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ORIGINAL INVESTIGATION

Selective effects of benzodiazepines on the acquisition of conditioned taste aversion compared to attenuation of neophobia in C57BL/6 mice

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Abstract
Introduction The effects of pre-conditioning administration of anxiolytic benzodiazepines on the acquisition of a conditioned taste aversion (CTA) and on the acquisition of attenuation of neophobia (AN) were investigated in C57BL/6 mice.
Materials and methods A CTA was induced by injecting lithium chloride (LiCl; 6 mEq·kg^{-1}) 30 min after the animal had imbibed a novel 0.5% saccharin solution. In other animals, neophobia was attenuated by a single access to the novel 0.5% saccharin solution, followed only by injection of saline.
Results and discussion Pre-conditioning administration of chlordiazepoxide (CDZ; 6–24 mg·kg^{-1}, i.p.) and alprazolam (0.3–1 mg·kg^{-1}, p.o.) resulted in a CTA that did not differ initially from that observed in vehicle-treated controls, but which showed faster extinction. The acquisition of AN was impaired only after the higher doses of CDZ (12–24 mg·kg^{-1}, i.p.) or alprazolam (1 mg·kg^{-1}, i.p.). The results show that in this test, altered acquisition of an aversive CTA memory by anxiolytic benzodiazepines is reflected in more rapid extinction. Moreover, at low doses, these drugs showed selectivity for weakening CTA learning compared to AN learning. Evidence is discussed that selective weakening of aversive memory formation is a clinically relevant effect of anxiolytic benzodiazepines.

Keywords Conditioned taste aversion · Attenuation of neophobia · Acquisition · Anxiolytics · Benzodiazepines · Chlordiazepoxide · Alprazolam · Mouse

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Introduction

Anxiety disorders are major health problems that affect a large proportion of the population at some time. Fear and anxiety are clearly related, but whereas fear is a normal response to a threatening stimulus, anxiety is considered to be a disorder when fear is excessive or induced by cues or situations that are not threatening to non-anxious individuals. Human and animal studies indicate that there are both genetic and environmental influences on fear and anxiety (Boomsma et al. 2005; Merikangas and Low 2005; Wigger et al. 2004). Recent research has emphasized that among environmental influences, learning plays a marked role in the acquisition and extinction of fear (Barad 2005; Davis 1990, 2002; Delgado et al. 2006; Ressler et al. 2004; Sotres-Bayon et al. 2006; Sullivan et al. 2004). By classical Pavlovian conditioning, a neutral stimulus that is followed by an aversive unconditioned stimulus (US) will become a conditioned stimulus (CS) that elicits fear as shown by its ability to induce a conditioned response (CR). By processes of generalization, stimuli similar to the CS may also elicit fear. Weakening of conditioned fear may involve forgetting of CS–US associations, but progresses more rapidly by the process of extinction that occurs when the CS is experienced in the absence of US. Several lines of evidence indicate that extinction is an additional learning process rather than an erasure of the original fear conditioning (Berman and Dudai 2001; Bouton

2002; Myers and Davis 2002; Phelps et al. 2004; Rescorla 1996).

The important role of conditioning in the acquisition and extinction of fear strengthens the possibility that the etiology of anxiety disorders may involve fear conditioning. For example, in patients with anxiety disorders, the acquisition or generalization of conditioned fear could be too strong, the extinction of conditioned fear could be too weak, or the patient may have simply experienced too many aversive, traumatic events. A recent meta-analysis of some of these aspects indicated a modest increase in the acquisition of fear conditioning and in conditioned responding during extinction in anxiety patients (Lissek et al. 2005). This strengthens the view that the effects of anxiolytic drugs on the mechanisms of fear conditioning could be of clinical therapeutic relevance.

The purpose of the present experiments was therefore to determine the effects of typical benzodiazepine anxiolytics on acquisition in the conditioned taste aversion (CTA) paradigm where a novel taste, after pairing with the aversive consequences of a US, lithium chloride (LiCl) injection which induces visceral malaise, becomes a CS (Welzl et al. 2001; Yamamoto and Fujimoto 1991; Yamamoto et al. 1994). The conditioned fear of the CS is shown by avoidance of it when it is subsequently offered. The CTA is a well-established association learning and memory paradigm involving activation of the gustatory insular cortex and amygdala (Bermudez-Rattoni 2004; Yamamoto and Fujimoto 1991; Yamamoto et al. 1994). As a comparison, we also determined effects on the acquisition of AN, a type of appetitive or safe taste learning (Ramirez-Lugo et al. 2006). The acquisition of AN describes the fact that after unpunished exposure to a novel taste, animals show less avoidance of it than animals that are naïve to this taste.

