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ORIGINAL ARTICLE

Low copulatory activity in selectively bred Sardinian alcohol-nonpreferring (sNP) relative to alcohol-preferring (sP) rats

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Abstract

Background. There is a growing consensus that similar neural mechanisms are involved in the reinforcing properties of natural rewards, like food and sex, and drugs of abuse. Rat lines selectively bred for high and low oral alcohol intake and preference have been useful for understanding factors contributing to excessive alcohol intake and may constitute proper animal models for investigating the neurobiological basis of natural rewarding stimuli.

Methods. The present study evaluated copulatory behavior in alcohol and sexually naïve Sardinian alcohol-preferring (sP) and -nonpreferring (sNP) male rats in three consecutive copulatory behavior tests.

Results. The main finding was that, under the conditions used in this study, sNP rats were sexually inactive relative to sP rats. To gain more information about the sexual behavior in sP rats, Wistar rats were included as an external reference strain. Only minor differences between sP and Wistar rats were revealed.

Conclusions. The reason behind the low copulatory activity of sNP rats remains to be elucidated, but may in part be mediated by innate differences in brain transmitter systems. The comparison between sP and Wistar rats may also suggest that the inherent proclivity to excessive alcohol drinking in sP rats may mainly be dependent on its anxiolytic properties, as previously proposed, and not changes in the reward system.

Key words: *Addiction, drugs of abuse, ejaculation, natural reward, Sardinian alcohol-preferring and non-preferring rats, sexual behavior, Wistar rat*

Introduction

There is a growing consensus that similar mechanisms are involved in the reinforcing properties of natural rewards, like food and sex, and drugs of abuse (1–5). The demand for an improved understanding of the basal functioning of the brain reward system, especially with regard to motivational aspects and choice of reinforcer, has recently been emphasized (1,4,5).

The mesocorticolimbic dopamine system with cell bodies in the ventral tegmental area and projections to

areas including the nucleus accumbens, amygdala, and the prefrontal cortex is highly conserved in evolution, a natural constituent of vital life sustaining behaviors (6), and a key component in reward and addiction processes (2,3,7). It is well acknowledged that sexual activity (8) and intake of palatable food (9) and drugs of abuse including alcohol (10) result in elevated levels of extracellular dopamine in areas associated with the mesocorticolimbic dopamine system, including the nucleus accumbens. In studies of basic mechanisms of eliciting reward-driven behavior

it is important to differentiate the appetitive phase (seeking the reinforcer) from the act of consumption. Natural and sexual selection processes are likely to be involved in the development of appetitive and consummatory activity with some key neuronal trajectories shared between species.

The development of rat lines selectively bred for high and low oral alcohol intake and preference has been a useful strategy for understanding factors contributing to excessive alcohol intake (11–16). These lines were bred for the same phenotypes, i.e. high or low voluntary alcohol intake and preference under the standard, home-cage, two-bottle free-choice paradigm with continuous access to alcohol, water, and food (17–19). One common aim of these selective breeding programs has been to determine behavioral (20), neurobiological (21), and genetic (22) characteristics associated with selection for high and low voluntary alcohol intake and preference. Considering the role of the brain reward system in reinforcing properties of natural rewards as well as drugs of abuse it is possible that selective breeding for high alcohol intake affects also other reward-related behaviors. The selectively bred alcohol-preferring and non-preferring lines may therefore represent possible models for studies of similarities between natural rewarding stimuli and drugs of abuse (23–25). Here the Sardinian alcohol-preferring (sP) and -nonpreferring (sNP) rats (16) were used to study some aspects of this fairly unexplored topic. The aim of the present study was to investigate if selective breeding of sP and sNP rats has also resulted in differences in copulatory behavior. Wistar rats were included as an external reference strain.

