Is behavioral sensitization to ethanol associated with contextual conditioning in mice?

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Behavioral sensitization to drugs of abuse seems to involve learning processes. In mice, ethanol-induced locomotor sensitization is potentiated by repeated pairing of ethanol (EtOH) injections and the testing chamber. The present study aimed to test: (1) the association between the performance in a contextual conditioning task and the development of behavioral sensitization to EtOH in mice; (2) whether EtOH sensitization would be expressed in a different testing environment. Male albino Swiss mice ($n=72$) were initially submitted to a contextual fear conditioning task. After 2 weeks without manipulation, the animals received daily i.p. injections of 2.2 g/kg EtOH ($n=52$) or saline ($n=20$), for 21 days. They were tested weekly for locomotor activity in activity cages. After 1 week of withdrawal, all mice received 2.2 g/kg EtOH and had their locomotor activity recorded in an open-field. According to the locomotor behavior displayed along the 21-day treatment, EtOH-treated mice were classified as sensitized ($n=15$) or non-sensitized ($n=15$). When these subgroups and saline-treated mice were compared for the freezing response in the conditioning test, sensitized mice displayed a greater freezing time than non-sensitized mice. When challenged with EtOH in the open-field, none of the EtOH-treated subgroups expressed behavioral sensitization. These results suggest that the development of EtOH sensitization seems to be positively associated with contextual learning, and further confirms that the expression of sensitization is highly dependent on contextual cues. *Behavioural Pharmacology* 14:129–136 © 2003 Lippincott Williams & Wilkins.

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Introduction

Repeated administration of several drugs of abuse may induce a potentiation of some drug-induced behavioral responses, a phenomenon known as behavioral sensitization. Psychostimulants, morphine and low doses of ethanol (EtOH) are able to induce sensitization to their psychomotor stimulating effects (Trujillo and Akil, 1995). Since the brain circuitry involved in psychomotor stimulation may be closely related, or even identical to the circuitry involved in brain reward (Wise and Bozarth, 1987), psychomotor sensitization could reflect a sensitized activation of brain reward systems, contributing to drug dependence (Robinson and Berridge, 1993; Roberts *et al.*, 2000).

Sensitization could be considered a simple manifestation of learning and memory, resulting in the facilitation of drug-induced responses after repeated experience (Stewart and Badiani, 1993). However, the development and expression of behavioral sensitization seem to be largely influenced by contextual cues surrounding drug administration (Robinson *et al.*, 1998; see also Stewart and Vezina, 1988). The repeated pairing of drug injections with the testing chamber, for example, seems to potentiate and facilitate the expression of sensitization in that context (Cunningham and Noble, 1992; Badiani *et al.*, 1995, 2000). Neurochemical correlates of behavioral sensitization to psychostimulants – such as the enhancement of glutamatergic transmission at the nucleus accumbens – also seem to be affected by context-related factors (Bell *et al.*, 2000). These data have led several authors to propose that more complex associative learning and memory processes may play an important role in drug sensitization in general. One possible explanation is that neuroadaptations underlying behavioral sensitization may be closely related to those mediating learning and memory processes (Trujillo and Akil, 1995; Tzschentke and Schmidt, 1998).

Several studies have shown that tolerance to some effects of ethanol (EtOH) may be highly related to context-dependent processes (Lê *et al.*, 1979; Crowell *et al.*, 1981;

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Melchior and Tabakoff, 1985). Working with genetically selected mouse lines, Radcliffe *et al.* (1998) showed that contextual fear conditioning was positively correlated with acute functional tolerance to EtOH. One of the few studies that examined the importance of contextual conditioning on EtOH sensitization showed that mice receiving repeated EtOH injections in the testing chamber displayed locomotor sensitization, while mice that received the same treatment – but unpaired to the testing chamber – did not (Cunningham and Noble, 1992). Notably, two mice strains (C57BL/6 and DBA/2) that are known to display different responses to the stimulant effects of EtOH and EtOH sensitization (Phillips *et al.*, 1994; Lessov *et al.*, 2001) also showed different responses in learning and memory tasks related to contextual learning (Paylor *et al.*, 1994).

