Behavioral profiling of multiple pairs of rats selectively bred for high and low alcohol intake using the MCSF test

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Abstract

Genetic aspects of alcoholism have been modeled using rats selectively bred for extremes of alcohol preference and voluntary alcohol intake. These lines show similar alcohol drinking phenotypes but have different genetic and environmental backgrounds and may therefore display diverse behavioral traits as seen in human alcoholics. The multivariate concentric square field™ (MCSF) test is designed to provoke exploration and behaviors associated with risk assessment, risk taking and shelter seeking in a novel environment. The aim was to use the MCSF to characterize behavioral profiles in rat lines from selective breeding programs in the United States (P/NP, HAD1/LAD1, HAD2/LAD2), Italy (sP/sNP) and Finland (AA/ANA). The open field and elevated plus maze tests were used as reference tests. There were substantial differences within some of the pairs of selectively bred rat lines as well as between all alcohol-preferring rats. The most pronounced differences within the pairs of lines were between AA and ANA rats and between sP and sNP rats followed by intermediate differences between P and NP rats and minor differences comparing HAD and LAD rats. Among all preferring lines, P, HAD1 and HAD2 rats shared similar behavioral profiles, while AA and sP rats were quite different from each other and the others. No single trait appeared to form a common 'pathway' associated with a high alcohol

drinking phenotype among all of the alcohol-preferring lines of rats. The marked behavioral differences found in the different alcohol-preferring lines may mimic the heterogeneity observed among human alcoholic subtypes.

Keywords

Alcohol non-preferring rats; alcohol-preferring rats; elevated plus maze (EPM); multivariate concentric square field™ (MCSF); open field (OF); selective breeding

INTRODUCTION

The development of animal models using selective breeding has been useful as a strategy for understanding the genetic, environmental and neurobiological underpinnings of excessive alcohol intake and dependence (Grahame 2000). To this end, several different lines of rats have been selectively bred for high and low oral alcohol preference and intake, including the University of Chile alcohol-drinking/non-drinking (UChB/UChA) rats (Mardones & Segovia-Riquelme 1983), the Alko alcohol/non-alcohol (AA/ANA) rats (Eriksson 1968), the Indiana alcohol-preferring/non-preferring (P/NP) rats and the two replicate lines of high/low alcohol-drinking (HAD1-2/LAD1-2) rats (Li *et al.* 1987; Li, Lumeng & Doolittle 1993), the Sardinian alcohol-preferring/non-preferring (sP/sNP) rats (Colombo *et al.* 2006) and the Warsaw high-/low-preferring (WHP/WLP) rats (Dyr & Kostowski 2008). These lines were bred for the same phenotypes, i.e. high or low alcohol preference and consumption under a standard, home cage, two-bottle free choice paradigm with continuous access to alcohol, water and food. One common goal of all of these selective breeding programs has been to compare the high and low alcohol-drinking lines to determine behavioral characteristics associated with selection for extremes of alcohol preference in alcohol naive animals.

The multivariate concentric square field™ (MCSF) test (Meyerson *et al.* 2006) has an ethological foundation and is designed to provoke exploration and behaviors associated with risk assessment, risk taking and shelter seeking by rodents in a novel environment. The arena involves a variety of zones, including sheltered, open and elevated areas, exploratory incentives, areas with different illumination and corridors enclosed by walls. The purpose of this multivariate design is to gather information that, taken together, enables a behavioral profiling of the animal in one and the same test situation (Meyerson *et al.* 2006).

The MCSF test has been useful for behavioral profiling of lines selectively bred for high and low alcohol intake, i.e. the AA and ANA rats (Roman *et al.* 2007) and the sP and sNP rats (Roman & Colombo 2009). The present investigation replicates and extends this work by comparing five different pairs of selectively bred lines of rats: the AA/ANA, sP/sNP, P/NP and the two replicate HAD1-2/LAD1-2 lines. The AA/ANA, sP/sNP and P/NP rats were generated from outbred Wistar rats, while the AA/ANA rats were raised from a mixed background including Wistar rats (Hilakivi *et al.* 1984; Murphy *et al.* 2002; Colombo *et al.* 2006). The HAD1-2 and LAD1-2 rats were bred from N/Nih rats, a heterogeneous foundation stock generated by crossing eight different inbred rat strains (Li *et al.* 1993). Therefore, Wistar rats and N/Nih rats were also evaluated in the present investigation along with the selectively bred lines.

A notable feature of this study is that all of the lines were tested concurrently, at the same age, by the same experimenters and in the same laboratory. This allowed direct comparison not just within each pair of lines but also across all of the lines selectively bred for differential alcohol drinking. The main goal was to use the MCSF test as well as the elevated plus maze (EPM) and open field (OF) tests to (i) compare each pair of lines; (ii) replicate previous results obtained from testing AA/ANA and sP/sNP rats; and (iii) investigate possible conformity in behavioral patterns linked to the alcohol-preferring and alcohol non-preferring phenotypes.

MATERIALS AND METHODS

Animals and housing

Adult male alcohol-naive AA and ANA rats [generation S97; National Institute for Health and Welfare (THL), Helsinki, Finland; $n = 12$/group], sP and sNP rats (generation S71; Charles River Laboratories, Calco, Italy; $n = 16$/group), P and NP rats (generation S64-65), HAD1 and LAD1 rats (generation S54) and HAD2 and LAD2 rats (generation S52; Indiana University School of Medicine, Indianapolis, IN; $n = 12$/group) were used. Age-matched, male Wistar rats (Hsd:WI; Harlan, Indianapolis, IN) and N/Nih rats (Indiana University School of Medicine, Indianapolis, IN) were also included ($n = 12$/group). The rats (12–14 weeks old when tested) were housed two per cage in acrylic cages ($45 \times 23 \times 20$ cm) with wood-chip bedding in a temperature-controlled and humidity-controlled animal room under a reversed 12-hour light/dark cycle. All rats were maintained for at least 3 weeks prior to behavioral testing. Research protocols were approved by the School of Science Institutional Animal Care and Use Committee and are in accordance with the guidelines of the National Institutes of Health, the Guidelines for the Care and Use of Mammals in Neuroscience and Behavioral Research (National Research Council of the National Academies 2003) and the European Communities Council Directive (86/609/EEC).

Procedure

During the week before testing, rats were handled, weighed and adapted to the bucket used to transport them from the home cage to the testing rooms. The MCSF was the main test for this investigation, with the OF and EPM tests serving as reference tests. Each animal was tested only once in each of the three tests over three consecutive days with the MCSF test completed on the first day, the OF test on the second and finally the EPM test on the third day. The sequential order of the three tests was based on a pilot study indicating that the MCSF test was most sensitive to previous experience and should be performed first to avoid carry-over effects (Augustsson 2004). The pairs of lines were tested in the following order: AA/ANA, P/NP, HAD1-2/LAD1-2 and N/Nih, and sP/sNP and Wistar. Testing was performed in separate rooms during the dark period of the light/dark cycle. Between rats, the apparatus was wiped with 10% alcohol solution and allowed to dry.

