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Psychosocial stress alters ethanol's effect on open field behaviors

Gregory Blakley, Larissa A. Pohorecky *

Center of Alcohol Studies, Rutgers University, 607 Allison Road, Piscataway, NJ 08854-1100, USA

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Abstract

Psychosocial stress, including social rank status, has been shown to alter spontaneously occurring behaviors in rodents as well as the behavioral effects of drugs of abuse. In this study, rats were repeatedly evaluated in a modified open field following: their initial exposure, and after intraperitoneal injections of saline and 0.75 g/kg ethanol (EtOH). All subjects were first tested while under single housing conditions, then again following 35 days of differential housing (singly or 3 rats/cage) with social status determined by scoring agonistic behavior at triad formation. The data suggest that (1) future subordinate rats differed with respect to specific aspects of behavior displayed in a 'novel' open field arena, (2) future subordinate rats were more emotional since they showed greater "anxiety-like" behavior and less exploratory behavior, (3) subordinate rats were more impaired by the saline injection stress, (4) subordinate rats were more sensitive to the depressant effects of EtOH, (5) grooming behavior did not show habituation, in contrast to the other behaviors, but showed sensitization on the second test. Overall, subordinate rats may have differed from their cage mates in innate anxiety, and this may underlie their distinct response to both stressors and EtOH. Furthermore, while EtOH had mostly stimulant effects in naive rats, psychosocial stress and/or repeated testing resulted in enhancement of EtOH's depressant effects.

Keywords: Rank status; Singly housing; Habituation; Modified open field test; Locomotor activity; Anxiety behavior; Rearing behavior; Headpoke behavior; Grooming behavior

1. Introduction

Psychosocial stress, like other more extensively investigated stressors, has profound effects on the physiology and behavior of rodents. A prominent behavioral effect of psychosocial stress is its depressant effect on locomotor activity (Blanchard, 2001; Moragrega et al., 2003; Pohorecky, 2006). Stressors, including psychosocial stressors, have also been reported to alter the response to drugs. Singly housed rodents tend to be more sensitive to certain drugs than group-housed rodents. For example, singly housed mice were more sensitive to pentobarbital (Ohdo et al., 1989; Dong et al., 1999) but less sensitive to amphetamine (del Pozo et al., 1978; Cheeta et al., 2001; Coudereau et al., 1999). On the other hand, subordinate monkeys generally were more sensitive to amphetamine compared to dominant animals (Gambill and Kornetsky, 1976) and chronic treatment with clomipramine or mianserin enhanced the hierarchical status of subdominants with little if any effect in dominant rats (Mitchell and Redfern, 1992). In the same vein, we have shown that the effect of EtOH on open field behaviors of rats varies with rank status of rats housed in groups of three (triad-housed) (Pohorecky, 2006). In that study, singly housed rats were more resistant to EtOH's effect on locomotor activity compared to triad-housed rats, and the anxiolytic effect of EtOH was more prominent in dominant rats. However, it is not known whether the altered drug sensitivity in these studies was innate or whether it developed as a consequence of chronic psychosocial stress.

Ethanol (EtOH) can have stimulant and/or depressant behavioral effects (Pohorecky, 1977; Davidson et al., 2002; King et al., 2002). In rodents, the stimulant action of low doses of EtOH has generally been assessed using locomotor behavior, with higher doses depressing this behavior (Paivarinta and Korpi, 1993; Agabio et al., 2001; Rodd et al., 2004). The locomotor stimulant effect of EtOH can be influenced by a rat's behavioral responsiveness to novelty. For instance, EtOH has a greater activating effect in rats that show high locomotor activity when placed in a novel environment, compared to low novelty reacting rats (Gingras and Cools, 1996). These authors

* Corresponding author. Tel.: +1 732 445 3593; fax: +1 732 445 3500.
E-mail address: larissa@rci.rutgers.edu (L.A. Pohorecky).

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also found that mild stress (habituation to novel environment) enhanced the stimulant effect of EtOH exclusively in the high novelty responders, but had no effect on the low novelty responders. Furthermore, we have shown that the anxiolytic-like effect of a low dose of EtOH was dependent on a subject's individual characteristics, possibly reflecting differences in previously experienced stress and/or in their adaptive coping response (Pohorecky, 2006). For instance, EtOH's behavioral effects were more prominent in triad-housed dominant rats and in singly housed rats.

That there is an interaction of EtOH and stress is attested to by numerous studies (for reviews see Pohorecky, 1990, 1991; Sayette, 1999). A particularly relevant study found significant individual variability in the development of tolerance to the locomotor stimulant effects of EtOH. Rats that were predisposed to develop sensitization to repeated exposure to mild stressors were found to be less sensitive to the locomotor stimulant effects of EtOH (Gingras and Cools, 1996). The aim of the present study was to further examine the effect of psychosocial stress on the sensitivity of rats to EtOH. To this end, the behavioral responsiveness to EtOH was compared in a modified open field prior to and after chronic psychosocial stress. Group housed male rats readily establish a social hierarchy. The dominant rat displays distinct behaviors towards its cage mates, and subdominant and subordinate rats display defensive behaviors, but the subdominant rat generally also challenges the dominant rat (Blanchard et al., 1991, 1993; Pohorecky et al., 1995, 1999). Compared to dominant animals, subdominant animals generally display lower locomotor activity, enhanced anxiety, and reduced sexual activity (Raab et al., 1986; Hilakivi-Clarke and Lister, 1992; Koolhaas et al., 1997; Shively et al., 1997). In view of a recent report that habituation factors may alter the locomotor activation produced by cocaine (Carey et al., 2005), we also determined whether differences in adaptation to the test environment may influence sensitivity to EtOH of psychosocially stressed rats.

