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Microstructural Analysis of Rat Ethanol and Water Drinking Patterns Using a Modified Operant Self-administration Model

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Abstract

Background—Ethanol drinking pattern has emerged as an important factor in the development, maintenance, and health consequences of alcohol use disorders in humans. The goal of these studies was to further our understanding of this important factor through refinement of an operant rodent model of ethanol consumption capable of drinking pattern microstructural analysis. We evaluated measures of total consumption, appetitive behavior, and drinking microstructure for ethanol and water at baseline and assessed alterations induced by two treatments previously shown to significantly alter gross ethanol appetitive and consummatory behaviors in opposing directions.

Methods—Male Long Evans rats were trained on an FR1 operant paradigm which allowed for continuous liquid access until an 8 second pause in consumption resulted in termination of liquid access. Total appetitive and consummatory behaviors were assessed in addition to microstructural drinking pattern for both ethanol and water during a five day baseline drinking period, after chronic intermittent ethanol vapor exposure, and following administration of a cannabinoid receptor antagonist SR141716a.

Results—As in previous operant studies, ethanol vapor exposure resulted in increases in ethanol-directed responding, total consumption, and rate of intake. Further, striking differential alterations to ethanol and water bout size, duration, and lick pattern occurred consistent with alterations in hedonic evaluation. Vapor additionally specifically reduced the number of ethanol-directed lever presses which did not result in subsequent consumption. SR141716a administration reversed many of these effects.

Conclusions—The addition of microstructural analysis to operant self-administration by rodents provides a powerful and translational tool for the detection of specific alterations in ethanol drinking pattern which may enable insights into neural mechanisms underlying specific components of drug consumption.

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Keywords

ethanol; microstructure; operant self-administration; drinking pattern; ethanol vapor

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1. INTRODUCTION

Operant self-administration has served for decades as a valuable tool in evaluating mechanisms underlying the drive to seek out and consume ethanol. Many operant studies examine components of ethanol directed behaviors through a focus on the total amount of ethanol consumed [1, 2] and appetitive responses performed, such as lever presses or nose-pokes [3–5]. Though such measures remain critical components in alcohol research, the specific patterns of ethanol intake have also emerged as important factors. For example, individual drinking patterns are potent indicators for the development and maintenance of alcohol abuse-like behaviors in both humans [6–8] and non-human primate models [9]. In the most general terms, patterns of consumption across the short term, such as individual days or hours, consist of frequency of drinking events (“wanting” of ethanol) and amount consumed per each event (“liking” of ethanol) [10]. These patterns can be evaluated over weeks to months or years as in human studies; but, potential pharmacotherapies typically act to reduce long-term ethanol intake through alteration of one or both of these short-term components. The short-term consumption patterns during these individual drinking sessions thus underlie the long-term intake patterns which emerge during periods of abuse and relapse. That consumption pattern within individual drinking sessions can impact therapeutic efficacy of treatments for alcohol use disorders in humans [11] adds further importance to understanding the neural mechanisms which underlie this pattern. Thus, evaluation of short-term ethanol drinking patterns within rodent self-administration has reemerged as an area of active interest in basic research [12–21].

In rodents, total liquid intake is broken into temporally distinct clusters of licks, termed licking ‘bouts.’ Individual rats may consume identical volumes of liquid but differ significantly with respect to moment-by-moment patterns (microstructure) that are related to the “wanting” (bout number and time between individual bouts (interbout-interval)) and “liking” (bout size, bout duration, intrabout licking rate) of the substance [22]. This drinking microstructure can reflect the relative hedonic value of the liquid, its caloric value, prior experiences (either positive or negative) with the substance, post-ingestive feedback from the gut, and the physiological state of the animal [22–28]. For highly palatable, hedonically valuable solutions like sucrose, a monotonic relationship exists between concentration and bout size [29], which contrasts with the typical inverted U-shaped relationship found between increasing concentrations and total intake volume [30]. An inverse relationship in bout size occurs in consumption of aversive substances like quinine (bitter taste) [27]. Additionally, even the rate and pattern of individual licks within a bout of consumption reflect this complex hedonic evaluation or relative “liking” of the substance. For example, the induction of conditioned taste aversion results in increasing amounts of a slower, ‘hesitant’ licking behavior characterized by long pauses between licks [26]. Drinking microstructure has most frequently been assessed during limited access (10–120 min), but importantly these relationships have been demonstrated over periods of up to 23h access

[31]. Thus, the analysis of drinking microstructure provides unique insights into specific components of consumption related to both “wanting” and the shifting hedonic values or “liking” for a substance. Further, as sucrose-directed licking microstructure has been shown to undergo distinct alterations following administration of a D2 or D1 antagonist [32], analysis of this pattern may also eventually contribute to understanding the relative contribution of distinct neurobiological mechanisms to specific components of ethanol intake.

Previous work has demonstrated treatment specific effects on ethanol bout frequency and structure using non-operant models of self-administration and operant models that procedurally separate appetitive (lever press) and consummatory behaviors [17–19, 21, 33–40]. However, concurrent evaluation of ethanol and water drinking microstructure at the millisecond level paired with an ongoing seeking component has not been performed. The concurrent analysis of total intake, appetitive behavior, and consummatory microstructure is of value in the full examination of neural mechanisms regulating interactions between ethanol seeking, evaluation, and consumption. A fixed ratio operant self-administration session is composed of a series of distinct periods of consumption, each unambiguously initiated by an appetitive response. As these consummatory periods are typically restricted by experimental design to a limited access period/amount of liquid following a response, the precise pattern of consumption as it relates to each seeking action cannot be evaluated within a typical operant session. We sought to address this limitation through modification of a classic FR1 operant procedure to allow for uninterrupted liquid access until a pause in licking behavior (selected based upon previous microstructural work within rats [25]) terminated the consummatory bout. The intake pattern was assessed for both ethanol and water during initial introduction to ethanol self-administration and following pharmacological manipulations previously demonstrated to significantly alter total ethanol consumption in opposing directions. As ethanol and water drinking patterns have been previously reported as most obviously distinct in the first 4–6h of self-administration [12], we further sought to evaluate if the pattern of appetitive and consummatory behaviors changed over time through use of a prolonged 6h session. Our microstructural analysis of ethanol drinking reveals unique outcomes associated with both chronic ethanol exposure and endocannabinoid receptor modulation.

