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ORIGINAL INVESTIGATION

Free versus forced exposure to an elevated plus-maze: evidence for new behavioral interpretations during test and retest

Vincent Roy · Pierre Chapillon · Mustapha Jeljeli · Jean Caston · Catherine Belzung

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Abstract
Rationale The rodent elevated plus-maze is based on an approach/avoidance conflict between secure closed arms and aversive open arms that can be measured to assess anxiety. Despite this apparent simplicity, several discrepancies emerge from the interpretation of an animal's behavior in the maze, especially when considering the one-trial tolerance effect.
Objectives and methods In order to bring new elements of interpretation, we compared the behavior of rats exposed to the standard version of the test (forced exposure) to the behavior of rats that were allowed to freely explore the apparatus. We also compared the effects of testing/retesting and chlordiazepoxide in these two situations.
Results Our results confirm that open-arm avoidance is a natural tendency and therefore that it is not learned during initial exposure to the maze. In addition, comparison of the two situations suggests that some of the open-arm entries during a forced confrontation with the maze are better interpreted as attempts to avoid the whole situation, rather than as indications of a low level of anxiety. Finally, the one-trial tolerance effect was partially reduced in the free-exposure situation.
Conclusions Our results contradict the hypothesis that there is acquisition of a phobic-like response to open arms during trial 1. Rather, they are discussed in line with the hypotheses by Rodgers and Shepherd (*Psychopharmacology* (Berl) 113:237–242, 1993) and Bertoglio and Carobrez (*Behav Brain Res* 108:197–203, 2000) concerning the acquisition of spatial information about the whole apparatus, leading on trial 2 to an unbalanced approach/avoidance conflict and to the inefficiency of anxiolytic drugs.

Keywords Elevated plus-maze · One-trial tolerance · Free exposure · Anxiety · Emotional reactivity · Rats

Introduction

In the field of behavioral neurosciences, the elevated plus-maze is currently the most widely used model to evaluate rodent anxiety-like behaviors. Based on the initial work by Montgomery (1955) on the relationship between fear induced by novelty and exploratory behavior, the elevated plus-maze was developed by Handley and Mithani (1984) as an animal test for state anxiety; it was validated by Pellow et al. (1985) in rats, and by Lister (1987) in mice. Briefly, the elevated plus-maze makes it possible to measure "anxiety-like behaviors" based on the a priori postulate that rodents exposed to the apparatus will respond to a conflict between safe parts of the maze that are closed and protected, and aversive parts of the maze that are open, unprotected, elevated, and more brightly lit.

V. Roy (✉) · P. Chapillon · J. Caston
UPRES PSY-NCA, EA4306,
Laboratoire de Psychologie et Neurosciences de la Cognition et de l'Affectivité, European Institute for Peptide Research (IFRMP 23), Université de Rouen,
76821 Mont-Saint-Aignan, Cedex, France
e-mail: vincent.roy@univ-rouen.fr

M. Jeljeli
Laboratoire de Physiologie Intégrée,
Faculté des Sciences de Bizerte,
7021 Jarzouna, Tunisia

C. Belzung
INSERM U930,
Université François Rabelais de Tours,
Parc Grandmont,
37200 Tours, France

The elevated plus-maze has been extensively used in a wide range of research fields in order to examine the effects of a variety of procedures (such as lesions, early stimulation, toxic substances) on anxiety-like behaviors (Carobrez and Bertoglio 2005); it is also the standard for screening the efficiency of pharmacological compounds (Rodgers 1997). As stated by Carobrez and Bertoglio (2005), the apparent simplicity, low cost and simple testing procedure of the elevated plus-maze has helped to make this device a valuable tool for the fields of the animal neurosciences and pharmacology.

However, despite its very wide utilization, our understanding of the behavior in the elevated plus-maze remains far from complete, and interpretations of the parameters that are measured in the test are questionable. For instance, the test has been pharmacologically validated and it is generally admitted that parameters such the number of open-arm entries, open-arm time, or associated ratios are related to anxiety behaviors (Lister 1987; Pellow et al. 1985). However, whether avoidance of open arms is really an unconditioned process, or whether it is rather something acquired during exposure to the test, is still very much an open question (Carobrez and Bertoglio 2005; Holmes and Rodgers 1999). Indeed, rodent aversion for open space is supposed to be natural; but the fact that open-arm entries actually decrease during exposure to the plus-maze argues in favor of some kind of learning of open-arm avoidance. This is especially relevant with respect to some potential explanations of the one-trial tolerance effect, consisting of a high avoidance of open arms and a decreased effectiveness for some anxiolytic drugs during re-exposure to the maze (e.g., Calzavara et al. 2005; Holmes and Rodgers 1999; Rodgers et al. 1996a, b, 1997; Rodgers and Shepherd 1993).

Other parameters such as closed-arm entries or total arm entries have been related to locomotion or activity in the maze. This was the case in several studies that used behavioral approaches along with principal component analyses, where closed-arm or total arm entries loaded on a factor that was defined as locomotion or activity (Cruz et al. 1994; Espejo 1997; Rodgers and Johnson 1995). However, it has also been shown that it may not be so simple to make a clear dissociation between anxiety and locomotion in the plus-maze (Dawson et al. 1995; Weiss et al. 1998). Indeed, when processes such as exploratory drive or motivation to escape are taken into account, the view of a simple motor activity factor for these parameters becomes inappropriate. The dissociation between locomotion driven by fear and that driven by exploration is a complex problem, that has already been extensively discussed with respect to open-field behavior—with no real solution (Boissier and Simon 1969; Denenberg 1969; Misslin et al. 1976). In order to overcome these confounding effects, the addition of ethological parameters has been strongly suggested (Carobrez and Bertoglio 2005; Rodgers and Dalvi 1997; Roy and Chapillon 2004; Weiss et al. 1998), nevertheless, the interpretation still remains complex. The fact that animals are forcibly exposed to the elevated plus-maze seems to be an important feature to consider (Belzung 1999; Belzung and Griebel 2001).

