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A phenotype-driven ENU mutagenesis screen for the identification of dominant mutations involved in alcohol consumption

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Abstract The aim of this study was the application of a phenotype-driven *N*-ethyl-*N*-nitrosourea (ENU) mutagenesis screen in mice for the identification of dominant mutations involved in the regulation and modulation of alcohol-drinking behavior. The chemical mutagen ENU was utilized in the generation of 131 male ENU-mutant C57BL/6J mice (G0). These ENU-treated mice were paired with wild-type C57BL/6J mice to generate G1 and subsequent generations. In total, 3327 mice were generated. Starting with G1, mice were screened for voluntary oral self-administration of 10% (v/v) alcohol vs. water in a two-bottle paradigm. From these mice, after a total period of 5 weeks of drinking, 43 mutants fulfilled the criteria of an "alcohol phenotype," that is, high or low ethanol intake. They were then selected for breeding and tested in a "confirmation cross" (G2–G4) for inheritance. Although we did not establish stable high or low drinking lines, several results were obtained in the context of alcohol consumption. First, female mice drank more alcohol than their male counterparts. Second, the former demonstrated greater infertility. Third, all animals displayed relatively stable alcohol intake, although significantly different in two different laboratories. Finally, seasonal and monthly variability was observed, with the highest alcohol consumption occurring in spring and the lowest in autumn. In conclusion, it seems difficult to identify dominant mutations involved in the modulation or regulation of voluntary alcohol consumption via a phenotype-driven ENU mutagenesis screen. In accordance with the findings from knockout studies, we suggest that mainly recessive mutations contribute to an alcohol-drinking or alcohol-avoiding phenotype.

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Introduction

One broad type of a genetic screen is a phenotype-driven one, in which mutations are generated at random across the genome and offspring are screened for phenotypes of interest. Mutant phenotypes that are heritable can then be mapped to the mutated region of the genome; then the region is narrowed down and the precise gene that is affected can be pinpointed. This kind of approach has driven genetics for years but has so far not been applied to the genetics of alcoholism. In mice, the chemical *N*-ethyl-*N*-nitrosourea (ENU) is the mutagen of choice for such screens and there have been several large-scale ENU-based screens carried out worldwide (Hrabé de Angelis et al. 2000; Nolan et al. 2000).

It is well documented that the ENU technique generates random point mutations in spermatogonal stem cells (e.g., Barbaric et al. 2007), with an average frequency of up to 1:700/locus/gamete (e.g., Soewarto et al. 2003). Phenotype-driven screens for dominant mutations as proposed here may be performed on a large scale in mice because the phenotypes can be identified in the first offspring generation of a treated animal. However, only about 1% of the offspring reveal any sort of mutant phenotype and good quantitative screening tools are needed in order to detect what could be a fairly subtle effect (Rossant 2003).

In this study we established the basics for a phenotype-driven approach in order to identify genes involved in voluntary alcohol consumption. The behavioral screening test is the bottleneck of a phenotype-driven approach and only tests that reliably measure a specific behavior are ultimately suitable in this respect. The home cage two-bottle free-choice test, measuring alcohol intake, preference, and total fluid intake, appears to be ideal because alcohol drinking in mice is a very stable behavior that can be easily and reliably measured across different laboratories (Crabbe et al. 1999; Spanagel et al. 2002; Wahlsten et al. 2003). This test is most appropriate for measuring alcohol reinforcement but has only limited applications with regard to other drugs because of noncomparable pharmacokinetics and aversive olfactory or taste characteristics (e.g., Pawlak and Schwarting 2002; Sanchis-Segura and Spanagel 2006). Thus, we worked out specific methodologic issues for conducting an "alcohol intake screen" in randomly ENU-mutagenized mice, including appropriate mouse strain, adapted ENU treatment, behavioral screen, reproducibility, and data management. According to our pilot studies, the most suitable inbred mouse strain for an ENU mutagenesis alcohol screen appears to be the C57BL/6J strain from The Jackson Laboratory (Bar Harbor, ME) since this strain displays a high oral intake of pharmacologically relevant concentrations of ethanol and exhibits neither a floor nor ceiling intake of ethanol. Therefore, we worked out an ENU-treatment and behavioral testing protocol for C57BL/6J mice to identify dominant mutations that are involved in the regulation and/or modulation of alcohol-drinking behavior.

