .040 main effect of trial. B) Average post-ethanol rotarod performance (% of baseline, Mean $\pm$ SEM, n's = 8). C) Relationship between BEC and average post-ethanol rotarod performance, r = -.63, p < .001 (excluding point (183, 79), r = -.41, p = .020).

Fig. 6. Assessment of tolerance to THC. A) Raw body temperature values ^{@@@} p < .001 vs baseline. B) Normalized change in body temperature post-THC (Z-Score, Mean $\pm$ SEM, n's = 8). C) Relationship between normalized change in body temperature and either cumulative week 2 THC dose consumed (mg/kg, solid line, r = .41, p = .027) or cumulative week 2 ethanol intake (g/kg, dashed line, r = .18, p = .341), excluding mice with normalized temperature changes $\geq$ 2.5 SD. D) Normalized change in body temperature (Z-Score, Mean $\pm$ SEM) as a function of edible THC dose consumed on day 11, ^{**} p = .007 vs < 12 mg/kg.

Table 1 Daily EtOH, Dough, and THC Provided, and Type of Assessment

Day	PND	Admin Route	EtOH Dose	Dough Volume	THC Dose	Assessment
0	26	Self-Admin	-	5 mg/g	-	Habituation
1	27	Self-Admin	20% (v/v)	5 mg/g	3 mg/kg	Locomotor Activity
2	28	Self-Admin	20% (v/v)	5 mg/g	3 mg/kg	Locomotor Activity
3	29	Self-Admin	20% (v/v)	5 mg/g	3 mg/kg	Locomotor Activity
4	30	Self-Admin	20% (v/v)	10 mg/g	6 mg/kg	Locomotor Activity
5	31	Self-Admin	20% (v/v)	10 mg/g	6 mg/kg	Locomotor Activity
6	32	-	-	-	-	-
7	33	-	-	-	-	-
8	34	Self-Admin	20% (v/v)	5 mg/g	6 mg/kg	Locomotor Activity
9	35	Self-Admin	20% (v/v)	5 mg/g	6 mg/kg	Locomotor Activity
10	36	Self-Admin	20% (v/v)	10 mg/g	12 mg/kg	Locomotor Activity
11	37	Self-Admin	20% (v/v)	10 mg/g	12 mg/kg	Locomotor Activity
12	38	Injection	2.5 g/kg	-	-	Rotarod, Blood Sample
		Injection	-	-	10 mg/kg	Temperature
13	39	-	-	-	-	Brain Extraction

Note: THC Dose is maximum dose achievable with full dough consumption. Dough contained 0.023-0.090 g/kg of ethanol, depending on THC dose provided.

Table 2. Percent of Dough Consumed (Mean $\pm$ SEM)

Volume (Max Dose)		5 mg/g (3 mg/kg)			10 mg/g (6 mg/kg)		5 mg/g (6 mg/kg)		10 mg/g (12 mg/kg)	
Day		1	2	3	4	5	8	9	10	11
Dough	Fluid/Order									
Control	Con-Water	100.00	100.00	100.00	100.00	100.00	100.00	100.00	100.00	100.00
		± 0.00	± 0.00	± 0.00	± 0.00	± 0.00	± 0.00	± 0.00	± 0.00	± 0.00
	Con-EtOH	100.00	100.00	100.00	100.00		100.00	100.00	100.00	100.00
		± 0.00	± 0.00	± 0.00	± 0.00	94.34 ± 5.66	± 0.00	± 0.00	± 0.00	± 0.00
THC	Water-Con	100.00	100.00	100.00	100.00	100.00	100.00	100.00	100.00	100.00
		± 0.00	± 0.00	± 0.00	± 0.00	± 0.00	± 0.00	± 0.00	± 0.00	± 0.00
	EtOH-Con	100.00	89.38	100.00	98.98	100.00	100.00	100.00	98.74	100.00
		± 0.00	± 10.63	± 0.00	± 1.03	± 0.00	± 0.00	± 0.00	± 1.26	± 0.00
THC	THC-Water	98.26	100.00	89.73	79.04*	82.11*	86.18	81.96	44.75****	20.66***
		± 1.74	± 0.00	± 9.41	± 8.46	± 8.94	± 8.41	± 9.55	± 9.25	± 6.02

THC-EtOH	88.64*	77.93	82.94	63.31*	70.43	80.93	83.23	56.43**	56.05**
	±5.55	±11.93	±12.68	±14.32	±11.83	±12.33	±12.36	±11.70	±12.27
Water-THC	97.65	89.13	100.00	100.00	94.23	100.00	97.89	83.89	61.51**
	±2.35	±10.88	±0.00	±0.00	±4.55	±0.00	±2.11	±11.16	±12.83
EtOH-THC	87.43	86.35	100.00	81.58	100.00	84.99	65.49**	63.45***	48.51***
	±8.08	±9.71	±0.00	±8.71	±0.00	±10.05	±10.56	±8.09	±12.97

Note: THC vs Control * $p < .05$ ** $p < .01$ *** $p < .001$, t-test (2-tailed)

Accepted Article

A Body Temperature

B Normalized Δ Temperature

C Δ Temp x Self-Administration

D Normalized Δ Temperature