Working with outbred mice, we have previously shown that in a population of EtOH-treated mice it was possible to distinguish subpopulations that sensitize and do not sensitize to the stimulant effect of EtOH (Masur and Boerngen, 1980; Masur *et al.*, 1986; Souza-Formigoni *et al.*, 1999). These subpopulations of sensitized and non-sensitized mice also showed functionally relevant differences in the *N*-methyl-D-aspartate (NMDA)-mediated glutamatergic system (Quadros *et al.*, 2002). Since NMDA receptors are very important for several learning and memory processes, including contextual and spatial learning (Morris *et al.*, 1986; Ward *et al.*, 1990), it was hypothesized that contextual conditioning might be associated with individual ability to develop behavioral sensitization to EtOH. Therefore, the aims of the present study were to determine: (1) whether an association exists between performance in a contextual fear conditioning task and development of behavioral sensitization to EtOH in mice; and (2) whether previously acquired EtOH sensitization would still be expressed in a different testing environment.

Methods

Subjects

Seventy-two male albino Swiss mice, approximately 90 days old at the beginning of the experiment, were used in this study. They were kept in a temperature-controlled colony room (22°C ± 1°C), with lights on between 07.00 and 19.00 hours, with free access to food and water. All animal procedures were carried out in accordance with the National Institutes of Health (NIH) *Principles of Laboratory Animal Care* (1985).

Contextual fear conditioning

The conditioning apparatus consisted of a 22 × 21 × 22 cm compartment, with black walls and white visual patterns on the walls. The top of the apparatus was made of transparent acrylic, and the floor consisted of a metal grid (each rod measuring 0.4 cm in diameter and placed 1.2 cm apart from each other) connected to a shock generator and control module (model 7551, Ugo Basile, Varese, Italy).

Each mouse was transported individually to the test room, and was immediately put in the conditioning compartment for the training procedure. The mice were allowed to explore the environment for 2 min, when the first electric shock was delivered to the animal's paws (0.3 mA for 1 s), followed by two further shocks at 30 s intervals. After the last footshock, the animal was allowed another 1 min exploration in the compartment before it was taken back to the colony room.

Twenty-four hours later, mice were submitted to the contextual conditioning test. They were again transported individually to the test room, and immediately put in the same conditioning apparatus. No shock was delivered during this test session. Freezing time, or the time during which the animal was in complete immobility, including an absence of vibrissa movements and sniffing (Bouton and Bolles, 1980), was recorded minute by minute, for 5 min.

Ethanol treatment and locomotor activity tests

Two weeks after the contextual fear conditioning procedure, the same 72 mice were tested individually in Opto-Varimex activity cages (Columbus Instruments, Columbus, Ohio, USA), which detect locomotion by interruptions of horizontal photoelectric beams. On the first day, animals were subjected to a 15 min test for basal locomotor activity scores, without any drug treatment. They were then divided into two groups, equated in terms of basal activity, and assigned to daily ethanol (2.2 g/kg, 15% w/v in 0.9% NaCl, i.p.) ($n = 52$) or saline ($n = 20$) treatments, which started 48 h after the basal test and lasted for 21 days. Mice were taken to the test room at least 1 hour before receiving each daily injection, and locomotor activity was measured for 15 min immediately after the injection on treatment days 1 (acute), 7, 14 and 21. All procedures were carried out in the afternoon (between 13.00 and 17.00 hours).