Materials and methods

ENU injection and initial breeding

We performed the animal studies under the license of the Regierung von Oberbayern and Landespräsidium Karlsruhe, Germany. For this study we used male C57BL/6J (The Jackson Laboratory) mice. The C57BL/6J mice were injected intraperitoneally with three weekly doses of 90 mg/kg ENU (Serva, Heidelberg, Germany) at approximately 10–14 weeks of age. Following a fertility test, three groups of ENU mice were transferred from Munich (M) to the Central Institute of Mental Health (CIMH) in Mannheim: M1 = 40 (July 2003), M2 = 30 (December 2004), and M3 = 61 animals (June 2005). The total of 131 male mice injected with ENU were termed ENU-founder generation (G0). The G0 mice were used exclusively for breeding purposes to produce offspring (G1) paired with wild-type C57BL/6J females (The Jackson Laboratory). The number of offspring per mutagenized G0 male was limited to 200 mice because clustered mutations can appear owing to the low number of remaining spermatogonal stem cells.

Animal husbandry

Drinking experiments were carried out between September 2003 and September 2006. The experiments were conducted in two different laboratories (see "Stability of intake in two different laboratories" below): First, at Abbott in Ludwigshafen (State of Rheinland-Pfalz) between September 2003 and June 2004, and later on, the experimental setup moved completely to the new laboratories of the CIMH (Mannheim, State of Baden-Wuerttemberg) between July 2004 and September 2006. During drinking experiments, all mice were housed individually (cage floor size: length 22.5 × width 16.5 × height 14 cm) under standard laboratory conditions with bedding, a standard diet, and tap water *ad libitum*. The housing room was maintained on a 12-h light:dark cycle (lights on: 0700–1900 h). All mice were handled only one day per week when changing the cages. Ambient temperature was 22 ± 1°C. Humidity varied between approximately 50% and 60%. Thus, the housing conditions were identical in both laboratories, with the exception that food was changed to extruded pellets (December 2004) soon after having moved to the second laboratory. The weather conditions (temperature, humidity) throughout the whole period of the drinking experiments were recorded retrospectively (www.wetter.com). Throughout the whole period of experiments in both laboratories, the same person, except for holidays, always conducted the experiments (i.e., changed the cages, changed and weighed the bottles, handled the animals).

Ethanol consumption assessment

Ethanol consumption was assessed with single-caged G1 mice using a two-bottle free-choice paradigm in the home cage. The same procedure was used for G2-G4 mice, respectively. When starting the experiments to gauge their "alcohol phenotype," the age of the mice was approximately 7–8 weeks (93.1%), while 6.9% were up to 12 weeks old due to a transfer to another laboratory (see below).

In the first week, two bottles, one empty and the other containing tap water, were available in the home cage to habituate the animals to the new condition. The placement of the bottles was changed every other day to avoid any side preferences and to promote seeking behavior. The ethanol-drinking experiment started in the second week. The empty bottle was replaced by another bottle containing 10% (v/v) ethanol and alcohol consumption was measured

for a period of 3 weeks (Phase 1, weeks 2–4) followed by 1 week of data analysis. All mice fulfilling the criteria for "high" or "low" ethanol intake (see below) during Phase 1 were tested for an additional 2 weeks (Phase 2, weeks 6–7), which was identical to the procedure of Phase 1, followed again by 1 week of data analysis. All mice had free access to both bottles during the entire course of the experiment of this two-bottle voluntary oral self-administration test. The tap water for the 10% (v/v) ethanol bottles was the same as for the water-only bottles.

Spillage and evaporation were minimized by the use of self-made glass cannulae in combination with a small plastic bottle. Under these conditions, ethanol concentration in a given solution remains constant for at least 1 week when measured with an alcoholmeter (data not shown). Bottles were weighed twice per week (every 3 or 4 days, respectively) using an electronic scale accurate to 0.01 g, which was directly linked up with a data management system. All drinking solutions were renewed after weighing. The placement of the bottles was reversed after each renewal so as to avoid any side preference effects. The difference between the weight of the freshly refilled bottles and the subsequent weight measure of the used bottles was taken as the index of fluid consumption. The formula for determining individual ethanol consumption measured in grams per day was as follows:

$$\text{ethanol (g/kg/day)} = \frac{\text{intake/day(mg)} \times 10\text{(ethanol)}}{100\text{ (water)}} \times 0.8\ \text{(density)} \times \frac{1000\text{(g)}}{\text{body weight (g)}}$$