The MCSF test

The MCSF apparatus and its defined zones are shown in Fig. 1a, and the parameters measured are listed in Table 1 and Appendix S1. Each animal was released in the CENTER facing the wall between the CENTER and BRIDGE, and the test session lasted 20 minutes.

The approximate light conditions (lx) in the MCSF arena were as follows: DCR: < 1; CENTER: < 20; CORRIDORs and HURDLE: < 10; SLOPE: < 30; BRIDGE: 600–650.

The OF test

The OF arena was a square field (90×90 cm). Lines divided the arena into a 6×6 array of small squares (15×15 cm) used to score number of crossings. Note, the center portions of the OF and MCSF were of different shapes and relative sizes. The parameters tested are described in Table 2A and Appendix S1. Each rat was released in the peripheral zone, and the test session lasted 10 minutes. The approximate light intensity in the OF arena was 35 lx.

The EPM test

The EPM apparatus (AccuScan, Columbus, OH) was white acrylic plastic with two open arms (50×10 cm) at right angles to two wall-enclosed and covered arms ($50 \times 10 \times 50$ cm), raised 90 cm off the floor. The parameters tested are listed in Table 2B and Appendix S1. Each rat was released in the center facing an open arm, and the test session lasted 5 minutes. The light conditions on the open and closed arms were approximately 45 lx and 10 lx, respectively.

Behavioral recordings

The animals were monitored by video cameras, while the observer watched remotely from an adjacent room. In the MCSF test, the numbers of stretched attend postures (SAPs; from the CORRIDORs into the CENTER and from the CORRIDOR into the SLOPE) and rearings were recorded by direct observation, and the number of head dips into the hurdle hole board was noted. Scoring was performed using the Observer XT 8.0 (Noldus Information Technology, Wageningen, the Netherlands). Visits to the defined zones were only scored as such if both hind legs had crossed over into that section.

Statistical analysis

The majority of the data was not normally distributed, and analysis was done using non-parametric statistics. The Mann–Whitney U-test or Kruskal–Wallis test was used where appropriate. The Pearson Chi-square test was used for analysis of occurrence, i.e. the number of animals entering a zone or performing a behavior. Differences were considered statistically significant at P 0.05. Statistica 8.0 (StatSoft Inc., Tulsa, OK) was used for the statistical analyses.

A trend analysis, conceptually similar to a multivariate analysis of variance, was used for the primary analyses of the MCSF data. For each of five functional behavioral categories, related variables were combined into single composite dependent variables. Individual rank values were summarized within the functional categories general activity (TOTAL ACTIVITY, FRQ TOTCORR and CENTER, and DUR/FRQ TOTCORR), exploratory activity (DUR TOTCORR, CENTER and HURDLE, REARING, and number of PHOTOCELL COUNTS in the hole board), risk assessment (SAP to CENTER and SLOPE, and DUR/FRQ SLOPE and BRIDGE ENTRANCE), risk-taking behavior (FRQ BRIDGE and CENTRAL CIRCLE, DUR BRIDGE and CENTRAL CIRCLE, and DUR/FRQ BRIDGE and CENTRAL CIRCLE) and shelter-seeking behavior (FRQ, DUR, and

DUR/FRQ DCR). The individuals were ranked against each other, either within each selectively bred pair of rats or within the group of preferring rats and the group of non-preferring rats. The rank values were then summed into a sum rank for each functional category and analyzed.

An additional multivariate data analysis was used to examine MCSF performance. A partial least squares to latent structures (PLS) analysis (Eriksson *et al.* 2006) was used to investigate the similarity between all the alcohol-preferring lines. SIMCA-P+ 12.0 (Umetrics AB, Umeå, Sweden) was used.

RESULTS

Descriptive statistics

Tables with a complete listing of the means ± standard errors of the mean (SEM) for each line are provided in Appendix S1. Tables 1 and 2 show the MCSF, OF and EPM parameters for which there were significant differences between the high and low drinking lines. There were many more differences between the sP and sNP lines and between the AA and ANA lines compared with the P/NP, HAD1/LAD1 or HAD2/LAD2 pairs of lines. This pattern was confirmed by the trend analysis and PLS analysis.

Within pair comparisons

AA and ANA rats

MCSF test: Figure 1b illustrates the number of visits to the different zones in relation to the activity of each individual. AA rats made significantly more percentage visits to the CENTER and CENTRAL CIRCLE than ANA rats. Furthermore, AA rats made significantly fewer visits to the DCR, HURDLE and CORRIDORs than ANA rats. The trend analysis for AA and ANA rats is shown in Fig. 2a. AA rats were characterized by significantly lower general activity, exploration and shelter-seeking behavior than ANA rats. Furthermore, AA rats had significantly higher risk assessment and risk-taking behavior compared with ANA rats.

OF test: A summary of the significant differences between AA and ANA rats in the OF test is shown in Table 2A and reveals that AA rats spent more time per visit in the center than ANA rats.

EPM test: A summary of the significant differences between AA and ANA rats in the EPM test is shown in Table 2B. The AA rats had higher open arm and center activity and lower closed arm activity than the ANA rats.

sP and sNP rats

MCSF test: sP rats made significantly more percentage visits to the CORRIDORs and DCR than sNP rats. Furthermore, sP rats made significantly fewer visits to the risk areas SLOPE, BRIDGE ENTRANCE, BRIDGE and CENTRAL CIRCLE than sNP rats (Fig. 1b). The trend analysis (Fig. 2b) indicated that general activity, exploration and risk-taking behavior

were significantly lower in sP than in sNP rats. Furthermore, risk assessment was significantly higher in sP rats compared with sNP rats.

OF test: Fewer sP than sNP rats visited the center, and sP rats had lower center activity than sNP rats. Moreover, sP rats were less active compared with sNP rats (Table 2A).

EPM test: Fewer sP than sNP rats visited the open arms of the EPM. The sP rats had lower open arm activity and higher closed arm activity than the sNP rats. Furthermore, sP rats were less active than sNP rats (Table 2B).

P and NP rats

MCSF test: P rats made significantly fewer visits to the CENTER compared with NP rats. Moreover, P rats made significantly more visits to the risk areas SLOPE, BRIDGE ENTRANCE and BRIDGE compared with NP rats (Fig. 1b). The trend analysis (Fig. 2c) indicated that P rats were characterized by significantly lower general activity and risk-taking behavior compared with NP rats, while exploration, risk assessment and shelter-seeking behavior did not differ between the two groups.