2. METHODS

2.1 Animals

Adult male Long Evans rats weighing 200 grams at arrival (7–8 weeks old) were purchased from a commercial supplier (Harlan Laboratories, Indianapolis, IN). A total of 16 animals were used. Animals were housed in groups of 4 on a reverse light/dark cycle (lights off at 9am) under standard conditions. Animals were habituated to housing environment for one week before initiation of behavioral testing and were handled and weighed daily throughout the study. Rodent chow and tap water remained available ad libitum in the animal home cage for the duration of the study except when in the operant chamber. 10–12 grams of chow was available within the chamber during operant sessions. All procedures were approved by

the Wake Forest Institutional Animal Care and Use Committee and were consistent with the NIH Guide for the Care and Use of Animals. No rats were excluded from study.

2.2 Operant Self-Administration

Operant self-administration occurred as daily six-hour long sessions, seven days per week, initiating between 09–11am. The operant self-administration chambers (Med Associates, St. Albans, VT) were housed within sound-attenuating chambers (Med Associates, St. Albans, VT) as previously described [41]. Each chamber contained a house light which remained on for the duration of the session. Operant chambers contained two levers located on opposing side walls; each lever was associated with one sipper bottle, the access point for which was located 2.25 inches away from the lever on the same side of the chamber. Successful completion of a lever press resulted in retraction of both levers and the associated sipper tube lowering into the chamber. The opposing sipper tube was therefore inaccessible until bout termination. Following an 8 second pause in sipper tube contacts the sipper tube was then retracted and both levers reinserted into the chamber. Commercially available software (MedPC, Med Associates) recorded data and controlled levers and sipper tube access.

Following the completion of an operant session, each chamber interior was cleaned with 70% ethanol, while waste trays, food bowls, and sipper bottles were cleaned with dish soap and hot water. To assess fluid intake, bottles were weighed before and following each operant session and grams/kilogram ethanol consumption calculated based on each animal's weight prior to the session. As sessions were run daily, no tail bloods were collected for blood ethanol concentration determination in order to avoid disruption of subsequent animal behavior.

2.2.1 Training—Animals were initially trained on an FR1 schedule for 16 second access to 10 % sucrose in water. Following a two day learning period with this solution, a modified Sucrose substitution procedure [42] was utilized over a 2 week period. Briefly, ethanol was gradually increased from 0% to 10% while sucrose was decreased until animals reliably pressed for access to a 10% ethanol solution in water. Early within the second week, the protocol was altered from a 16 second access to the 8 second bout-termination criterion discussed below. Additionally, one sucrose/ethanol bottle was replaced with a bottle containing solely tap water at this time. Following introduction of the water tube, 10% ethanol and water sipper tubes alternated sides daily to prevent side bias from influencing results.

2.2.2 Operant Testing—Baseline responding was measured over a 5 day period. Rats then underwent a 10 day chronic intermittent ethanol (CIE) vapor exposure and 3 day abstinence period (described below). Following this CIE exposure, animals returned to the operant chambers for 5 additional sessions. Following this experiment, one cohort (n=7) of animals underwent an additional 4 days of operant self-administration. On day 1–3 each animal received an I.P. injection of vehicle 30 minutes prior to session start to acclimate subjects to injection. Day 3 alone was used in data analysis. On day 4 animals received a 3mg/kg injection of SR141716a 30 minutes prior to session start. Rats were run for the entire 6hr session on all days. On the final day of vehicle and SR141617a test day only the

first 2hr of the session were evaluated to match the approximate 2hr half-life of the drug [43] and ensure any alterations in behavior were a direct result of drug administration.

2.2.3 Microstructural Analysis—Initiation of a discrete drinking bout was defined as the performance of an individual lever press followed by at least one lick to the sipper tube and bout termination defined as an 8 second pause in licks to the sipper tube. Typical microstructural analysis utilizes pauses of >1s to indicate bout termination, however such a brief period is not viable within an operant model which must allow for lever retraction/sipper tube insertion. Spector et al. observed in rats that while using bout termination criteria of greater than 1s decreased total bout number, no qualitative differences in microstructure were detected until termination pause criteria reached a 30s duration [25, 27]. With this in mind, a criterion which occurred within the period Spector termed a 'moderate' termination range (1–10s) was selected. This allowed reasonable time for rats to: 1) properly re-orientate towards the sipper following a lever press, 2) account for the time required for the sipper tube to be lowered and removed from the access point within the cage (1–2 seconds total), and 3) perform postural adjustments for a variety of behaviors associated with ingestion of a taste-aversive substance (including: gapes, chin rubs, face washing, and head shakes [26]) between individual licks without an incidental termination of a continuous consumption bout occurring. The time between one bout and the next press of the same lever which resulted in consumption was defined as the 'interbout-interval' (IBI) for that liquid. Interlick-interval (ILI) describes the time between individual licks during a single bout. Latency to lick refers to the time between a lever press and the first lick of the individual bout. In the event a rat failed to lick for a give substance, a maximal latency/ILI for the evaluated range was assigned.

Grams/kilogram ethanol/hour consumed was calculated as following. The amount consumed per lick was calculated from the average total licks/session (across all animals) divided by the average total milliliters consumed during the session. We corrected the volume of ethanol or water consumed during a session for spillage by subtracting 0.1ml from the final measure, consistent with the amount of liquid that spills during handling of the sipper bottles (personal observation). This calculation resulted in an approximate 4.89 μ l consumed per lick, with this volume in good agreement with previous studies demonstrating rats consume liquids in the range of 4–8 μ l/lick [44]. This volume/lick multiplied by the number of licks within each hour was then used to determine the grams/kilogram ethanol/hour for each animal. Notably, when volume/lick was calculated for individual animals, no significant change in volume consumed per lick was found following CIE vapor exposure ($p > .05$, $t = 1.69$, t-test).

2.3 Chronic Intermittent Ethanol (CIE) Vapor Chamber Exposure

Ethanol vapor exposure was accomplished by ethanol inhalation using a method similar to that used in other studies [45]. All animals were placed in the home cage within large, custom-built Plexiglas chambers (Triad Plastics, Winston-Salem, NC) at the beginning of the light cycle (lights on at 9 pm EST). Ethanol vapor, produced by submerging an air stone in 95% ethanol, was mixed with room air and was pumped into the chambers. Animals were exposed to the ethanol vapor 12 h/day, followed by room air for 12h/day, over 10

consecutive days. Animals then underwent 72 hours abstinence from ethanol. During this time animals were exposed to room air only. No supplemental ethanol doses or alcohol dehydrogenase inhibitors were used at any time. Tail blood was taken at the end of some exposure periods to determine blood ethanol concentrations (BECs) through use of a standard, commercially available assay (Carolina Liquid Chemistries Corporation, Brea, CA). Animals reached an average blood ethanol concentration of 250 mg/dl.