2. Methods

2.1. Subjects and housing

The subjects were male adult Long Evans rats weighing approximately 300 g at the start of the experiment (Harlan Sprague Dawley, Indianapolis, Indiana). Purina chow and water were available ad libitum throughout the study. The vivarium was kept at 21 ± 1 °C, with controlled humidity and a reverse light/dark cycle (12 h each, lights off at 12:30 PM). To adapt to our vivarium conditions, rats were initially individually housed in hanging wire-mesh stainless steel cages for 14 days. Assignment of rats to group housing was based on body weight, that is, the body weight of the rats assigned to a triad differed by less than 5%. Rats were weighed prior to group housing and then weekly for the duration of the study. The housing cages were made of Plexiglas and had a wire mesh floor. One of the cage walls had either one (single cages) or 2 (triad cages) 1-cm opening for drinking spouts. The cages for individually housed rats were square (25 cm × 25 cm × 30 cm), and those for the triad housed rats were rectangular (26 cm wide × 82 cm long × 30 cm high) so that the floor space per rat was relatively similar, 625 cm^2 vs. 710 cm^2 for single and triad-housed, respectively. The triad-housed rats were separated from each other by two removable Plexiglas cage dividers. The bottom of the dividers consisted of a 6-cm-high wire mesh screen that allowed rats to maintain sensory contact even when separated. These dividers were removed daily for a 1-h period that allowed the rats to interact and reinforce their social hierarchy. All animal facilities are certified by AAALAC, and the experimental protocols were approved by the Rutgers University review Committee for the use of Animal Subjects, and all principles of laboratory care were adhered to.

2.2. Agonistic behavior rating

Agonistic behaviors were first assessed at the time triads were formed. On day 1 of the study, subjects were placed into a novel triad cage and the social interactions were recorded during the first 30 min of group-housing. Agonistic behaviors were scored using an expanded version of the method originally described by Peterson and Pohorecky (1989), and subsequently modified and expanded (Pohorecky et al., 1999, 2004a). Twenty-three different behaviors were scored and subsequently grouped for analysis into four major categories: self-centered (rearing, self-grooming, genital grooming), social (approach, sniff body, sniff genitals, groom other, mount other), defensive (defensive upright, immobility, vocalization, flight/attempt to jump out of the cage) and aggressive (piloerection, aggressive push-under, pounce on, nip other, cage mark, offensive block or pacing, defensive/offensive back kick, lateral threat, on top, roll-tumble interaction). These behaviors have been described in greater detail previously (Pohorecky et al., 2004a). Lateral threat behavior was defined as the initiation of a broadside approach and push against the flank of the conspecific, and piloerection. The offset of lateral threat behavior involved the termination of approach or physical contact. Roll-tumble fights were defined as the initiation of a vigorous attack in which both conspecifics were in motion. On top behavior was defined as initiating and maintaining a position in which a rat straddled a supine conspecific. The offset of these first two offensive aggressive behaviors was the termination of physical contact between the two conspecifics. Freezing behavior was defined as the rigid maintenance of a fixed position. Defensive upright behavior was defined as the maintenance of a position in which both forepaws were off the cage floor, with shoulders clearly raised above the hips. Based on these behavioral assessments, along with changes in body weights determined 24 h after triad formation, individual members of a triad were designated as dominant, subdominant, and subordinated rats. The dominant rat displayed offensive aggressive behaviors towards its cage mates. Unlike the subdominant rat that did not display subordinate behavior, the subordinate rat rapidly learned to display such behaviors (freezing and vocalization). As soon as the subordinate rat signaled submission during its first interaction with the dominant, the latter then generally ignored it. The subordinate rat remained immobile in one corner of the

cage during agonistic interactions of its cage mates. The subdominant rat, on the other hand, was involved in aggressive interactions with the dominant rat, and sometimes displayed displaced aggression against the subordinate rat. The frequency of 22 kHz ultrasonic vocalizations was used to confirm non-dominant rats. Ultrasonic vocalizations were detected using a BAT Detector (QMC Inc., London, UK). The combined use of behavioral scores, ultrasonic and audible vocalizations, with the body weight changes 24 h after triad formation, formed the basis for assigning rank status within a triad (Pohorecky et al., 2004b). In cases where rank status was difficult to establish at triad formation because of the lack of agonistic interactions, the triad was either discarded or a given rat was exchanged (a total of two), so that differences rank were noted. To further support the previous assignments of rank status, at the end of the study carcasses were examined for size and location of any bodily wounds. Agonistic behavioral observations were also carried out at 1–2-week intervals to verify the stability of rank assignments.

2.3. Modified open field test

The modified open field arena was 100 cm × 100 cm, with the floor elevated 10 cm above the ground level. The floor was divided into 16 quadrants, and every other square had a 4-cm diameter centrally located hole (a total of 6 around the perimeter and 2 in the center of the field). The frequency and duration of rearing, headpoke, center entry, and crossing activity of the test subject were quantified over a 10-min period with the aid of a computer equipped with a manually operated interface (Pohorecky et al., 1999). Crossing behavior, a measure of locomotion, was counted when the rat crossed with all four legs from one quadrant to another. Center behavior was counted when all four legs of the rat were in one of the four center quadrants (total area 50 cm × 50 cm). A rear was counted when a rat stood on its hind limbs, with its forelimbs completely off the floor. Headpoke behavior, a measure of exploration, was counted when an animal inserted its head below eye level into a hole in the floor. To minimize test environment generated anxiety, we conducted open field testing during the rat's dark phase, between 1:00 and 5:00 PM.