OF test: P rats made more crossings than NP rats (Table 2A).

EPM test: P rats spent longer time in the center compared with NP rats (Table 2B).

HAD1 and LAD1 rats

MCSF test: HAD1 and LAD1 rats did not differ in number of visits to the different zones (Fig. 1b). The trend analysis (Fig. 2d) detected no significant differences in general activity, exploration, risk assessment, risk taking and shelter-seeking behavior.

OF test: HAD1 rats had lower activity in the center of the OF compared with LAD1 rats. Furthermore, HAD1 rats were characterized by lower general activity compared with LAD1 rats (Table 2A).

EPM test: HAD1 rats spent more time on the closed arms and less time in the center compared with the LAD1 rats (Table 2B).

HAD2 and LAD2 rats

MCSF test: HAD2 and LAD2 rats did not differ in number of visits to the different zones (Fig. 1b). The trend analysis (Fig. 2e) revealed significantly lower risk assessment in HAD2 rats than LAD2 rats and a tendency for a higher general activity in HAD2 rats compared with LAD2 rats ($P = 0.07$). No line differences were found for exploration, risk taking and shelter-seeking behavior.

OF test: HAD2 rats had higher activity and spent less time per visit in the periphery than LAD2 rats (Table 2A).

EPM test: HAD2 rats spent less time in total and shorter time per visit on the open arms compared with the LAD2 rats (Table 2B).

Wistar and N/Nih rats—The results from the MCSF, OF and EPM tests in Wistar and N/Nih rats are shown in Appendix S1 (Tables S9 and S10). Figure 1b illustrates the number of visits to the different zones in relation to the activity of each individual in Wistar and N/Nih rats, respectively.

Comparisons within all of the alcohol-preferring and within all of the alcohol non-preferring lines

Trend analysis—Among the alcohol-preferring lines (Fig. 3a), overall differences were detected for general activity, exploratory activity, risk taking and shelter-seeking behavior. sP rats had significantly lower general activity than all other preferring lines. HAD1 rats were characterized by significantly higher exploratory activity compared with the other preferring rat lines. AA rats displayed significantly higher risk-taking behavior than all other lines, and sP rats were found to have significantly lower risk-taking behavior compared with all other preferring rat lines. Finally, AA rats displayed significantly lower shelter-seeking behavior than all other preferring lines, and P rats seek less shelter than HAD2 rats.

Among the non-preferring lines (Fig. 3b), overall differences were detected for general activity and risk-assessment behavior. NP rats were significantly more active than all other non-preferring lines, while LAD2 rats were significantly less active compared with the other non-preferring lines. ANA rats showed lower risk-assessment behavior compared with the other non-preferring lines, while LAD 2 rats displayed significantly higher risk-assessment behavior compared with the other non-preferring lines. Finally, NP rats showed less risk assessment than sNP rats.

PLS analysis—The PLS analysis revealed similarities between the groups of alcohol-preferring lines (Fig. 4). Groups of animals that load closer together on the PLS plot show greater similarity than groups that are located farther away. The greatest similarity in MCSF performance was found between P, HAD1 and HAD2 rats. The sP rats, loading alone in the upper left quadrant, and the AA rats, loading in the lower right quadrant, were different. The loading of sP rats was associated with latency measures and duration in the CORRIDORs (generally longer in sP rats), and parameters for shelter-seeking behavior. The loading of AA rats was associated with parameters for risk-taking behavior (generally more pronounced). Parameters for general activity loaded in the same quadrant as P, HAD1 and HAD2 rats. The picture from the PLS analysis supports the results from the trend analysis (Fig. 3a).

Impulsive-like behavior and anxiety-like behavior—The latency to first enter the BRIDGE in relation to first enter the SLOPE (the SLOPE/BRIDGE interval; see Table 1) can be viewed as a measure of impulsive-like behavior. After entering the SLOPE, the animal has to assess the risk of entering the BRIDGE. Animals displaying impulsive-like behavior will have a shorter interval in entering the risk area. A significant difference was detected when comparing sP and sNP rats, implying higher impulsive-like behavior in sP than sNP rats. The relationship between time spent in the sheltered area (DCR) and the risk

area (BRIDGE) in relation to the time spent in both zones (the shelter/risk index, see Table 1) can be viewed as one measure of anxiety-like behavior. The sP rats had a significantly larger index than the sNP rats, thus implying higher anxiety-like behavior in sP rats. No other significant line differences were found.

DISCUSSION

In the present investigation, the majority of the alcohol-preferring and non-preferring pairs of selectively bred rats available worldwide were tested. Great differences were found within some of the pairs of selectively bred rat lines as well as between all alcohol-preferring and all alcohol non-preferring rats, despite their evident phenotypes regarding voluntary alcohol intake. The most pronounced differences within the pairs of selectively bred lines were found when comparing AA and ANA rats, and sP and sNP rats. Minor differences were revealed when comparing HAD1 and LAD1, and HAD2 and LAD2 rats. Differences between P and NP rats were intermediate. Thus, there was marked heterogeneity in behavioral traits associated with high alcohol drinking.

The different pairs of selectively bred preferring and non-preferring lines originate from different foundation stocks. The outbred Wistar colony used to raise the P/NP lines was the WrmWRC(WI)BR stock, a closed colony housed at the Walter Reed Army Institute of Research and is now extinct (Murphy *et al.* 2002). The Wistar stock used to breed the sP/sNP lines was purchased from Morini (San Polo d'Enza, RE, Italy) (Colombo *et al.* 2006). It is likely that these Wistar stocks were genetically and phenotypically different from each other. The AA/ANA lines were originally derived from a foundation stock that included Wistar and Sprague–Dawley rats and were later crossed with F1 hybrids from a Lewis and Norwegian Brown cross (Sommer, Hyytiä & Kiianmaa 2006). The HAD1-2/LAD1-2 rats were derived from the heterogeneous N/Nih stock (Murphy *et al.* 2002). Because the gene pools are different among the various foundation stocks, different genes of varied behavioral traits likely cosegregated with the genes for alcohol preference. This is the most cogent reason for the highly varied behavioral dispositions that we now observe in the five different lines of alcohol-preferring rats.

Another distinct possibility is that many of the differences observed between the lines are because of chance changes in frequency and fixation of alleles resulting from inbreeding. Inbreeding, omnipresent in closed animal colonies and roughly proportional to the number of generations without interbreeding with other lines or strains, would alter genes in a random fashion. Inbreeding would tend to yield behavioral differences between lines that are unrelated to the selection pressure originally applied from drinking differences (Crabbe *et al.* 1990). Suggestive that inbreeding plays a role here is that the older (in terms of number of generations of selective breeding), and therefore presumably more inbred lines (sP/sNP and AA/ANA) showed more behavioral differences than the newer (HAD/LAD) lines.