2.4 Drugs

SR141716a (5-(4-chlorophenyl)-1-(2,4-dichlorophenyl)-4-methyl-N-1-piperidinyl-1H-pyrazole-3-carboxamide) (Cayman Chemicals, Ann Arbor, MI) was dissolved in one drop of Tween 80 and then diluted in saline solution. SR141716a or vehicle alone was administered in a 3mg/kg intraperitoneal injection 30 min before the self-administration session.

2.5 Statistics

Statistical analysis was performed by using GraphPad Prism (GraphPad software, San Diego, CA). In the CIE vapor exposure experiment data were collapsed across a five day baseline or post-treatment period for each rat. For the SR141716a experiment one vehicle treatment day was compared to one drug injection day. The mean value for each animal was used for analysis of all variables save interbout-interval (see above for bout definition). The median IBI per animal was used due to the relatively low number of individual bouts per session, leading to highly variable mean IBI. Unless otherwise noted, all data sets were analyzed with two-way repeated measures ANOVAs, with all statistically significant effects ($p < .05$) followed by Bonferroni's *post hoc* test.

3. RESULTS

3.1 BASELINE RELATIONSHIPS BETWEEN TOTAL INTAKE AND BOUT NUMBER/SIZE

The relationship between total intake and bout number and size at baseline were assessed for both ethanol and water. Total ethanol licks did not correlate with the number of bouts ($r^2 = 0.14$; $p > .05$) (Fig. 1a), but rather with mean bout size ($r^2 = 0.83$; $p < .001$) (Fig. 1b). Notably, there was no relationship between ethanol bout number and size ($r^2 = 0.001$; $p > .05$) (Fig. 1c). Water consumption exhibited a pattern of relationships that contrasted with ethanol. Total water licks significantly correlated with bout number ($r^2 = 0.27$; $p < .05$) (Fig. 1d), but not bout size ($r^2 = 0.11$; $p > .05$) (Fig. 1e). Additionally, a significant negative relationship between bout number and size was found ($r^2 = 0.27$; $p < .05$) (Fig. 1f) in which larger water bouts were associated with the performance of a fewer number of bouts. These data suggest that total ethanol and water intake during this initial phase of testing may be under the influence of differing regulatory mechanisms.

3.2 CHRONIC INTERMITTENT ETHANOL VAPOR EXPOSURE DIFFERENTIALLY REGULATES WATER AND ETHANOL APPETITIVE AND CONSUMMATORY BEHAVIORS

3.2.1 TOTAL CONSUMPTION—Two-way ANOVA of the total number of licks detected significant main effects for both liquid type ($F(1, 30) = 16.36$, $p < .001$) and vapor exposure ($F(1, 30) = 11.99$, $p < .01$). *Post hoc* test found a specific increase in ethanol lick number following vapor exposure ($p < .01$) (Fig. 2a). Analysis of the lick ratio (ethanol licks/total

licks) confirmed this significant shift towards ethanol-directed consumption from $21.88\% \pm 4.03$ at baseline to $33.24\% \pm 6.67$ post-vapor ($p < .01$, $t=3.187$ $df=15$, t-test) (Fig. 2b). Ethanol intake (g/kg) also significantly increased following vapor exposure (baseline: 0.517 ± 0.078 ; post-vapor: 0.875 ± 0.177) ($p < .05$, $t = 2.927$, $df = 15$, t-test) (Fig. 2c).

To examine any shift in the temporal pattern of ethanol intake following vapor exposure, we analyzed consumption patterns for each hour. Two-way analyses detected a generalized increase in ethanol-directed consumption across session time (time: $F(5, 90) = 1.873$, $p > .05$; vapor: $F(1, 90) = 23.48$, $p < .001$) (Fig. 2d). In contrast, there was a significant effect of session hour for water-directed licks ($F(5,90) = 10.99$, $p < .001$) but no impact of vapor exposure ($F(5, 90) = 2.143$, $p > .05$) (Fig. 2e). These data indicate exposure to CIE vapor resulted in a specific increase in total ethanol consumption which occurred equally across the 6hr session.

3.2.2 BOUT NUMBER—Liquid consumption over the course of the session was divided into distinct bouts composed of an individual lever press followed by drinking (here defined as at least 1 lick). Two-way ANOVA (main factors: liquid and vapor exposure) found significantly fewer ethanol bouts occurred overall in comparison to water ($F(1, 30) = 13.98$, $p < .001$). This was accompanied by a significantly longer interbout-interval for ethanol versus water both at baseline and following vapor exposure ($F(1, 30) = 16.37$, $p < .001$) (Fig. 3a,b). Vapor exposure significantly increased both ethanol and water bout number compared to baseline ($F(1, 30) = 49.33$, $p < .001$) (Fig. 3a). This generalized increase was confirmed by CIE vapor inducing no change in the ethanol:water bout ratio (Baseline: 38.21 ± 1.96 , Post-Vapor: 39.02 ± 2.63 ; $p > .05$, $t = 0.5055$, $df = 15$, t-test). In contrast to bout number, a significant interaction was found between liquid type and vapor exposure for the interbout-interval ($F(1, 30) = 5.615$, $p < .01$) which *post hoc* test demonstrated to be a specific reduction in ethanol IBI (Ethanol: $p < .001$; Water: $p > .05$) (Fig. 3b). Given this specific decrease in ethanol IBI, we reasoned CIE vapor may have resulted in an increased drive to seek ethanol. Using the appetitive measure of the latency to first lick, two-way ANOVA found a significant impact of vapor ($F(1, 30) = 22.65$, $p < .001$), but not liquid ($F(1, 30) = 3.242$, $p > .05$) or any interaction between these factors ($F(1, 30) = 3.018$, $p > .05$). Based on our *a priori* hypothesis that increased bout frequency (a measure which has been used in feeding literature to evaluate appetitive behaviors [28]) may suggest ethanol-specific seeking-related behaviors, a *post hoc* test was performed which confirmed a selective reduction in ethanol ($p < .001$), but not water ($p > .05$), latency to first lick (Fig. 3c). Despite a general trend, there was no significant effect of either of these main factors for another appetitive measure, the response latency to first lever press ($p > 0.07$, $t=1.919$ $df=15$).