2.4. Experimental procedures

The study was run as two consecutive cohorts (11 triads and 10 singly housed, total of 43 rats). The overall chronology of this study is shown in Table 1. The second column in this table indicates the tests that are being reported here. Rats were initially pre-tested for ingestion of a 6% solution of EtOH over a 15-day period, and were then tested in the open field after a 9-day recovery period. The first test was a 'novel' open field test, with rats tested as described above. On the second test, rats were injected with saline (the volume being commensurate with that of the EtOH injection to be used on the following day) and immediately tested, and on third test rats were injected with 0.75 g/kg EtOH (10% w/v solution in saline) and again tested immediately afterwards. We selected the 0.75 g/kg dose of EtOH since we found that the 0.5 g/kg had a robust stimulant effect on locomotor behavior, wile a 1 g/kg dose had little if any effect. Thirty-four days later, rats were assigned and transferred to triad or individual new cages described above. After 35 days of differential housing, rats were again tested in the 'novel' open field. On the subsequent 2 days, they were tested after being treated with saline and then with EtOH, respectively, as described for the pre-triad phase. As indicated in the overall chronology, all rats were also tested for voluntary EtOH intake over a 15-day period ending 9 days prior to both open field tests, these data will be published separately.

Table 1
Schedule of experimental procedures

Overall chronology	Present study chronology	Measures taken and behavioral tests
Days 1–18		Vivarium acclimatization
Days 19–34		Choice of 6% EtOH vs. water for half of the animals
Days 35–43		Recovery days
Days 44	Pre-triad—OFA	Open field testing, "novel"
Days 45	—OFA	Open field testing, saline injection
Days 46	—OFA	Open field testing, EtOH injection
Days 47–79		Recovery
Day 80		Pre-triad body weight and triad formation
Day 81		24-h body weight change
Days 82–88		Acclimatization to new housing
Days 89–104		Choice of 6% EtOH vs. water for half of the animals
Days 105–114		Recovery days
Day 115	Triad—OFA	Open field testing, "novel"
Day 116	—OFA	Open field testing, saline injection
Day 117	—OFA	Open field testing, EtOH injection

2.5. Statistical data analysis

The data are presented as means and standard errors of the means. The data were analyzed using StatView version 5. A two-way ANOVA was employed for the analysis of social interaction and body weights data, while a four × two × two repeated measures mixed design ANOVA was employed for the open field data. The between subjects factor was the rats' housing and rank status (dominant, subdominant, subordinate, single). The repeated measure parameters were test phase (pre-triad and triad) and drug treatment (saline and EtOH). When appropriate, a post-hoc Fischer's test was employed to determine group differences, with significance set at $P \leq 0.05$.

3. Results

3.1. Behavioral assessment, body weight changes and ingestion of ethanol by triad and singly housed rats

When unfamiliar, previously singly housed, rats are placed together in a cage, they initially explore the new environment and then begin to interact with each other. Generally, within the next 5–20 min the social ranking within the triad becomes

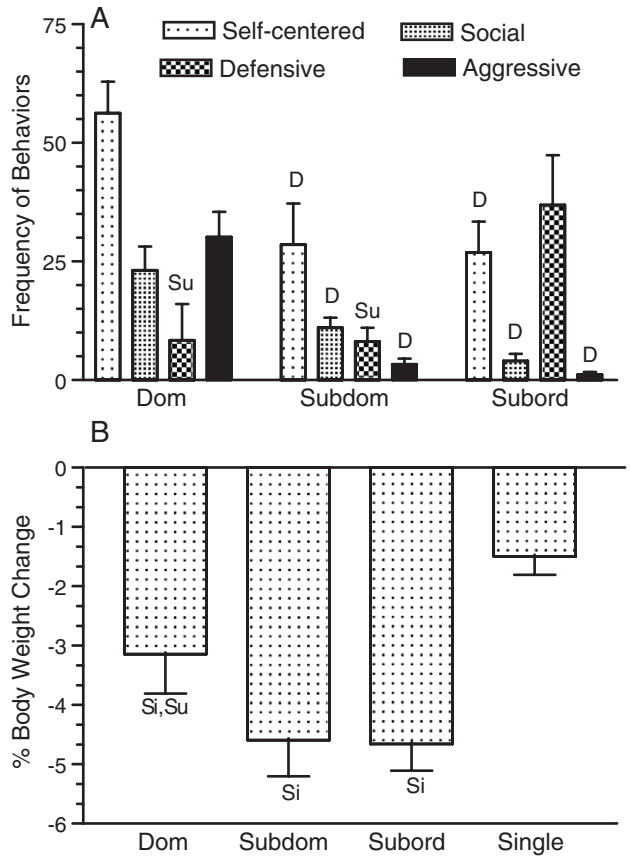

Fig. 1. (A) Frequency of offensive behaviors displayed by triad-housed rats. Behaviors were assessed during the initial 30-min test performed when triads were formed. (B) Change in body weight of triad- and singly housed rats after the initial 24-h of differential housing. Data are shown as the mean score±S.E.M. for groups of 11 triad and 10 singly housed subjects. Letters (D=dominant, Su=subordinate) above individual bars denote statistically significant difference between experimental groups ($P \leq 0.05$).

clear. Rats subsequently designated as dominants differed significantly from their cage mates with respect to all behaviors that were displayed during the 30-min assessment period (Fig. 1A). There were rank related differences in self-centered ($F2,33=5.015$, $P=0.0125$), social ($F2,33=8.782$, $P=0.0009$), defensive ($F2,33=4.640$, $P=0.0168$), and aggressive ($F2,33=26.210$, $P<0.0001$) behaviors. Dominant rats performed more self-centered and social behaviors compared to subdominant ($P=0.019$) and subordinate rats ($P=0.0082$), and also engaged in more social behaviors compared to subdominant ($P=0.0129$) and subordinate ($P=0.0002$) rats. On the other hand, subordinate rats engaged in more defensive behaviors compared to both dominant ($P=0.0131$) and subdominant ($P=0.0122$) rats. And most significantly, the dominant rats showed most of the aggressive behaviors towards their cage mates ($P<0.0001$, with respect to subordinates).