Ethanol challenge in the open-field

One week after the end of the 21-day treatment, all mice received a challenge injection of 2.2 g/kg ethanol and were tested for locomotor activity in a different context – an open-field apparatus. The open-field arena consisted of a circular wooden arena (40 cm in diameter and 50 cm high) with an open top. The floor was divided into 18 sectors, and locomotor activity was scored as the number of lines crossed by the animal in a 5 min session (Costa *et al.*, 2001). Five minutes after injection, each mouse was set in the open-field apparatus, and its locomotor activity was recorded during 5 min by observers who were unaware of grouping and treatment conditions.

Classification

Based on the locomotor activity scores displayed by EtOH-treated mice during the 21-day treatment, it was possible to classify two behaviorally distinct groups of mice, which showed different locomotor responses to chronic EtOH treatment. EtOH-treated mice with activity scores in the upper 30% of the scores distribution on day 21 were classified as 'sensitized' ($n = 15$), whereas those in the lower 30% were classified as 'non-sensitized' ($n = 15$), as previously described (Souza-Formigoni *et al.*, 1999; Quadros *et al.*, 2002). Retrospective analyses indicated that animals classified as sensitized or non-sensitized on day 21 were also in the upper or lower 30% of the distribution either on day 7 and/or on day 14.

Ethanol challenge in activity cages

In order to confirm that EtOH sensitization was still present after 1 week of withdrawal from the 21-day treatment, mice that had been used in a previous experiment (Quadros *et al.*, 2002) were tested. These mice had received 3 weeks of daily 2.4 g/kg EtOH ($n = 36$) or saline ($n = 16$) injections and weekly locomotor activity tests in Opto-Varimex activity cages, as described above. They were also challenged with 0.25 mg/kg MK-801 (i.p.) and saline as part of a different study (for details see Quadros *et al.*, 2002).

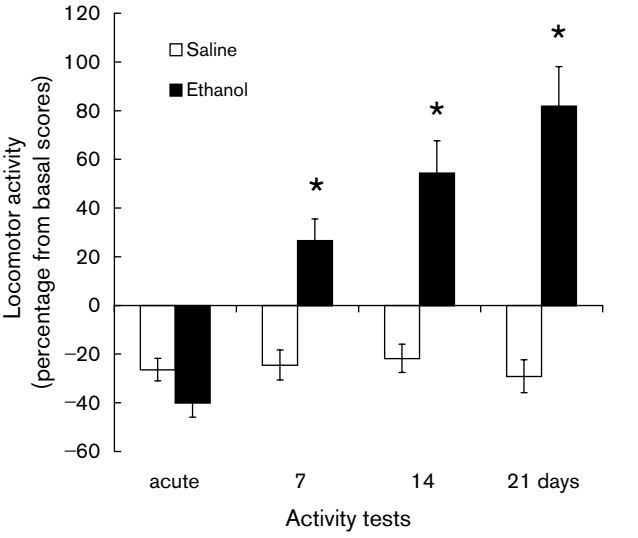
After the saline challenge, these mice were kept for 1 week with no treatment ('withdrawal'). After the withdrawal period, all mice were challenged with 2.4 g/kg EtOH and had their locomotor activity recorded in Opto-Varimex activity cages for 15 min (counts were recorded in three sessions of 5 min each for comparison with the open-field challenge data).