Selection of putative "alcohol phenotypes"

The conditions for selecting suspected "alcohol phenotype" mutants were defined as follows: (1) The individual alcohol intake should be $\geq$ 1.5 standard deviations (SD) below or above the total mean alcohol intake for all tested mutants of a given ENU group (M1–M3); (2) 4 or more of 6 (Phase 1, two measures per week for 3 weeks) and 8 or more of 10 (Phase 2, two measures per week for an additional 2 weeks) measures should fulfill criterion 1; (3) the selected animals should not display any evident alterations with regard to their body weight and spontaneous behavior, respectively. Only those mice that fulfilled all criteria as putative "alcohol phenotypes" after Phase 2 were selected for a confirmation cross.

Confirmation cross

Only mice showing either high or low alcohol consumption on 8 over 10 measure points above or below 1.5 SD of the mean of the respective ENU group were considered as putative candidates for further examination (Phase 2). The selected putative "alcohol phenotype" mutants were bred with wild-type C57BL/6J mice to generate the next generation at an age ranging between 15/16 (> 90%) or 20 weeks (< 10%). Then, the inheritance of their "alcohol phenotype" was sought to be confirmed in the next generation in the same two-bottle free-choice paradigm as used before, i.e., confirmation cross of G2 and G3, respectively. Only such confirmed extreme "alcohol phenotypes" were deemed appropriate to undergo further behavioural and genetic analyses.

Seasonal and monthly effects of drinking

Seasonal effects were analyzed for all mice. The definition of the four seasons was as follows: winter = January-March; spring = April-June; summer = July-September; autumn = October-December. When two seasons during the drinking period overlapped, only one season was determined by the number of days in which most of the drinking experiment occurred. Monthly effects were also investigated and analyzed separately. Both analyses were carried out for Phase 1.

Stability of intake in two different laboratories

The study was carried out in two different laboratories. Due to limited capacity of our laboratories at CIMH, the drinking experiments of the first mice ($n = 660$) were carried out in the laboratories of the company Abbott in Ludwigshafen (State of Rheinland-Pfalz) between September 2003 and June 2004. Later, the experimental setup moved completely to the new laboratories at CIMH (Mannheim, State of Baden-Wuerttemberg) and the experiment continued between July 2004 and September 2006 when the majority of mice ($n = 2667$) were analyzed. This analysis was carried out for Phase 1.

Statistics

The mean $\pm$ 1.5 SD of each sample was calculated. Mice with alcohol consumption $\geq$ 1.5 SD below or $\geq$ 1.5 SD above the mean were considered as interesting for further study. Statistics were calculated with χ^2, t tests, or analysis of variance (ANOVA) followed by post-hoc comparisons with Bonferroni. Data are expressed as the mean $\pm$ SD or as SEM.

Results

From the original breeding, all of the 131 ENU-injected C57BL/6J males were each bred with four wild-type C57BL/6J females; this breeding produced 2129 G1 mice.

The confirmation cross of G1 paired with wild-type C57BL/6J females generated 980 mice for G2, 194 mice for G3, and 24 mice for G4. Thus, a total of 3327 individuals was obtained. As expected, an even gender distribution was found: 1724 males (51.8%) and 1603 females (48.2%).

Alcohol intake and gender effects

All mice were screened for alcohol consumption (10% v/v). An examination of the distribution of ethanol consumption (g/kg/day) in all males ($n = 1724$) and females ($n = 1603$) revealed stable drinking intake for both sexes during the period of three consecutive weeks (Phase 1), measured twice per week (Fig. 1). In males, the overall mean ± SD of this distribution was 8.9 ± 3.7 g/kg/day, whereas in females it was 14.4 ± 5.5 g/kg/day. ANOVA with repeated measures showed significant interaction, time, and gender effects in alcohol intake ($p < 0.001$). These gender-specific alcohol consumptions were post-hoc significant for all six time measures in Phase 1 ($p < 0.001$).

After completion of Phase 1, 307 of these mice from all generations fulfilled the criteria of suspected "alcohol phenotype" (see Materials and methods). The statistical definition of a putative "alcohol phenotype" after Phase 1 is shown for all mice in Fig. 2. Analyses of these 307 mice by gender showed that 171 males (55.7%) and 136 females (44.3%) were assessed as putative mutants. These selected mice were tested again for 2 weeks (Phase 2). Only those mice that still fulfilled all criteria as a putative "alcohol phenotype" at the end of Phase 2 (data not shown) were selected for a confirmation cross.