All rats lost some weight 24-h after they had been transferred to their new housing cages ($F3,39=7.459$, $P=0.0005$). While rats did not differ in body weight prior to differential housing, there were small but significant losses in body weight in all of the rats housed in triads (Fig. 1B). Dominant rats lost significantly more body weight compared to singly housed rats ($P=0.0388$), but significantly less compared to subordinate rats ($P=0.050$). Subdominant and subordinate rats also lost more body weight compared to singly housed rats ($P=0.0003$ and $P=0.0002$, respectively).

There were no housing/rank differences in the daily ingestion of EtOH at either the pre-triad or triad tests. Daily intake of EtOH (g/kg) however was significantly higher at the pre-triad compared to the triad tests for both the triad (0.924±0.042 and 0.425±0.019, for the pre- and triad phases, respectively) and singly housed rats (0.893±0.116 and 0.438±0.036, for the pre- and triad phases, respectively) ($F1,44=69.166$, $P<0.0001$).

3.2. Crossing behavior

3.2.1. 'Novel' test

During the 'novel' phase, there was no main effect of housing/rank on crossing frequency in the open field test, but there was a main effect of test phase ($F1,39=34.507$, $P<0.0001$) (Table 2). Crossing frequency was lower at the pre-triad compared to the triad phase. This increase in crossing frequency from the pre- to the triad phase was significant for dominant, subdominant and singly housed rats ($P<0.0001$, $P=0.0103$ and $P=0.0191$, respectively), but not in subordinate rats. At the triad phase, dominant and subdominant rats were

Table 2
Behavior of rats during 'novel' open field tests

Housing/rank	Test phase	Crossing frequency	Center duration	Headpoke duration	Rearing duration	Groom duration
Dominant	Pre-triad	59.36±4.33	5.87±2.31	17.04±2.28	46.46±4.05	1.39±0.78
	Triad	83.46±5.78* (Sb)	5.84±0.65	13.09±0.63	31.04±3.31*	8.71±1.75* (SD)
Subdominant	Pre-triad	55.82±4.34	4.66±1.46	13.25±1.86	46.87±3.16	1.85±0.59
	Triad	80.09±3.89* (Sb)	7.59±1.91	10.54±1.66	26.99±3.50*	36.12±8.67*
Subordinate	Pre-triad	60.82±3.47	1.01±0.76	11.35±0.77	53.68±2.79	5.06±1.66
	Triad	65.82±6.28	8.99±3.36*	11.66±1.94	32.47±1.39*	28.10±5.96*
Single	Pre-triad	57.00±3.95	3.99±1.68	14.40±3.23	56.80±3.40	2.73±1.17
	Triad	75.10±5.46*	3.00±0.54	10.37±1.60	35.45±3.21*	13.12±2.25* (SD)

Rats that were subsequently triad- or singly housed rats were tested in the open field 80 days prior to differential housing, and subsequently again 35 days after differential housing. The open field test was 10 min long. Data are presented as means±S.E.M. for groups of 11 triads and 10 singly housed rats. (*) Indicates significant difference at $P<0.05$ compared to the pre-triad phase. (SD) and (Sb) indicate significant differences at $P<0.05$ from subdominant and subordinate rats, respectively.

more active than the subordinate rats ($P=0.0255$ and $P=0.050$, respectively); pre-triad there were no housing/rank differences. The percent increase in crossing frequency from pre-triad to triad was 43.2±8.8% for dominants, 53.5±14.2% for subdominants, 36.9±13.2% for singly housed, and only 9.8±0.1% for subordinate rats.

3.2.2. Saline/ethanol test

A four×two×two repeated measures ANOVA identified the significant effects of housing/rank ($F3,78=13.773$, $P<0.0001$), test phase ($F1,78=144.978$, $P<0.0001$), and EtOH treatment ($F1,78=8.000$, $P=0.0059$) on crossing frequency (Fig. 2A), rank and housing and EtOH treatment