Considering these previous points, what the elevated plus-maze is really measuring can also be questioned from a more conceptual point of view. For instance, the current view of a conflict between "safe" and "aversive" parts of the maze in order to explain the animal's behavior raises some unresolved questions. In particular, the possibility that open-arm avoidance may be learned rather than unconditioned (e.g., Carobrez and Bertoglio 2005; Holmes and Rodgers 1999) has some awkward consequences. If open-arm avoidance actually has to be learned, this would mean that the "approach/avoidance conflict" which is theoretically supposed to explain the behavior could not be properly effective at the time a rodent is first introduced into the maze. The rodent would initially have to explore and learn the different parts of the maze *before* the "approach/avoidance conflict" can really be invoked in order to explain its behavior. Since other processes such as fear could interfere with a forced exploration, even for the supposedly "safe" closed arms, the plus-maze should be viewed not so much as a conflict test, but rather as an exploration test involving a conflict that is progressively resolved according to various coping strategies and progressive exploration of the maze. This view might help to interpret atypical behaviors such as a rapid open-arm entry followed by a long bout of freezing.

In order to contribute some new elements to these issues, we have conducted experiments in which rats were subjected either to a classical version of the elevated plus-maze (with forcible exposure), or to a free version in which the animals were allowed to freely explore the maze from the end of a closed arm and gradually discover the aversive parts of the maze. The aim of this comparison was notably to test whether avoidance of open arms corresponds to an unconditioned or to a learned process; but also to obtain new information about the real value of open-arm entries for the animal in terms of approach/avoidance conflict. In addition, since free exploration of the apparatus gives an opportunity for the animal to elaborate a progressive exploration of the maze, with no behavioral interferences such as locomotion in an attempt to escape from the apparatus (Belzung 1999; Belzung and Griebel 2001), we expected a higher value for some ethological parameters such as closed-arm returns and stretched attend postures (SAPs). Finally, in order to bring the information from this free exploration paradigm to bear on the one-trial tolerance effect, some animals were re-exposed to the maze 24 h after initial exposure and the effects of chlordiazepoxide (CDZ) were also evaluated during both trials 1 and 2.

Materials and methods

Animals

DA/Han rats (also referred as Dark Agouti) were tested in our study. This strain is not very widely used but it has been well characterized in our laboratory, especially with respect to emotional reactivity tests (Chapillon et al. 2002; Patin et al. 2005; Roy and Chapillon 2002, 2004; Roy et al. 2003); moreover, it has been described as "anxious" compared to other strains such as Sprague–Dawley or Hooded Lister (King 1999; Mechan et al. 2002). This latter aspect makes the strain relevant for our study, especially in order to avoid ceiling effects for emotional behaviors and to obtain anxiolytic effects for CDZ treatments.

A total of 93 DA/Han male rats (2–4 months) from our own breeding colony were tested. Throughout the experiment, the animals were maintained in an air-conditioned room (21±2°C) under a 12-h light–dark cycle (lights on at 00h00). Groups of three animals (±1) were maintained in standard polycarbonate cages (40×28×18 cm) with free access to food and water.

The research reported in this paper was conducted in accordance with the guide for care and use of laboratory animals established by the National Institute of Health of the United States of America (1996) and with applicable guidelines from the French Ministry of Agriculture.

Behavioral testing and experiments

Animals were tested during the first part of the dark phase (between 14h00 and 18h00). The day before testing, animals were weighed and isolated for habituation in beige-painted wooden boxes (38 cm×38 cm×28 cm). These boxes were previously used in our laboratory as a "familiar compartment" for the free exploration paradigm (Roy and Chapillon 2004). The floor of each box was covered with sawdust and animals were given free access to food and water. Each box had a small removable door (8 cm×12 cm) on one of its sides. All tests were video-recorded and analyzed with Etholog 2.25 (Ottoni 2000).

Experiment 1: forced (center) vs. free exposure

In the first experiment, we compared the behavior of DA/Han rats forcibly exposed to a standard version of the elevated plus-maze (n=12), with that of DA/Han rats freely exposed to the elevated plus-maze (n=11). Forcibly exposed rats were taken from their habituation cage and placed in the middle part of the maze, facing an open arm, and allowed to explore the maze for 5 min. In the free-exposure condition, the rats were allowed to freely explore the maze from their habituation box that was positioned next to the maze and connected to the end of a closed arm by the small removable door. The door was removed and the rats' behavior was then recorded for as long as necessary to obtain 5 min of presence in the maze after the initial entrance. At the end of trial 1, the forcibly and freely exposed rats were taken back to their familiarization cages and re-exposed 24 h later (trial 2) to exactly the same condition (forcible or free exposure, respectively).

Animals from the free-exposure condition that did not enter the maze for 5 min after 20 min of test were not included in the statistical analyses. In experiment 1, only one animal was removed from the statistical analyses due to this criterion.

Experiment 2: forced (end of a closed arm) vs. free exposure

In experiment 2, we also compared forcibly and freely exposed rats. However, this time, the rats from the forced condition were introduced into the plus-maze by the end of the same closed arm that was used for free exploration. This procedure was used in order that both forcibly and freely exposed rats had an exploration of the maze that began with a secure closed arm, rather than from the ambiguous central part for one group and from the end of a closed arm for the other group. In addition, in order to better understand the behavior of the rats in the free-exposure version of the test, we modulated the animals' anxiety by using chlordiazepoxide (CDZ). CDZ was chosen since it is a well-known anti-anxiety compound whose effects have been well evaluated in the standard version of the plus-maze. Finally, since CDZ is prone to the one-trial tolerance effect in the forced exposure condition, the effects of CDZ in the free-exposure condition were also evaluated during a second exposure to the maze (trial 2), 24 h after the initial exposure.