Finally, animal handling and other routines vary in different breeding laboratories (Wahlsten *et al.* 2003), and early environmental and social influences have been suggested to be as important as genetics in determining later life behavior (Lathe 2004). These factors may induce variation and individual differences produced in various ways including by epigenetic

mechanisms. Differences in spontaneous behaviors such as exploration, risk assessment and risk taking are probably conserved during the process of selective breeding. The exception should be if a specific behavioral trait(s) is truly associated with preference for alcohol in which case both the behavior and alcohol intake will differ between preferring and non-preferring rats. The underlying neural mechanisms involved in alcohol intake are likely more limited compared to those factors responsible for behaviors of importance for the species overall behavioral repertoire, which often is necessary for survival. It may well be that only a small part of the functions regulating spontaneous behavior is associated with alcohol preference and intake. The following sections are summaries of the within and between line comparisons found in the present investigation.

AA and ANA rats

The results from the MCSF test reveal that AA rats generally are less active, explorative and shelter seeking and more risk assessing and risk taking compared with ANA rats. These results replicate a previously published comparison of the AA and ANA rats using the MCSF test, in spite of the use of a smaller arena in the previous study (Roman *et al.* 2007). The profiles are here interpreted as higher risk taking and lower anxiety-like behavior in AA compared with ANA rats. In agreement with the findings in the MCSF, higher open arm activity was observed in the EPM test and minor indications for higher center activity were noted in the OF test in AA rats relative to ANA rats. Taken together, these findings are in agreement with previous studies that compared the AA and ANA rats using a variety of tests (Knapp *et al.* 1997; Möller *et al.* 1997; Sandbak *et al.* 1998; Roman *et al.* 2005).

sP and sNP rats

The results from the MCSF test reveal lower general activity, exploratory drive and risk-taking behavior and higher risk-assessment behavior in sP rats relative to sNP rats. The profile of the sP rats is interpreted as higher vigilance and anxiety-related behaviors compared with the sNP rats. The lower activity and risk-taking behavior in sP rats is also a consistent finding in the OF and EPM tests. Lower activity and exploratory drive have previously been demonstrated in sP rats compared with sNP rats (Colombo *et al.* 1998; Agabio *et al.* 2001). The profiles obtained using the MCSF test replicate a previous study in which sP and sNP rats were investigated (Roman & Colombo 2009). sP rats have also been characterized by higher anxiety-related behaviors in various behavioral tests (Colombo *et al.* 1995; Richter *et al.* 2000; Leggio *et al.* 2003).

P and NP rats

The results from the MCSF test reveal lower general activity and to some extent also lower exploration in P rats than in NP rats. When their lower general activity was taken into account, P rats made more percentage visits to the risk areas compared with NP rats, which suggested higher risk-taking behavior. However, when differences in activity were not taken into account (as in the trend analysis), lower risk-taking behavior was observed in P than in NP rats. Observations with the OF and EPM tests did not support any line differences in risk-taking behavior and offered little support for higher anxiety-like behavior in P rats. These findings contrast significantly with previous and recent reports demonstrating higher anxiety-like behavior in P rats compared with NP rats (Stewart *et al.* 1993; Hwang *et al.*

2004; Pandey *et al*. 2005; Zhang *et al*. 2010), but see also Badishtov *et al*. (1995) and Viglinskaya *et al*. (1995) who found no differences between P and NP rats. The factors that underlie the discrepancy between the present results and previous reports of higher anxiety-like behavior in P rats are unclear at this time and could include undefined differences in procedural variables. Herein, rats were tested in the EPM and OF after testing in the MCSF, and it cannot be excluded that this sequence of testing somehow affected the results. It is of interest to note that considerable instability in genetic differences in EPM behavior in mice has been observed, even when investigators have gone to great lengths to equalize testing procedures (Wahlsten *et al*. 2003). Genetic drift may also play a role.

HAD and LAD rats

The results from the MCSF test reveal only minor differences between HAD and LAD rats. A consistent finding from the MCSF and OF tests is that HAD1 rats are less risk taking in an open area (MCSF CENTRAL CIRCLE and OF center) than LAD1 rats. Furthermore, HAD2 rats are found to be less risk assessing than LAD2 rats, while no such difference was found when comparing HAD1 and LAD1 rats. The general lack of differences is in agreement with previous studies in which, for instance, no differences in anxiety-related behaviors between HAD and LAD rats have been found (Viglinskaya *et al*. 1995; Overstreet *et al*. 1997; Hwang *et al*. 2004). More recently, HAD1 and HAD2 rats were found to discount delayed and probabilistic rewards more steeply than LAD1 and LAD2 rats, which was interpreted as higher impulsive-like behavior in the HAD rats (Wilhelm & Mitchell 2008). The lower risk assessment behavior and shorter SLOPE/BRIDGE interval (higher impulsive-like behavior) in HAD2 rats relative to LAD2 rats demonstrated herein is in agreement with this finding.

Comparisons within all of the alcohol-preferring lines

General activity was similar in all lines except the sP rats, which were characterized by the lowest activity. Explorative drive was highest in the HAD1 rats and more similar in the other lines. No differences in risk assessment behavior were revealed among the different lines. With regard to risk-taking behavior, AA rats showed the highest, while sP rats showed the lowest. Shelter-seeking behavior was lowest in AA rats and highest in HAD2 rats. The PLS analysis revealed similar behavioral strategies in P, HAD1 and HAD2 rats, while the behavioral strategies of AA and sP rats were quite different from each other and the others. Thus, AA rats constitute one subgroup with more pronounced risk-taking behavior, and sP rats exert another subgroup characterized by lower activity, explorative drive and lower risk-taking behavior. From this pattern, it can be concluded that the different alcohol-preferring lines share no common behavioral profile.

This is one of the few studies in which behavioral traits in addition to alcohol drinking/self-administration have been concurrently investigated in so many pairs of selectively bred alcohol-preferring and non-preferring rodent lines. Perhaps the most consistent behavioral finding among different alcohol-preferring lines is elevated consumption of sweet-tasting solutions compared with the non-preferring lines, previously observed in all of the pairs of selectively bred rats except the sP/sNP lines (Murphy *et al*. 2002; Colombo *et al*. 2006; Sommer *et al*. 2006). With regard to neurochemical factors underlying opposite alcohol consumption behavior in preferring and non-preferring lines, a number of differences have

been demonstrated. For example, P, sP and HAD rats seem to have a dysfunction in the serotonin system not shared by the AA rats. Furthermore, the mesolimbic dopamine pathway appears to be of less importance for the positive reinforcing properties of alcohol in AA rats than in P, HAD and sP rats (Murphy *et al.* 2002). Thus, differences in neurochemical and neurobiological factors have been found, which may underlie divergent expressions of explorative strategies and behavioral profiles in the different pairs of selected lines.