Results

Twenty-one-day ethanol treatment and locomotor activity

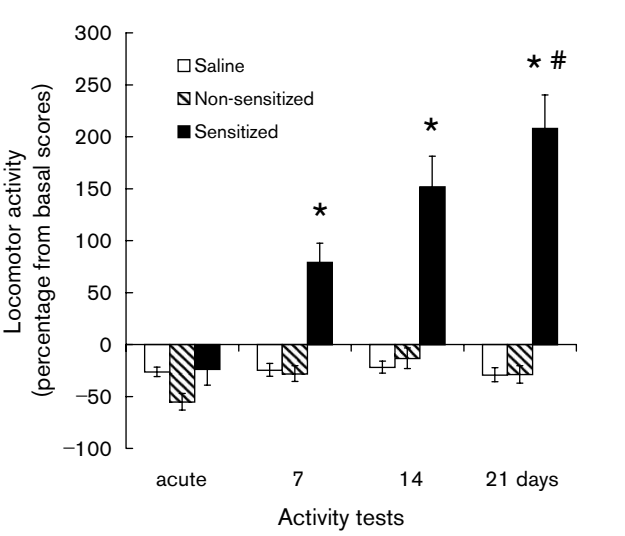
As a group, EtOH-treated mice developed behavioral sensitization to the stimulant effect of EtOH (Fig. 1). However, closer inspection of the behavioral data showed that after chronic treatment with ethanol some mice developed behavioral sensitization, while others did not (Fig. 2). When data from sensitized ($n = 15$), non-sensitized ($n = 15$) and saline-treated control mice ($n = 20$) were compared, a two-way analysis of variance (ANOVA) with repeated measures revealed significant effects of Group ($F(2,47) = 34.72$, $P < 0.001$), Time ($F(3,141) = 71.74$, $P < 0.001$) and a significant Group × Time interaction ($F(6,141) = 48.65$, $P < 0.001$). Duncan's new multiple range tests for the Group factor indicated that sensitized mice were statistically different from both non-sensitized and control mice ($P < 0.001$), while the non-sensitized group was similar to the saline controls ($P > 0.7$). Separate one-way ANOVAs for each test day revealed significant group differences on days 7 ($F(2,47) = 30.48$, $P < 0.001$), 14 ($F(2,47) = 30.55$, $P < 0.001$), and 21 ($F(2,47) = 57.64$, $P < 0.001$). These analyses also revealed that sensitized, non-sensitized and saline-treated mice were not different in their basal

Fig. 1



Mean (±SEM) locomotor activity scores for ethanol- ($n=52$) and saline-treated ($n=20$) mice across the 21-day treatment. EtOH-treated mice showed significantly higher locomotor activity levels than their own acute scores; they also differed from saline-treated mice at each marked test (* $P<0.01$).

Fig. 2



Mean (±SEM) locomotor activity scores for sensitized ($n=15$), non-sensitized ($n=15$) and saline-treated ($n=20$) mice across the 21-day treatment. EtOH-sensitized mice showed higher activity levels than both non-sensitized and saline-treated mice (* $P<0.01$); activity scores of sensitized mice on day 21 are higher than their own scores in every other test (# $P<0.01$).

activity scores during the first exposure to the activity cage, 48 h prior to the beginning of the treatment (mean locomotor activity scores ± SEM of sensitized group: 965.5 ± 276.1; non-sensitized: 1171.7 ± 283.9; and saline controls: 1098.8 ± 311.7) ($F(2,47) = 1.92$, NS). EtOH-sensitized animals were significantly more active than the other two groups on days 7, 14 and 21 ($P < 0.001$). Within-group analyses confirmed that activity scores for sensitized animals on day 21 were significantly higher than their own scores on every preceding test day ($P < 0.005$).

Contextual fear conditioning

Saline and EtOH treatment groups presented similar freezing times during the contextual fear conditioning test. The mean (± SEM) freezing time was 27.30 ± 3.24 s/min for saline controls, and 29.31 ± 2.44 s/min for EtOH-treated mice. However, when sensitized, non-sensitized and control mice were compared using a two-way ANOVA with repeated measures (each minute of the test measure was considered as one repeated measure), there were significant main effects of Group ($F(2,47) = 4.96$, $P < 0.02$) and Minute ($F(4,188) = 11.23$, $P < 0.001$), but no Group × Minute interaction. Duncan's new multiple range tests for the Group factor indicated that mice classified as sensitized displayed significantly more freezing behavior than both non-sensitized and control mice ($P < 0.02$), as shown in Fig. 3.