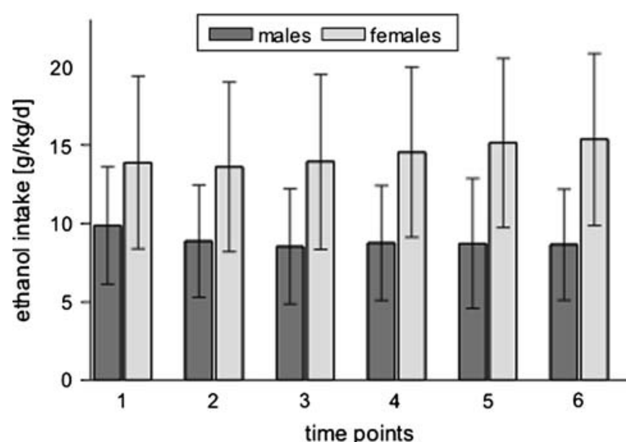

Fig. 1 Ethanol intake of male and female mice over 3 weeks, with two measures per week. All 3327 mice, which were derived from ENU-treated animals (G1) and subsequent generations (G2–G4) are shown. The mean ± SD ethanol intake of 1724 males and 1603 females is shown

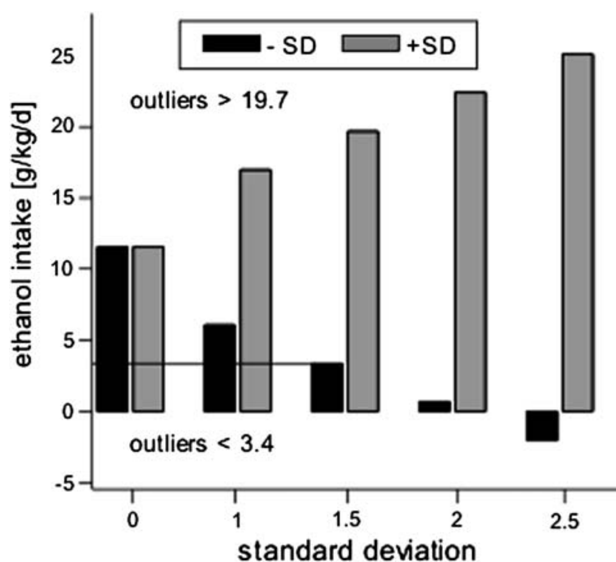

Fig. 2 Definition of outliers for alcohol intake of 3 weeks of drinking for all mice is displayed in one graph for simplicity. Note: if the threshold had been set at more than 2 SD, then either a ceiling effect or negative values would have been obtained

Confirmation cross

For a confirmation cross of G1 mice, 30 animals that fulfilled the criteria were considered putative carriers of a mutation of interest and therefore selected for breeding with wild-type C57BL/6J mice to generate G2 offspring. Overall, 980 mice were obtained in G2 and tested for the phenotype of interest with the same protocol used for G1. One of 12 selected G1 females (8.3%) was able to produce viable offspring, whereas 14 of 18 selected G1 males generated viable offspring (77.8%) (Table 1).

In the confirmation cross of G2 mice, 11 animals fulfilled the criteria and were bred with wild-type C57BL/6J mice to generate 194 G3 offspring. In contrast to G1, 2 of 2 selected G2 females (100 %) were able to produce viable offspring, whereas only 4 of 9 selected G2 males generated viable offspring (44.4%). The confirmation crosses of G3 mice produced two animals (1 female, 1 male) suspected as putative mutants. Only the male G3 mouse generated viable G4 offspring (Table 1).

When performing different analyses of these 43 putative "alcohol phenotypes" selected after Phase 2, which were subsequently used for a confirmation cross, additional fertility analyses were performed. Of those 43 mice, 21 mice (9 males, 12 females) produced no offspring at all, whereas 22 mice (19 males, 3 females) generated viable offspring (males 67.9% and females 32.1%, respectively). A χ^2 test revealed a significant difference for this distribution of viable offspring by gender ($\chi^2 = 7.14$, $p = 0.01$).