Material and methods

Animals

Adult alcohol and sexually naïve sP and sNP male rats (kept under specific pathogen and opportunistic free conditions at Charles River Laboratories, Calco, Italy; generation S65; $n = 15$ rats per group) and age-matched male Wistar rats (Sca:WI; Scanbur BK AB, Sollentuna, Sweden; $n = 10$) were studied. This study was initially designed to compare copulatory activity in sP and sNP rats; however, the large degree of sexual inactivity observed in sNP rats in several preliminary experiments forced us to include a set of Wistar rats as reference strain. Wistar rats were preferred over other strains of outbred rats as they constituted the foundation stock from which the selective breeding of sP and sNP rats was started (16). It should, however, be noted that the Wistar rats used herein were from a different supplier, and vendor-dependent differences between Wistar rats exist (26–29). Twenty-four female Wistar

rats (HanTac:WH; Taconic, Ejby, Denmark), not related to the male Wistar rats, weighing approximately 300 g, were used as stimulus females. All rats were housed three per cage in transparent cages ($59 \times 38 \times 20$ cm) containing wood-chip bedding material and paper sheets as enrichment. The cages were placed in temperature-controlled ($22 \pm 1^\circ\text{C}$) and humidity-controlled ($50\% \pm 10\%$) housing cabinets with a reversed 12-hour light/dark cycle (lights off between 7.00 a.m. and 7.00 p.m.). The rats were maintained on rat chow (R36; Lantmännen, Kimstad, Sweden) and water *ad libitum*.

The females used as stimuli in the copulatory behavior test were brought into estrus by hormone treatment. They were ovariectomized under isoflurane anesthesia and allowed to recover for 10 days. The hormone treatment consisted of subcutaneous administrations of 25 $\mu\text{g}/\text{kg}$ of estradiol benzoate (Sigma-Aldrich, St. Louis, MO, USA) in olive oil 48 h before progesterone, and 1 mg/rat of progesterone (Sigma-Aldrich) in olive oil 4–6 h before use. Prior to the assessment of copulatory behavior, all male rats had undergone behavioral testing (30), which was completed at approximately 20 weeks of age. The sexual behavior test was initiated at 30 weeks of age.

All animal experiments were approved by the Uppsala Animal Ethical Committee and followed the guidelines of the Swedish Legislation on Animal Experimentation (Animal Welfare Act SFS1998:56) and the European Communities Council Directive (86/609/EEC).

Copulatory behavior test

Male copulatory behavior with receptive females was scored in three tests. The first two tests, which were considered as learning tests, lasted for 15 min, and the animals had two test-free days between each test. The third test, which served to investigate copulatory behavior in sexually experienced rats, was performed one week after the second test. The third test lasted for a maximum of 30 min but was interrupted after scoring the post-ejaculatory interval. The tests were performed in a wooden cage ($60 \times 35 \times 35$ cm) with a transparent front, used to score copulatory behavior only, under dim illumination during the dark phase of the light/dark cycle. The male rat was allowed to habituate to the cage for 5 min before the receptive female was introduced. Each female was used for two to three males, and the females were alternated between the three copulatory tests. Based on established protocols (31–33), parameters according to the ethogram in Table I were scored by direct observation by an experienced, blinded observer or calculated based on the scored behaviors.

Table I. Ethogram of the behaviors scored by direct observation or calculated during the copulatory activity tests.

Parameters	Definitions
Latency mount	The time (s) from the introduction of the receptive female until the first mount was observed
Latency intromission	The time (s) from the introduction of the receptive female until the first intromission was observed
Latency ejaculation	The time (s) from the introduction of the receptive female until an ejaculation was observed
Frequency mount	The total number of observed mounts
Frequency intromission	The total number of observed intromissions
Frequency ejaculation ^a	The total number of observed ejaculations
Post-ejaculatory interval	The time (s) from ejaculation until the next observed intromission
Mounts + intromissions	The sum of observed mounts and intromissions
Mounts + intromissions/min	The sum of observed mounts and intromissions calculated per minute
Intromission ratio	The number of observed intromissions calculated in relation to the total number of observed mounts and intromissions
Time from first intromission to ejaculation	The elapsed time (s) from the first observed intromission until an observed ejaculation
Mounts + intromissions/ejaculation	The sum of mounts and intromissions required to reach ejaculation
Inter-intromission rate III	The latency (s) from the introduction of the receptive female until ejaculation divided by the total number of intromissions
Copulatory rate	The sum of mounts and intromissions divided by the elapsed time (s) from the first mount until an observed ejaculation