Ethanol sensitization in 'high'- and 'low'-freezing responders

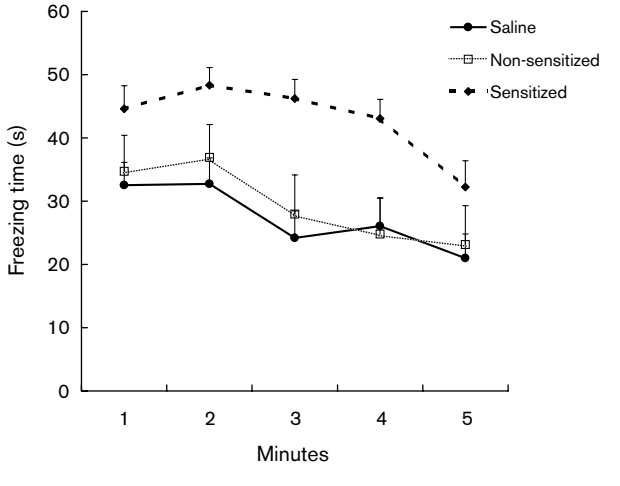
In order to further probe possible relationships between EtOH sensitization and freezing responses, the same data were reanalyzed using freezing behavior during the contextual fear conditioning test as the grouping variable. EtOH-treated mice ($n = 52$) with freezing scores in the upper 30% of the distribution were classified as 'high-freezing responders' ($n = 15$), while those in the lower 30% of the distribution were classified as 'low-freezing responders' ($n = 15$). 'High'- and 'low'-freezing responders were significantly different in freezing scores; mean (± SEM) freezing time for high responders was 49 ± 0.82 s/min, while for low responders it was 7 ± 0.79 s/min ($P < 0.001$). A two-way ANOVA for EtOH-induced locomotor activity along the four activity tests showed that high-freezing responders presented significantly higher locomotor activity levels than both the low-freezing responders and the saline group (Group effect: $F(2,47) = 6.87$, $P < 0.01$), as shown in Fig. 4 (Test and Group × Test interaction were also significant: $F(3,141) = 43.6$, $P < 0.01$, and $F(6,141) = 15.34$, $P < 0.01$, respectively).

Separate one-way ANOVAs for each test day indicated a significant Group effect on days 7, 14 and 21 ($P < 0.02$), as shown in Fig. 4. On days 14 and 21, high-freezing responders displayed higher locomotor activity when compared to low responders and saline-treated animals ($P < 0.03$).

Locomotor response to ethanol challenge in the open-field

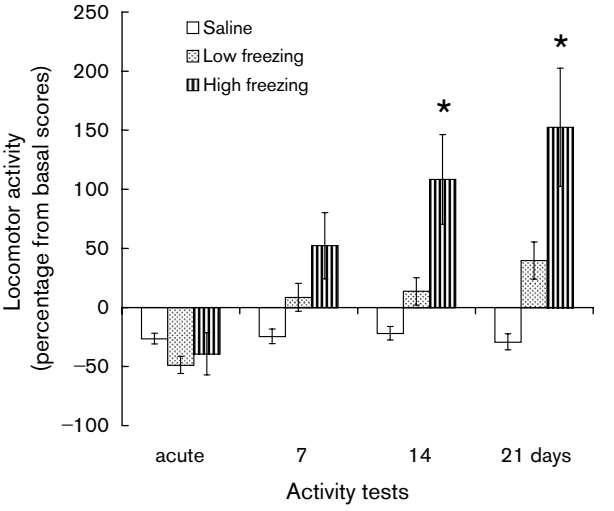
When mice pretreatment with EtOH ($n = 52$) or saline ($n = 20$) were given an EtOH challenge injection and