Seventy rats with a mean weight of 218.0±28.4 g were used in experiment 2. These rats were tested on trial 1 in the forced or in the free condition with either an IP injection of NaCl (9‰), a 2.5 mg/kg or a 5.0 mg/kg IP injection of chlordiazepoxide hydrochloride (Sigma). On trial 2, the rats that were tested on trial 1 with NaCl were randomly tested under NaCl, 2.5 mg/kg or 5.0 mg/kg dose of chlordiazepoxide (CDZ). Fresh CDZ (Sigma) was mixed daily and dissolved into NaCl (9‰) so as to inject a volume of 0.2 ml for a 200 g rat.

Five freely exposed animals in experiment 2 were discarded from statistical analyses due to the time criterion mentioned above. The numbers of rats per group in experiment 2 are presented in Table 1.

Elevated plus-maze

The elevated plus-maze was used in conditions close to previous work in our laboratory (Roy and Chapillon 2004). The maze was made of clear painted wood, the arms were 50 cm long and 10 cm wide, and the apparatus was elevated at a height of 50 cm. The closed arms were surrounded by a 50 cm wall while open arms had 0.5 cm edges in order to

Table 1 Number of rats per group (NaCl, 2.5 mg/kg and 5.0 mg/kg of CDZ) in the forced exposure and in the free-exposure conditions during trial 1 and trial 2 of Experiment 2

Condition	Trial 1	Trial 2
Forced	NaCl (n=22)	NaCl (n=8)
		CDZ 2.5 (n=7)
		CDZ 5.0 (n=7)
	CDZ 2.5 (n=9)	–
	CDZ 5.0 (n=8)	–
Free	NaCl (n=17)	NaCl (n=7)
		CDZ 2.5 (n=5)
		CDZ 5.0 (n=5)
	CDZ 2.5 (n=7)	–
	CDZ 5.0 (n=7)	–

maximize open-arm entries (Treit et al. 1993). Red light (5 lx on open arms and center, 1 lx at the extremities of closed arms) was chosen to provide low anxiogenic conditions. Closed arms and open arms were divided into a proximal and a distal part and part-entries were observed so as to obtain a more sensitive measure of locomotion within the maze. Time and number of entries in the different parts of the maze and numbers of rears, were recorded as standard measures. Time in the open parts of the maze was also presented as % in tables [(time open/session duration)×100]. A four-paw criterion validated an entrance into an arm, and the animal was considered out of the arm when two paws had left the arm. Ethological measures included protected stretched attend postures (pSAPs), closed-arm returns (the rat enters the central part with two paws and then goes back into the same closed arm), head scans (the rat points its head into an open arm with its body still in the center or in a closed arm of the maze) and head dipping (Roy and Chapillon 2004).

Statistical analyses

Data were analyzed with Statistica software (5.1). Data are presented as mean±SEM and were analyzed with ANOVA since homogeneity of variance was respected for a majority of the parameters analyzed. Follow-up analyses of simple main effects of significant interactions were analyzed using Tukey's HSD tests for different ns (Spjotvoll/Stoline).

Results

Experiment 1—forced versus free exposure during test and retest

Results were analyzed using a two-way ANOVA with condition (free or forced exposure) as a principal factor and trials 1/2 as repeated measures.

The data for Experiment 1 are shown in Table 2. First, statistical analyses indicated a significant effect of interaction between condition and test repetition for time in the open arms ($F_{1,\ 21}$=5.46; p<0.05) and number of open-arm entries ($F_{1,\ 21}$=7.00; p<0.05). For both parameters there was no significant main effect for condition and repetition. Follow-up comparisons for open-arm time showed no significant difference between animals from the two conditions during trial 1 and trial 2; the p value for the comparison between forcibly and freely exposed rats on trial 1 was 0.08. In the case of open-arm entries (Fig. 1a), follow-up comparisons indicated that freely exposed rats entered the open arms less frequently than forcibly exposed rats during the first trial (p<0.01). During trial 2, this effect was abolished since open-arm entries were significantly reduced in forcibly exposed rats compared to trial 1 (p<0.05).

No significant effect was obtained for the time spent in the closed arms and in the center of the maze. However, it can be noted that freely exposed rats during trial 1 spent more time in the closed arms and less time the center part of the maze (though not significantly). This difference was completely abolished during trial 2.

Total activity in the maze was not significantly different among the two conditions (Fig. 1b) but the rats' activity was lower during trial 2 than during trial 1 ($F_{1,\ 21}$=11.14; p<0.01). The number of rears was not affected by the condition and by trial repetition.

A significant main effect for condition ($F_{1,\ 21}$=4.93; p<0.05) and for trial repetition ($F_{1,\ 21}$=6.13; p<0.05) was observed for closed-arm returns. These behaviors were more frequent in the free-exposure condition and during trial 1. Similarly, protected SAPs (Fig. 1c) were more frequent in freely exposed rats ($F_{1,\ 21}$=8.49; p<0.01) and during trial 1 ($F_{1,\ 21}$=10.93; p<0.01).

Head scan and head-dipping behaviors were less frequent during trial 2 (respectively $F_{1,\ 21}$=13.40; p<0.01 and $F_{1,\ 21}$=8.81; p<0.001).

Experiment 2—chlordiazepoxide (CDZ) effects in the forced and in the free procedures during test and retest

Chlordiazepoxide effects during trial 1

Results for this second experiment were first analyzed using a two-way ANOVA with condition (free or forced exposure) and CDZ dose (0, 2.5, and 5.0 mg/kg) as principal factors. This statistical protocol allowed the evaluation of CDZ effects on trial 1 in both forced and free conditions. The results are presented in Table 3.