Differences in the above-cited factors may also underlie heterogeneity in alcohol-seeking behaviors among the preferring lines despite their similarities in meeting the criteria for their selective breeding—high voluntary alcohol intake and preference in the continuous-access, two-bottle choice test. For instance, when alcohol availability is contingent on lever pressing, the alcohol-preferring lines differ, with HAD1>P > sP > HAD2 > AA in magnitude of operant responding for alcohol (Files *et al.* 1997; Samson *et al.* 1998; Vacca *et al.* 2002). Furthermore, line differences have been observed in the tendencies to display and magnitudes of the alcohol deprivation effect (ADE), a temporary increase in voluntary alcohol intake following a period of alcohol abstinence (Sinclair & Li 1989; Agabio *et al.* 2000; Serra *et al.* 2003; Vengeliene *et al.* 2003; Rodd *et al.* 2009). Thus, selective breeding for alcohol preference has resulted in diverse behavioral outcomes among the different selectively bred lines in aspects of alcohol-seeking behavior in addition to exploratory strategies and behavioral profiles as highlighted here with the MCSF test.

General discussion

Since alcohol dependence involves several different components, e.g. gene × environment and gene × gene interactions, heterogeneity among humans as well as experimental animals is to be expected. In a recent study that attempted to classify different subtypes of alcoholics (Moss, Chen &Yi 2007), five different clusters or subtypes among alcohol-dependent individuals were identified and differed according to time of onset, genetic load, presence of psychiatric comorbidity including other drug use disorders and levels of psychosocial functioning. These different subtypes illustrate the heterogeneity among alcohol-dependent individuals, which cannot be captured in one single animal model. The need for developing animal models that reflect this heterogeneity has recently been emphasized (Crabbe 2010). Among the alcoholism-related phenotypes and endophenotypes considered to be important targets for genetic studies using rodent models was behavioral under control—encompassing the constructs of impulsivity, novelty seeking and risk taking (Crabbe 2010), which are reflected in how rats explore the MCSF.

The results presented herein clearly indicate that although the five preferring lines were selected for a similar high alcohol-drinking phenotype, the different foundation stocks used together with different breeding environments have produced lines with different exploratory strategies and behavioral profiles. An analogy could be drawn between the heterogeneity observed among human alcoholic subtypes and that seen among the different alcohol-preferring lines. From a translational perspective, it is tempting to speculate about the different alcohol-preferring rats somehow mimicking the alcoholic subtypes. In analogy with the human population sample (Moss *et al.* 2007), different profiles exist in the different

preferring rat lines, with AA and sP rats, respectively, exerting the opposing extremes in several aspects. Our results suggest that behavioral traits such as high anxiety or risk taking/impulsivity are not required for high alcohol drinking or preference. However, the possibility that either high anxiety or high risk taking/impulsivity, when present, can contribute quantitatively to high alcohol preference cannot be excluded.

Overall, the current findings argue against a single behavioral 'pathway' to high alcohol intake, detectable with the MCSF, shared among all the selectively bred lines of rats. In the realm of translational research, the behavioral profiles of the five alcohol-preferring lines are suggested to emulate the attributes of different alcoholic subtypes. Thus, the different alcohol-preferring lines represent valuable models for the heterogeneity of alcohol dependence in humans.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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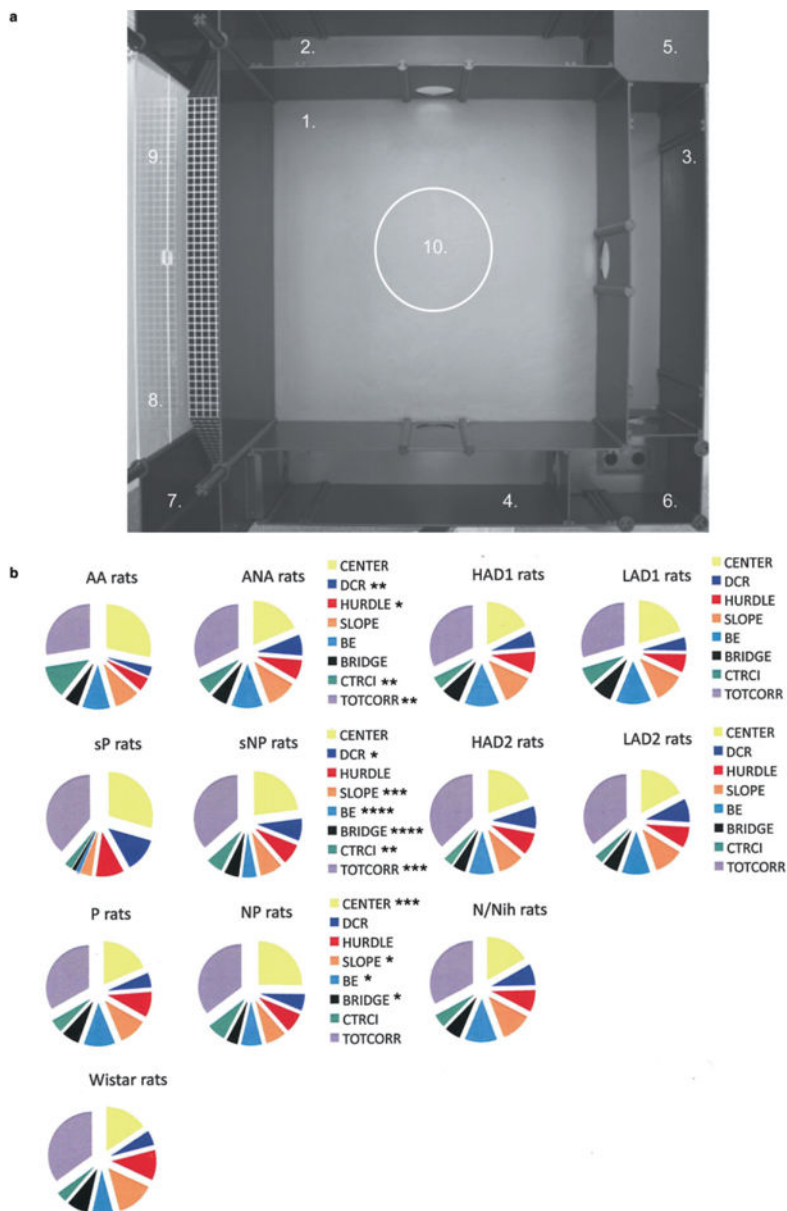
Figure 1.
(a) The MCSF arena (100 × 100 cm) and the defined zones (Roman & Colombo 2009) numbered as follows: 1, CENTER, 70 × 70 cm, open area; 2–4, CORRIDORs, transit areas; 5, dark corner room (DCR), area for shelter seeking; 6, HURDLE, high passage to hole board with photocell to count head dips, exploratory incentive; 7, SLOPE, leading up to BRIDGE, risk assessment area; 8, BRIDGE ENTRANCE, risk assessment area; 9, BRIDGE, elevated and illuminated, risk area; 10, CENTRAL CIRCLE, 25 cm diameter, risk area. (b) The percentage number of visits to the CENTER, dark corner room (DCR), HURDLE, SLOPE, BRIDGE ENTRANCE (BE), BRIDGE, CENTRAL CIRCLE (CTRCI) and the CORRIDORs (TOTCORR) in the MCSF test in AA and ANA rats, sP and sNP rats, P and NP rats, all originally related to outbred Wistar rats, and HAD1 and LAD1 rats and HAD2 and LAD2 rats, derived from N/Nih rats. *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$, ****P