Unlike the increase in total ethanol consumption seen across the six hour session, CIE vapor impacted bout number in a time-dependent manner. Two-way ANOVA detected a significant interaction between vapor and time for both ethanol ($F(5, 90) = 4.44$, $p < .01$) and water ($F(5, 90) = 4.30$, $p < .01$). CIE vapor significantly increased bout number during the early portion of the session alone, specifically during hours 1 ($p < .001$, *post hoc* test) and 2 ($p < .05$) for ethanol (Fig. 3d), and hours 1 ($p < .001$), 2 ($p < .05$), and 3 ($p < .05$) for water (Fig. 3e). Interbout-interval across time was not evaluated as large variability occurred

due to some rats failing to reliably initiate more than one drinking bout per liquid per hour during the latter portions of the session. In contrast to bout number, the latency to first lick displayed a generalized decrease across session time as two-way ANOVA detected a significant effect of time (time: $F(5, 90) = 4.434, p > .01$) and vapor exposure ($F(1, 90) = 15.62, p > .001$) but no significant interaction between factors ($p < .05, F = 0.6728$).

3.2.3 BOUT COMPOSITION—We speculated that the specific increase in ethanol consumption accompanied by the generalized increase in bout number may be a result of vapor-induced alterations in bout size. In support of this hypothesis, we found a significant interaction between liquid type and vapor exposure (two-way ANOVA, $F(1, 30) = 21.89, p < .001$); and *post hoc* test demonstrated that CIE significantly increased ethanol ($p < .05$) and decreased water-directed bout size ($p < .01$) (Fig. 4a). Given this, we predicted a parallel shift in the average length of time animals required to complete an individual bout (bout duration). Interestingly, this was significantly reduced for water and not altered for ethanol (liquid X vapor interaction: $F(1, 30) = 15.55, p < .001$; *post hoc* test ethanol: $p > .05$, water: $p < .01$) (Fig. 4b). This may have occurred as a result of a significant increase in lick rate (licks/minute) for ethanol following CIE vapor exposure which was notably accompanied by a decrease in water lick rate (two-way ANOVA Liquid X Vapor interaction: $F(1, 30) = 25.75, p < .001$; *post hoc* test ethanol: $p < .01$; water: $p < .01$) (Fig. 5c). As number of licks appeared to more directly reflect alterations in bout size, we selected this variable to evaluate across session time. Vapor-induced changes in bout size remained relatively consistent across session time for both liquids (factor: time; ethanol: $F(5, 90) = 0.4567, p > .05$; water: $F(5, 90) = 1.276, p > .05$) with vapor exposure resulting in a time-independent increase in ethanol bout size ($F(1, 90) = 10.04, p < .01$) (Fig. 4d) and decrease in water bout size ($F(1, 90) = 11.25, p < .01$) (Fig. 4e). These data confirm the ethanol specific increase in total consumption was due to an increase in both ethanol bout number and size. Conversely, a decrease in water bout size and duration was offset by increased bout number to maintain total water consumption.

3.2.4 INTRABOUT COMPOSITION—The contrasting results in bout size and duration paired with the increased lick rate found above (Fig. 4a,b,c) may have been contributed to by selective alterations in the time between individual licks (interlick intervals or ILIs) within the drinking bouts. Figure 5a and 5b show the frequency distribution of intrabout ethanol and water ILIs expressed as the percent total. For ethanol, two-way ANOVA detected a significant interaction between ILI duration range and vapor exposure ($F(15, 240) = 6.149, p < .001$) which was determined by *post hoc* test to be driven by alterations within the 121–130ms ($p < .001$), 131–140ms ($p < .01$), 151–160ms ($p < .05$), and 161–170ms ($p < .05$) ranges. Vapor exposure was not found to be a significant factor for the water ILIs ($F(1, 240) = 0.0000, p > .05$) although there was an overall significant impact of ILI duration range ($F(15, 240) = 87.90, p < .001$). These data indicate vapor exposure resulted in an ethanol-specific significant leftward shift in ILI duration, indicative of an overall decrease in the time between individual ethanol-directed licks. For a typical liquid meal [27], the majority of ILIs are less than 250ms; and a significant shift of the mean of ILIs 250ms towards latencies >250ms is thought to indicate a disruption in the central pattern generator for licking behavior rather than any alteration in hedonic value of the liquid [26,

46]. Consistent with previous work, most intrabout ILIs for both liquids in our study were found to be $< 250\text{ms}$ ($>84\%$ ethanol, $>90\%$ water at baseline). Further, ethanol ILIs within this range were found to be significantly shorter than water (factor: liquid $F(1, 30) = 6.576$, $p < .05$), with no impact of vapor exposure on this measure ($F(1, 30) = 1.9741$, $p > .05$) (Fig. 5c). These data indicate any ethanol-induced disruption to the central pattern generator is unlikely to have occurred.