Further analyses of the 43 mice with a putative "alcohol phenotype" revealed a differential gender pattern. For

Table 1 All mice that fulfilled the criteria as an "alcohol phenotype" and were used for breeding (see Materials and methods for details)

G1	G2	G3
M 95 - high (29)	F 328 - high (6)	
F 132 - high (0)		
M 164 - low (136)	M 466 - low (0)	
M 220 - high (0)		
M 275 - low (84)		
M 276 - low (170)	M 594 - low (0)	
	M 942 - low (92)	F 1364 - high (0)
		M 1577 - low (24)
F 299 - high (0)		
F 314 - high (0)		
M 318 - low (125)	M 889 - low (20)	
M 345 - low (163)	F 593 - high (18)	
F 461 - low (0)		
F 470 - high (0)		
F 471 - high (0)		
M 475 - low (67)	M 1301 - high (0)	
F 849 - low (0)		
M 897 - low (0)		
F 983 - low (0)		
M 1043 - low (38)	M 1538 - low (27)	
	M 1588 - low (0)	
F 1084 - low (10)	M 1651 - high (0)	
M 1164 - low (21)		
M 1187 - high (33)		
M 1284 - high (54)		
M 1339 - low (8)		
M 1390 - high (23)	M 1620 - high (20)	
F 1399 - low (0)		
F 1404 - high (0)		
F 1438 - high (0)		
M 1458 - low (19)		
M 1506 - low (0)		
M 1608 - low (0)		
30 (980)	11 (194)	2 (24)

For each mouse, the sex (M = male, F = female), sample number, extreme drinking behavior (high or low), and total number of offspring (in parenthesis) are presented. If a high or low drinker generated offspring that were also putative "alcohol phenotypes," they are shown in the next generations (G2 and G3)

males, 20 mice were putative low and 8 were putative high "alcohol phenotypes". In contrast, for females, 5 mice were putative low and 10 were putative high "alcohol phenotypes". A χ^2 test revealed a significant difference for this distribution of "alcohol phenotype" by gender ($\chi^2 = 4.36$, $p = 0.05$).

Analyses of the 22 viable mice showed that 12 mice generated offspring without any putative mutants that were of interest for further breeding, while the other 10 mice produced 13 offspring with a suspected "alcohol phenotype (Table 1)." Of those 13 mice, 9 showed the same extreme drinking behavior as their mutated parent, whereas 4 mice showed an opposite extreme drinking intake as compared to their mutated parent (Table 1). Thus, the success rate of the confirmation cross was 9 of 1194 (G2-G4) offspring (0.75%).

Seasonal and monthly effects of drinking

The drinking behavior was analyzed with regard to the time of the year for all mice collapsed for all time points. The highest consumption was observed in spring, followed by winter, with the lowest intakes in summer and autumn (Table 2). One-factorial ANOVA analysis showed seasonal effects in alcohol intake ($p < 0.001$). Post-hoc comparisons revealed that drinking in spring was significantly higher compared to all other seasons (p values < 0.001). Alcohol intake during winter was substantially higher than in autumn ($p = 0.023$), while no differences were observed between summer and autumn.

Monthly analyses showed the highest alcohol intake in April, and the lowest in August (Fig. 3). One-factorial ANOVA analysis showed monthly effects in alcohol intake ($p < 0.001$). Post-hoc comparisons revealed 39 significant and 27 nonsignificant comparisons between months, which are shown in detail in Table 3. The weather conditions (temperature, humidity) did not show any substantial seasonal peculiarities by eye-sight analysis (values could be retrieved only in a graph retrospectively). The highest average temperature was recorded in June/July each year and the lowest average temperature between December and February. The highest average relative humidity was recorded in October/November each year and the lowest average relative humidity between March and June (data not shown).