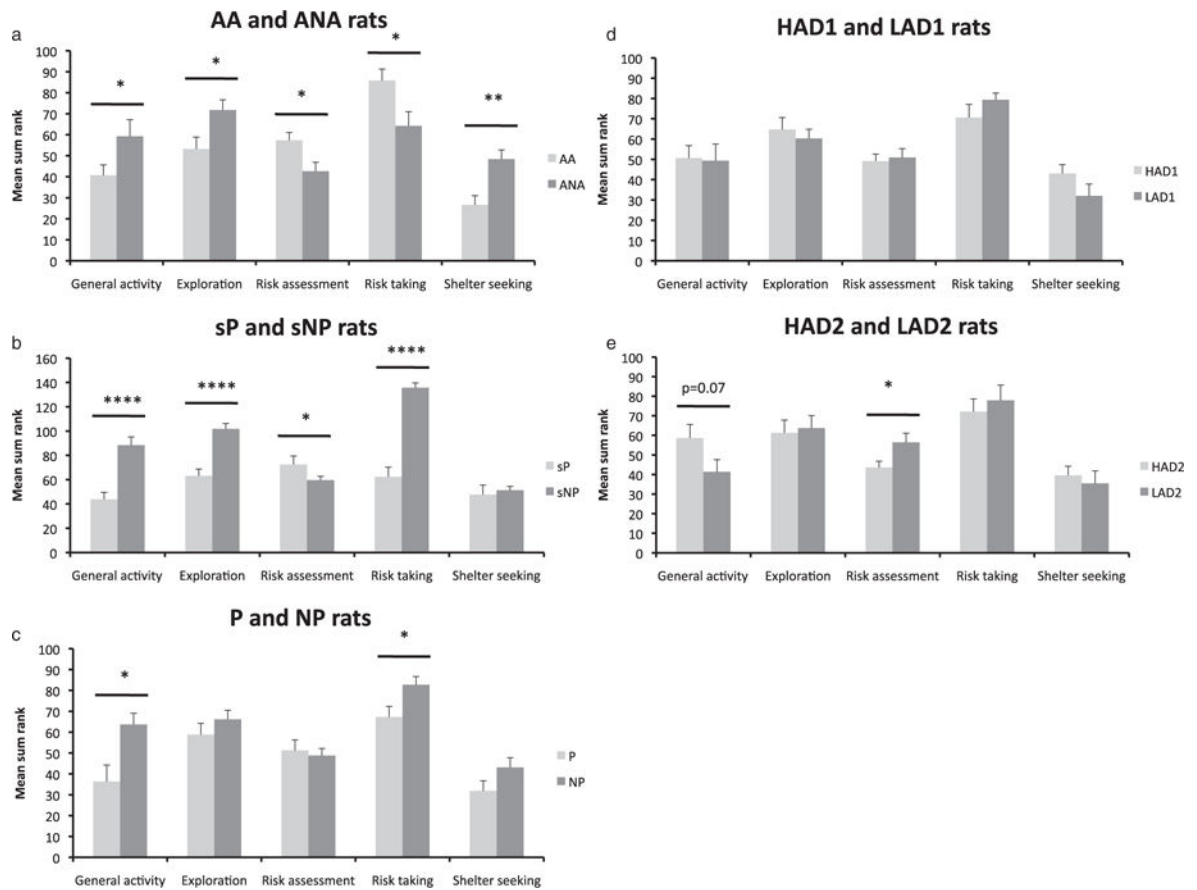< 0.0001 comparing the respective alcohol-preferring and non-preferring lines (Mann–Whitney *U*-test)

Figure 2.
The trend analysis for AA and ANA rats (a), sP and sNP rats (b), P and NP rats (c), HAD1 and LAD1 rats (d), and HAD2 and LAD2 rats (e). Values represent mean ± SEM. *P 0.05, **$P < 0.01$, ***$P < 0.001$, ****$P < 0.0001$ when comparing the respective alcohol-preferring and alcohol non-preferring rats (Mann–Whitney U-test)
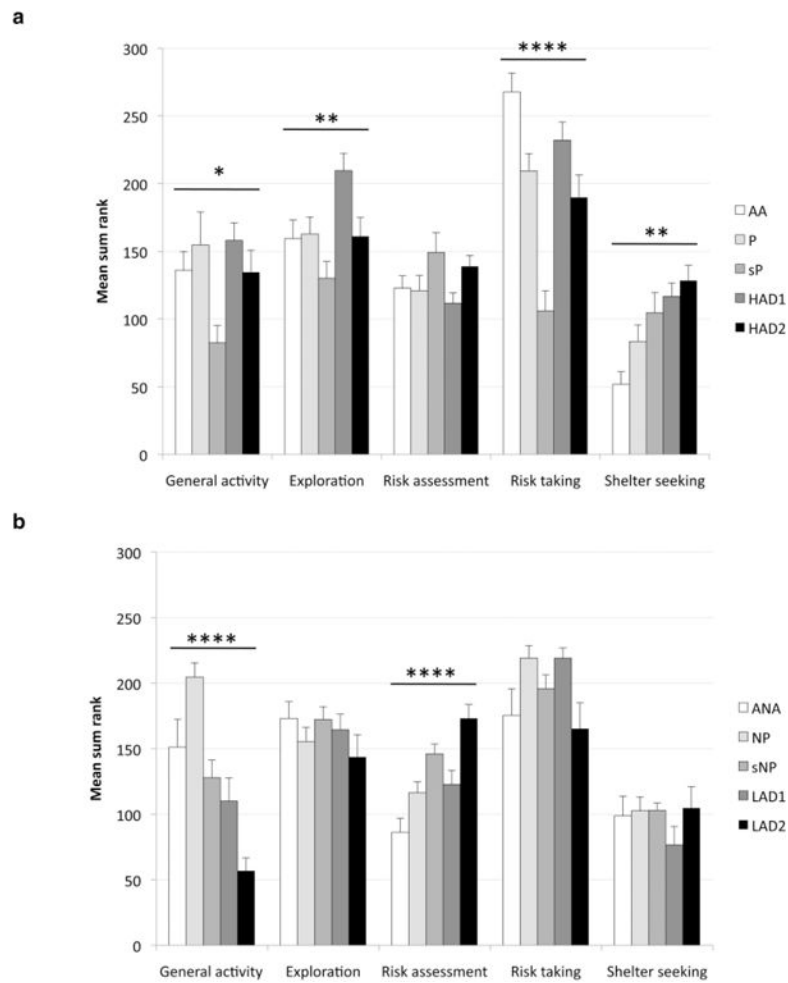
Figure 3.
The trend analysis for alcohol-preferring (a) and alcohol non-preferring (b) lines. Values represent mean ± SEM. *P 0.05, **$P < 0.01$, ****$P < 0.0001$ comparing all alcohol-preferring and non-preferring lines, respectively (Kruskal–Wallis test). For specific differences between the preferring and non-preferring lines, respectively, please see the Results section