Significant main effects for condition ($F_{1,64}$=7.00; p<0.05) and dose ($F_{2,64}$=18.62; p<0.001) were present for the time in open arms; there was no significant

Table 2 Behaviors of rats forcibly exposed (n=12) or freely exposed (n=11) to the elevated plus-maze in trial 1 and trial 2

	Forced (n=12)		Free (n=11)		
	Trial 1	Trial 2	Trial 1	Trial 2	
Time in open arms (s)	15.92±4.99	3.42±2.34	1.72±1.16	7.27±7.27	c
% Time spent in open arms	5.31%±1.66	0.58%±0.39	1.14%±0.78	2.42%±2.42	c
Open-arm entries	1.58±0.45	0.42±0.29°	0.18±0.12*	0.36±0.36	c
Time in closed arms (s)	178.33±10.07	209.42±14.59	213.36±10.45	205.45±11.57	
Time in the center (s)	105.75±8.62	87.17±13.92	84.91±9.84	87.27±9.79	
Total activity	34.25±1.82	28.75±8.62	32.18±2.50	27.27±2.09	b
Number of rears	12.33±1.06	10.00±0.92	10.00±0.82	9.09±0.74	
Closed-arm returns	1.08±0.40	0.25±0.13	1.91±.051	1.18±0.30	a, b
Protected SAPs	2.08±0.42	1.25±0.25	4.18±0.55	2.63±0.69	a, b
Head scans	11.58±0.79	8.33±1.08	12.09±1.15	9.18±1.14	b
Head dips	5.42±1.16	1.83±0.60	4.45±0.61	3.27±0.66	b

Data are given as mean±SEM

(a) Significant main effect of condition, *(b)* significant main effect of trial repetition, *(c)* significant interaction between condition and trial repetition

*p<0.05, comparison of forcibly and freely exposed groups on the same trial; °p<0.05 comparison of trial 1 and trial 2 in the same condition of exposure

interaction between these factors. Forcibly exposed animals spent more time in the open arms; and follow-up comparisons showed that CDZ doses (2.5 and 5.0 mg/kg) increased open-arm time (respectively p<0.05 and p<0.001). Results for the number of open-arm entries were similar (Fig. 2a): significant main effects of condition ($F_{1,64}$=6.81; p<0.05) and dose ($F_{2,64}$=12.44; p<0.001) were observed. Forcibly exposed animals entered open arms more frequently; and both CDZ doses (2.5 and 5.0 mg/kg) increased open-arm entries in comparison to NaCl (respectively, p<0.05 and p<0.001). No significant interaction effect was noted for open-arm entries.

The opposite effects were observed for the time spent in closed arms. Significant main effects for condition ($F_{1,64}$= 14.52; p<0.001) and dose ($F_{2,64}$=6.89; p<0.001) were obtained, and this time freely exposed animals were the ones spending more time in closed arms. In addition, CDZ reduced closed-arm time but only the 5.0 mg/kg dose was significantly different from NaCl (p<0.05). The p value for the 2.5 mg/kg dose in comparison to NaCl was 0.051. Freely exposed rats also spent less time in the center of the maze compared to forcibly exposed rats ($F_{1,64}$=7.83; p<0.01); CDZ had no effect on this parameter.

Total activity in the maze was not significantly different between the two conditions (Fig. 2b). It was globally enhanced by CDZ ($F_{2,64}$=3.91; p<0.05) but follow-up comparisons showed no significant difference.

Closed-arm returns were roughly two times more frequent in the freely exposed group, but this result was not statistically significant (p=0.10). Protected SAPs were more frequent in freely exposed rats, but this result was again not statistically significant (Fig. 2c). In addition, CDZ reduced pSAPs ($F_{2,64}$=3.88; p<0.05), and the follow-up comparisons showed a significant effect at the dose of 5.0 mg/kg (p<0.05).

Finally, no significant effect was observed for the number of rears and for the number of head scans. However, head dipping was significantly affected by the condition ($F_{1,64}$= 12.39; p<0.001) and by CDZ dose ($F_{2,64}$=12.47; p<0.001). Head dipping was less frequent in freely exposed animals and was enhanced at 2.5 and 5.0 mg/kg of CDZ (respectively p<0.05 and p<0.001).

Chlordiazepoxide effects during trial 2 in forced and free-exposed rats

Animals injected with NaCl on trial 1 were randomly ascribed for trial 2 to one of the following groups: NaCl, CDZ (2.5 mg/kg) or CDZ (5.0 mg/kg). Since behavior on day 1 was not exactly the same in animals from these groups (though no significant difference was obtained), a score was calculated from the difference between trial 2 and trial 1 for each behavior (see Table 4). This score was then analyzed by a two-way ANOVA with condition (free or forced exposure) and CDZ dose (NaCl, 2.5 and 5.0 mg/kg) as principal factors.

The trial 2–trial 1 difference for the time spent into the open arms was higher in freely exposed rats (significant main effect of condition: $F_{1,33}$=16.25; p<0.001) and in rats injected with a 5.0 mg/kg of CDZ (significant main effect of dose: $F_{2,33}$=5.98; p<0.01 and follow-up comparison with NaCl rats: p<0.05). The interaction between condition and dose was also significant ($F_{2,33}$=7.87; p<0.01) and explained by a significantly higher trial 2–trial 1 difference in rats that were freely exposed and treated with 5.0 mg/kg of CDZ compared to rats from the same condition but injected with