It has been previously proposed that the percentage of total ILIs which fall within a subpopulation of ILIs $250\text{--}2000\text{ms}$ may act as a measure of liquid palatability, particularly for taste-aversive substances (such as ethanol in rodents [47]). This subpopulation may reflect the rodent performing taste-aversive related behaviors during an ongoing bout as opposed to intentional bout termination [26, 28]. A significantly larger percentage of ILIs fell within this range for ethanol (16.30%) compared to water (7.84%) at baseline ($p < .001$, $t = 4.331$, $df = 15$, t-test). Following vapor exposure, a significant interaction between liquid and vapor was detected by two-way ANOVA ($F(1, 30) = 9.118$, $p < .01$) with the percentage of ILIs $>250\text{ms}$ reduced for ethanol bouts alone (ethanol: $p < .001$; water: $p > .05$) (Fig. 5d). Our current study expanded this range from $250\text{--}2000\text{ms}$ to $250\text{--}8000\text{ms}$ to accommodate our bout termination criterion. While baseline ILIs within the $2000\text{--}8000\text{ms}$ range composed only $\sim 2\%$ and 0.5% of all ethanol and water ILIs, respectively, this population was still found to be significantly reduced following vapor exposure for ethanol alone (two-way ANOVA vapor X liquid interaction: $F(1, 30) = 4.576$, $p < .05$; *post hoc* test ethanol: $p < .05$, water: $p > .05$). To confirm our results were not driven by this expanded population alone, intervals restricted to those falling within the more validated $250\text{--}2000\text{ms}$ range were assessed. The percent of ILIs within this range was also significantly reduced for ethanol alone (two-way ANOVA vapor X liquid interaction: $F(1, 30) = 5.615$, $p < .05$; *post hoc* test ethanol: $p < .05$, water: $p > .05$). 'Missed licks' are strongly represented by ILIs within the $250\text{--}500\text{ms}$ range [48], but there was no impact of vapor ($F(1, 30) = 0.3173$, $p > .05$) or liquid ($F(1, 30) = 1.703$, $p > .05$) for intervals within this range, indicating a change in the rate of missed licks did not contribute to this finding. Finally, we evaluated the timing of the longer ILIs within the bout. ILIs within the $>250\text{ms}$ range were found to occur equally during the first and last half of individual ethanol bouts at baseline ($p > .05$, $t = 0.8368$, $df = 15$) (Fig. 5e). These data indicate that ILIs $>250\text{ms}$ were evenly dispersed across bouts rather than signaling any specific pause related to bout initiation or termination. Further, the percentage of intervals which fell within the $250\text{--}8000\text{ms}$ range was consistent for ethanol across the 6hr session (vapor: $p < .01$, $F = 9.394$; time: $p > .05$, $F = 1.543$). Overall, our findings show that dependence resulted in an ethanol-selective decrease in the longer ILIs typically associated with responses related to low-palatability.

3.2.5 INCOMPLETE BEHAVIORAL SEQUENCES—During our bout analysis, we detected the consistent appearance of lever presses which did not result in subsequent consumption. These lever presses were termed 'Incomplete Behavioral Sequences' (IBS) and analyzed separately from true bouts (see methods). Two-way ANOVA found a significantly larger percentage of ethanol-directed bouts qualified as an IBS ($F(1, 30) = 5.125$, $p < .05$). Further, a significant main effect of CIE vapor exposure ($F(1, 30) = 8.732$, $p < .01$) was found by *post hoc* test to be due to a specific reduction in the percentage of ethanol-directed

IBS lever presses ($p < .05$) with no significant change occurring in water-directed lever presses (water $p > .05$) (Fig. 6a). Interestingly, despite the higher IBS occurrence rate in ethanol, significant correlations were found between the percent of total ethanol and water lever presses which qualified as an IBS both at baseline ($p > .01$, $r^2 = 0.49$) (Fig. 6b) and following vapor exposure ($p > .05$, $r^2 = 0.32$) (Fig. 6c). This correlation may suggest that IBS events vary between individual rats with some rats prone to perform high or low amounts of this behavior regardless of liquid. This IBS percentage was not impacted by time for either liquid (factor: time, ethanol: $F(5, 90) = 1.352$, $p > .05$; water: $F(5, 90) = 1.397$, $p > .05$), and vapor-induced alterations were seen across the 6hr session for ethanol alone (factor: vapor, ethanol: $F(1, 90) = 11.66$, $p < .01$; water: $F(1, 90) = 0.6023$, $p > .05$) (Fig. 6d,e). Vapor exposure thus specifically increased the likelihood an ethanol-associated lever press would result in a consummatory bout.

3.3 EXPERIMENT 3: SR141716A SELECTIVELY REDUCES DRINKING MICROSTRUCTURE ASSOCIATED WITH ETHANOL 'LIKING'

3.3.1 CONSUMPTION—We next examined whether a pharmacological treatment previously demonstrated to reduce ethanol intake in other models would similarly alter ethanol drinking microstructure. Consistent with these studies, a 3mg/kg dose of the CB1 antagonist SR141716a significantly and selectively reduced total ethanol consumption as measured both by grams/kilogram ethanol (vehicle = 0.67 ± 0.16 g/kg, SR141716a = 0.11 ± 0.07 g/kg) ($p < .01$, $t = 4.790$, $df = 6$, t-test) and by two-way ANOVA of total licks performed (drug $\times$ liquid interaction: $F(1, 12) = 5.129$, $p < .05$; *post hoc* test ethanol: $p < .05$; water: $p > .05$) (Fig. 7a).

3.3.2 BOUT COMPOSITION—There was a general decrease in the total number of bouts associated with this part of the experiment that can likely be attributed to the injection procedure and time period assessed (see methods). Regardless, SR141716a only modestly reduced ethanol-directed bout number, but the effect was not significant (two-way ANOVA vapor: $F(1, 12) = 0.4384$, $p > .05$) (Fig. 7b). Of note, there was no significant impact of the liquid type factor detected during this experiment ($F(1, 12) = 0.0000$, $p > .05$) which contrasts with the lower number of ethanol compared to water-directed bouts found previously, likely due to the factors mentioned above. Interbout-interval was not analyzed for this experiment due to the relatively low number of bouts which occurred for each liquid. SR141716a did not significantly alter the latency to first lick for either ethanol or water (repeated measures two-way ANOVA factor liquid: $F(1, 12) = 0.2559$, $p > .05$; factor drug: $F(1, 12) = 0.8303$, $p > .05$; factor interaction: $F(1, 12) = 1.258$, $p > .05$), but increased the apparent variability in this measure. To provide a more powerful evaluation of potential locomotor effects [20, 39, 40], we analyzed latency to first lick at any sipper tube and latency to first response at any lever (ethanol and water combined). Despite considerable trends due to increased variability, SR141716a did not significantly alter either this combined latency to first lick ($p > .05$, $t = 0.9023$ $df = 13$) (Fig. 7c) or the response latency to first lever press ($p > .05$, $t = 1.440$, $df = 6$) (Fig. 7d). Together, these two results suggest that SR141716a did not significantly reduce locomotor activity, represented by response and lick latencies, at the dose used here.