Figure 4.
The partial least squares to latent structures (PLS) analysis showing the relationship between the alcohol-preferring lines, i.e. AA, sP, P, HAD1 (H1) and HAD2 (H2) and the MCSF parameters important for the loading of the respective group. Parameters located further away from the origin are of greater importance. TOTACT, sum of visits to all zones in the arena; TOTCORR, sum of performance in the CORRIDORs

Table 1

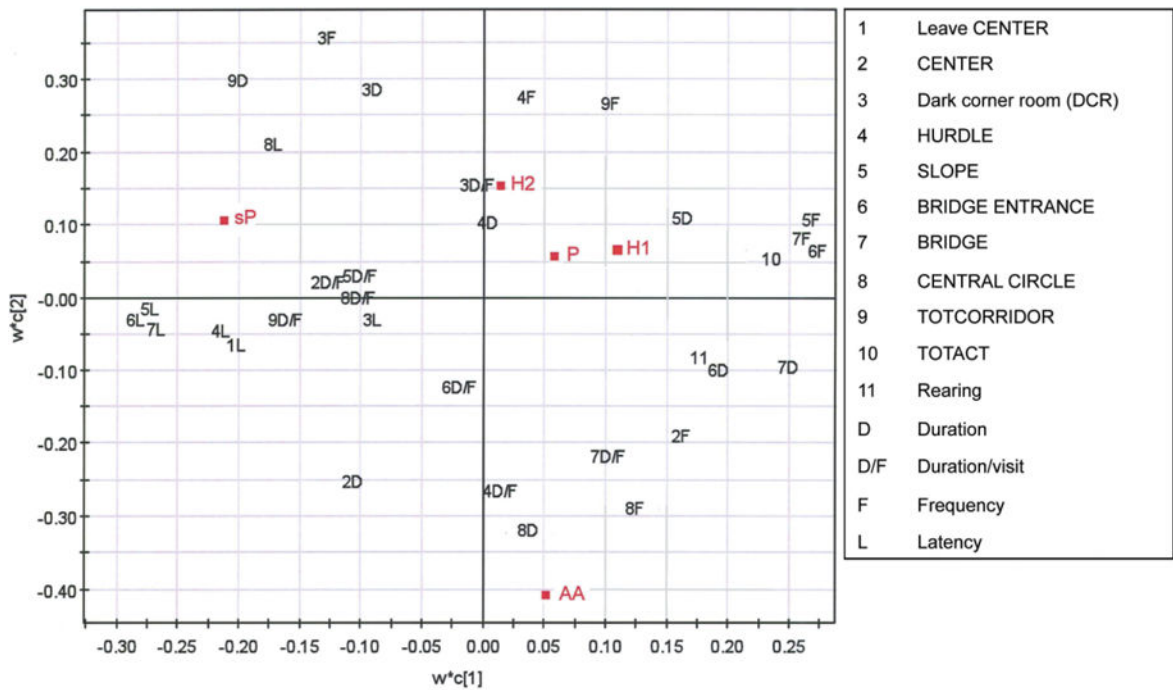A summary table over MCSF parameters for which there were significant differences between high and low drinking lines.

Functional categories	Parameters	P versus NP	AA versus ANA	sP versus sNP	H1 versus L1	H2 versus L2
General activity	TOTACT	P < NP *	AA < ANA *	sP < sNP ****		
	FRQ TOTCORR	P < NP *	AA < ANA *	sP < sNP **		H2 >L2 *
	FRQ CENTER	P < NP *		sP < sNP ***		
	DUR CENTER		AA > ANA ***			
	DUR/FRQ CENTER	P > NP *	AA > ANA *	sP > sNP **		
Exploratory activity	LAT LEAVE	P > NP **	AA > ANA ****	sP > sNP ***		
	DUR TOTCORR	P > NP *		sP > sNP ***		
	DUR/FRQ TOTCORR	P > NP **	AA > ANA *	sP > sNP ****		
	LAT HURDLE		AA > ANA **	sP > sNP **		H2 < L2 *
	FRQ HURDLE		AA < ANA **			
	DUR HURDLE			sP < sNP *		
	DUR/FRQ HURDLE		AA > ANA **	sP < sNP **	H1 < L1 ****	H2 < L2 **
	FRQ HEAD DIP		AA < ANA *			
	# ZONES VISITED			sP < sNP **		
	OCC VISIT ALL ZONES			sP < sNP ###		
	REARING			sP < sNP ****		
Risk assessment	LAT SLOPE		AA > ANA *	sP > sNP ***		H2 < L2 *
	FRQ SLOPE		AA < ANA **	sP < sNP ****		
	DUR SLOPE			sP < sNP ****		
	DUR/FRQ SLOPE		AA > ANA ***			
	OCC SLOPE			sP < sNP ##		
	LAT BRIDGE ENTRANCE		AA > ANA *	sP > sNP ***		H2 < L2 **
	FRQ BRIDGE ENTRANCE		AA < ANA **	sP < sNP ****		
	DUR BRIDGE ENTRANCE			sP < sNP **		