Materials and methods

Materials

Drugs

LiCl (Sigma-Aldrich) and chlordiazepoxide (CDZ) hydrochloride (Sigma-Aldrich) were dissolved freshly on the morning of the experiment in saline. The doses used were 6 $mEq \cdot kg^{-1}$ of LiCl and 3, 6, 12, or 24 $mg \cdot kg^{-1}$ i.p. of CDZ HCl. Because of its poor aqueous solubility, alprazolam (synthesized at Novartis Pharma AG, Basel, Switzerland) was finely ground in 0.5% methyl cellulose (Amimed, BioConcept, Allschwil, Switzerland) and administered per os. Since the aim of the present experiments was to determine the effect of each drug on CTA compared to AN and since no conclusions are based on any between-drugs comparison, the different routes of administration do not affect the conclusions of the experiments. Alprazolam doses were initially 0.1, 0.3, 1, and 3 $mg \cdot kg^{-1}$, but since saccharin intake during the initial saccharin exposure was markedly reduced by the 3-$mg \cdot kg^{-1}$ dose (see "Results"), the effect of this dose on CTA and AN acquisition could not be assessed. Sodium saccharin (Sigma) was dissolved in distilled water at a concentration of 0.5% (*w*/*v*).

LiCl or saline was injected 30 min after the end of saccharin access on the conditioning day. CDZ or saline vehicle was injected intraperitoneally in a volume of 10 $mg \cdot kg^{-1}$ 30 min before saccharin access on the conditioning day, and alprazolam or 0.5% methyl cellulose vehicle was administered per os 60 min before the saccharin access.

Animals

To test the effect of benzodiazepines, we selected C57BL/6 mice, a highly inbred mouse strain of low emotionality, responsive to benzodiazepines in some, but not all, studies (Crabbe et al. 1998; Crawley et al. 1997; Griebel et al. 2000), and often used to investigate interaction of genes and behavior. Male C57BL/6 mice weighing 20–25 g were obtained from Charles River Ltd, Germany. They were housed in individual cages at 22°C in a temperature-regulated room on a 12-h light/dark cycle (lights on at 0600). Water and food were available ad libitum before the start of the experiment. Subsequently, water was available on a restricted schedule as described below. Experiments were performed during the light phase of the cycle. The studies were performed according to protocols approved by the Veterinary Authority of the Canton of Basel-Stadt.

Experimental conditions

Training period to accustom animals to limited drinking access

Animals had limited access to water (2×30 $min \cdot day^{-1}$) and were trained to drink from modified 15-ml Falcon polypropylene conical tubes (Becton-Dickinson; D'Adamo et al. 2004). Fluid consumption was recorded by weighing the Falcon tubes to an accuracy of 0.01 g before and after the drinking period. The training period lasted 2–3 days. All subsequent experimental manipulations concerned the morning drinking period, with water always being given for 30 min in the afternoon drinking period.

Conditioning trial

A CTA experiment and an AN experiment were performed in parallel. In the CTA experiment, animals received LiCl after saccharin access and thus learned to avoid saccharin, whereas in the AN experiment, animals received only saline after saccharin access and thus acquired a learned AN to the saccharin.

In each experiment, drug or vehicle was administered before saccharin access on the conditioning day. CDZ or saline vehicle was injected intraperitoneally in a volume of 10 ml·kg^{-1} 30 min prior to saccharin access. Alprazolam or 0.5% methyl cellulose vehicle was administered per os 60 min before saccharin access. Saccharin solution (0.5% in water) was then offered for 30 min in a single Falcon tube. Saccharin consumption was recorded by weighing the tube before and after the drinking period. Thirty minutes later (i.e., 60 min after the start of saccharin access), the animals were injected with LiCl or saline, respectively (10 ml·kg^{-1}, i.p.) and returned to their cages.

Animals in a saccharin-naïve control group, which had no opportunity to learn anything about saccharin before the first preference test, were injected with vehicle before a 30-min access to drinking water followed by saline injection.

Memory expression and extinction trials

Beginning on the day after conditioning, during the 30-min morning session, all animals were offered two tubes simultaneously: one filled with tap water the other with 0.5% saccharin solution. Fluid consumption was recorded by weighing the Falcon tubes before and after the drinking period. The aversion index was calculated as:

$$\mathrm{AI}\% = 100 \times \text{water intake}/(\text{water intake} + \text{saccharin intake})\%.$$

The aversion index (AI) thus ranged from 0 (for 100% saccharin preference) to 100 (for 100% saccharin aversion). As a ratio between water intake and total intake, the AI is independent of general increases or decreases of motor behavior. In the afternoon, to reduce the severity of water restriction, water was offered for 30 min. These procedures were repeated daily throughout the extinction period. On weekends, only water was offered for 1 h per day.