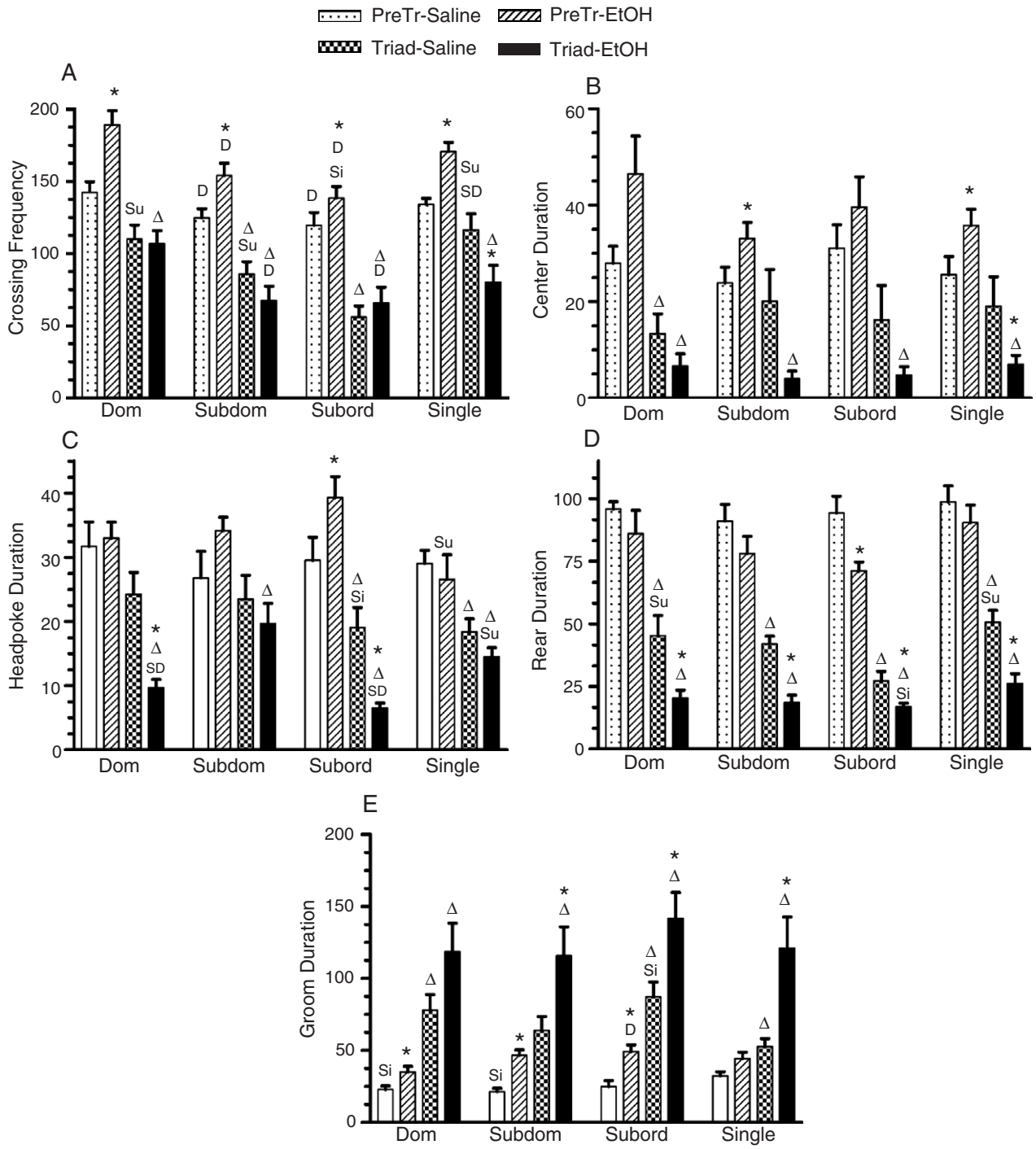

Fig. 2. Open field behaviors of rats tested after 35 days of differential housing. Frequency of crossing behavior (A), and duration of center (B), headpoke (C) rearing (D), and grooming (E) behaviors were evaluated during 10-min tests in a modified open field apparatus. Data are shown as the mean score±S.E.M. for groups of 11 triad and 10 singly housed subjects. The duration measure is in seconds. Letters above individual bars denote statistically significant difference between experimental groups, (D) with dominant group, (SD) subdominant, (Su) subordinate and (Si) singly housed groups. (△) Designates statistically significant difference between pre-triad and triad phases. (*) Designates statistically significant effect of EtOH treatment compared to saline treatment. Statistical significance was set at $P\leq 0.05$.

were between factors, and test phase was the repeated measure. Furthermore, the interactions of test phase × EtOH ($F1,78=37.855$, $P<0.0001$), and of housing/rank condition × test phase × EtOH ($F3,78=3.212$, $P=0.0274$) were also significant. These interaction effects indicate differences in locomotor activity at the two test phases were strongly dependent on the housing/rank status of the subjects and on the effect of EtOH. Pre-triad the saline groups did not differ in crossing behavior. Except for the singly housed rats, crossing behavior of saline rats was lower at the triad compared to the pre-triad phase. This decline was larger for the subordinate (−53.0± 6.55%, $P<0.0001$) compared to the dominant (−19.4±9.4%, $P=0.0159$) and subdominant rats (−27.9±9.0%, $P=0.0017$), but was not significant for singly housed rats (−12.4±8.9%). As a result, at the triad phase locomotor frequency of subordinate rats was lower than that of the other saline-treated rats ($P=0.0002$, $p=0.0260$ and $P<0.0001$, for dominant, subdominant and singly housed rats), while in subdominant rats it was lower than in singly housed rats ($P=0.0260$). It appears that, at both the 'novel' and saline tests, the subordinate rats differed in crossing behavior from their cage mates.

The interaction effects mentioned above also indicate that the effect of EtOH differed with test phase and housing/rank status. Compared to saline treatment, EtOH increased crossing frequency of all four groups at the pre-triad phase ($P=0.0019$, $P=0.0018$, $P=0.0280$ and $P=0.0010$, for dominant, subdominant, subordinate and singly housed rats, respectively). However, at the triad phase, the frequency of crossing behavior of all EtOH-treated rats was significantly lower than at the pre-triad test ($P<0.0001$ for all groups). Furthermore, in contrast to the pre-triad phase, EtOH no longer had a stimulant effect on crossing frequency, and EtOH actually depressed locomotor activity of singly housed rats ($P=0.0089$). While the EtOH dominant rats crossed more frequently than the corresponding subdominant and subordinate rats at both pre-triad ($P=0.0039$ and $P=0.0081$) and triad phases($P=0.0088$ and $P=0.0117$, respectively), subordinate rats crossed less frequently than the singly housed at the pre-triad phase ($P=0.0094$).

3.3. Center behavior

3.3.1. 'Novel' test

There was a significant effect of test phase on center duration ($F1,39=4.566$, $P=0.0385$) and a significant interaction of housing/rank and test phase ($F3,39=3.101$, $P=0.0376$) (Table 2). Only the subordinate rats spent significantly more time in the center of the open field at the triad phase compared to the pre-triad phase ($P=0.0308$); there were no significant group differences at either test phase.

3.3.2. Saline/ethanol test

A four × two × two repeated measures ANOVA identified a significant main effect of test phase ($F1,78=66.213$, $P<0.0001$) on center duration, and the interaction of test phase × EtOH was also significant ($F1,78=28.116$, $P<0.0001$) (Fig. 2B). There were no housing/rank differences at either test phase in the saline-treated rats. Center duration tended to be longer during the pre-triad phase compared to the triad phase, but the change was significant only for the dominant rats ($P=0.0080$). While pre-triad EtOH tended to increase center duration, this effect reached statistical significance only for subdominant and singly housed rats ($P=0.0126$ and $P=0.0463$, respectively). Conversely, EtOH dramatically depressed center duration of all rats at the triad phase. However, again because of the large individual variability, this decline was significant only for singly housed rats ($P=0.0463$), with a similar trend in subdominant and subordinate rats ($P=0.0544$ and $P=0.0588$, respectively).