^aThe number of ejaculations was only scored in the first two tests, which lasted for 15 min each. Only one sP rat ejaculated twice, and this was in the first test. The third test lasted for a maximum of 30 min but was interrupted after scoring the post-ejaculatory interval.

Statistical analyses

Data were tested for normality using the Shapiro–Wilk’s *W* test. Copulatory behavior data were not normally distributed, and therefore non-parametric statistical methods were used. The learning effect was evaluated by comparing latency and frequency measures over the three tests using the Friedman test followed by the Wilcoxon signed rank test when appropriate. For these analyses all values were included, and latency measures were set at 900 s if the behavior was not performed. The number of animals performing mounts, intromissions, and ejaculations was evaluated with the chi-square test. The third test was analyzed for performed behaviors, and thus if a behavior did not occur this was considered a missing value. Group comparisons of performance in the third test were performed using the Kruskal–Wallis test and/or the Mann–Whitney *U* test. Differences were considered statistically significant at $p \leq 0.05$. Statistica 10.0 (StatSoft Inc., Tulsa, OK, USA) was used for the statistical analyses.

Results

Acknowledging that copulatory activity is a product of two individuals interacting, the behavior of the females was not scored since, after hormonal priming,

all females were sexually proceptive and displayed receptive behaviors including lordosis.

Performance over time

The main finding in the present study is a very low copulatory activity in male sNP rats (Figure 1). Thus, fewer sNP than sP rats engaged in copulatory behavior in all three tests. In the first and second test, only 2 out of 15 sNP males mounted, and in the third test this number was increased to 3 males, which was significantly fewer (chi-square = 8.89, $df = 1$, $p < 0.01$; chi-square = 13.39, $df = 1$, $p < 0.001$; chi-square = 13.39, $df = 1$, $p < 0.001$, respectively) when compared with sP rats. In the second and third test, no sNP rats achieved intromission (chi-square = 9.13, $df = 1$, $p < 0.01$; chi-square = 17.37, $df = 1$, $p < 0.0001$) or ejaculation (chi-square = 10.91, $df = 1$, $p < 0.001$). Based on this sexual inactivity, sNP rats were excluded from further detailed comparison with the sP line. To get information about the copulatory behavior in sP rats, a detailed statistical analysis using Wistar rats as reference strain was performed.

There were significant differences over time in sP and Wistar rats in latency to first mount (sP Friedman ANOVA ($n = 15$, $df = 2$) = 5.71, $p = 0.058$; Wistar Friedman ANOVA ($n = 10$, $df = 2$) = 15.08,

