Published in final edited form as:

Alcohol Clin Exp Res. 2012 August ; 36(8): 1330–1339. doi:10.1111/j.1530-0277.2012.01749.x.

Ethanol drinking microstructure of a High Drinking in the Dark selected mouse line

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Abstract

Background—The High Drinking in the Dark (HDID) selected mouse line was bred for high blood ethanol concentration (BEC) following the limited access Drinking in the Dark (DID) test and is a genetic animal model of binge-like drinking. The present study examines the microstructure of ethanol drinking in these mice and their control line during three versions of the DID test to determine how drinking structure differences might relate to overall intake and BEC.

Methods—Male mice from the HDID-1 replicate line and HS/Npt progenitor stock were tested in separate experiments on 2- and 4-day versions of the DID test, and on a 2-day two-bottle choice DID test with 20% ethanol and water. Testing took place in home cages connected to a continuous fluid intake monitoring system (BioDAQ) and drinking during the DID test was analyzed for drinking microstructure.

Results—HDID-1 mice had more drinking bouts, shorter interbout interval, larger bout size, greater total ethanol intake, and higher BECs than HS/Npt mice on the 2nd day of the 2-day DID test. The 4-day DID test showed greater bout size, total ethanol intake, and BEC in the HDID-1 mice than the HS/Npt mice. Total ethanol intake and BECs for the HDID-1 mice in the DID tests averaged 2.6–3.0 g/kg and 0.4–0.5 mg/mL, respectively. The two-bottle choice test showed no genotype differences in drinking microstructure or total consumption, but did show greater preference for the ethanol solution in HDID-1 mice than HS/Npt.

Conclusions—These results suggest that inherent differences in ethanol drinking structure between the HDID-1 and HS/Npt mice, especially the larger bout size in the HDID-1 mice, contribute to the difference in intake during the standard DID test.

Keywords

Binge drinking; selected lines; drinking pattern; genetics

Introduction

Binge drinking as defined by the National Institute on Alcohol Abuse and Alcoholism is a pattern of drinking that results in blood alcohol levels at or above the legal limit (0.08 gram %, or 0.8 mg ethanol/mL blood) (NIAAA Winter Newsletter, 2004) and this type of consumption is associated with a variety of both short- and long-term risks (e.g. Dawson et al., 2008; Flowers et al., 2008; Hutton et al., 2008). Although binge drinking is often present in alcoholism, individuals without a diagnosis of an alcohol use disorder still binge drink, making it an extremely pervasive issue. Modeling binge-like drinking in animals requires an experimental paradigm that results in intoxicating blood ethanol concentrations (BECs),

something that frequently has been difficult to obtain in common drinking procedures such as 24-hour access, two-bottle choice tests even when using high-drinking animals (Dole and Gentry, 1984), though there are exceptions (Matson and Grahame, 2011). The Drinking in the Dark (DID) test takes advantage of the natural tendency for rodents to engage in consummatory behaviors during the dark phase of the circadian period (Armstrong, 1980). Providing limited access to a single ethanol solution during the circadian dark enables mice to drink relatively large quantities of ethanol and to reach intoxicating BECs over a short period of time (Rhodes et al., 2005). Since its introduction, the DID test has been fairly widely employed by a number of laboratories to explore the neurobiology and pharmacology of binge-like drinking (for review, see Crabbe et al. 2011a; for a bibliography, see <http://www.scripps.edu/cnad/inia/methodology.html>). The High Drinking in the Dark (HDID) selected mouse lines were bred for high BECs following a short version of the DID test and consequently show a drinking phenotype that is reminiscent of binge drinking in humans. These animals drink enough during the DID test to show behavioral intoxication (Crabbe et al., 2009). Additionally, HDID mice do not differ from unselected control mice in consumption of other novel tastants such as saccharin and quinine during preference tests (Crabbe et al., 2011b), indicating that their high-drinking phenotype is somewhat specific to ethanol.