Statistics

Statistical tests were performed using the program SYSTAT (SPSS Inc., version 10). Values are shown as mean ± SEM. Effects of drug treatment on acquisition and neophobia were compared using one-way analysis of variances (ANOVA). If ANOVA showed a significant effect of treatment, then individual groups were compared to the control group by means of Dunnett's multiple comparison (two-tailed). Effects of drug treatment on extinction and AN were compared using two-way ANOVA with drug treatment and day as factors. In the case of two-factor ANOVA, the most conservative p value was taken after Greenhouse–Geisser or Huynh–Feldt correction.

Results

Experiment 1. Effects of CDZ at doses of 3 and 6 mg·kg^{-1} on acquisition of CTA and AN

Effects in the CTA experiment

Saccharin intake during the conditioning trial One factor ANOVA showed a significant effect of treatment group [$F(2,27) = 18.3$, $p<10^{-5}$] (Table 1). Compared to the vehicle-treated group, intake was significantly increased in the 3-mg·kg^{-1} CDZ group and in the 6-mg·kg^{-1} CDZ group (two-tailed Dunnett's tests, $p<10^{-3}$ and $p<10^{-5}$, respectively). Also, when intake was expressed relative to body weight, one-factor ANOVA showed a significant effect of treatment group [$F(2,27) = 16.6$, $p<10^{-4}$). Compared to the vehicle-treated group, intake was significantly increased in both the 3-mg·kg^{-1} CDZ group and the 6-mg·kg^{-1} CDZ group (two-tailed Dunnett's tests, $p<10^{-3}$ and $p<10^{-4}$, respectively).

Acquisition and extinction of the CTA LiCl treatment resulted in acquisition of a CTA. As expected, the results (Fig. 1a) indicated that compared to the saccharin-naïve control group, LiCl treatment caused a long-lasting increase of the AI. Comparison of the AI data of the vehicle-treated conditioned group and the saccharin-naïve group by two-factor ANOVA (group and day as a repeated factor) showed a significant effect of group [$F(1,15) = 88.1$, $p<10^{-6}$], day

Table 1 Saccharin intake in the CTA conditioning trial of mice that received 3 or 6 mg·kg^{-1} of CDZ or vehicle before access to saccharin

Treatment group	Saccharin intake	
	ml	ml·10g^{-1} body weight
Vehicle	0.94±0.11	0.39±0.05
3 mg·kg^{-1} CDZ	1.64±0.13*	0.70±0.06*
6 mg·kg^{-1} CDZ	1.85±0.10*	0.78±0.04*

Values are shown as mean ± SEM

*$p<0.001$ (two-tailed Dunnett's tests)