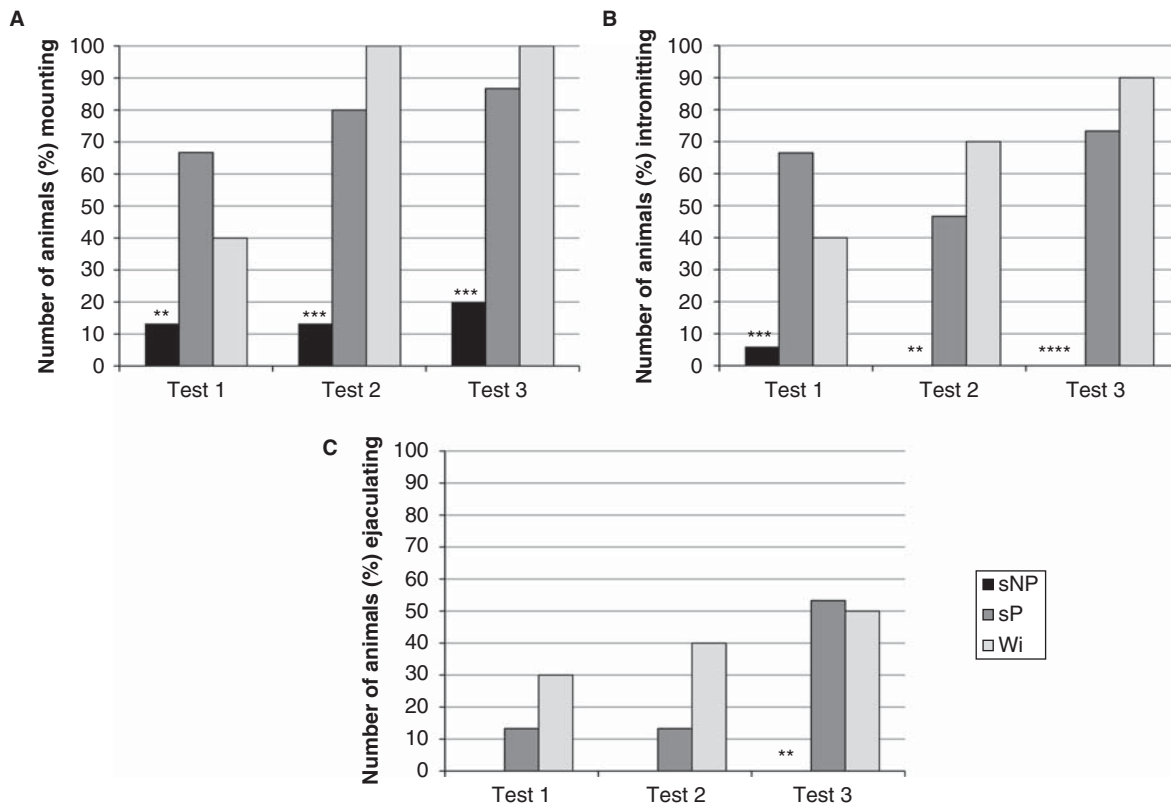


Figure 1. The percentage number of sNP ($n = 15$) and sP ($n = 15$) rats mounting (A), intromitting (B), and achieving ejaculation (C) over the three copulatory behavior tests. Due to the low number of sNP rats engaging in copulatory activity also in the third test, they were excluded from further detailed statistical analyses of copulatory behavior. Wistar ($n = 10$) rats were included as a reference strain. ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$ compared to sP rats (chi-square test).

$p < 0.001$), latency to first intromission (sP Friedman ANOVA n.s.; Wistar Friedman ANOVA ($n = 10$, $df = 2$) = 6.59, $p < 0.05$) and latency to first ejaculation (sP Friedman ANOVA ($n = 15$, $df = 2$) = 11.38, $p < 0.01$; Wistar Friedman ANOVA n.s.) (Figure 2). In Wistar rats, the latency to first mount and intromission, respectively, was significantly shorter in the second (mount $Z = 2.29$, $p < 0.05$; intromission $Z = 2.02$, $p < 0.05$) and third (mount $Z = 2.80$, $p < 0.01$; intromission $Z = 2.55$, $p < 0.05$) test relative to the first test. Likewise, in the third test, the latency to ejaculation was shorter ($Z = 2.52$, $p < 0.05$) when compared with the first test in sP rats. Moreover, when comparing sP and Wistar males, Wistar rats had shorter latency to the first mount in the third test ($U = 37.5$, $p < 0.05$; Figure 2A).