Functional categories	Parameters	P versus NP	AA versus ANA	sP versus sNP	H1 versus L1	H2 versus L2
	DUR/FRQ BRIDGE ENTRANCE		AA > ANA *			
	OCC BRIDGE ENTRANCE			sP < sNP##		
	SAP TO CENTER			sP > sNP**		
	OCC SAP TO CENTER			sP > sNP##		
	SAP TO SLOPE			sP > sNP****		H2 < L2 *
Risk taking	LAT BRIDGE		AA > ANA *	sP > sNP**		H2 < L2 **
	FRQ BRIDGE		AA < ANA **	sP < sNP****		
	DUR BRIDGE			sP < sNP****		
	DUR/FRQ BRIDGE		AA > ANA *			
	OCC BRIDGE			sP < sNP###		
	FRQ CTRCI		AA > ANA *	sP < sNP*	H1 < L1 *	
	DUR CTRCI		AA > ANA **	sP < sNP*	H1 < L1 **	
	DUR/FRQ CTRCI		AA > ANA **		H1 < L1 **	
	OCC CTRCI			sP < sNP##		
Shelter seeking	LAT DCR		AA > ANA ***			
	FRQ DCR		AA < ANA ***			
	DUR DCR		AA < ANA *			
Anxiety-like behavior	DUR SHELTER/RISK INDEX			sP > sNP***		
Impulsive-like behavior	SLOPE/BRIDGE INTERVAL			sP > sNP**		

Behavioral parameters, recorded during the 20-minute trial of the MCSF test, for which there were significant differences within the respective high and low drinking pair of selectively bred rats. Occurrence (OCC) indicates the zones that were not visited by all animals in each group or the behaviors that were not performed by all animals in each group ($n = 12$–16/group). The sum of frequencies to the three CORRIDORs (FRQ TOTCORR) and to all zones (TOTACT) was used for assessment of general locomotor activity. The total time spent in the three CORRIDORs was given the denomination DUR TOTCORR. The shelter/risk index is calculated from the difference in time spent in the dark corner room (DCR) and on the BRIDGE, relative to the total time spent in the two zones and is used as one way of interpreting anxiety-like behavior. A positive value indicates that the animals spent more time in the DCR than on the BRIDGE and is interpreted as higher anxiety-like behavior. The SLOPE/BRIDGE interval reveals how long time it takes the animals to enter the BRIDGE in relation to first entering the SLOPE and is here used as one way of interpreting impulsive-like behavior. Thus, a value close to zero indicates less risk assessment and a fast risk-taking response.

*
  P 0.05,

**
  $P < 0.01$,

***
  $P < 0.001$,

****
P < 0.0001 comparing each pair of selectively bred rats (Mann—Whitney *U*-test);

#
P 0.05,

##
P < 0.01,

###
P < 0.001 comparing each pair of selectively bred rats (Pearson Chi-square test). Raw data values are shown in Appendix S1. CTRCI = central circle; DCR = dark corner room; DUR = duration (s); DUR/FRQ = duration per visit (s); FRQ = frequency; LAT = latency (s); OCC = occurrence; SAP = stretched attend posture; TOTACT = total activity, i.e. the sum of all frequencies; TOTCORR = total corridor, i.e. the sum of all corridors.

Table 2

A summary table of OF parameters (A) and EPM parameters (B) for which there were significant differences between high and low drinking lines.

Functional categories	Parameters	P versus NP	AA versus ANA	sP versus sNP	H1 versus L1	H2 versus L2
A. Open field						
General activity	TOTACT			sP < sNP****	H1 < L1***	H2 > L2*
	CROSS	P > NP***		sP < sNP****	H1 < L1***	H2 > L2***
Risk taking	FRQ CENTER			sP < sNP***	H1 < L1***	H2 > L2*
	DUR CENTER			sP < sNP***	H1 < L1****	
	DUR/FRQ CENTER		AA > ANA*		H1 < L1*	
	OCC CENTER			sP < sNP##		
	%DUR CENTER			sP < sNP****	H1 < L1****	
Thigmotaxis	FRQ PERIPHERY			sP < sNP****	H1 < L1***	H2 > L2*
	DUR PERIPHERY			sP > sNP****	H1 > L1****	
	DUR/FRQ PERIPHERY			sP > sNP****	H1 > L1***	H2 < L2*
	%DUR PERIPHERY			sP > sNP****	H1 > L1****	
B. Elevated plus maze						
General activity	TOTACT		AA < ANA****	sP < sNP****		
Exploratory activity	LAT LEAVE	P > NP*				
Risk assessment	FRQ CENTER		AA < ANA****	sP < sNP***		
	DUR CENTER	P > NP*	AA > ANA*		H1 < L1**	
	DUR/FRQ CENTER		AA > ANA****	sP > sNP**		
	%DUR CENTER	P > NP*	AA > ANA*		H1 < L1**	
Risk taking	LAT OPEN					H2 > L2*
	FRQ OPEN			sP < sNP*		
	DUR OPEN		AA > ANA*	sP < sNP*		H2 < L2*
	DUR/FRQ OPEN		AA > ANA**			H2 < L2*
	OCC OPEN			sP < sNP###		

Functional categories	Parameters	P versus NP	AA versus ANA	sP versus sNP	H1 versus L1	H2 versus L2
	%DUR OPEN		AA > ANA *	sP < sNP ****		
	%FRQ OPEN			sP < sNP ****		
"Shelter" seeking	FRQ CLOSED		AA < ANA ****	sP < sNP *		
	DUR CLOSED		AA < ANA *	sP > sNP **	H1 > L1 *	
	DUR/FRQ CLOSED			sP > sNP ***	H1 > L1 **	
	%DUR CLOSED		AA < ANA *	sP > sNP **	H1 > L1 *	

Behavioral parameters, recorded during the 10-minute trial of the OF test (A) and the 5-minute trial of the EPM test (B), for which there were significant differences within the respective high and low drinking pair of selectively bred rats. Occurrence (OCC) indicates the zones that were not visited by all animals in each group or the behaviors that were not performed by all animals in each group ($n = 11$–16/group). In the OF test, duration and frequency of visits into a peripheral zone, 15 cm from each of the four walls of the arena was used for assessment of thigmotaxis, whereas duration and frequency of visits into the central part (60×60 cm) revealed center activity. The total number of lines crossed (CROSSINGS) and the sum of visits to the periphery and center (TOTACT) were used as measures of general locomotor activity. In the EPM test, the sum of visits to the open and closed arms (TOTACT) was used for assessment of general locomotor activity.

*
P 0.05,

**
$P < 0.01$,

***
$P < 0.001$,

****
$P < 0.0001$ comparing each pair of selectively bred rats (Mann–Whitney U-test);

#
P 0.05,

##
$P < 0.01$,

###
$P < 0.001$ comparing each pair of selectively bred rats (Pearson Chi-square test). Raw data values are shown in Appendix S1 Tables. CROSS = number of lines crossed in the OF; DUR = duration (s); DUR/FRQ = duration per visit (s); FRQ = frequency; LAT = latency (s); LAT LEAVE = time to leave the periphery area of the OF or center area of the EPM at the beginning of the session; OCC = occurrence; TOTACT = total activity, i.e. the sum of entries into the center and periphery of the OF or into the open and closed arms of the EPM.