3.3.3 INTRABOUT MICROSTRUCTURE—As distinct alterations to ‘appetitive behavior’ versus total consumption were detected, we predicted SR141716a may act through a specific reduction in ethanol bout size or duration. Bout size and duration were found by two-way ANOVA to be impacted by SR141716a treatment (bout size: $F(1, 12) = 8.499, p < .05$; bout duration: $F(1, 12) = 4.840, p < .05$), but not by liquid type or an interaction between factors (bout size: liquid: $F(1, 12) = 0.3878, p > .05$, interaction: $F(1, 12) = 3.138, p > .05$; bout duration: liquid: $F(1, 12) = 0.3908, p > .05$, interaction: $F(1, 12) = 2.673, p > .05$). Based on our *a priori* hypothesis above, a *post hoc* test was performed which confirmed this ethanol-specific change in bout size (ethanol: $p < .05$, water: $p > .05$) (Fig. 7e) and duration (ethanol: $p < .05$, water: $p > .05$) (Fig. 7f) without causing any significant alteration in water bout composition. No change in licks/minute was found for ethanol or water (factor drug: $F(1, 12) = 2.600, p > .05$; factor liquid: $F(1, 12) = 0.4465, p > .05$). Due to the unexpectedly large decrease in consumption following SR141716a [20], we were unable to evaluate the percentage of total ILIs falling within the ‘hesitant’ range of 250–8000ms. However, SR141716a did not affect mean of ILIs > 250ms for either ethanol or water (factor drug: $F(1, 12) = 1.219, p > .05$; factor liquid: $F(1, 12) = 2.401, p > .05$), suggesting a lack of effect on the central pattern generator.

3.3.4 INCOMPLETE BEHAVIORAL SEQUENCES—As in experiment 1, all lever presses which did not result in subsequent consumption were qualified as IBS events and analyzed separately from true bouts (see methods). SR141716a administration did not alter IBS event frequency ($p > .05$; $F(1, 12) = 0.7274$) (Fig. 7f). Further, in contrast to the previous experiment, liquid type did not significantly impact the difference in the percentage of total lever presses which qualified as IBS events ($p > .05$; $F(1, 12) = 0.03731$) suggesting again that the injection procedure itself may have modified these measures.

4. DISCUSSION

The main aim of these studies was to evaluate the drinking microstructure associated with ethanol consumption on the level of individual licks using an extended-duration, operant ‘choice’ paradigm. At baseline, total intake of ethanol and water correlated with distinct aspects of behavior. These results are consistent with several previous studies showing that changes in total ethanol consumption do not necessarily occur alongside changes in bout number [18, 33–35] and may indicate ethanol and water consumption are mediated by distinct regulatory mechanisms. Importantly, we show that ethanol dependence induced by chronic intermittent vapor exposure (CIE) altered not only total ethanol consumption, consumption rate, and appetitive responding as shown by several previous studies [3, 5, 21, 49], but also modified drinking microstructure on the level of individual licks. Ethanol vapor exposure was found to result in a leftward shift of ILIs overall and to specifically reduce the percentage of ILIs falling within the ‘low-palpability’ interval range. Importantly, these alterations occurred for ethanol but not water consumption, indicating a specific shift in the relative hedonic value or “liking” of ethanol. The CB1 receptor antagonist SR141716a resulted in a significant decrease in ethanol bout size and duration without impacting total number of bouts, latency to first lever press, response latency, lick rate, or IBS responding. No alterations in water-directed behaviors were detected. Administration of a CB1

antagonist within this model thus resulted in an overall reduction in ethanol “liking” but not “wanting.” Together, our study shows that an analysis integrating common measures of ethanol intake with more detailed drinking microstructure can offer a unique understanding of the neurobehavioral control of ethanol consumption.

As pattern of ethanol consumption has increasingly emerged as a factor of importance for the understanding and treatment of alcohol use disorders (AUDs), microstructural analysis of drinking has emerged as an area of interest. Extensive work has characterized baseline patterns of ethanol consumption and the impact of pharmacological manipulations on these measures within primarily non-dependent animals. These studies have utilized a variety of experimenter-defined criteria to define the termination of a drinking bout or lick-run, most typically defined as a pause in licking or lever press behavior lasting one to several minutes. Our study is unique in that duration of sipper access following a lever press was determined by the animal itself until a pause in consumption similar to those validated in feeding studies occurred [25]. Despite this methodological distinction, the general pattern of ethanol consumption reported here fits well with these previous studies. For example, the largest amount of lever pressing/consumption occurred within the early portion of the drinking session. During the first session hour at baseline $35.3 \pm 2.6\%$ of bouts and $37.6 \pm 5.9\%$ of licks for ethanol occurred, compared to the 8–17% of these behaviors in each subsequent hour. Water followed a similar ‘loading’ pattern. These findings are in agreement with the general trend seen in both operant [17, 19, 35] and non-operant [21, 34] drinking studies. A novel finding was CIE vapor exposure altered ethanol intake, but not lever presses/bout number, equally across the session rather than during the ‘loading’ period alone.

Ethanol bout size was another previously assessed component of microstructure altered within this study. An ethanol-specific increase occurred following CIE vapor exposure which was not related to an increase in bout duration, but rather resulted from an increase in lick number and rate. A similar increase in bout size but not duration was found in mice following a low systemic dose of the neurosteroid alloprenanolone [33]. Operant schedule itself has been shown to alter these consumption parameters. The transitioning from a FR4 to a RR4 or RR8 schedule of reinforcement in non-dependent mice increased the size and duration of runs of licks, as well as decreased lick rate [17]. The increase in lick rate following CIE reported here agrees with that seen in C57BL/6J mice following CIE vapor exposure [21]. Although all these treatments increased ethanol bout size, the CIE-specific alterations to bout size and lick rate, but not duration, may indicate ethanol dependence affects bout composition through mechanisms distinct from those involved in pharmacological manipulation of drinking in non-dependent animals.

We further evaluated drinking bout characteristics following a pharmacological challenge known to decrease ethanol consumption. SR141716a significantly reduced ethanol bout size and duration without altering lick rate. These results are consistent with previous work with SR141716a in Long-Evans rats within an operant self-administration paradigm that procedurally segregates lever pressing and drinking. In this study, both the size and duration of the first lick run was decreased with no impact on lick rate or the number of lick runs [20]. Interestingly, these drinking alterations were seen for both ethanol and sucrose. This is contrasted with our study in which no change in water-related behaviors occurred. These

results may support role of CB1 activity in the consumption of hedonically rewarding substances [50, 51]. These effects appear to be receptor-specific as mecamylamine, a nicotinic receptor antagonist, reduces ethanol drinking bout frequency but not intrabout composition [36], and raclopride, a dopamine D2 receptor antagonist, decreases total ethanol intake via a specific reduction in number of lick runs [38]. All together, these data strongly support the idea that specific pharmacological interventions can be aimed at reducing ethanol intake through a number of distinct mechanisms. A more thorough understanding of the precise components of ethanol-related behaviors impacted by a treatment may therefore enable more personalized interventions within a clinical setting.