Stability of intake in different laboratories

We analyzed whether the drinking intake was influenced by the two different laboratories for all mice. An

Table 2 Seasonal effects on voluntary alcohol consumption for all mice collapsed for all time points

	Mean (g/kg/day)	SD	p values
Winter (w)	11.53	5.4	< 0.001 (s); $= 0.023$ (a)
Spring (s)	12.38	5.8	< 0.001 (w, su, a)
Summer (su)	11.38	5.5	< 0.001 (s)
Autumn (a)	11.21	5.2	< 0.001 (s); $= 0.023$ (w)

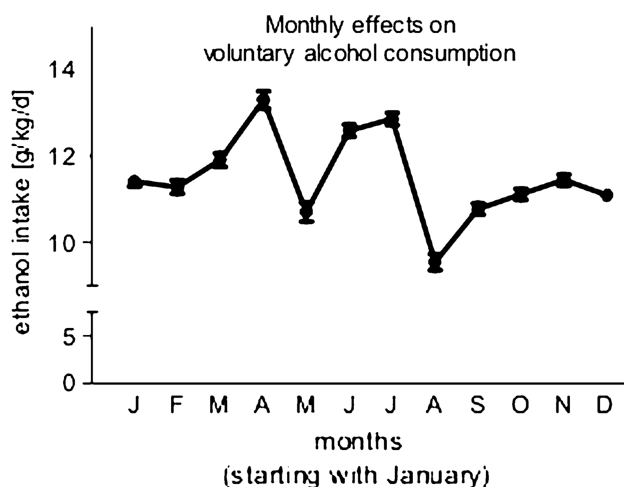

Fig. 3 Monthly effects on voluntary alcohol consumption for all mice collapsed for all time points. The vertical bar displays the total mean intake. Data are presented as mean ± SEM. Please see Table 3 for detailed statistics

independent *t* test showed that animals subjected to the experimental conditions at Abbott (12.5 ± 5.3 g/kg/day) drank significantly more alcohol than animals tested at the CIMH (11.7 ± 5.5 g/kg/day; $p < 0.001$). The weather conditions for both laboratories were comparable (if not identical) because both cities are in close proximity to each other, divided only by the river Rhein.

Discussion

Here we report on the first phenotype-driven ENU mutagenesis program for the identification of mutations involved in the modulation or regulation of voluntary alcohol consumption. The primary result demonstrated that offspring from ENU-treated mice showed a large variability in their voluntary oral self-administration of alcohol, with some mice showing stable extreme (high or low) intake patterns for a period of up to 6 weeks. However, when these individual mice were bred in confirmation crosses to establish stable lines, ultimately only nine offspring with the same extreme drinking behavior as their parents became apparent, but this did not occur consistently. Thus, after screening more than 3300 C57BL/6J mice for their drinking behavior, it was not possible to produce even one single stable line of extreme alcohol-drinking mice. At least several or perhaps even the majority of animals ought to have displayed extreme drinking behavior in a fashion similar to their mutated parent in a confirmation cross. It may be quite possible that the polygenic trait of excessive alcohol consumption is controlled mainly by recessive alleles (Melo et al. 1996), which would be one probable explanation for the failure in establishing a stable line of extreme alcohol intake following a screen for dominant mutations. This suggestion is in line with findings from a variety of genetic modification studies with respect to voluntary alcohol consumption. Thus, a very recent comprehensive review of 93 genes in genetically engineered mice demonstrated the impact of gene deletion on alcohol consumption without a gene dose effect in a way that heterozygous mice did not differ from their wild types (Crabbe et al. 2006).

Despite this failure, some other results derived from our large-scale study do merit further consideration. The amount of daily alcohol intake in males and females was comparable to that of other groups who also used C57BL/6J mice (Chester et al. 2006; Crabbe et al. 1999; Middaugh et al. 1999, 2003). In addition, we observed that female mice drank significantly more alcohol than their male counterparts in our drinking paradigm, which is also in line with previous findings (Chester et al. 2006; Middaugh et al. 1999). Furthermore, a significantly higher number of males as opposed to females showed a putative low

Table 3 Significant *p* values for the monthly effects on voluntary alcohol consumption of Fig. 3 analyzed by Bonferroni post-hoc tests

	Jan	Feb	Mar	Apr	May	Jun	Jul	Aug	Sep	Oct	Nov	Dec
Jan	n/a											
Feb		n/a										
Mar			n/a									
Apr			<0.001	n/a								
May			<0.001	<0.001	n/a							
Jun	<0.001	<0.001	<0.05		<0.001	n/a						
Jul	<0.001	<0.001	<0.001		<0.001		n/a					
Aug	<0.001	<0.001	<0.001	<0.001	<0.01	<0.001	<0.001	n/a				
Sep	<0.05		<0.001	<0.001		<0.001	<0.001	<0.001	n/a			
Oct			<0.01	<0.001		<0.001	<0.001	<0.001		n/a		
Nov				<0.001		<0.001	<0.001	<0.001	<.05		n/a	
Dec			<0.01	<0.001		<0.001	<0.001	<0.001				n/a