Although the potential utility of the HDID line is clear given their readiness to drink to intoxication, there is much that is not yet known about their behavior. Of particular interest is the patterning and microstructure of their drinking during the DID test and how these factors might promote the high BECs that are achieved. Microstructure refers to the component parts of a delineated period of ethanol consumption, such as bouts (temporally-associated bursts of drinking) and the intervals between them; these elements determine total intake. The microstructure of drinking during the 4 day DID test has been previously assessed in male and female C57BL/6J mice using lickometers (Rhodes et al., 2007), but the use of only one strain of mice did not allow for relating drinking patterning to genotype-driven intake. Bout analysis measures are of potential importance for understanding genetic models of high ethanol consumption. Bout size, specifically, may be related to excessive intake. Non-human primate studies have demonstrated that a 'gulping' (large bout size) phenotype is predictive of future heavy drinking (Grant et al., 2008). Similarly, multiple high-drinking rat selected lines have been shown to have greater bout sizes than their corresponding low-drinking lines (for review see Samson, 2000). Genetic contributions to bout size have been suggested by bout size differences between inbred mouse strains, although the relationship between larger bout size and greater total intake was not seen for C57BL/6J and DBA/2J mice (Fidler et al., 2011).

The patterning of drinking over the course of the DID test is of interest because the number, size, and timing of drinking bouts during the 4 hour test has an obvious potential impact on the BEC measured at the end. Initial studies of DID in C57BL/6J mice showed stable rates of consumption throughout the 4 hr test (Rhodes et al., 2007). However, one could conceive of a situation where high BECs are due in part to the sequestering of drinking in the latter portion of the test, or to larger bout sizes during this period. Analysis of ethanol intake during the first 2 hours and last 2 hours of the 4 hour test from the S₀ and S₁₁ selection generations suggested that HDID animals consumed slightly more during the second half of the test (Crabbe et al., 2009). Analysis of the five most recent generations shows that this disparate consumption does not appear to vary systematically with generation, and that roughly equivalent quantities of ethanol are consumed during the first and second halves of the limited access test (42% vs. 58% of total consumption occurring on average during the first and second halves, respectively) (unpublished data). Nonetheless, this does not rule out the possibility that differences in timing and structure of drinking bouts within these 2 hour periods are a factor in the resulting high BECs. Also of interest are potential differences in

microstructure or patterning that occur between the 2- and 4-day versions of the DID test. In the 2-day DID test, ethanol is provided for 2 hr on the first day and 4 hr on the second day. The 4-day DID test provides 2 hr of ethanol access for three days and then 4 hr access on the final day. It has been shown that intake on Day 1 of the 4-day DID test does not correlate well with that on Days 2-4, while Days 2-4 correlate well with each other (Rhodes et al., 2005). Given this concordance in drinking across the later test days, a 2-day version of the test was used to expedite the HDID selection (Crabbe et al., 2009). However, 4-day and longer DID procedures are often employed elsewhere (e.g. Navarro et al., 2009; Nuutinen et al., 2011) and we thus chose to examine how drinking structure might change across days with the repeated ethanol experience of the 4-day test.

The experiments described here examined the microstructure of ethanol consumption during two variants of the DID test in the HDID-1 selected line and the progenitor heterogeneous stock (HS/Npt or HS). We hypothesized that there would be differences between the two lines in the microstructural components of drinking (e.g. number of bouts, bout size, interbout interval). Given past findings suggesting greater bout size in higher drinking animals, we were particularly interested in bout size and hypothesized that larger ethanol bouts in the HDID-1 than the HS might in part explain their greater intake during the DID test. Finally, a two-bottle choice DID test between water and ethanol was used to assess whether there were microstructural differences in drinking between ethanol and water when both were available.

Materials and Methods

Animals and Husbandry

A total of 95 naïve male mice from the HDID-1 selected line and HS progenitor stock were used for these studies (HDID-1: 24.03 ± 2.87 g, aged 72 ± 15 days at start of testing; HS: 26.27 ± 4.24 g, aged 72 ± 14 days at start of testing). Mice were bred and housed in the Veterinary Medical Unit at the Veterans Affairs Medical Center (Portland, OR). Experiment 1 used HDID-1 mice from selection generation 19, Experiment 2 used mice from selection generation 20, and Experiment 3 used mice from selection generation 21. HS mice are the starting population from which the HDID-1 mice were selected and are the product of a systematic 8-way cross of inbred strains described in detail elsewhere (Crabbe et al., 2009). HS mice are maintained without selection and are used as a control comparator line for the HDID animals because HDID selection was unidirectional and a corresponding low-drinking line does not exist. Mice were kept on a reverse 12 h/12h light/dark cycle with lights on at 2130h for all experiments. At the start of each experiment animals were singly housed in polycarbonate shoebox cages on Bed-o-cob bedding and provided continuous access to food (5001 Purina rodent chow, PMI Nutrition International, Brentwood, MO) and water unless otherwise specified. Room temperature was maintained at $21 \pm 1^\circ\text{C}$. All procedures were approved by the local Institutional Animal Care and Use Committee and were conducted in accordance with the NIH Guidelines for the Care and Use of Laboratory Animals.