3.4. Headpoke behavior

3.4.1. 'Novel' test

During the 'novel' test, there were no statistically significant effects of either housing/rank, test phase or drug treatment on headpoke behavior (Table 2).

3.4.2. Saline/ethanol test

With headpoke duration the four × two × two repeated measures ANOVA identified a significant effect of EtOH ($F1,78=20.439$, $P<0.0001$), and significant interactions of rank × test phase ($F3,78=3.176$, $P=0.0287$) and of EtOH × test phase ($F1,78=20.439$, $P<0.0001$) (Fig. 2C). Headpoke duration of saline-treated rats did not differ in terms of housing/rank at the pre-triad test phase. However, saline subordinate and singly housed rats spent less time headpoking at the triad phase compared to the pre-triad phase ($P=0.0403$ and $P=0.0017$). EtOH treatment increased headpoke duration in subordinate rats ($P=0.0154$) at the pre-triad phase, making their headpoking being longer than that of the correspondingly treated singly housed rats ($P=0.0050$). Overall, EtOH-treated rats headpoked longer at the pre-triad compared to triad phase ($P=0.0001$, $P<0.0001$, $P=0.0001$ and $P=0.0088$ for dominant, subdominant, subordinate and singly housed rats, respectively). EtOH treatment at the triad phase significantly depressed headpoke duration of dominant ($P=0.0110$) and subordinate ($P=0.0073$) rats. At the triad phase, EtOH subdominant rats spent more time headpoking than the correspondingly dominant and subordinate rats ($P=0.0006$, and $P<0.0001$, respectively); EtOH subordinate rats spent less time headpoking than the corresponding singly housed rats ($P=0.0059$).

3.5. Rearing behavior

3.5.1. 'Novel' test

There was a statistically significant effect of test phase ($F1,39=99.830$, $P<0.0001$) on rearing duration (Table 2). All rats spent less time rearing at the triad compared to the pre-triad test phase ($P=0.0010$, $P=0002$, $P<0.0001$ and $P=0.0056$ for dominant, subdominant, subordinate and singly housed rats).

3.5.2. Saline/ethanol test

Similar to crossing, the four × two × two repeated measures ANOVA identified significant effects of housing/rank on rearing

duration ($F3,78=3.930$, $P=0.0115$), test phase ($F1,78=357.968$, $P<0.0001$), and EtOH ($F1,78=50.265$, $P<0.0001$), but there were no significant interaction effects (Fig. 2D). Rearing duration was shorter at the triad compared to the pre-triad phase for all groups irrespective of drug treatment ($P=0.0001$ for all groups). Pre-triad, there were no housing/rank differences in rearing duration after saline treatment. At the triad phase, saline subordinate rats spent less time rearing than did the dominant and singly housed rats ($P=0.0195$, $P=0.0040$, respectively), with a similar trend for subdominant rats ($P=0.0543$). While pre-triad, EtOH decreased rearing duration of subordinate rats ($P=0.0107$), it did so in all the groups at the triad phase ($P=0.0251$, $P=0.0003$, $P=0.0267$ and $P=0.0003$, for dominant, subdominant, subordinate and singly housed rats, respectively). EtOH-treated rats did not differ with respect to housing/rank status at the pre-triad phase, however at the triad phase the subordinate rats spent less time rearing than the singly housed rats ($P=0.0337$).

3.6. Grooming behavior

3.6.1. 'Novel' test

With grooming duration was very low pre-triad but increased dramatically during the triad phase ($P=0.0039$, $P=0.0020$, $P=0.0054$ and $P=0.0029$, for dominant, subdominant, subordinate and singly housed rats, respectively) (Table 2). At the triad phase subdominant rats groomed longer than did dominant and singly housed rats ($P=0.0010$ and $P=0.0047$, respectively).

3.6.2. Saline/ethanol test

For grooming duration, the four × two × two repeated measures ANOVA identified a significant effects of test phase ($F1,78=107.245$, $P<0.0001$) and of EtOH treatment ($F1,78=49.721$, $P<0.0001$), and significant interaction effects for test phase × EtOH ($F1,78=11.990$, $P=0.0009$) (Fig. 2E). Pre-triad the saline singly housed rats groomed longer than the corresponding dominant and subdominant rats ($P=0.047$ and $P=0.0190$, respectively). All saline rats groomed longer at the triad compared to the pre-triad phase ($P<0.0001$, $P=0.0003$, $P<0.0001$, and $P=0.0034$, for dominant, subdominant, subordinate and singly housed rats, respectively). At the triad phase, saline subordinate rats groomed longer than singly housed rats ($P=0.0155$). Overall, treatment with EtOH prolonged grooming behavior in all groups. Pre-triad EtOH treatment prolonged grooming of dominant and subdominant rats ($P=0.0280$ and $P=0.0003$, respectively), and at the triad phase EtOH enhanced grooming of all rats except for the dominant rats ($P=0.0030$, $P=0.0238$ and $P=0.0175$, in subdominant, subordinate and singly housed rats).

4. Discussion

The present findings confirm our previous results (Pohorecky, 2006) that psychosocial stress modifies the acute behavioral effects of EtOH. EtOH had a stimulant action on most of the open field behaviors while rats were individually housed prior to differential housing. Subsequently, however, this same dose of EtOH depressed behaviors at the triad test, though the triad-housed rats were insensitive to the locomotor depressant effect of EtOH. Conversely, EtOH stimulated grooming behavior irrespective of housing/rank status or of test phase. Interestingly, future subordinate rats tended to differ from the other experimental groups. Compared to the other groups, these animals were either less sensitive to the stimulant effect of EtOH (headpoke behavior), or more sensitive to the depressant effect of EtOH (rearing behavior).