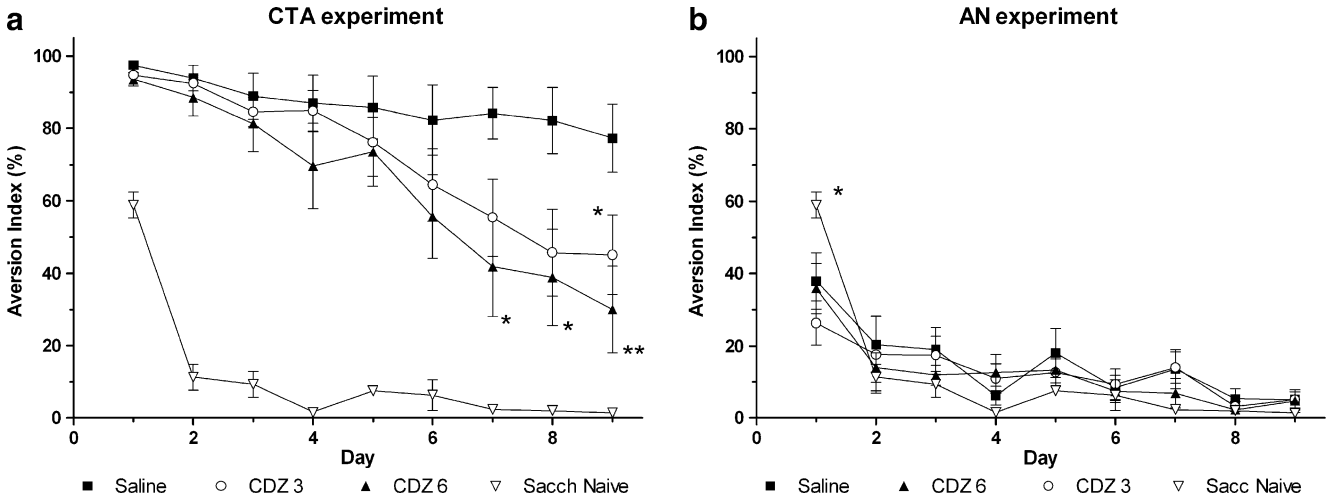

Fig. 1 **a** Acquisition of a CTA under the influence of CDZ (3–6 mg·kg^{-1}) results in a CTA that shows faster extinction. **b** Acquisition of AN is unaltered by treatment with CDZ (3–6 mg·kg^{-1}) before saccharin exposure. The graph shows the daily aversion indices of groups of eight to ten mice that received the indicated dose of CDZ or saline vehicle intraperitoneally before drinking saccharin in the conditioning trial, as well as the results of the saccharin-naïve control group. *p<0.05, **p<0.01 vs saline vehicle group (Dunnett's multiple comparison tests, two-tailed)

[F(8,120) = 27.5, p<10^{-7}] and a significant interaction of these factors [F(8,120) = 9.36, p<0.001]. The groups differed significantly at each individual time point (two-tailed t tests, p<0.001 in each case).

CDZ (3–6 mg·kg^{-1}) treatment resulted in a CTA that showed faster extinction (Fig. 1a). The effect of CDZ was analyzed by two-factor ANOVA (group and day as repeated factors) of the AI data of animals that had received LiCl after saccharin access. This showed no significant effect of CDZ group [F(2,26) = 2.08, p>0.1], but a highly significant effect of day [F(8,208) = 28.8, p<10^{-8}] and a significant interaction of CDZ group × day [F(16,208) = 3.13, p<0.05]. Multiple comparisons of the CDZ groups to the vehicle group at individual time points showed in the 6-mg·kg^{-1} CDZ group significant reductions of AI (two-tailed Dunnett's tests) on days 7, 8, and 9 after conditioning and in the 3-mg·kg^{-1} CDZ group at 9 days.

Effects in the AN experiment

Saccharin intake during the conditioning trial One-factor ANOVA of saccharin intake during the conditioning trial revealed a significant effect of pre-conditioning treatment [F(2,27) = 6.98, p<0.01] (Table 2). Compared to the vehicle-treated group, saccharin intake was increased significantly in the 6-mg·kg^{-1} CDZ group. Also, when intake was expressed relative to body weight, one-factor ANOVA showed a significant effect of treatment group [F(2,27) = 6.61, p<0.005]. Compared to the vehicle-treated group, intake was significantly increased in the 6-mg·kg^{-1} CDZ group.

Acquisition of AN A single unpunished saccharin exposure resulted in AN (Fig. 1b). The day 1 data indicate that one exposure to saccharin alone resulted in an AI that was significantly attenuated compared to that of the saccharin-naïve group that had never experienced saccharin before the first preference test. Thus, the day 1 AI of the vehicle group was significantly less than that of the saccharin-naïve group (p<0.05, two-tailed t test).

CDZ (3–6 mg·kg^{-1}) treatment did not alter the acquisition of AN (Fig. 1b). The effect of CDZ was analyzed by one-factor ANOVA (CDZ group as factor) of the day 1 AI data of animals that had received saline after saccharin access. This showed no significant effect of CDZ group [F(2,27) = 0.78, p>0.4].

In these groups, with repeated saccharin exposure in daily preference tests, the saccharin preference increased, but this showed no effect of CDZ treatment group. Thus, two-factor ANOVA of the AIs over all testing days showed

Table 2 Saccharin intake in the AN conditioning trial of mice that received 3 or 6 mg·kg^{-1} of CDZ or vehicle before access to saccharin

Treatment group	Saccharin intake	
	ml	ml·10g^{-1} body weight
Vehicle	1.21±0.11	0.50±0.05
3 mg·kg^{-1} CDZ	1.35±0.13	0.56±0.06
6 mg·kg^{-1} CDZ	1.75±0.07*	0.73±0.03*

Values are shown as mean ± SEM

*p<0.01 (two-tailed Dunnett's test)

a significant effect of day [$F(8,208) = 11.0$, $p<10^{-6}$], but no effect of CDZ treatment group [$F(2,26) = 0.32$, $p>0.7$] and no interaction of CDZ treatment group × day [$F(16,208) = 0.48$, $p>0.8$].