"alcohol phenotype," while females revealed a distribution toward a putative high "alcohol phenotype." However, this different gender pattern may better be accounted for by considering methodologic issues. Thus, in the present study the average mean of alcohol intake to identify putative low or high "alcohol phenotypes" in the groups M1–M3 was not calculated independently for gender. Because of the typical lower alcohol intake of male compared to female mice, it was more likely that more males would be detected as putative low "alcohol phenotypes" due to the average mean being higher than if it had been calculated for males only. Conversely, the average mean collapsed for both genders was lower than for the average mean for females only. Therefore, we suggest that the uneven gender distribution of low and high "alcohol phenotypes" would have vanished if separate means for each gender had been calculated.

Most importantly, we observed differences in drinking behavior with respect to the four seasons and all months of the year. There have been reports about seasonal drinking effects in humans (Del Rio et al. 2002; Uitenbroek 1996) but, to the best of our knowledge, not for mice. This may be an interesting finding with regard to further studies, as there is evidence for considerable seasonal variation in consumption (20%) in a general human population (Lemmens and Knibbe 1993). Potentially even more fascinating, the general tendency for consumption was highest in the spring season and lowest in autumn (Lemmens and Knibbe 1993). Unexpectedly, this seasonal pattern is identical to the one of the present mice, although it ought to be noted that the difference between spring and autumn was approximately 10%. Behavioral seasonal variations that may occur in laboratory animals reared under standard conditions and in a constant 12-h dark/12-h light cycle are poorly investigated. However, it is worthwhile to note that the impact of environmental stressors on behavior in rodents does also underlie a similar seasonal pattern (Aksoy et al. 2003; Meyer et al. 2006). The impact of environmental stressors on alcohol consumption in mice has been well documented and, therefore, we suggest that the seasonal variations observed in alcohol consumption could be due to circannual changes of the neuroendocrine stress system.

The stability of alcohol intake in different laboratories was unintentionally tested because of the initial limited capacity of the laboratories of our institute (CIMH). The results demonstrated significantly higher alcohol consumption in the Abbott laboratory compared with that at the new facilities of the CIMH later on. However, the difference of 6.4% between both locations appears to be negligible when compared with a similar variability of three different laboratories for the same mouse strain in another study (Crabbe et al. 1999). While the latter experiment revealed no substantial laboratory effects with 16 C57BL/6J mice, our study with more than 3300 mice had a much higher statistical power providing a highly significant result, although both studies showed comparable percentage differences between laboratories. Thus, despite the significant difference in the present experiment, we should emphasize that only minor variations occur with respect to voluntary alcohol consumption in rodents when data from different laboratories are compared.

Finally, it is important to note that there was a strong effect on the fertility of the ENU-mutated mice. Approximately 50% of the mice used in a confirmation cross produced no viable offspring, which may be explained by the fact that the mutagen ENU is also a toxin and carcinogen. The present results are in line with data showing that most G1 hybrid animals tolerate ENU in a satisfactory fashion, but that inbred strains of mice vary in their longevity and in their ability to recover fertility after treatment with ENU (Justice et al. 2000). However, there are no reports available pertaining to significantly reduced fertility in ENU-mutated female versus male mice as observed in the present study. We speculate one possible reason could be that there are more genes involved in the reproduction processes in females than in males (Naz and Rajesh 2005). Subsequently, due to the complexity of female fertility, the probability of a random ENU-induced mutation in these genes is greater, resulting in a proportionally higher infertility in females. Finally, one critical point for fertility is the dose of ENU. Unpublished data from our group has shown that after an intraperitoneal injection of 3×90 mg/kg, which was identical to the dose used here, 52% of C3HeB/FeJ male mice became infertile. This infertility rate was higher than in the present study, but strain differences may account, at least partially, for these differences. Even more, 4–5-month old C57BL/6J female mice from G1 were used for further breeding. It is our experience that C57BL/6J female mice at this age can have a lower fertility rate, especially when they are used for the first breeding cycle.

Alcohol drinking is a complex behavior with a variety of environmental and genetic factors. The present findings imply that it is mainly recessive mutations that contribute to alcohol intake. What is more, given the high statistical power by testing more than 3300 mice, we can draw some further solid conclusions with respect to voluntary alcohol consumption. First, female mice do drink more alcohol than male mice; second, alcohol consumption varies across different laboratories, however, the variations are below 10%; and, finally, alcohol consumption in mice demonstrates similar seasonal changes as is observed in humans.