The behaviors of rodents in an open field are believed to reflect exploratory behavior. Their response to novelty involves arousal, emotionality, as well as stress response mechanisms (Dai et al., 1995). However, prolonged or repeated exposure to any environment results in the decline of the behavioral activity initially shown in that environment. In the present study, habituation of spontaneously occurring behaviors differed with respect to the specific type of behavior, test phase, as well as housing and rank status. Locomotor, center and grooming behaviors were higher at the 'novel' triad compared to the 'novel' pre-triad test for all rats except the future subordinates. On the other hand, headpoke behavior generally did not change, except again for subordinate rats, while rearing behavior declined from the pre-triad to the triad 'novel' tests for all rats. These differences in response with test phase may reflect differences in habituation of individual behaviors. The data also indicate that behavioral habituation in an open field depends on the subject's housing and social status. For example, subordinates did not display habituation in crossing behavior while all the other groups did, and conversely, they did show habituation in headpoke behavior while the other groups did not. That habituation can be altered by housing conditions has been previously reported. Westenbroek et al. (2005) found that pair-housed control rats, but not singly housed or stressed pair-housed rats, displayed rapid habituation to an open field. Our findings support the evidence that behavioral habituation in an open field can vary with the subject's housing condition and prior stress experience.

Future subordinate rats differed in various respects from their cage mates at the pre-triad 'novel' test. Locomotor activity of the subordinate group was almost identical at the pre- and triad phases, although for the other groups it was significantly higher at the triad phase. Also, future subordinates were more anxious compared to the other rats. At the saline tests, the subordinate group showed a greater decline in locomotor activity at the triad compared to the pre-triad phase compared to the other groups. Furthermore, the future subordinate group was least sensitive to EtOH's locomotor stimulant effect compared to the other groups. These findings suggest (1) that future subordinate rats differed with respect to specific aspects of behavior displayed at the 'novel' open field test, (2) future subordinate rats were more emotional since they tended to show greater "anxiety-like" behavior in the initial pre-triad 'novel' test, and (3) future subordinate rats differed in sensitivity to EtOH. The greater 'anxiety-like' behavior of subordinate rats is supported by data we have obtained using different tests believed to reflect anxiety-like behavior (Steensland et al., 2005; Pohorecky et al.,

unpublished data). It is likely that the greater innate anxiety of subordinate rats may underlie their distinct response to stressors and to EtOH.

We found no differences in locomotor activity between dominant and singly housed rats at the 'novel' triad test, which supports previous reports (Brain and Benton, 1979; del Pozo et al., 1978; Michel and Tirelli, 2002). Also in accord with our findings, other investigators have found that in socially housed animals, the dominant animals were more active than the subdominant animals irrespective of species tested (Fuchs and Flugge, 2002; Bartolomucci et al., 2001; Blanchard, 2001, Hilakivi-Clarke and Lister, 1992; Schaefer and Michael, 1991), as were rodents defeated in a resident-intruder test (Meerlo et al., 1996; Bartolomucci et al., 2003). The defeat-induced decline in locomotor activity has been found to last up to 7 days after a single defeat (Meerlo et al., 1996), though changes in anxiety were reported to be transient (de Jong et al., 2005).

An injection of saline significantly enhanced all open field behaviors at the pre-triad test compared to the no-injection 'novel' test (Table 2 and Fig. 2). However, except for grooming behavior, this saline-induced behavioral activation was smaller or absent at the triad phase. Thus, at the triad saline test subordinate rats had lower locomotor, rearing and headpoke behaviors, compared to their cage mates, while the first two behaviors did not differ at the pre-triad phase, possibly due to a greater stressor sensitivity of subordinate rats.

In confirmation of previous findings (Paivarinta and Korpi, 1993; Gingras and Cools, 1996; Rodd et al., 2004; Pohorecky, 2006), an acute EtOH challenge was found to stimulate locomotor activity in our modified open field test. The future dominant rats showed the most prominent locomotor stimulant effect of EtOH compared to the other groups. If confirmed, this finding may further support the existence of innate differences in sensitivity to EtOH.

Unexpectedly, the response to EtOH differed significantly at the triad compared to the pre-triad test. Locomotor behavior was not altered by EtOH in triad rats, but was depressed in singly housed rats. Recently, we reported that a 0.5 g/kg dose of EtOH enhanced most open field behaviors of triad but not singly housed rats, while a 1 g/kg dose of EtOH was overall ineffective or depressant (only rearing behavior) (Pohorecky, 2006). Using singly housed rats, others have reported that a 1 g/kg dose of EtOH depressed locomotor activity (Spanagel and Holter, 2000). The explanation of the depressant effect of EtOH on all behaviors at the triad test is challenging, especially since it was evident in both triad and singly housed rats. One possibility, is the significant age difference of animals at the two tests, since the triad test was performed 115 days after the pre-triad test. While sensitivity to EtOH changes with age (Casoli et al., 2001; Wang et al., 2003), it is not known whether the neurobiological substrates for the stimulant and depressant effects of EtOH (Gingras and Cools, 1996) are similarly affected. Second, both open field tests took place after all rats underwent some additional tests, including EtOH intake, 9 days prior to either testing. It should be stressed that *all* rats underwent the same procedures within the same time frames, nevertheless, previous testing may have influenced the response to both saline and EtOH treatments in the open field test (Koide et al., 2000; Montkowski et al., 1997). Repeated handling and testing may have differentially affected the stimulant and depressant actions of EtOH, enhancing tolerance to its excitatory effects while sensitizing animals to its depressant effects (Gingras and Cools, 1996). Somewhat surprisingly, the intake of EtOH by all rats was lower at the triad phase compared to the pre-triad phase, again possibly as a consequence of age difference of the subjects. Because ingestion of EtOH was substantially lower at the triad ingestion test, a potential carry over effect would not explain a greater depressant effect of EtOH, since it should have resulted in a smaller, not greater, effect of the drug. The lower intake of EtOH at the triad phase may be due to its slower elimination from the organism (Seitz et al., 1989).