The number of intromissions was higher ($Z = 2.24$, $p < 0.05$) in the third test relative to the first test in Wistar rats, with a trend close to statistical significance also for sP rats ($Z = 1.91$, $p = 0.056$) (Figure 3). The sum of mounts and intromissions was higher ($Z = 2.40$, $p < 0.05$) in the third test compared with the first test in Wistar rats, while no such difference was found in sP rats.

Assessment of copulatory behavior in sexually experienced sP and Wistar rats

Eighty-seven percent of the sP rats and 100% of the Wistar rats mounted, while the corresponding values for intromissions were 73% (sP) and 90% (Wistar), and for ejaculations 53% (sP) and 50% (Wistar) (Figure 1). More detailed studies of performed behaviors in the third copulatory behavior test in sP and Wistar rats revealed a difference in inter-intromission rate ($U = 6.0$, $p < 0.05$) (Table II). Moreover, there were trends toward longer latency to first mount ($p = 0.088$) and fewer mounts and intromissions per minute ($p = 0.067$), and longer post-ejaculatory interval ($p = 0.065$) in sP rats relative to Wistar rats (Table II).

Discussion

The aim of this study was to determine if the response to a natural rewarding stimulus, i.e. copulatory activity, differed in rats selectively bred for opposite alcohol preference and consumption. The main finding was an almost absence of copulatory activity in the