Ethanol Consumption Recording

During all experiments, mice were housed in specialized polycarbonate shoebox cages connected to the BioDAQ Episodic Intake Monitoring System (Research Diets Inc., New Brunswick, NJ). Cages have seven cutouts (2 on each end and 3 on one side) where liquid hoppers can be attached. During habituation for all experiments and during testing for Experiments 1 and 2, liquid hoppers were attached to the center position on the side and all other cutouts were covered by stainless steel panels. During testing for Experiment 3, water and ethanol hoppers were attached to the left and right positions on the side, with left-right

position of each solution counterbalanced across animals. Water was returned to the center position for the period between the limited access testing sessions on Days 1 and 2. Food was provided in a hopper in the center of the stainless steel wire cage tops. Water and ethanol were provided in polycarbonate bottles with stainless steel ball bearing-tip sipper tubes (8mm spout diameter). Bottles had a volume of approximately 70 ml, but did not allow for specific volumetric measurements. The BioDAQ system provides a novel means of recording ethanol consumption in laboratory animals and detailed information about the system and its data collection method can be found at the website of the vendor (<http://www.researchdiets.com/biodaq/index.htm>). Briefly, weight sensors recorded the weight of the ethanol bottle once every second and registered the initiation of drinking when triggered by the mouse applying a 0.08g or greater force to the bottle/hopper unit. When a new stable weight was recorded for five consecutive seconds, the system considered drinking to be terminated and the change in weight of the bottle was recorded with a sensitivity of 0.01g. The raw data output obtained from the system included the exact time of each initiation of drinking and the weight of the bottle at that time. Fluid leakage from the bottles during the limited access test was negligible, as determined by recordings made from ethanol and water bottles attached to control cages during pilot studies.

Experiment 1: 2-day Drinking in the Dark—The 2-day DID test was chosen for microstructure evaluation because of its use as the test procedure for the selection of the HDID lines. This experiment used 36 mice ($n=18/\text{line}$) and testing was conducted in 3 passes with 12 mice per pass. Mice were singly housed in BioDAQ cages and allowed to acclimate for 1-2 weeks. On Day 1 of testing, mice were weighed at least 1 hour before alcohol access began. At 3 hours into the dark cycle, water bottles were removed and replaced with bottles containing 20% ethanol (Decon Laboratories Inc., King of Prussia, PA) solution (v/v in tap water). Ethanol bottles were left in place for 2 hours and then removed and water bottles were returned. Ethanol consumption was continuously recorded during the drinking session by the BioDAQ system. On Day 2, the procedure was identical to Day 1, but ethanol bottles were left in place for 4 hours. At the end of the 4 hours, a 20 μL blood sample was taken from the periorbital sinus of each animal to be analyzed for ethanol content via gas chromatography as described below.

Experiment 2: 4-day Drinking in the Dark—The 4-day DID test version was also used to assess whether drinking microstructure on the final test day (Day 4) was affected by repeated ethanol experience. This experiment used 35 mice ($n=17\text{-}18/\text{line}$) and testing was carried out in 3 passes with 11-12 mice per pass. Mice were habituated to the BioDAQ cages for 2 weeks before testing. Testing procedure on Days 1-3 was identical to that of Day 1 of Experiment 1, and Day 4 testing was the same as Day 2 of Experiment 1, including blood-sampling procedures.

Experiment 3: Two-bottle choice Drinking in the Dark—A 2-day, two-bottle choice version of the DID test was used to assess whether the presence of water during the DID test affected ethanol drinking microstructure, and whether the microstructure of water drinking differed between genotypes. This experiment used 24 mice ($n=12/\text{line}$) and testing was carried out in 2 passes with 12 mice per pass. Mice were habituated to the BioDAQ cages for 2 weeks before testing. Testing procedure was identical to that of Experiment 1, except that a water bottle was present during each ethanol drinking session.

Bout Analysis

Each drinking period was divided into 30-min blocks and the number of bouts, average bout length, and average bout size (g/kg consumed) were calculated for each block, as well as totaled across the entire drinking period. Average interbout interval was calculated for the

entire drinking period. For Experiment 3, drinking variables were determined for each fluid type. The minimum bout criteria were at least a 0.02g change in weight of the ethanol (or water) bottle followed by at least 1 minute of no change. The g/kg ethanol intake in bouts and total g/kg intake were calculated for each block and for the total drinking period. All bout analysis was conducted using Microsoft Excel (Microsoft Office version 2007) and the time-marked raw weight change output of the BioDAQ system.