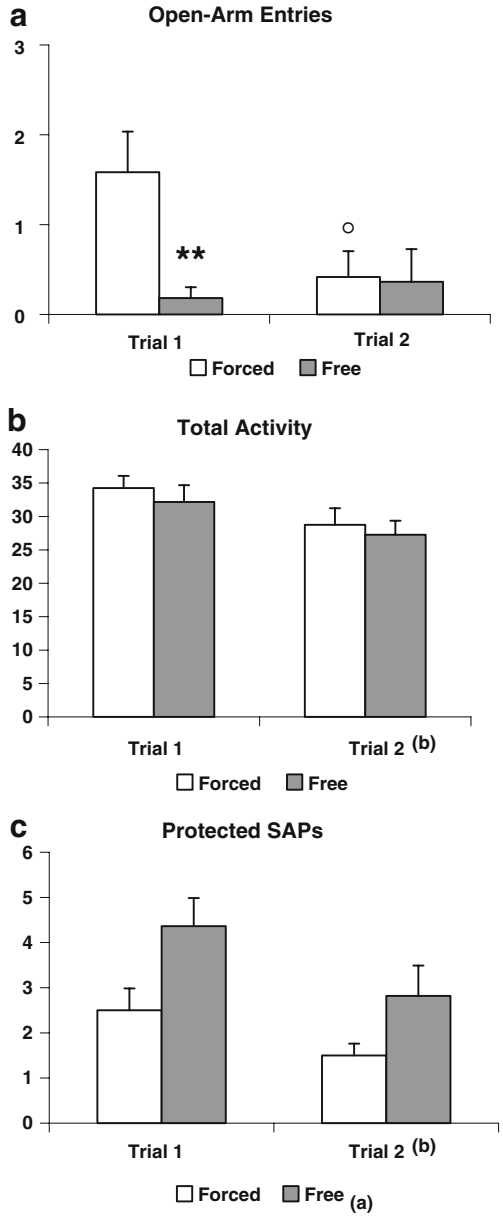

Fig. 1 Open-arm entries, total activity and protected saps in rats from the forced exposure (n=12) and in rats from the free-exposure (n=11) conditions. Data are given as mean±SEM. Statistics: **a** significant main effect of condition, **b** significant main effect of trial repetition. Follow-up comparisons: *p<0.05, comparison of forcibly and freely exposed rats on the same trial; °p<0.05 comparison of trial 1 and trial 2 in the same condition of exposure

NaCl (p<0.01) and compared to rats forcibly exposed and injected with 5.0 mg/kg of CDZ (p<0.001).

Results for the trial 2–trial 1 difference in open-arm entries yielded similar results. Significant main effects for condition ($F_{1,33}$=19.96; p<0.001), CDZ dose ($F_{2,33}$=6.99; p<0.01) and the interaction between the two factors ($F_{2,33}$=4.63; p<0.01) were observed. As seen in Fig. 3a, the trial 2–trial 1 difference was positive in freely exposed rats and significantly higher than in forcibly exposed rats. In addition, the difference was higher in rats injected with CDZ 5.0 mg/kg (p<0.01 compared to NaCl) and the interaction was explained by significant differences between rats freely exposed to the maze and treated with 5.0 mg/kg of CDZ compared to rats from the same condition but injected with NaCl (p<0.01) and compared to rats injected with 5.0 mg/kg of CDZ but forcibly exposed to the maze (p<0.01). Conversely, the trial 2–trial 1 difference for closed-arm time was significantly lower in freely exposed rats ($F_{1,33}$=8.93; p<0.01). No other main effect or interaction effect was noted for this parameter as well as for time in the center and total activity (Fig. 3b) and protected SAPs (Fig. 3c). Other parameters such as head scans and rears were also more frequent during trial 2 in freely exposed rats while less frequent in forcibly exposed rats. A significant main effect of condition was thus observed for these parameters (respectively, $F_{1,33}$=7.21; p<0.05 and $F_{1,33}$=7.60; p<0.01).

Head-dipping behavior paralleled open-arm entries since condition, CDZ dose and interaction between the two factors both showed significant effects (respectively, $F_{1,33}$=7.61; p<0.01, $F_{2,33}$=4.97; p<0.05, $F_{2,33}$=7.42; p<0.01). Head dips on trial 2 were more frequent in freely exposed rats and in the CDZ 5.0 mg/kg dose (p<0.05 compared to the NaCl condition). In addition, the interaction was explained by a higher trial 2–trial 1 difference for head dipping in freely exposed rats injected with 5.0 mg/kg of CDZ compared to forcibly exposed rats also injected with 5.0 mg/kg of CDZ (p<0.01) or compared to NaCl freely exposed rats (p<0.01).

Closed-arm returns on trial 2 were constant in forcibly exposed animals, but significantly reduced in the free-exposure condition ($F_{1,33}$=8.21; p<0.01). There was no CDZ dose effect but a significant main effect for interaction ($F_{2,33}$=7.67; p<0.01); follow-up comparisons showed that freely exposed rats from the CDZ 5.0 mg/kg dose realized less closed-arm returns than CDZ 5.0 mg/kg rats exposed by force (p<0.05), and NaCl rats freely exposed (p<0.05).

Discussion

The aim of our experiments was to contribute some new observations in order to aid the interpretation of rodents' behavior in the widely used elevated plus-maze. To this end, we compared the behavior of rats exposed to the standard version of the test (i.e., forced exposure) to the behavior of rats that were allowed to freely explore the apparatus from a familiar cage. Our experiments provide several interesting points of discussion.

Open-arm avoidance is unconditioned

Firstly, the observation of rats that explore the maze freely shows that the avoidance of open arms is clearly an

Table 3 CDZ effects in rats forcibly exposed or freely exposed to the elevated plus-maze

	Controls		CDZ 2.5 mg/kg		CDZ 5.0 mg/kg		
	Forced (n=22)	Free (n=17)	Forced (n=9)	Free (n=7)	Forced (n=8)	Free (n=7)	
Time in open arms (s)	13.73±3.03	5.00±2.80	31.22±9.64	19.57±6.59	55.25±7.91	34.86±11.84	a, b *, °°°
% Time spent in open arms	4.58%±1.01	1.67%±0.93	10.41%±3.21	6.52%±2.20	18.42%±2.64	11.62%±3.95	a, b *, °°°
Open-arm entries	1.45±0.28	0.47±0.23	2.55±0.65	2.14±0.67	4.13±0.72	2.29±0.84	a, b *, °°°
Time in closed arms (s)	175.64±5.83	216.94±11.03	141.33±15.82	187.86±13.95	147.88±13.19	173.29±11.43	a, b, °
Time in the center (s)	110.64±5.43	78.06±9.64	127.44±11.74	92.57±11.96	96.88±15.18	91.86±4.32	a
Total activity	31.59±1.30	30.00±2.59	31.67±2.98	41.00±5.16	37.13±4.09	39.71±4.79	b
Number of rears	11.14±1.16	6.71±1.05	10.56±1.59	10.57±2.17	8.63±1.53	6.71±0.56	
Closed-arm returns	0.64±0.19	1.35±0.38	0.89±0.48	1.00±0.58	0.63±0.42	1.57±0.69	
Protected SAPs	2.27±0.30	3.65±0.66	3.11±0.59	3.57±0.53	1.38±0.50	1.86±0.46	b °
Head scans	12.68±0.65	10.35±1.11	12.11±1.05	12.71±1.67	10.00±0.65	12.43±1.17	
Head dips	4.73±0.49	3.18±0.70	9.22±1.87	5.14±1.50	11.00±1.10	6.86±1.77	a, b *, °°°