A novel aspect of this work was evaluating ethanol bout structure at the level of individual licks. CIE vapor shifted ethanol ILIs leftwards, resulting in shorter intervals between licks. When combined with the increase seen in bout size, this may suggest an increase in ethanol “liking” [22]. CIE specifically decreased the percent of ethanol ILIs >250ms, a range of intervals proposed to represent ‘hesitant’ licking associated with low liquid palatability, reflecting the time required to perform low-palatability associated behaviors such as gapes, chin rubs, and head shaking [18, 26, 52]. Though we expanded this range to 250–8000ms to reflect our selected bout termination criteria, this decrease occurred both during our extended period and within this more heavily validated range of 250–2000ms alone. These results lend further support to CIE vapor increasing the hedonic evaluation or “liking” of ethanol in rats which administer even low to moderate levels of ethanol. This increased “liking” fits well with the proposed role of CIE vapor exposure to both increase the rewarding and reduce sensitivity to the aversive effects of ethanol [3, 21, 49, 53–58]. Analysis of licking microstructure may serve as another important tool in evaluating any shift in the balance between the relative impact of the aversive and rewarding properties of ethanol following dependence.

A surprising finding was that $48.3 \pm 4.2\%$ and $30.8 \pm 3.6\%$ of ethanol and water lever presses, respectively, resulted in no subsequent liquid ingestion by Long-Evans rats. These events were termed ‘Incomplete Behavioral Sequences’ or IBS events. The occurrence of these IBS events is consistent with observations made for operant ethanol self-administration by C57Bl6/J mice [17]. Our current data suggests this phenomenon is not an ethanol-specific event as animals which performed high or low levels of IBS events tended to do so for both liquids. This may indicate this behavior varies between individuals within an outbred strain and may potentially be under genetic control. A further novel result was that CIE vapor, but SR141716a administration, significantly altered the frequency of these events for ethanol. This may indicate that while neural circuitry regulating these events during ethanol seeking/consumption is impacted by CIE vapor, the CB1 receptor is not critically involved. Of further note, these results imply the efficacy of any pharmacological intervention which impacts the frequency of these events may be significantly under-valued within operant studies which do not directly confirm consumption following reinforcer delivery.

An ethanol-specific impact of CIE vapor exposure on appetitive behavior was also found. This was determined by a reduction in latency to first lick within bouts. This was accompanied by a trend towards a significant decrease in response latency to the first lever

press. This disparity in appetitive measures is not totally unexpected given the position of the ethanol and water levers were alternated daily. Thus a potential period of 'indecisiveness' on initial lever selection may have contributed to response latency measure not reaching full significance. While these measures have been most highly validated in operant paradigms utilizing higher lever press requirements [17–20, 35, 36], the latency to first lick has been validated as a measure of ethanol seeking behavior even in non-operant forms of ethanol self-administration [18, 37]. A similar latency is further used in the study of dopamine regulation of non-operant behavior [59–61]. That this decrease was ethanol specific lends further support to this serving as a measure of seeking versus changes in overall locomotor behavior. This increase in ethanol “wanting” is consistent with the increase in progressive ratio break-point for ethanol previously found following vapor exposure and withdrawal [62].

The ethanol-specific change in latency to lick was accompanied by a non-liquid specific increase in bout number. This result was unexpected as several studies have demonstrated ethanol-specific increases in lever pressing following dependence [3–5]. Methodological differences related to concurrently available water and ethanol alternating location daily may underlie this divergence as animals were forced to 'sample' at each lever during the morning to form lever-liquid association. As this elevation in bout number persisted beyond the time this association would be reasonably expected to occur, it is of interest to speculate that changes in impulsive and/or habitual behaviors may have contributed to this prolongation. Chronic ethanol exposure has been demonstrated to significantly increase impulsive decision making [63], which may manifest within our experiments as a reduced ability to inhibit non-reward directed pressing when a liquid is available at each lever. CIE vapor exposure also facilitates the transition from goal-directed to habitual responding in mice [64] and so may have reduced the behavioral flexibility required to shift attention each day to the ethanol-specific lever. Thus, despite impaired inhibition in lever press behavior overall, rats could have maintained an increased drive to initiate ethanol consumption specifically, paired with more rapid termination of water bouts as detected by decreased bout size and duration. Further studies are needed to parse out the specific mechanisms underlying the generalized increase in bout number paired with liquid-specific alterations in latency to lick found in these experiments.

In contrast to vapor exposure, SR141716a administration did not alter the latency to first lick or lever response latency, suggesting a lack of impact on our selected appetitive behaviors. Of note, SR141716a produced a large increase in variability for response latency. These findings are in keeping with previous results which demonstrated both a lack of effect on latency to first lick and a notable increase in variability in the response latency which did not reaching significance [20]. Following this lack of significant effect, latency to first lick for both liquids was further evaluated in combination to assess any potential generalized change in locomotor function [20, 39, 40]. Our present results agree with other studies which indicate a lack of impairment induced by SR141716a at this dose [65, 66]. This lack of apparent effect on appetitive behavior appears at odds with previous findings that CB1 antagonism reduces progressive ratio responding for ethanol and sucrose [65] and operant responding for food and ethanol [4, 20, 66]. It is important to note that these previous operant studies used a much greater response requirement (FR10). Of particular note, the

study by Freedland *et al.* found SR141716a similarly failed to impact response latency, latency to first lick, or lick rate, but significantly impaired the ability to complete a RR16 lever press requirement [20]. It is thus tempting to speculate CB1 antagonism may more heavily impact the 'maintenance' of appetitive and/or consummatory behaviors rather than their initiation. Regardless, while the response requirement sipper model offers multiple excellent measures of appetitive responding, our current model emphasized consumption. This emphasis was an intentional result of our goal to generate detailed analysis of microstructural patterns. Related to this point, SR141716a resulted in a more dramatic decrease in ethanol consumption than that previously seen [20], resulting in the majority of animals performing less than 25 total licks for ethanol, making more detailed analysis problematic. Thus, as the general results from our current study fit well with those previously found using the 'sipper' tube method [20] we elected not to evaluate the impact of SR141716a treatment on additional animals. This may have further limited our ability to detect subtle alterations in appetitive behavior.