BEC Determination

Blood samples were processed and analyzed according to previously published methods (Rustay and Crabbe, 2004). Briefly, blood samples were dispensed into a microcentrifuge tube containing 50 μ L zinc sulfate and placed on ice until all samples had been collected. They were then processed with 300 μ L distilled water and 50 μ L of barium hydroxide and centrifuged at 12,000 rpm for 5 minutes. The supernatant was removed and frozen until analyzed by gas chromatography (Model 6890N, Agilent Technologies, Santa Clara, CA) as compared to a standard ethanol concentration curve (0.2369-4.932 mg/ml). All samples from a given experiment were analyzed at the same time and using the same standard curve.

Statistics

Statistical analyses were carried out using Systat (version 13, Systat Software, Inc, Chicago, IL). DID 30-min block data for Experiments 1 and 2 were initially analyzed separately for the first and final day by repeated measures ANOVA with genotype as the between subjects factor and time block as the repeated measure. With the exception of the few cases discussed below in the results section, there were no significant genotype by time interactions. We also performed exploratory data analysis of drinking during the first half and second half of the final day for each experiment via repeated measures ANOVA. In nearly all instances (excluding total g/kg consumption and average bout duration in Experiment 2) there were no significant block by genotype interactions. We therefore chose to average results across the entire 4 hr drinking period and data were subsequently analyzed as daily totals by one-way ANOVA with genotype as the factor for Experiments 1 and 2. In addition, we analyzed drinking data during the first 2 hr of drinking on the final day of Experiment 1 and during Day 2 of Experiment 2, as these are procedurally identical time periods and allow for comparison between the 2- and 4-day DID test. For Experiment 3, we initially used two separate repeated measures analyses by time block to assess ethanol and water data individually. Daily totals were then analyzed by two-way ANOVA with genotype as the between groups factor and fluid type as the within groups factor. Ethanol and water intake and bout size for Experiment 3 were analyzed using values of milliliters of fluid consumed per kilogram of body weight to facilitate comparisons between fluid types. Ethanol preference, expressed as the ratio of 20% ethanol solution to total fluid intake, and total fluid intake were analyzed by repeated measures ANOVA with genotype as the between groups factor and test day as the repeated measure. BEC data were analyzed by one-way ANOVA with genotype as the factor. Significance for all tests was set at $\alpha=0.05$ unless otherwise specified and a Bonferroni correction for multiple comparisons was used for post-hoc analyses.

Results

Experiment 1: 2-day Drinking in the Dark

Figure 1 shows the g/kg ethanol intake over 30 min blocks on Days 1 and 2. Figure 2 shows the mean values for these and other indices averaged over the entire 4 hour test period on Day 2. Day 1 repeated measure analyses showed no significant genotype by time interaction for any measure. When averaged over the entire drinking period, there were no significant differences in drinking between the genotypes, with HDID-1 and HS mice not differing

significantly in their total g/kg intake ($F_{1,34}=0.738$, $p=0.396$) (fig. 1), number of drinking bouts, bout size, interbout interval, or average bout length (all $F_{1,29-34}<1$, NS: data not shown). On Day 2, repeated measures analyses again showed no significant genotype by time interaction for g/kg intake (fig. 1) or any other measure. However, analyses for the whole 4 hr drinking period showed that HDID-1 mice drank in significantly more bouts than HS ($F_{1,34}=4.775$, $p=0.036$) (fig. 2A) and had shorter average interbout intervals than HS mice ($F_{1,34}=11.538$, $p=0.002$) (fig. 2B). Although bout duration did not differ significantly between lines ($F_{1,34}=0.000$, $p=0.999$) (fig. 2C), the HDID-1 animals had a larger average bout size (g/kg ethanol consumed per bout) than the HS animals ($F_{1,34}=7.534$, $p=0.01$) (fig. 2D). There was also a main effect of genotype for total g/kg ethanol intake, with HDID-1 mice having greater intake than HS ($F_{1,34}=13.823$, $p=0.001$) (fig. 2E). This last index included ethanol intake occurring outside of defined bouts, which did not differ between genotypes (data not shown). BEC of the HDID mice at the end of the 4 hour test was also significantly greater than HS mice ($F_{1,34}=9.489$, $p=0.004$) (fig. 2F). Analysis of the first 2 hr of Day 2 showed no main effect of genotype for number of bouts or average bout length (all $F_{1,34} \leq 0.683$, NS) (average interbout interval could not be accurately computed for individual times bins and was only included in total session analyses), but did show larger bout size ($F_{1,34}=5.095$, $p=0.031$) and greater total g/kg intake ($F_{1,34}=6.968$, $p=0.012$) in HDID-1 than HS.