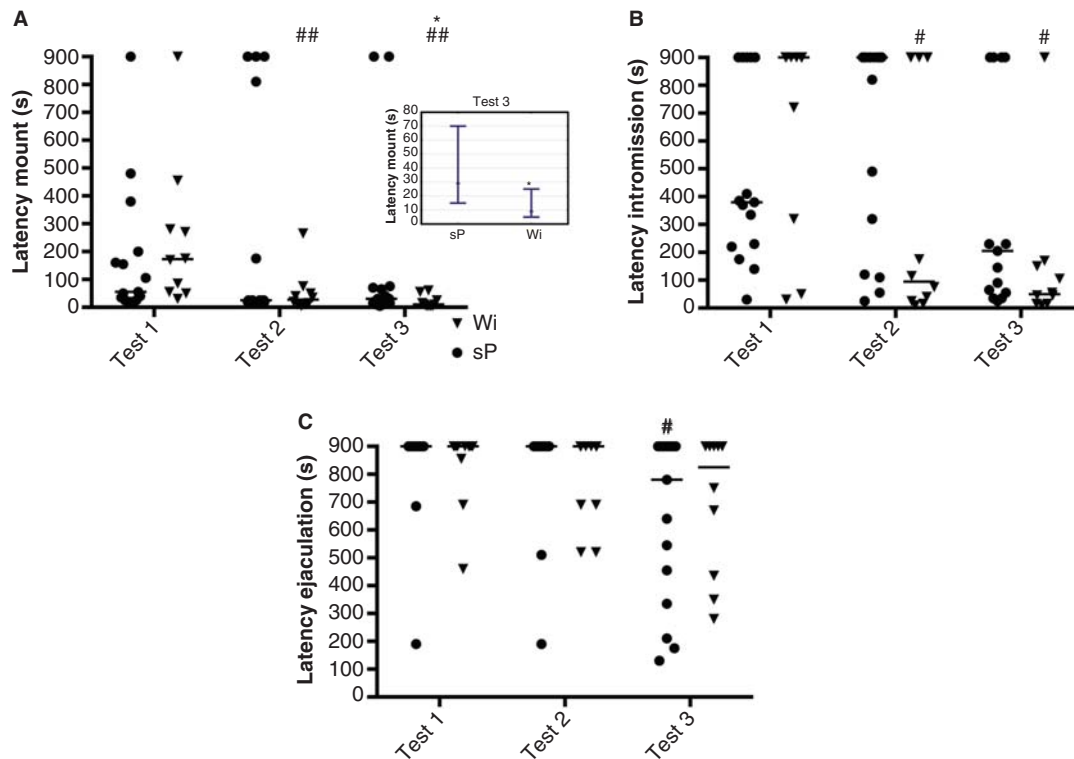


Figure 2. Latency to first mount (A), intromission (B), and ejaculation (C) in the three copulatory behavior tests in male sP ($n = 15$) and Wistar ($n = 10$) rats. The latency to first mount (A) in the third test is also shown in the insert for a better illustration of the difference between sP and Wistar rats. Values represent individual data points with the median value marked as a line, and in the insert values represent median and quartile range. * $p < 0.05$ comparing sP and Wistar rats (Mann–Whitney U test); # $p < 0.05$, ## $p < 0.01$ compared to the first copulatory behavior test within the respective group (Wilcoxon signed rank test).

alcohol-nonpreferring sNP rats compared with -preferring sP rats. Even after three tests, no sNP rat had intromissions or achieved ejaculation, and based on this finding they are considered sexually inactive (33), also termed non-copulators (34).

Copulatory behavior, like drug-taking behavior, is comprised of appetitive and consummatory acts (35). The mount and intromission latencies are considered measures of appetitive acts or sexual motivation (33,36). Ejaculation represents a consummatory act, while the interpretation of post-ejaculatory interval is less clear (33). Non-copulators are males that do not mate even after they are tested repeatedly with sexually receptive females, and are found in several species including rats and mice (33,34,37). The finding that sNP rats do not display sexual motivation or engage in consummatory sexual behavior after three tests may suggest characteristics of non-copulators. Since the females used were primed and therefore displayed proceptive and receptive behaviors it is considered unlikely that the behavior of the female rats is the cause for the low copulatory activity observed in the sNP rats. In addition, since the sNP line is maintained over generations, these rats obviously copulate under other circumstances; the

mating technique adopted in the breeding procedure includes indeed long periods of time (up to one week) during which male and female sNP rats are housed together in the same cage.

When compared with Wistar rats, sP rats displayed low sexual behavior in initial tests but increased their copulatory activity over successive testing. This pattern of increasing copulatory activity over time has been attributed to distraction or fear generated by the novelty of the test situation (34). Beside inherent proclivity to excessive alcohol drinking, sP rats display more anxiety-related behaviors compared with sNP rats and, when tested, sometimes also compared with Wistar rats (20,30,38–41). In a more complex setting, i.e. the multivariate concentric square field™ test, sP rats are characterized by low general activity and exploratory drive, and low risk-taking behavior compared with sNP rats (20,30) as well as other selectively bred alcohol-preferring rat lines (20). Since voluntarily consumed alcohol ameliorated different anxiety-related behaviors (41,42), it has been proposed that sP rats consume alcohol for its anxiolytic properties (16,41). When sexually experienced, i.e. in the third test, the copulatory behavior of sP rats was similar to that of Wistar rats. Thus, explorative strategies and