Fig. 3



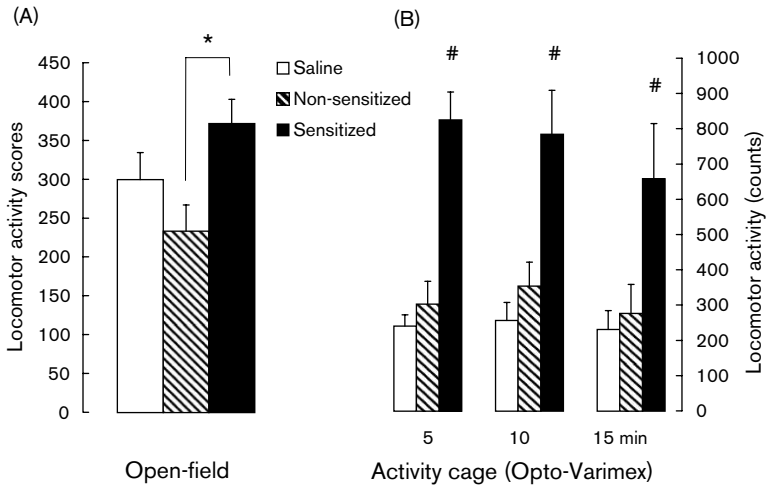
Mean (± SEM) freezing time of sensitized ($n = 15$), non-sensitized ($n = 15$) and saline-treated ($n = 20$) mice in each minute of the 5 min contextual fear conditioning test carried out prior to any drug treatment. EtOH-sensitized mice showed higher freezing scores than both other groups ($P < 0.02$).

Fig. 4



Mean (± SEM) locomotor activity scores (in Opto-Varimex cages) of mice classified according to freezing scores in the contextual fear conditioning test. High freezing responders (animals with the highest 30% of freezing scores, $n = 15$) showed higher activity than low freezing responders (animals with the lowest 30% of freezing scores, $n = 15$) and saline-treated mice ($n = 20$) on days 14 and 21 (* $P < 0.05$).

Fig. 5



After 1 week of treatment withdrawal, all mice were challenged with EtOH. (A) Mean (± SEM) locomotor activity scores displayed by sensitized ($n=15$), non-sensitized ($n=15$) and saline-treated ($n=20$) mice challenged with 2.2 g/kg ethanol (i.p.) and tested in the open-field for 5 min, after 1 week withdrawal from the 21-day treatment. No sensitization was expressed by any of the EtOH-treated groups when compared with the acute ethanol-induced response from saline pretreatment mice. However, EtOH-sensitized mice showed higher activity scores than non-sensitized mice (* $P<0.01$). (B) Mean (± SEM) locomotor activity scores displayed by sensitized ($n=10$), non-sensitized ($n=9$) and saline-treated ($n=16$) mice challenged with 2.4 g/kg ethanol (i.p.) and tested for locomotor activity in Opto-Varimex cages (the same used during the 21-day treatment), as a separate study. Data shown refer to each 5 min period from the 15 min test session. When challenged in the same testing environment previously paired to ethanol, sensitized mice showed higher activity levels than both saline and non-sensitized mice (# $P<0.001$).

their locomotor activity was measured in the open-field, no sensitization was observed: EtOH-pretreatment mice were not different from saline-treated mice ($F(1,70) = 0.67$. NS).

When only the selected EtOH subgroups (sensitized and non-sensitized) and the control group were analyzed, however, there was a significant Group effect ($F(2,47) = 4.50$, $P < 0.05$). Duncan's new multiple range tests indicated that neither of the EtOH-treated subgroups differed from the saline-treated group, indicating that no sensitization was expressed even by sensitized mice. However, sensitized animals presented higher locomotor activity scores than non-sensitized animals ($P < 0.01$), as shown in Fig. 5A.

Ethanol challenge in activity cages

When EtOH-treated mice ($n = 36$) were challenged in Opto-Varimex activity cages after 1 week of withdrawal, as part of a separate experiment, they presented significantly higher activity levels than saline-treated ($n = 16$) mice receiving EtOH for the first time ($F(1,47) = 7.73$. $P < 0.01$). When sensitized ($n = 10$), non-sensitized ($n = 9$) and saline-treated mice were compared, sensitized mice presented higher activity levels than both other groups in any of the 5 min periods of the total 15 min test, as shown in Fig. 5B (only the Group effect was significant in the two-way ANOVA: $F(2,30) = 16.8$, $P < 0.001$). Therefore, EtOH-sensitized mice still expressed behavioral sensitization after 1 week of withdrawal from treatment, as long as they were tested in the same testing environment used during the treatment phase.