Experiment 2: 4-day Drinking in the Dark

Figure 3 shows g/kg ethanol intake over 30 min blocks across Days 1-4. Figure 4 shows the mean values of these and other indices over the entire 4 hour test period on Day 4. On Day 1, HDID-1 animals had a greater total ethanol intake ($F_{1,33}=4.979$, $p=0.033$) (fig. 3) and a significantly greater bout size than HS ($F_{1,33}=8.454$, $p=0.006$). HDID-1 and HS mice did not differ significantly in number of bouts, average interbout interval, or average bout duration (all $F_{1,32-34}<1$, NS: data not shown), and there was a significant genotype by time interaction only for the number of bouts ($F_{3,99}=3.225$, $p=0.026$). This interaction appeared to be due to a trend towards HDID-1 mice having a greater number of bouts than HS mice during the final 30 min of drinking, but this difference did not reach statistical significance ($p=0.056$). Day 4 showed a similar pattern of differences in microstructure between the genotypes, though there were no significant genotype by time interactions (all $F<1$). HDID-1 and HS animals again did not differ significantly in number of bouts (fig. 4A), average interbout interval (fig. 4B), or average bout duration (fig. 4C) (all $F_{1,32-33}<1$, $p \leq 0.288$). Bout size was significantly greater in HDID-1 animals than HS ($F_{1,33}=7.697$, $p=0.009$) (fig. 4D), as was total g/kg intake ($F_{1,33}=5.032$, $p=0.032$) (fig. 4E). BEC following the 4 hour drinking period on Day 4 was also significantly higher in HDID-1 mice than HS ($F_{1,33}=8.240$, $p=0.007$) (fig. 4F). Analysis of Day 2 showed a similar pattern of results to that seen during the comparison time period on Day 2 of Experiment 1 (see above). Number of bouts and average bout duration did not differ between the genotypes (all $F_{1,31-32} \leq 0.928$, NS), but HDID-1 mice did have significantly larger bout size ($F_{1,31}=6.157$, $p=0.019$) and greater total g/kg intake ($F_{1,32}=4.851$, $p=0.035$) than HS mice.

Experiment 3: Two-bottle choice Drinking in the Dark

Figure 5 shows mL/kg intake of the 20% ethanol solution (A) and water (B) over 30 min blocks across Days 1 and 2. The inset in panel A shows g/kg dose of ethanol consumed. Water intake was greater than ethanol intake, as expected, and it also apparently varied more across time blocks as well. Repeated measures ANOVAs for intake (fig. 5) and the other microstructure elements conducted within each fluid and day separately yielded multiple instances of genotype by time block interactions, but clear temporal patterns were not detected for any measure. We therefore for simplicity's sake show the Day 2 data averaged across all time blocks in Figures 6 (ethanol) and 7 (water). Analysis of daily totals from Day

1 showed a main effect of genotype and a significant genotype by fluid type interaction for number of bouts ($F_{1,22}=5.191$, $p=0.033$; $F_{1,22}=7.488$, $p=0.012$) and total mL/kg intake ($F_{1,22}=5.356$, $p=0.03$; $F_{1,22}=8.736$, $p=0.007$). Follow-up analyses showed that HS animals had more water bouts than HDID-1 animals ($F_{1,22}=10.336$, $p=0.004$) and greater water intake ($F_{1,22}=7.945$, $p=0.01$), with no significant differences in ethanol intake or number of bouts. A main effect of fluid type was seen for all measures except for bout duration (all $F_{1,22} \leq 5.039$, $p < 0.035$). Water bouts were larger, greater in number, and had a shorter average interbout interval than ethanol bouts.

There were no main effects of genotype on Day 2 (all $F_{1,22} \leq 2.441$, $p \geq 0.132$) (fig. 6 and 7). Total mL/kg intake showed a significant genotype by fluid type interaction ($F_{1,22}=4.379$, $p=0.048$). Follow-up analyses showed that ethanol consumption did not differ significantly between the genotypes ($p > 0.05$) (fig. 6E), but there was a trend towards greater water intake by HS animals than HDID-1 ($F_{1,22}=3.56$, $p=0.072$) (fig. 7E). No other significant genotype by fluid type interactions were seen (all $F_{1,22} \leq 2.74$, $p \geq 0.112$). A main effect of fluid type was again seen for all measures except bout duration (all $F_{1,22} \geq 18.212$, $p < 0.001$). Similar to Day 1, water bouts were larger than ethanol bouts, greater in number, and the interbout interval between water bouts was shorter than between ethanol bouts.