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References

Aksoy A, Schulz D, Yilmaz A, Canbeyli R (2004) Seasonal variability in behavioral despair in female rats. Int J Neurosci 12:1513–1520

Barbaric I, Wells S, Russ A, Dear TN (2007) Spectrum of ENU-induced mutations in phenotype-driven and gene-driven screens in the mouse. Environ Mol Mutagen 48:124–142

Chester JA, de Paula Barrenha G, DeMaria A, Finegan A (2006) Different effects of stress on alcohol drinking behaviour in male and female mice selectively bred for high alcohol preference. Alcohol Alcohol 41:44–53

Crabbe JN, Wahlsten D, Dudek BC (1999) Genetics of mouse behavior: interactions with laboratory environment. Science 284:1670–1672

Crabbe JC, Phillips TJ, Harris RA, Arends MA, Koob GF (2006) Alcohol-related genes: contributions from studies with genetically engineered mice. Addict Biol 11:195–269

Del Rio MC, Prada C, Alvarez FJ (2002) Drinking habits throughout the seasons of the year in the Spanish population. J Stud Alcohol 63:577–580

Hrabé de Angelis M, Flaswinkel H, Fuchs H, Rathkolb B, Soewarto D, et al. (2000) Genome-wide, large-scale production of mutant mice by ENU mutagenesis. Nat Genet 25:444–447

Justice MJ, Carpenter DA, Favor J, Neuhauser-Klaus A, Hrabé de Angelis M, et al. (2000) Effects of ENU dosage on mouse strains. Mamm Genome 11:484–488

Lemmens PH, Knibbe RA (1993) Seasonal variation in survey and sales estimates of alcohol consumption. J Stud Alcohol 54:157–163

Melo JA, Shendure J, Pociask K, Silver LM (1996) Identification of sex-specific quantitative trait loci controlling alcohol preference in C57BL/6 mice. Nat Genet 13:147–153

Meyer L, Caston J, Mensah-Nyagan AG (2006) Seasonal variation of the impact of a stressful procedure on open field behaviour and blood corticosterone in laboratory mice. Behav Brain Res 167:342–348

Middaugh LD, Kelley BM, Bandy ALE, McGroarty KK (1999) Ethanol consumption by C57BL/6 mice: influence of gender and procedural variables. Alcohol 17:175–183

Middaugh LD, Szumlinski KK, Van Patten Y, Marlowe ALB, Kalivas PW (2003) Chronic ethanol consumption by C57BL/6 mice promotes tolerance to its interoceptive cues and increases extracellular dopamine, an effect blocked by naltrexone. Alcohol Clin Exp Res 27:1892–1900

Naz RK, Rajesh C (2005) Gene knockouts that cause female infertility: search for novel contraceptive targets. Front Biosci 10:2447–2459

Nolan PM, Peters J, Strivens M, Rogers D, Hagan J, et al. (2000) A systematic, genome-wide, phenotype-driven mutagenesis programme for gene function studies in the mouse. Nat Genet 25:440–443

Pawlak CR, Schwarting RK (2002) Object preference and nicotine consumption in rats with high vs. low rearing activity in a novel open field. Pharmacol Biochem Behav 73:679–687

Rossant J (2003) Genetics: a balancing act. Nature 425:29–32

Sanchis-Segura C, Spanagel R (2006) Behavioural assessment of drug reinforcement and addictive features in rodents: an overview. Addict Biol 11:2–38

Soewarto D, Blanquet V, Hrabé de Angelis M (2003) Random ENU mutagenesis. Methods Mol Biol 209:249–266

Spanagel R, Sigmund S, Cowen M, Schroff KC, Schumann G, et al. (2002) The neuronal nitric oxide synthase (nNOS) gene is critically involved in neurobehavioral effects of alcohol. J Neurosci 22:8676–8683

Uitenbroek DG (1996) Seasonal variation in alcohol use. J Stud Alcohol 57:47–52

Wahlsten D, Metten P, Phillips TJ, Boehm SL II, Burkhart-Kasch S, et al. (2003) Different data from different labs: lessons from studies of gene-environment interaction. J Neurobiol 54:283–311