Discussion

The most interesting finding of this study was that mice classified as sensitized to the stimulant effect of EtOH presented higher freezing scores than non-sensitized mice in a contextual fear conditioning task carried out prior to any EtOH treatment. Although several other studies have shown the importance of contextual cues for behavioral sensitization to be induced and/or expressed, to our knowledge these data provide the first indication that the ability to perform a contextual learning task may be closely associated with the susceptibility to develop behavioral sensitization to EtOH.

These data point in the same direction as results obtained by Radcliffe *et al.* (1998) working with acute functional tolerance to EtOH. Those authors reported that mice showing high acute functional tolerance (HAFT) to EtOH presented higher freezing response in the contextual fear conditioning test than LAFT (low acute functional tolerance) mice. They also suggested that the correlation between tolerance and memory processes may be due to an involvement of similar or closely related neural circuitry in both phenomena (Radcliffe *et al.*, 1998). Our data are in accordance with the idea that behavioral sensitization to EtOH may also share some of these common pathways for neuroplasticity.

Pharmacological experiments support the idea of a common pathway for plasticity processes by showing that glutamatergic NMDA receptor antagonists are able to reduce or block several of these phenomena. NMDA antagonists have been shown to block several memory and learning tasks (Morris *et al.*, 1986; Venable and Kelly, 1990; Ward *et al.*, 1990;), several types of tolerance (Wu *et al.* 1993; for a review, see Trujillo and Akil, 1995) and also behavioral sensitization to several drugs of abuse (see Sripada *et al.* 2001 for a review on psychostimulant-induced sensitization; for EtOH-sensitization: Broadbent and Weitemier, 1999; Camarini *et al.*, 2000). If similar cellular mechanisms underlie these phenomena, it seems reasonable to suggest that characteristics underlying the susceptibility to EtOH sensitization may overlap characteristics that determine contextual learning, as suggested by the present data.

It is interesting to note that the observed association between freezing and sensitization was also obtained when EtOH-treated mice were classified as 'high' or 'low' responders in terms of freezing response to the context: the 'high' responder group was found to show more sensitization than the 'low' responder group. This characteristic does not seem to be due to a generalized higher response to EtOH in high-freezing responders, since 'high' and 'low' mice presented similar responses to EtOH in the acute locomotor activity test and also in the EtOH challenge in the open-field (data not shown).

A second hypothesis for explaining the association between contextual conditioning and EtOH sensitization, which does not preclude the hypothesis of a common pathway for plasticity, concerns a specific role for contextual learning in the development of behavioral sensitization. Robinson *et al.* (1998) have shown that circumstances surrounding drug administration may interfere with the induction, or development, of drug-induced sensitization. These authors showed that the absence of drug-related stimuli (such as the presence of the experimenter, the needle prick and the specific association of the testing chamber to drug administration) was able to completely block the acquisition of sensitized rotational behavior induced by repeated amphetamine administration. If contextual cues are important in determining whether sensitization will or will not develop, it seems plausible that the variation in the ability to learn contextual cues may influence the ability to develop behavioral sensitization to EtOH, as observed in the present study.

Alternatively, it is also possible that common factors affecting both behavioral sensitization and contextual conditioning could play a role in the observed association of these phenomena. One of these putative factors might be stress reactivity. In the present study, it was not possible to rule out the possibility that the difference in freezing response could be related to different responses to footshock stress, and not to differences in learning ability, as suggested above. This possibility is interesting and relevant, since stress also influences ethanol sensitization (Phillips *et al.*, 1997; see also Hoshaw and Lewis, 2001). However, 'high'- and 'low'-freezing responders (as well as 'sensitized' and 'non-sensitized' mice) did not show different locomotor scores when first exposed to the activity cage with no drug treatment ('basal activity test'), suggesting that the animals did not react differently, at least to novelty stress. Nonetheless, since reactivity to novelty stress may be different from reactivity to other types of stressors (such as footshock or the ethanol injection procedures), more studies would be necessary to address this possibility.