Behavior in the central zone of an open field is believed to reflect emotional behavior, with decreased entries reflecting an anxiogenic-like effect, and conversely, higher entries reflecting an anxiolytic-like effect (Angrini et al., 1998; Ramos et al., 2003; Stefanski et al., 1992). Results from the triad 'novel' test did not indicate enhanced anxiety-like state for any of the groups. Pre-triad, EtOH had an anxiolytic-like effect in all rats, except the subordinates, while it tended to have an "anxiogenic-like" effect at the triad phase. As mentioned above, EtOH depressed locomotor behavior only in singly housed rats at the triad phase. This distinct response to EtOH may reflect differences in anxiety generated by the open field in triad vs. singly housed rats. For example, rats housed in triads may have experienced not only agarophobia (fear of large open space) but also separation from their cage mates, which was not an issue for the singly housed rats. Contact with group mates minimized stress during testing in a modified hole board apparatus, particularly in more anxious rats (Ohl et al., 2001). The greater anxiogenic-like response of subdominant and singly housed rats vs. dominant and subordinate rats (Fig. 2B), could reflect greater sensitivity to EtOH's depressant action and/or tolerance to its stimulant effect.

Headpoke and rearing behaviors are believed to reflect horizontal and vertical exploratory behavior (File and Wardill, 1975; Paulus et al., 1999). Both behaviors were similarly changed in all the experimental groups by both treatment procedures. A previous report indicated that EtOH treatment enhanced headpoke behavior (File and Wardill, 1975), but in our study this effect was evident only in the future subordinate rats. Conversely, EtOH depressed headpoke duration of dominant and subordinate rats, but had no effect in subdominant and singly housed rats. Rearing duration appeared to be particularly prone to habituation even in the untreated animals at the two 'novel' tests. Habituation was also evident after saline treatment, and was further enhanced by EtOH in all groups. EtOH depressed rearing duration at the triad test for all rats, but only in subordinate rats at the pre-triad test.

Grooming serves a variety of functions including body hygiene, social communication (pheromonal signaling), a displacement behavior, relaxation in stressful situations and wound healing (Spruijt et al., 1992), and can be influenced by stressors (van Erp et al., 1994). All rats groomed longer at the triad compared to the pre-triad phase irrespective of treatment.

This enhanced grooming behavior may reflect a de-stressing response to the test apparatus (Moyaho and Valencia, 2002). EtOH enhanced grooming duration of all rats at both tests phases. Duration of grooming at the triad test was prolonged even more by the EtOH treatment, while as discussed above, other behaviors were depressed by EtOH. That is, grooming appeared to show a sensitized response to EtOH. Considering that grooming duration was very brief during the 'novel' tests, particularly at the pre-triad 'novel' test, this enhancement by EtOH is even more remarkable. Since at low doses EtOH have an anxiolytic effect (Spanagel et al., 1995; Stewart et al., 1993; Pohorecky et al., unpublished data), the EtOH-enhanced grooming probably indicates that either grooming behavior serves another function under our experimental conditions, or that the enhanced grooming is part of the mechanism by which EtOH reduces anxiety.

Our evidence indicates that in rats habituation of open field behaviors is behavior specific. It is also apparent that habituation to both the stimulant and depressant effects of EtOH need to be considered when evaluating its effects. Clearly, the data emphasizes the importance of evaluating different behaviors since some of them did, while others did not, show habituation to EtOH. Interestingly, behaviors in a mildly stressful novel environment might predict future hierarchal status. For example, subordinate rats did not show habituation in the open field that was equivalent to the other groups. These rats were more emotional, and their response to EtOH also differed. Compared to the other groups, these animals were less sensitive to the stimulant effect of EtOH (headpoke behavior) and more sensitive to its depressant effect (rearing behavior). Our data also demonstrated that differentially housed rats varied widely in their ability to cope with stressors as well as in their response to EtOH. Potentially, differences in innate behavior may provide clues to a subject's sensitivity to drugs and in their ability to cope with stressors, since this model could be adapted to investigate the neurobiological mechanisms underlying such behavioral differences. It should be pointed out, however, that because of the design of our study there are potential interactive effects of pre- and post-testing, of repeated drug administration with both housing and ethanols' behavioral effects which will require further research to dissect.

In studies addressing social stress, researchers do not always consider the dimensions of the employed housing cages. Specifically, the housing cages should provide adequate and equivalent floor space per rat for both the individually and the socially housed animals. Crowding should be avoided because physically crowded animals will be unable to appropriately display agonistic behaviors that are essential for the development and display of social rank differences. Furthermore, future studies should examine the influence that differences in the level of stress (i.e., the severity of agonistic interactions) have on the behavior and physiology of group-housed animals. We had expected to also address this issue in the present study. However, this could not be accomplished because we had an uneven distribution of triads that differed in aggression levels. This was a consequence of the unpredictability in the aggression rats display between shipments. These shipment differences make it difficult to predict the distribution of high, moderate of very low aggressive triads in a given cohort since moderately aggressive triads tend to predominate.

Summarizing, there were significant differences in behavioral responses to both saline and EtOH at the pre-triad and triad phase phases. These differences indicate that environmental variables have a significant impact on EtOH's behavioral effects, including its anxiolytic action. However, since these differences were observed in both triad- and singly housed rats, these effects cannot be entirely ascribed to differential housing. Differences in the pattern of change from pre-triad to triad phases in locomotor and center behaviors, particularly in subordinate rats, suggest that distinct biobehavioral mechanisms underlie these open field behaviors. In conclusion, our studies show that the behavioral effects of EtOH are highly dependent on a number of variables. While housing and social status and prior exposure to EtOH represent some of these variables, habituation to the testing environment, and age differences appear to also important. Clearly, in studies of stress, and particularly social stress, the effect of age should be further investigated.

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