Experiment 2. Effects of CDZ at doses of 12 and 24 mg·kg^{-1}

Effects in the CTA experiment

Saccharin intake during the conditioning trial One-factor ANOVA of saccharin intake during the conditioning trial revealed a significant effect of pre-conditioning treatment [$F(2,25) = 4.51$, $p<0.05$] (Table 3). Compared to the vehicle-treated group, the 24-mg·kg^{-1} CDZ group showed significantly reduced intake. Also, when intake was expressed relative to body weight, one-factor ANOVA showed a significant effect of treatment group [$F(2,25) = 4.09$, $p<0.05$]. Compared to the vehicle-treated group, intake was significantly reduced in the 24-mg·kg^{-1} CDZ group.

Acquisition and extinction of the CTA LiCl treatment resulted in acquisition of the CTA. The results indicate that compared to the saccharin-naïve control group, LiCl treatment caused a long-lasting increase of the AI (Fig. 2a). Analysis of the AI data of the vehicle-treated conditioned group and the saccharin-naïve group by two-factor ANOVA (group and day as repeated factors) showed a significant effect of group [$F(1,15) = 41.9$, $p<0.10^{-4}$], day [$F(8,120) = 11.9$, $p<0.10^{-4}$] and a significant interaction of these factors [$F(8,120) = 3.34$, $p<0.05$]. The groups differed significantly at each individual time point (two-tailed t tests, $p<0.01$ in each case).

CDZ (12–24 mg·kg^{-1}) treatment resulted in a CTA that showed faster extinction (Fig. 2a). The effect of CDZ was analyzed by two-factor ANOVA (group and day as repeated factors) of the AI data of animals that had received LiCl after saccharin access. This showed significant effects of CDZ group [$F(2,25) = 3.98$, $p<0.05$] and day [$F(8,200) = 52.6$, $p<10^{-15}$] and a significant interaction of CDZ group × day [$F(16,200) = 5.92$, $p<10^{-4}$]. Multiple comparisons of the CDZ groups to the vehicle group at individual time points showed a significant reduction in AI in both CDZ groups (two-tailed Dunnett's tests) on days 7, 8, and 9 after conditioning.

Table 3 Saccharin intake in the CTA conditioning trial of mice that received 12 or 24 mg·kg^{-1} of CDZ or vehicle before access to saccharin

Treatment group	Saccharin intake	
	ml	ml·10g^{-1} body weight
Vehicle	1.37±0.10	0.62±0.05
12 mg·kg^{-1} CDZ	1.28±0.18	0.58±0.09
24 mg·kg^{-1} CDZ	0.82±0.11*	0.36±0.05*

Values are shown as mean ± SEM

*$p<0.05$ (two-tailed Dunnett's test)

Effects in the AN experiment

Saccharin intake during the conditioning trial Qualitatively, the effect of CDZ was to reduce saccharin intake at the highest dose, as in the CTA experiment, but this effect was not statistically significant (Table 4). Thus, one-factor ANOVA of saccharin intake during the conditioning trial revealed no significant effect of pre-conditioning treatment [$F(2,24) = 2.20$, $p>0.1$]. It should be noted that in the latter group, three animals were excluded from the experiment because they did not drink saccharin in the conditioning trial. Also, when intake was expressed relative to body weight, one-factor ANOVA showed no significant effect of pre-conditioning treatment [$F(2,24) = 2.44$, $p>0.1$].

Acquisition of AN A single unpunished saccharin exposure resulted in an AN (Fig. 2b). The day 1 data indicate that one exposure to saccharin alone resulted in an AI that was significantly attenuated compared to that of the saccharin-naïve group that had never experienced saccharin before the first preference test. Thus, the day 1 AI of the vehicle group was significantly less than that of the saccharin-naïve group ($p<0.001$, two-tailed t test).

CDZ (12–24 mg·kg^{-1}) inhibited the acquisition of AN (Fig. 2b). The effect of CDZ was analyzed by one-factor ANOVA (CDZ group as factor) of the AI data of animals that had received saline after saccharin access. This showed a significant effect of CDZ group [$F(2,24) = 11.92$, $p<0.001$]. Compared to the vehicle group, both CDZ groups had an increased day 1 AI (two-tailed Dunnett's tests, $p<0.001$).