Data are given as mean±SEM
a Significant main effect of condition, *b* significant main effect of CDZ dose
*$p<0.05$ for comparisons between the 2.5 mg/kg dose with the NaCl group and °$p<0.05$, °°°$p<0.01$ for comparisons between the 5.0 mg/kg dose with the NaCl group.

unconditioned tendency rather than a learned one. This issue was recently questioned by Carobrez and Bertoglio (2005), on the grounds that mice and rats enter open arms more frequently during the first minutes of the experiment than later on, which could reflect some kind of learning of open-arm avoidance. However, in our experiment with freely exposed rats, open-arm avoidance was almost complete. This certainly corresponds to an unconditioned avoidance process, especially since no deficit in exploratory drive was observed in these rats. Indeed, their locomotion was comparable to locomotion in forcibly exposed rats, and many behaviors directed towards open arms such as pSAPs or head scans also argue against the possibility that open arms were not detected. Therefore, rats with no previous experience of the maze have a specific avoidance with respect to the open arms; this disproves the hypothesis that a phobic-like fear of the open arms is acquired during trial 1 (File and Zangrossi 1993; File et al. 1993). It can be assumed that the open-arm aversion is due to ecologically relevant features such as the absence of thigmotaxis possibilities, or to luminosity contrast between the open arms and the protected part of the maze, as shown in previous studies (Griebel et al. 1993b; Hogg 1996; Treit et al. 1993). Moreover, the conclusion in favor of an unconditioned open-arm avoidance is perfectly in accordance with the original work by Montgomery (1955), in which rats strongly avoided open alleys they were allowed to explore freely.

Forced exposure induces open-arm entries

The second interesting point highlighted by our results is that rats entered the open arms more frequently when they were forcibly exposed to the maze than when they were tested in the free-exposure condition. Since we may suppose that rats from the former condition are also unconditionally averse to the open arms, this difference must be due to some features of the forced procedure.

An explanation may partly reside in the fact that when a rat (or a mouse) is initially confronted to the plus-maze, it does not have an instant representation of the whole apparatus, notably in terms of the relative danger represented by each part of the device. As a result, this animal will not respond immediately accordingly to the "approach/avoidance conflict" as defined a priori by the experimenter. In order to behave in accordance with this conflict, the animal will first need to explore the different parts of the maze (closed arms, centre, and open arms) and attribute to them some values in terms of potential danger. Afterwards, it will be able to respond by some kind of place preference for the protected parts as opposed to the unprotected parts.

In addition, it can also be argued that forcible exposure will induce a greater intensity of fear than free exposure (Misslin et al. 1982). Thus, it can reasonably be advanced that a significant part of the animal's activity during initial discovery of the apparatus is not driven by "curiosity" but rather by a search for a mean to escape from the maze. This hypothesis was previously put forward to interpret an animal's behavior during initial exposure to an open field (Boissier and Simon 1969; Denenberg 1969; Misslin et al. 1976; Roy and Chapillon 2004); it is also in agreement with the high secretion of corticosterone observed in response to an elevated plus-maze session (Rodgers et al. 1999).

The observation that ethological behaviors such as pSAPs or closed-arm returns were more frequent in the free than in the forced situation is also highly consistent with the above interpretation. In the free configuration the

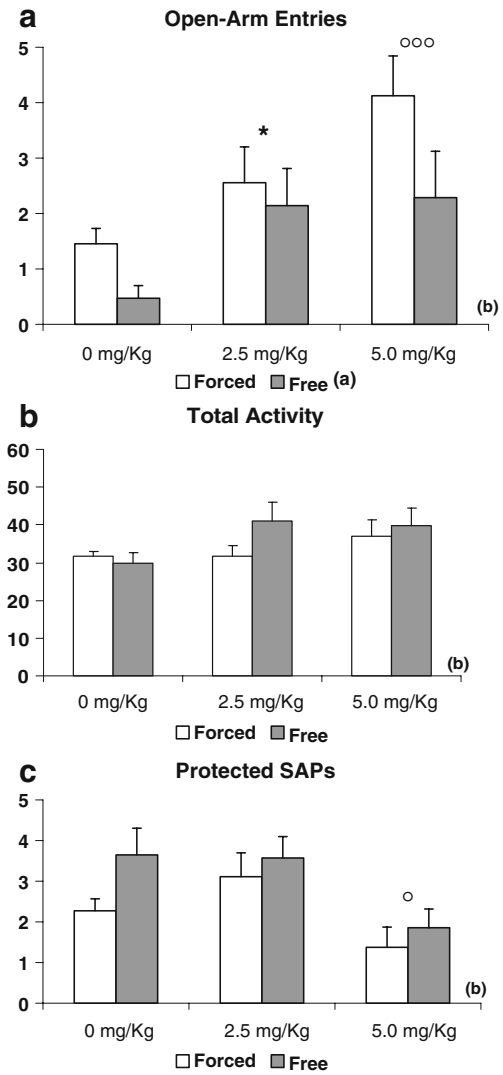

Fig. 2 Open-arm entries, total activity and protected saps in rats from the forced exposure and the free-exposure groups under 0 mg/kg, 2.5 mg/kg or 5.0 mg/kg doses of CDZ during trial 1 of Experiment 2. Data are given as mean±SEM. Statistics: **a** significant main effect of the condition (forced vs. free exposure), **b** significant main effect of CDZ dose; Follow-up comparisons: *$p<0.05$, comparison for 2.5 mg/kg vs. NaCl and °$p<0.05$, °°°$p<0.001$, comparisons for 5.0 mg/kg vs. NaCl