Previous microstructural studies performed within the homecage have demonstrated the overall pattern of consumption occurs in distinct temporal patterns for ethanol and water, with this divergence being most prominent during the first 6 session hours [12]. It is of note this divergence was not seen within our current study, suggesting either transition to the operant chamber or co-availability of ethanol and water may alter intake pattern. Genetic background also plays a role in liquid intake microstructure [14], and so differences between Sprague Dawley/Wistar rats and Long Evans may have also contributed. Our 6hr session time was initially selected to evaluate any such potential divergences in ethanol and water microstructure at baseline and assess any CIE vapor –induced alterations to these patterns. However, changes in bout number were the only time specific alteration in drinking pattern to emerge. These data seem consistent with studies in mice which demonstrate a two-hour self-administration session during the early dark period is sufficient to capture the most meaningful daily behaviors [20, 67]. Future studies of alterations in drinking microstructure will therefore likely focus on this first two-hour period.

The evolving number of known risk factors and predictors of AUDs in humans calls for an expansion of rodent models examining specific components of ethanol drinking. Examination of the microstructural pattern of intake along with total consummatory and appetitive behavior is of considerable interest as drinking pattern acts as a strong predictor for the development and maintenance of alcohol related problems [6–9]. Our current results are consistent with the large body of literature examining drinking pattern in both the homecage and operant paradigms which indicate specific treatments used to increase and decrease ethanol consumption result in distinct alterations in drinking microstructure. These specific alterations in behaviors underlying the “liking” and “wanting” aspects of ethanol intake may prove of clinical use as the development of personalized methods of therapeutic interventions become more prevalent (for recent review see [68]). A secondary aspect of the model used within these current experiments was evaluate microstructure using bout-pause criterion more closely related to the temporal patterns found within rodent feeding behaviors [25]. Though ethanol intake remains unique in many aspects, the well-established overlap of brain regions and neurotransmitters which regulate both feeding and drug consumption [69,

70] demonstrate that such theoretical consolidation is critical in the search for treatments which impact ethanol intake and not that of naturally rewarding substances.

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Highlights

- We evaluate drinking microstructure within a model of operant self-administration
- Initial patterns of EtOH vs water intake differ in operant self-administration
- CIE vapor alters total consummatory/appetitive behavior and drinking microstructure
- CB1 antagonist SR141716a reverses many of these CIE effects

Figure 1.

Total consumption for ethanol and water correlates with distinct aspects of behavior at baseline. Total ethanol consumption **A**) did not correlate with the number of bouts performed, but rather with **B**) average bout size. **C**) No correlation between ethanol bout number and size was found. An inverse in relationships were found for total water consumption, which correlated with **D**) bout number and **E**) not average bout size, with a correlation between **F**) bout number and size found. ($n=16$)

Figure 2.

Chronic intermittent ethanol vapor exposure specifically increases ethanol consumption. CIE vapor exposure resulted in **A**) a selective increase in total ethanol licks, **B**) an increase in the percentage of total licks which were ethanol-directed, **C**) an increase in the grams/kilogram ethanol consumed. Evaluation of consummatory behavior across the 6hr session determined a general increase across time in **D**) ethanol licks, with no change across time in **E**) water-directed licks. # = $p < .05$, ## = $p < .01$ t-test; +++ = $p < .001$ main factor impact; ** = $p < .01$ *post hoc* test. (n=16)

Figure 3.

Chronic intermittent ethanol vapor exposure increases total bout number and ethanol appetitive behavior. CIE vapor exposure resulted in **A**) a generalized increase in total bouts performed, **B**) an ethanol-selective decrease in the time between individual bouts, and **C**) an ethanol-selective decrease in latency to first lick within each bout. Evaluation of bout number across the 6hr session determined a specific increase in behavior during the first half of the session for both **D**) ethanol and **E**) water. *** = $p < .001$, * = $p < .05$ post hoc test. (n=16)

Figure 4.

Chronic intermittent ethanol vapor exposure alters bout composition. CIE vapor exposure resulted in **A)** a selective increase in average ethanol and decrease in average water bout size, **B)** a selective decrease in water bout duration, and **C)** a selective increase in ethanol and decrease in water lick rate (licks/minute). Across 6hr session time average bout size occurred equally for **D)** ethanol and **E)** water directed bouts. ++ = $p < .01$ main factor impact; * = $p < .05$, ** = $p < .01$ *post hoc test*. (n=16)

Figure 5.

Chronic intermittent ethanol vapor exposure specifically impacts ethanol interlick-intervals. Distribution of the percent of total ILIs which fell in each interval duration range for **A)** ethanol and **B)** water. CIE vapor exposure resulted in **C)** no alteration in ILIs less than 250 milliseconds, and **D)** a selective decrease in ethanol ILIs falling within the extended duration range of 251–8000ms **E)** which occurred with equal frequency throughout bout duration. // = change in axis scale; + = $p < .05$ main factor impact; * = $p < .05$, ** = $p < .01$, *** = $p < .001$ *post hoc test*. (n=16)

Figure 6.

Chronic intermittent ethanol vapor exposure impacts incomplete behavioral sequences. CIE vapor exposure resulted in **A)** a selective decrease in the percentage of total ethanol-directed lever presses which qualified as IBS events. A correlation was found between ethanol and water for the percentage of total lever presses which qualified as an IBS event during **B)** baseline and **C)** following vapor exposure. Evaluation across the 6hr session determined a general decrease in of IBS occurrence rate across time in **D)** ethanol but not **E)** water-directed lever presses. +++ = $p < .001$ main factor impact; * = $p < .05$ post hoc test. (n=16)

Figure 7.

CB1 antagonist administration modulates drinking microstructure. Relative to vehicle, SR141716a administration resulted in **A)** a selective decrease in total ethanol licks, **B)** no change in bout number, **C)** latency to first lick, **D)** or response latency, and **E)** ethanol-specific decrease in bout size and **F)** bout duration. **G)** no alteration in the percent of total lever presses which qualified as IBS events was found. * = $p < .05$ post hoc test. (n=7)