In these groups, with repeated saccharin exposure in daily preference tests, the saccharin preference increased. Two-factor ANOVA of the AIs over all testing days showed a significant effect of day [$F(8,192) = 39.4$, $p<10^{-15}$], CDZ treatment group [$F(2,24) = 5.83$, $p<0.01$], and a significant interaction of CDZ treatment group × day [$F(16,192) = 5.36$, $p<10^{-4}$]. Multiple comparisons of the CDZ groups to the vehicle group at individual time points showed a significant increase in AI in both CDZ groups (two-tailed Dunnett's tests) on the first and second day after conditioning.

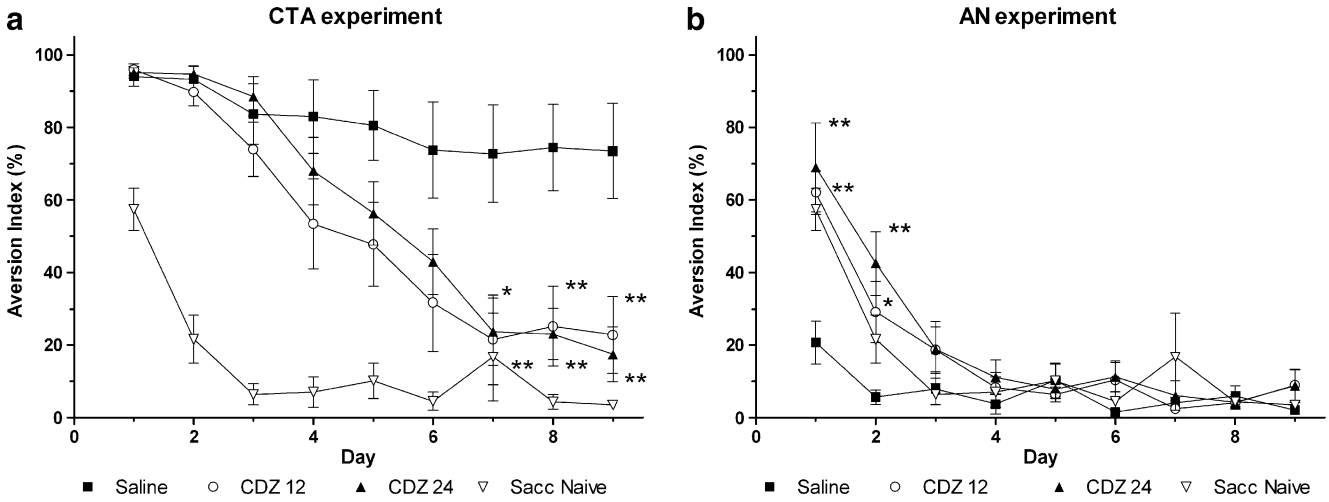

Fig. 2 **a** Acquisition of a CTA under the influence of CDZ (12–24 mg·kg^{-1}) results in a CTA that shows faster extinction. **b** Acquisition of AN was inhibited by treatment with CDZ (12–24 mg·kg^{-1}) before saccharin exposure The graph shows the daily aversion indices of groups of eight to ten mice that received the indicated dose of CDZ or saline vehicle intraperitoneally before drinking saccharin in the conditioning trial, as well as the results of the saccharin-naïve control group. *p<0.05, **p<0.01 vs saline vehicle group (Dunnett's multiple comparison tests, two-tailed)

Experiment 3. Effects of alprazolam at doses of 0.1 and 0.3 mg·kg^{-1}

Effects in the CTA experiment

Saccharin intake during the conditioning trial One-factor ANOVA showed no significant effect of treatment group [F(2,22) = 3.03, p > 0.05] (Table 5). When intake was expressed relative to body weight, one-factor ANOVA showed a significant effect of treatment group [F(2,22) = 3.53, p<0.05], but compared to the vehicle-treated group, intake was not significantly increased in either alprazolam-treated group (two-tailed Dunnett's tests, p>0.05).

Acquisition and extinction of the CTA LiCl treatment resulted in acquisition of a CTA (Fig. 3a). As expected, the results indicated that compared to the saccharin-naïve control group, LiCl treatment caused a long-lasting increase of the AI. Analysis of the AI data of the vehicle-treated conditioned group and the saccharin-naïve group by two-factor ANOVA (group and day as repeated factors) showed a significant effect of group [F(1,14) = 444.0, p<10^{-11}], day [F(8,112) = 22.4, p<10^{-5}], and a significant interaction of these factors [F(8,112) = 16.4, p<10^{-4}]. The groups differed significantly at each individual time point (two-tailed t tests, p<0.001 in each case).