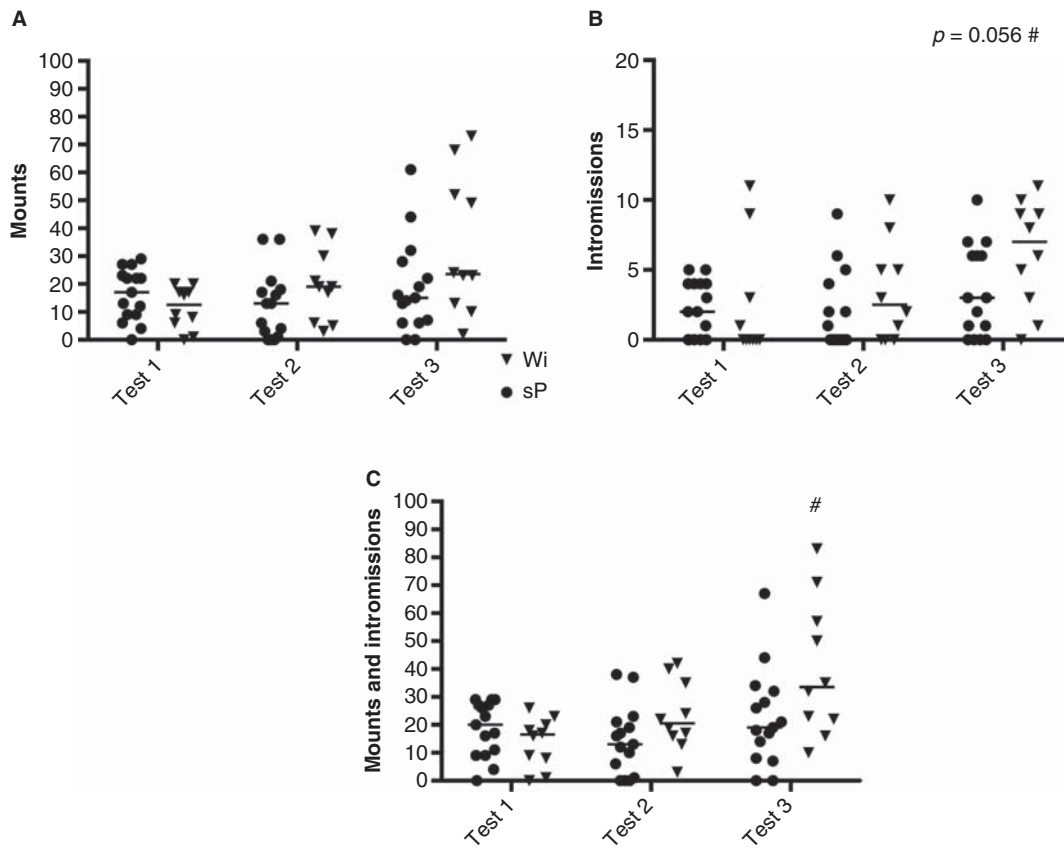


Figure 3. The number of mounts (A), intrusions (B), and sum of mounts and intrusions (C) in the three copulatory behavior tests in male sP ($n = 15$) and Wistar ($n = 10$) rats. Values represent individual data points with the median value marked as a line. # $p < 0.05$ compared to the first copulatory behavior test within the respective group (Wilcoxon signed rank test).

copulatory activity in sP rats display differences as the copulatory behavior in the third test was similar to that of the Wistar rats while no attenuation of anxiety-related behaviors in sP rats occurs with repeated testing [(30), and Roman and Colombo, unpublished observation].

A number of brain transmitter systems and specific brain areas have been linked to sexual function. The sP and sNP rats differ in a number of brain transmitter systems; these differences have been suggested to underlie their opposite alcohol-drinking phenotypes (21). For example, basal dopamine levels were higher in the nucleus accumbens shell in sP than in sNP and Wistar rats, and in the medial prefrontal cortex in sP rats relative to Wistar but not sNP rats (39). Dopamine denervation of the nucleus accumbens and infusion of the dopamine D2 antagonist raclopride both affect sexual behavior by delaying the initiation of mounting and intromitting without affecting the number of mounts and intrusions (43). Also the endocannabinoid system seems to play a role in the regulation of sexual behavior as the endocannabinoid anandamide was shown to induce copulatory behavior in non-copulating animals (44). The sP rats have

higher density of the cannabinoid receptor CB1 and higher levels of the endocannabinoids anandamide and 2-arachidonoylglycerol compared with sNP rats (45). These innate differences between sP and sNP rats could explain, at least in part, the differences in copulatory behavior observed herein.

In previous investigations of responses to a natural reward stimulus, such as palatable food, there were no differences in intake and in some cases also preference of intake of a chocolate-flavored drink (46), sucrose (46,47), or saccharin solutions (48) between sP and sNP rats. However, in these studies, the high reinforcing properties of these fluids might have overwhelmed any possible existing difference in reward sensitivity between sP and sNP rats. In addition, both sP and sNP rats initiated and maintained operant self-administration of sucrose (49). Therefore, on the basis of the relatively scarce amount of data available, at present it cannot be ruled out that sNP rats are characterized by a generally lower motivational drive (not limited solely to alcohol and sexual activity) than sP rats.