Though total ethanol intake did not differ between the genotypes on either day, there was a main effect of genotype on ethanol preference, with HDID-1 mice having a greater ethanol preference ratio than HS mice ($F_{1,22}=6.26$, $p=0.02$) (fig. 8A), though this did not reach a level of true preference for ethanol over water (i.e. an ethanol preference ratio greater than 50%). The difference in preference ratio was paralleled by the differences in water drinking between the genotypes (i.e. greater HS water intake on Day 1 and trend towards greater intake on Day 2 than HDID-1 mice), and also by greater total fluid intake by HS animals than HDID-1 ($F_{1,22}=8.285$, $p=0.009$) (fig. 8B). No animals of either genotype had a measurable BEC at the end of drinking on Day 2.

Discussion

The present findings show that mice selectively bred for high BEC following DID have a drinking microstructure that differs from their control line during both the 2- and 4-day version of this test. The 2-day DID showed genotypic differences in number of bouts, bout size, interbout interval, and total ethanol intake, but only on the 2nd day of drinking. The 4-day DID test showed fewer drinking microstructure differences overall between the genotypes; the only significant difference was larger bout sizes in the HDID-1 animals than the HS. When ethanol and water were offered in a 2-day DID preference test, no differences in ethanol drinking microstructure or total intake were seen between HDID-1 and HS mice, though HDID-1 mice did have a greater ethanol preference ratio than HS.

Of the microstructural drinking elements assessed, the most robust difference between genotypes was that of bout size, which was found to be larger in HDID-1 than HS mice in both the 2- and 4-day standard DID tests. Although Experiment 1 showed a difference between the genotypes for the number of bouts, the absence of this difference during Experiment 2 may suggest that this variable is more susceptible to influence by environmental factors and procedural variation than bout size. Comparison of drinking during the first 2 hr of the final day of Experiment 1 (2-day DID) and the second day of Experiment 2 (4-day DID) showed a similar pattern of microstructure differences between the genotypes (i.e. equal number of bouts, equal average bout duration, and larger bout size in HDID-1 than HS). Since the genotypic difference in number of bouts did not emerge until the second half of the drinking session on Day 2 of the 2-day DID (and was not seen at all during the 4-day DID test), it's possible that the timing of the initial experience with an

extended ethanol drinking session rather than cumulative ethanol exposure promotes the increase in number of bouts for HDID-1 mice. A modified, 3-day DID test wherein mice receive access for 2 hr on the first day and 4 hr on the second and third days (thereby replicating both the Day 2 extended access experience of the 2-day test and the total hours of ethanol access of the 4-day test) might therefore produce similar microstructure results to Experiment 1. Additionally, increasing the duration of ethanol access on the initial day of drinking could provide insight into the effects of longer drinking periods on the emergence of genotypic differences in the number of bouts.

As previously mentioned, there is evidence to suggest that larger bout size might be associated with excessive ethanol intake. Correlational analysis of drinking variables on the final days of testing showed that bout size and g/kg intake both significantly predicted BEC in Experiments 1 and 2 (data not shown). This relationship was observed for bout size and intake during both the first half and last half of the drinking session in Experiment 1, and only for these variables during the second half of the session in Experiment 2. The present bout size results are also consistent with the previous findings of both the non-human primate (Grant et al., 2008) and rat literatures (e.g. Files et al., 1998), in that the animals genetically predisposed to drink more (HDID-1) exhibited significantly larger bout size than the control animals. There is also some evidence to suggest that bout size potentially may be related to reward processes. One study of NaCl solution licking microstructure showed that bout size can be decreased in rats following treatment with the dopamine D2 receptor antagonist raclopride (Canu et al., 2010). Dopaminergic signaling has been shown to mediate in part both ethanol and sucrose DID during a 2-day procedure (Kamdar et al., 2007), though it is unknown whether this involvement extends to ethanol bout size. Identification of the specific neurotransmitter systems and brain regions critical for determining ethanol bout size may prove to be a fruitful area for future study.