"approach/avoidance conflict" is more clearly defined. The animal starts from a safe place with sawdust and odorant cues; it will then progressively explore the maze, starting from the less anxiogenic part of the maze and having the opportunity to enter the more anxiogenic parts (i.e., open arms). In such a situation, it seems that the approach/avoidance conflict is well defined, and this renders ethological behaviors particularly relevant (Roy and Chapillon 2004).

One-trial tolerance effects and chlordiazepoxide effects

Our results finally bring interesting information about the one-trial tolerance effects. Firstly, we may recall the clear demonstration that on their *second* exposure to the elevated plus-maze, rats or mice display a reduced number of open-arm entries (i.e., they exhibit open-arm avoidance); moreover, they no longer respond to anxiolytic treatments (e.g. Bertoglio and Carobrez 2002; Calzavara et al. 2005; Carobrez and Bertoglio 2005; Rodgers et al. 1997). The results of our experiments under forced exposition are consistent with this observation. Forcibly exposed rats in experiment 1 significantly decreased their open-arm entries between trial 1 and trial 2, and a similar tendency was observed in experiment 2 with rats from the NaCl condition. In addition, while CDZ effects were apparent in experiment 2 on trial 1 with 2.5 and 5.0 mg/kg doses, the same doses had no effect on trial 2 in forcibly exposed rats previously exposed to the maze with NaCl.

Concerning the free-exposure condition, both control rats from experiment 1 and NaCl rats from experiment 2 continued to avoid open arms during re-exposure to the maze, thus, confirming that open-arm avoidance is unconditioned. However, while CDZ had no effects during trial 2 in the force-exposed condition, it can be noticed that anxiolytic effects were observed in free-exposed rats during trial 2, particularly at the 5.0 mg/kg dose. This lack of a one-trial tolerance effect in the free situation is interesting, would seem to contradict the hypothesis of Gonzalez and File (1997) according to which a previous maze-experience leads to a desensitization of benzodiazepine receptors. However, this discrepancy could also arise from the fact that the free exploration situation refers to trait anxiety whereas the forced situation refers to state anxiety (Belzung and Berton 1997; Griebel et al. 1993a; Lister 1990). More experiments are needed to clarify this point.

Taken together, these results raise interesting questions about the one-trial tolerance effect and bring new elements of interpretation. It was previously proposed that the one-trial tolerance effect could result from a phobic-like fear of the open arms acquired during trial 1 (File and Zangrossi 1993; File et al. 1993). On the basis of our present results, we have argued that open-arm avoidance is unconditioned, already present on trial 1, and highly expressed whenever the test conditions allow.

A second hypothesis to account for the one-trial tolerance effect, related to the approach/avoidance conflict, was proposed by Rodgers et al. (1996b) and Rodgers and Shepherd (1993). According to these authors, some kind of learning of the spatial configuration of the maze occurs during trial 1, certainly including the aversive aspects of open arms, thereby, reducing the conflict on trial 2. In other words, during trial 1, the open arms would be losing their novel aspect and gaining aversive properties, thus unbalancing the approach/avoidance conflict on trial 2 and reducing anxiolytic drugs to inefficiency. Another closely related hypothesis by Bertoglio and Carobrez (2000) stipulates that animals need to

Table 4 Differences between trials 2 and 1 for the behaviors of rats (NaCl, 2.5 or 5.0 mg/kg CDZ) forcibly exposed or freely exposed to the elevated plus-maze

	Controls		CDZ 2.5 mg/kg		CDZ 5.0 mg/kg		
	Forced	Free	Forced	Free	Forced	Free	
Time in open arms (sec.)	0.63±3.71	1.14±8.98	−6.57±4.63	8.20±8.20	−4.62±5.12	49.60±11.94°°, ***	a, b$^{\#\#}$, c
% Time spent in open arms	0.21%±1.24	0.38%±2.99	−2.19%±1.54	2.73%±2.73	−1.62%±1.71	16.53%±3.98°°, ***	a, b$^{\#\#}$, c
Open-arm entries	−0.38±0.38	0.14±0.70	−0.71±0.61	0.60±0.60	−0.14±0.40	3.60±0.51°°, **	a, b$^{\#\#}$, c
Time in closed arms (sec.)	19.25±19.82	−0.29±23.67	10.43±22.32	−41.80±25.45	30.86±16.58	−52.80±11.44	a
Time in the center (sec.)	−19.88±17.66	−0.71±16.84	−3.86±18.87	33.60±21.82	−26.00±18.28	3.20±12.11	
Total Activity	−2.25±2.19	−2.57±4.38	−6.57±2.69	−3.60±2.09	−6.57±1.62	0.60±3.71	
Number of rears	−0.88±1.55	1.00±1.53	−2.71±2.31	1.80±1.36	−2.86±1.67	2.20±0.97	a
Closed-arm returns	−0.38±0.26	0.43±0.20	0.00±0.44	−1.60±0.68	0.29±0.36	−1.80±0.58°, *	a, c
Protected SAPs	−1.00±0.93	−2.00±0.69	−0.57±0.65	−0.20±1.46	−1.43±0.20	−3.40±1.21	
Head scans	−2.88±1.43	−0.14±2.37	−4.00±1.20	1.80±1.50	−3.43±1.31	−0.80±1.39	a
Head dips	−0.50±1.70	−2.86±1.52	−0.29±1.92	3.20±1.69	−1.71±0.71	7.80±1.07°°, **	a, b$^{\#}$, c