Alprazolam (0.1–0.3 mg·kg^{-1}) treatment resulted in a CTA that showed faster extinction (Fig. 3a). The effect of alprazolam was analyzed by two-factor ANOVA (group and day as repeated factors) of the AI data of animals that had received LiCl after saccharin access. This showed a significant effect of alprazolam group [F(2,22) = 8.78, p<0.002], a highly significant effect of day [F(8,176) = 19.5, p<10^{-5}], and a significant interaction of alprazolam group × day [F(16,176) = 4.21, p<0.004]. Multiple comparisons of the alprazolam-treated groups to the vehicle group at individual time points showed significant reductions of AI in the 0.3-mg·kg^{-1} alprazolam group on days4 to 9 after conditioning (two-tailed Dunnett's tests).

Table 4 Saccharin intake in the AN conditioning trial of mice that received 12 or 24 mg·kg^{-1} of CDZ or vehicle before access to saccharin

Treatment group	Saccharin intake	
	ml	ml·10g^{-1} body weight
Vehicle	1.39±0.10	0.62±0.05
12 mg·kg^{-1} CDZ	1.15±0.14	0.51±0.06
24 mg·kg^{-1} CDZ[a]	0.96±0.20	0.42±0.09

[a] Excluding three subjects that did not drink during the conditioning trial. Values are shown as mean ± SEM

Table 5 Saccharin intake in the CTA conditioning trial of mice that received 0.1 or 0.3 mg·kg^{-1} of alprazolam or vehicle before access to saccharin

Treatment group	Saccharin intake	
	ml	ml·10g^{-1} body weight
Vehicle	1.05±0.09	0.45±0.03
0.1 mg·kg^{-1} alprazolam	1.34±0.08	0.57±0.03
0.3 mg·kg^{-1} alprazolam	1.32±0.10	0.57±0.04

Values are shown as mean ± SEM

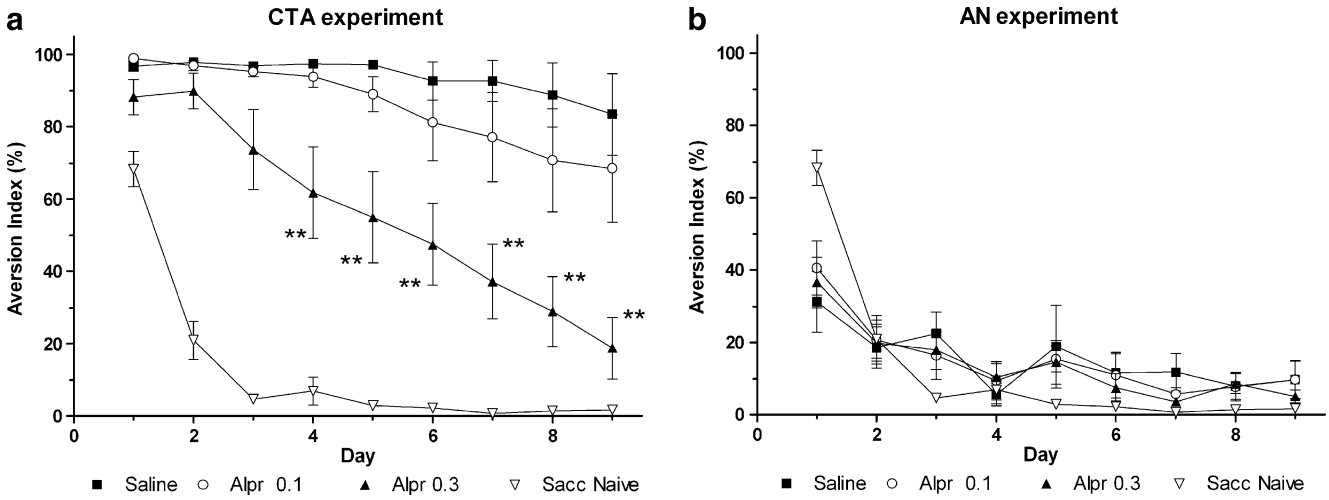

Fig. 3 **a** Acquisition of a CTA under the influence of alprazolam (0.1–0.3 mg·kg^{-1}) results in a CTA that shows faster extinction. **b** Acquisition of AN is unaltered by treatment with alprazolam (0.1–0.3 mg·kg^{-1}) before saccharin exposure. The graph shows the daily aversion indices of groups of eight to ten mice that received the indicated dose of alprazolam or methylcellulose vehicle per os before drinking saccharin in the conditioning trial, as well as the results of the saccharin-naïve control group. $^{**}p<0.01$ vs saline vehicle group (Dunnett's multiple comparison tests, two-tailed)

Effects in the AN experiment

Saccharin intake during the conditioning trial One-factor ANOVA of saccharin intake during the conditioning trial revealed no significant effect of pre-conditioning treatment [$F(2,21) = 1.54$, $p>0.2$] (Table 6). Also, when intake was expressed relative to body weight, one-factor ANOVA showed no significant effect of treatment group [$F(2,21) = 1.35$, $p>0.2$].