Interestingly, despite the greater ethanol bout size of the HDID-1 mice, the duration of their bouts did not differ significantly from that of the HS. This ability of the HDID-1 mice to consume significantly more ethanol than HS mice during the same amount of time indicates an apparent increased efficiency of drinking among these animals. There are several possible explanations for this. One possibility is that the lick rate of HDID-1 mice may be faster than that of HS. Lick rate is thought to be controlled by a central pattern generator (Travers et al., 1997) and there is evidence that it is under some degree of genetic control as inbred mouse strains have been shown to differ in the time taken between licks of water (Boughter et al., 2007; Johnson et al., 2010). Consequently, selection for high BEC following DID might have resulted in animals with a faster innate lick rate. Alternatively, lick rate might be comparable between the genotypes, but the HDID-1 mice might consume a greater volume of ethanol per lick, thereby resulting in the observed larger bout size. With the present studies we were not able to distinguish between these possibilities, but future work using a combination of the BioDAQ and lickometer systems may help to tease apart the underlying basis of the bout size difference between these genotypes. Additionally, the fact that both genotypes had large water bouts in Experiment 3 shows that HS animals are capable of drinking in large bouts, and that their 'sipping' phenotype observed in Experiments 1 and 2 appears to be somewhat specific to ethanol rather than a general inability to consume large quantities of fluid in a given bout.

Genetic differences in bout size are of potential interest to alcohol research for several reasons. It has been suggested that larger sized bouts may represent loss of control over drinking, a critical aspect of alcohol use disorders in humans, and could suggest deficits in the ability to terminate drinking (Samson, 2000). Bout size (and drinking microstructure in general) also represents an area of potential phenotypic concordance between preclinical animal work and human studies of alcohol consumption, thereby enhancing the possibility

of translating findings across species. Currently, rodent studies typically report intake in terms of g/kg dose of ethanol consumed over a given amount of time. Human intake, however, is often reported as drinks per day or other time period (Leeman et al., 2010). Drinking microstructure elements such as interbout interval, or bout duration have easily measured potential human correlates such as time between drinks or length of time taken to finish a drink. Furthermore, there is some evidence from the human literature to suggest that maximum alcohol dose per drinking session may be a better predictor of alcohol related problems than drinking frequency or annual intake (Bobak et al., 2004), indicating that the patterning of drinking is a critical area for research and that reducing bout size might be an important goal for pharmacological treatments.

It should be noted that both the g/kg ethanol intake and BECs seen here for the 2-day test are lower than what was measured by our laboratory during the selection tests for the generations of HDID mice used in these experiments (mean g/kg intake and BEC of male mice were 5.78 g/kg and 1.31 mg/ml, 6.59 g/kg and 1.50 mg/ml, and 5.70 g/kg and 1.23 mg/ml, respectively for generations 19-21; unpublished data). Consequently, few of the mice in these experiments probably drank to intoxication. However, the expected differences in total intake and BEC between the HDID and HS mice remain robust. We cannot rule out the possibility that the microstructure patterns we report here might differ if we were able to assess them during our own standard DID test where BECs would be higher. That intake (and BEC) was lower than during the standard drinking tube DID test in both HDID-1 and HS mice is likely an artifact of the cage design and drinking bottle differences arising from the use of the BioDAQ system. Most notably, the spout diameter of the BioDAQ sipper tubes is marginally wider than that used for selection, and accessing the sipper tube requires the animal to enter a slightly elevated alcove attached to the side of the cage. Spout orifice size has been shown to affect licking behavior in several strains of inbred mice, though fluid intake appeared to remain fairly consistent (Dotson and Spector, 2005). Additionally, the slight climb needed to reach the sipper tube represents an increase in the amount of effort expended in order to drink relative to standard home drinking procedures where the sipper tube extends into the center of the cage. Though not a perfect analogue, operant studies have shown that mild increases in response requirement (e.g. from one to four bar presses) will reduce total ethanol intake (Samson and Hodge 1996), so it seems probable that this aspect of the cage design may be having a similar effect. Finding a way to increase total intake in the BioDAQ system might reveal additional differences in the microstructure and pattern of drinking.