Data are given as mean±SEM

a Significant main effect of condition, *b* significant main effect of CDZ dose, *c* significant interaction between condition and CDZ dose

**p*<0.05, ***p*<0.01, ****p*<0.001 comparison of forcibly exposed and freely exposed groups with the same pharmacology; $^{\#}p$<0.05, $^{\#\#}p$<0.01 CDZ 5.0 mg/kg in comparison to NaCl; °*p*<0.05, °°*p*<0.01 CDZ 2.5 mg/kg or CDZ 5.0 mg/kg rats in comparison to NaCl rats from the same condition

experience two different aversive environments (open and closed arms) on trial 1, so as to learn to avoid open arms on trial 2. Once this aversion is learned, the approach/avoidance conflict is no longer operative and the anxiolytic drugs are no longer effective. In the light of our present results, we agree with these previous hypotheses. However, rather than the specific acquisition of open-arm avoidance during trial 1, we propose that what animals actually learn is that closed arms really are safe. Indeed, our results show that the open-arm avoidance is unconditioned and very pronounced. In addition, it has recently been demonstrated that the one-trial tolerance effect was altered neither by experience of an elevated maze where all four arms were open (Frussa-Filho and Ribeiro Rde 2002), nor by experience of confinement in open arms (Bertoglio and Carobrez 2000; Falter et al. 1992; Treit et al. 1993). Experience of a maze with four enclosed arms had exactly the same effect as experience of the standard plus-maze, leading the animal to an aversion of open arms during trial 2 (Frussa-Filho and Ribeiro Rde 2002). In addition, whereas Bertoglio and Carobrez (2002) found no effect of closed-arm confinement on the one-trial tolerance effect, it was found by Falter et al. (1992) that the confinement of rats in a closed arm for 20 min prior testing resulted in a reduced efficacy for chlordiazepoxide. This

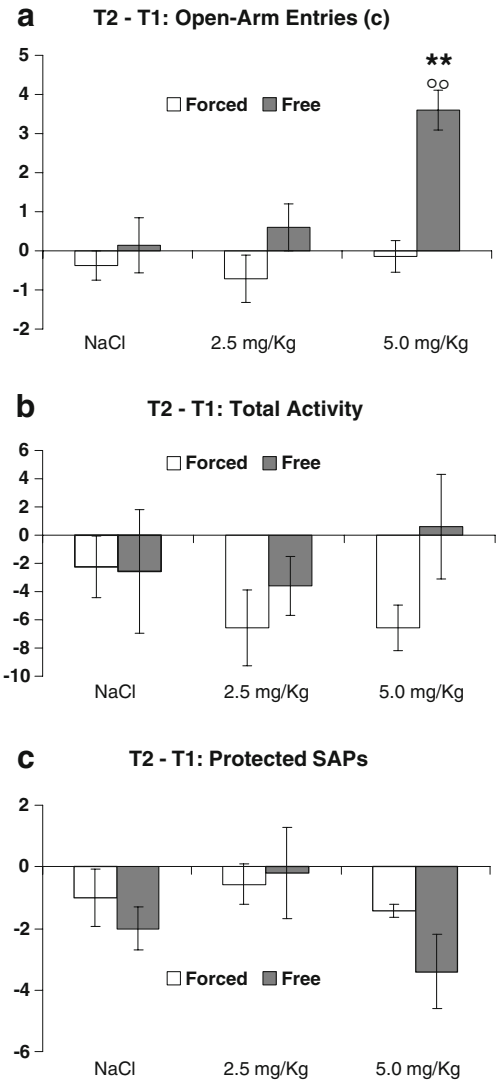

Fig. 3 Differences between Trial 2 and Trial 1 scores for open-arm entries (**a**), total activity (**b**), and protected SAPs in rats from the forced exposure and the free-exposure conditions under 0 mg/kg, 2.5 mg/kg or 5.0 mg/kg doses of CDZ. Data are given as mean±SEM. Statistics: **c** significant interaction between condition (forced vs. free exposure) and CDZ dose. Follow-up comparisons: ***p*<0.01, comparison of free and forced groups at the same dose; °°*p*<0.01, comparison of the 5.0 mg/kg CDZ dose towards NaCl

latter result was previously qualified as "awkward" by Holmes and Rodgers (1999) but is coherent with our proposition. Indeed, although the closed arms of a plus-maze may seem to be "protected" from the point of view of a human observer, they do represent a novel situation that could well generate fear or anxiety for an animal that has only experienced its home cage before. It is probable that during confinement to closed arms, the animal can learn their safeness, and then react with a clearer avoidance of open arms later when exposed to a conflict between the naturally feared open arms and the previously experienced safe closed arms. In the case of a naïve confrontation with the elevated plus-maze, we believe that the animals are initially threatened by both the unknown closed arms and the aversive open arms. After a moment of exploration, they will learn that closed arms are safe and they will likely reduce their exploration of open arms. This proposition is totally in agreement with what has been regularly observed in several studies with a minute to minute analysis (Carobrez and Bertoglio 2005; Rodgers et al. 1996b).

In conclusion, our results with the free-exposure situation demonstrate that open-arm avoidance is a natural tendency that does not need learning. In addition, our results strongly suggest that some of the initial open-arm entries during a forced confrontation with the elevated plus-maze are better interpreted as attempts to avoid the whole situation, rather than as indications of a low level of anxiety. We, therefore, propose that during initial exposure to a plus-maze, the approach/avoidance conflict is not clearly defined (from the animal's point of view); this conflict will be solved progressively as the animal becomes familiar with the closed arms. This explanation seems coherent with studies that have demonstrated a behavioral shift during the course of the initial trial (decreasing exploration of open arms), and with current observations about the one-trial tolerance effects. However, further studies are needed to validate our proposition and, in particular, to generalize this result to mice or to other strains of rats.

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