The present results therefore suggest that the development of EtOH sensitization seems to be positively associated with contextual learning, although further studies are necessary to determine possible mechanisms for this association. Curiously, an opposite association seems to be observed in C57 and DBA mice lines. While C57 mice are less sensitive to EtOH stimulation and sensitization than DBA mice (Phillips *et al.*, 1994; Lessov *et al.*, 2001), the former showed better contextual learning performance than the latter (Paylor *et al.*, 1994). However, one must notice that C57 and DBA mice strains present several other neurobiological differences that may be associated with the differential susceptibility of these mice to EtOH sensitization.

Blockade of the expression of ethanol sensitization

A second interesting finding of this study was that when all mice were challenged with EtOH in a different context (the open-field), after 1 week of withdrawal from EtOH or saline treatment, there was no sensitization in EtOH-treated animals. EtOH-sensitized mice did not present higher activity levels than their saline counterparts that received EtOH for the first time, indicating the lack of expression of behavioral sensitization in the open-field. This observation supports the idea that context-related cues may gain control over the expression of behavioral sensitization, as proposed by Stewart and Badiani (1993). The present data are in accordance with several studies reporting the reduction or complete blockade of the expression of sensitization when animals are challenged in an environment that had never been specifically paired to drug injections (Cunningham and Noble, 1992; Anagnostaras and Robinson, 1996; Robinson *et al.*, 1998).

The possibility that EtOH-sensitization could have disappeared after 1 week of termination of drug treatment seems unlikely, since this process seems to be a long-lasting phenomenon (Lessov and Phillips, 1998; Fish

et al., 2002). Data from a separate experiment confirmed that EtOH sensitization persisted for at least 1 week, as long as mice were challenged in the original drug-paired testing environment. In that experiment, activity scores from sensitized mice were approximately three times greater than saline-pretreatment mice receiving EtOH for the first time.

Interestingly, however, EtOH-sensitized and non-sensitized mice still presented different locomotor responses to EtOH when tested again in the open-field. Thus, while the alterations in the testing context were sufficient to block the expression of sensitization, sensitized mice still displayed higher locomotor activity than non-sensitized mice in the open-field (although neither groups differed from saline pretreatment animals). This difference does not seem to be due to a generalized higher response to novelty (the open-field) by sensitized mice, in comparison to their non-sensitized counterparts, since these groups presented similar locomotor responses in the first activity test in Opto-Varimex cages, as previously mentioned. Rather, the maintenance of this differential response to EtOH may be due to the fact that the remaining contextual cues surrounding drug administration – other than the testing context itself – could still play a role in the differential locomotor response of sensitized and non-sensitized mice in the open-field. Another likely possibility is that at least part of the differential response to EtOH in these subgroups could be due to context-independent pharmacological factors. These possibilities are currently under investigation.

In summary, this study demonstrated that mice showing high versus low levels of sensitization to EtOH showed correspondingly different freezing responses in a contextual fear conditioning task carried out prior to any drug treatment. Sensitized mice displayed higher freezing scores than non-sensitized mice, suggesting that a higher contextual learning performance may be positively associated with the development of EtOH sensitization. Furthermore, EtOH sensitization was completely blocked when animals were challenged in a different testing context, confirming an important role for contextual cues in the expression of sensitization. However, sensitized mice still presented a higher response to EtOH than non-sensitized mice in the altered testing context. More studies are necessary to understand the role of contextual conditioning in EtOH sensitization and the possible mechanisms that underlie the convergence of these phenomena.

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