The difference in ethanol bout size was apparently affected by the presence of concurrent access to water in Experiment 3. One possible explanation is that mice drank in combined ethanol and water bouts (i.e. rapid switching between fluid types) that were not apparent when looking at ethanol and water bouts separately, and that these combined bouts would show a genotypic difference in size. Alternatively, these results might suggest that HDID-1 preference drinking during continuous access tests and binge-like drinking such as the limited access DID test have fundamentally different bout structures which may relate to different underlying mechanisms of control. These traits are substantially, but not perfectly, genetically correlated across inbred strains (Rhodes et al., 2007). The lack of difference in ethanol intake between the genotypes observed in Experiment 3 is not surprising, as an earlier study looking at repeated limited access (2 hr) choice between 15% ethanol and water showed that HDID-1 mice needed upwards of 50 days to develop a significantly increased ethanol intake relative to HS (Crabbe et al. 2011b). The greater preference ratios for the ethanol solution we saw in Experiment 3 in the HDID-1 mice relative to the controls is also consistent with this previous study, where greater ethanol preference was observed from the beginning and throughout the repeated DID tests. It is possible that repeated two-bottle choice limited access exposure would result in an increase in bout size or number of bouts,

thus driving the increase in HDID-1 consumption that was ultimately observed in the earlier experiment.

In summary, the studies discussed here have shown systematic differences in how HDID-1 and HS mice drink ethanol during the DID test, with the HDID selection process having apparently produced mice that consistently drink in larger bouts than control animals when only ethanol is present. Other genotypic differences in ethanol drinking structure are also present, but they do not appear to be as robust and may be more affected by environmental and procedural variations. When given a choice between ethanol and water, HDID-1 and HS mice show similar bout structures for ethanol, suggesting possible different bout regulatory mechanisms for binge-like and preference drinking. However, HS mice do drink water in large bouts, demonstrating that the observed genotypic difference in ethanol bout size is fluid-type specific. Future DID studies employing a combination of continuous fluid intake recording techniques and varying the parameter of prior exposure to ethanol and/or water will be necessary to determine whether ethanol bout size differences are due to differences in lick rate, lick volume, or other factors.

Acknowledgments

These studies were supported by NIH-NIAAA grants AA13519, AA10760 and AA007468, and by the Department of Veterans Affairs and the Achievement Rewards for College Scientists Foundation. The authors thank Chris Cunningham for assistance with manuscript preparation, and Andy Jade Cameron for expert technical assistance with gas chromatography analyses.

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Fig. 1. Total g/kg ethanol intake per 30 min time block during the 2-day DID test. Mean $\pm$ SEM is shown. n=18 per genotype.

Fig. 2.

Summary of drinking variables on Day 2 of the 2-day DID test. Mean values were calculated for each genotype over the entire 4 hour drinking period. Variables are number of bouts (A), interbout interval (B), bout duration (C), bout size (D), total g/kg ethanol intake (E), and BEC after 4 hr of drinking (F). Asterisks denote values significantly different from HS/Npt. * indicates $p < 0.05$ and ** indicates $p < 0.01$. $n = 18$ per genotype.

Fig. 3. Total g/kg ethanol intake per 30 min time block during the 4-day DID test. Mean $\pm$ SEM is shown. n=17-18 per genotype.

Fig. 4. Summary of drinking variables on Day 4 of the 4-day DID test. Mean values were calculated for each genotype over the entire 4 hr drinking period. Variables are number of bouts (A), interbout interval (B), bout duration (C), bout size (D), total g/kg ethanol intake (E), and BEC after 4 hr of drinking (F). Asterisks denote values significantly different from HS/Npt. * indicates p<0.05 and ** indicates p<0.01. n=17-18 per genotype.

Fig. 5.

Panel A shows total mL/kg ethanol intake per 30 min time block during Days 1 and 2 of the two-bottle choice DID test and panel B shows total mL/kg water consumption. Mean $\pm$ SEM is shown. The inset of panel A shows total g/kg dose of ethanol consumed per 30 min time block across both days. $n=12$ per genotype.

Fig. 6. Summary of ethanol drinking variables on Day 2 of the two-bottle choice DID test. Mean values were calculated for each genotype over the entire 4 hr period. Variables are number of bouts (A), interbout interval (B), bout duration (C), bout size in mL/kg (D), and total mL/kg 20% ethanol solution intake (E). n=12 per genotype.

Fig. 7.

Summary of water drinking variables on Day 2 of the two-bottle choice DID test. Mean values were calculated for each genotype over the entire 4 hr period. Variables are number of bouts (A), interbout interval (B), bout duration (C), bout size in mL/kg (D), and total mL/kg intake (E). n=12 per genotype.

Fig. 8. Mean preference for the 20% ethanol solution as a percent of total fluid consumption (A) and total fluid consumption in mL (B) over 2 hr on Day 1 and 4 hr on Day 2. Asterisk denotes statistically significant effect of genotype. * indicates $p < 0.05$ and ** indicates $p < 0.01$. $n = 12$ per genotype.