The results in the present study differ markedly from those of a previous experiment where only

Table II. Copulatory behavior in sexually experienced sP and Wistar rats.

Parameters	sP				Wistar				
	Median	Min	Max	Occ	Median	Min	Max	Occ	
Latency mount	20.0	5.0	75.0	13/15	10.0	5.0	60.0	10/10	$U = 37.5, p = 0.088$
Frequency mount	16.0	6.0	61.0	13/15	23.5	2.0	73.0	10/10	
Mounts/min	1.1	0.4	2.4	13/15	1.6	0.2	2.4	10/10	
Latency intromission	90.0	25.0	1245.0	11/15	45.0	10.0	170.0	9/10	
Frequency intromission	6.0	1.0	10.0	11/15	8.0	1.0	11.0	9/10	
Intromissions/min	0.3	0.03	0.7	11/15	0.6	0.03	0.8	9/10	
Mounts + intromissions	21.0	7.0	67.0	13/15	33.5	10.0	83.0	10/10	
Mounts + intromissions/min	1.4	0.5	2.8	13/15	2.2	0.8	2.8	10/10	$U = 35.0, p = 0.067$
Intromission ratio	17.6	7.1	58.8	11/15	28.1	2.0	80.0	9/10	
Latency ejaculation	395.0	130.0	780.0	8/15	435.0	280.0	750.0	5/10	
Time from first intromission to ejaculation	280.0	40.0	725.0	8/15	425.0	225.0	735.0	5/10	
Mounts + intromissions/ejaculation	18.5	7.0	28.0	8/15	22.0	10.0	35.0	5/10	
Post-ejaculatory interval	312.5	230.0	520.0	8/15	245.0	190.0	340.0	5/10	$U = 7.0, p = 0.065$
Inter-intromission rate III ^a	98.2	54.5	130.0	8/15	48.3	43.8	74.4	5/10	$U = 6.0, p < 0.050$
Copulatory rate	0.06	0.03	0.1	8/15	0.05	0.03	0.06	5/10	

Copulatory behavior in sP and Wistar rats during the third sexual behavior test, i.e. when the rats were sexually experienced from two previous tests. Values are expressed as median, min, and max. Occurrence (Occ), i.e. the number of animals displaying each behavior, is shown since behaviors that did not occur were considered missing values.

^a $p < 0.05$ comparing sP and Wistar rats, additional trends are shown (Mann–Whitney U test).

modest differences between sP and sNP rats were found (38). However, the study by Cagiano et al. (38) was designed to study the impact of alcohol exposure during the perinatal period on subsequent behavioral development, and the animals had undergone perinatal treatment with alcohol or sucrose from gestational day 15 to postnatal day 7, which may explain the different outcome. Perinatal exposure to both alcohol and sugar-rich diets affect responses to drugs of abuse later in life (50,51), likely through adaptations in the reward-related mesostriatal dopaminergic system that also may impact on copulatory behavior.

In conclusion, the present investigation reveals profound differences in copulatory activity between the selectively bred alcohol-preferring sP and -nonpreferring sNP rats. sP rats, with an innate proclivity for high voluntary alcohol intake and high levels of anxiety-related behaviors, display sexual behavior similar to that of outbred Wistar rats when sexually experienced; sNP rats were considered sexually inactive, with characteristics of non-copulating males, suggesting a lower motivational drive. With regard to natural rewards, our results imply different responses following access to palatable food reward and a sexually receptive female in male sP and sNP rats. Considering the known differences between the different lines of rats selectively bred for high and low alcohol intake and preference it

would be of interest in future studies to compare sexual behavior also in other lines.

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Declaration of interest: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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