Acquisition of AN A single unpunished saccharin exposure resulted in an AN (Fig. 3b). The day1 data of Fig. 3b indicate that one exposure to saccharin alone resulted in an AI that was significantly attenuated compared to that of the saccharin-naïve group that had never experienced saccharin before the first preference test. Thus, the day1 AI of the vehicle group was significantly less than that of the saccharin-naïve group ($p<0.002$, two-tailed t test).

Table 6 Saccharin intake in the AN conditioning trial of mice that received 0.1 or 0.3 mg·kg^{-1} of alprazolam or vehicle before access to saccharin

Treatment group	Saccharin intake	
	ml	ml·10g^{-1} body weight
Vehicle	1.03±0.16	0.45±0.07
0.1 mg·kg^{-1} alprazolam	1.46±0.23	0.62±0.09
0.3 mg·kg^{-1} alprazolam	1.33±0.14	0.57±0.06

Values are shown as mean ± SEM

Alprazolam (0.1–0.3 mg·kg^{-1}) treatment did not alter the acquisition of AN (Fig. 3b). The effect of alprazolam was analyzed by one-factor ANOVA (alprazolam group as factor) of the day1 AI data of animals that had received saline after saccharin access. This showed no significant effect of alprazolam group [$F(2,21) = 0.37$, $p>0.6$].

In these groups, with repeated saccharin exposure in daily preference tests, the saccharin preference increased, but this showed no effect of CDZ treatment group. Thus, two-factor ANOVA of the AIs over all testing days showed a significant effect of day [$F(8,168) = 11.7$, $p<10^{-7}$], but no effect of alprazolam treatment group [$F(2,21) = 0.07$, $p>0.9$] and no interaction of alprazolam treatment group × day [$F(16,168) = 0.38$, $p>0.9$].

Experiment 4. Effects of alprazolam at a dose of 1 mg·kg^{-1}

Initially, 3 mg·kg^{-1} alprazolam CTA and AN groups were also included, but after this dose, only one animal from ten in the CTA experiment and one animal from ten in the AN experiment drank the saccharin solution during the conditioning trial. Therefore, only the results of the 1-mg·kg^{-1} dose are shown.

Effects in the CTA experiment

Saccharin intake during the conditioning trial Only four animals from ten drank saccharin solution during the conditioning trial after 1 mg·kg^{-1} alprazolam and nine of ten animals in the vehicle group (Table 7). There was no

Table 7 Saccharin intake in the CTA conditioning trial of mice that received 1 mg·kg^{-1} of alprazolam or vehicle before access to saccharin

Treatment group	Saccharin intake	
	ml	ml·10g^{-1} body weight
Vehicle	0.81±0.13	0.35±0.05
1 mg·kg^{-1} alprazolam	0.52±0.21	0.23±0.10

Values are shown as mean ± SEM

effect of alprazolam on the absolute intake or on the intake expressed relative to the body weight (*t* tests, $p>0.2$ in each case).

Acquisition and extinction of the CTA LiCl treatment resulted in acquisition of a CTA (Fig. 4a). As expected, the results indicated that compared to the saccharin-naïve control group, LiCl treatment caused a long-lasting increase of the AI. Comparison of the AI data of the vehicle-treated conditioned group and the saccharin-naïve group by two-factor ANOVA (group and day as repeated factors) showed a significant effect of group [$F(1,15) = 209.9$, $p<10^{-9}$], day [$F(8,120) = 18.2$, $p<10^{-4}$], and a significant interaction of these factors [$F(8,120) = 7.33$, $p<0.004$]. The groups differed significantly at each individual time point (two-tailed *t* tests, $p<0.002$ in each case).

Alprazolam (1 mg·kg^{-1}) treatment resulted in a CTA that showed faster extinction (Fig. 4a). The effect of alprazolam was analyzed by two-factor ANOVA (group and day as repeated factors) of the AI data of animals that had received LiCl after saccharin access. This showed a significant effect of alprazolam group [$F(1,11) = 20.1$, $p<0.001$], a significant effect of day [$F(8,88) = 16.7$, $p<10^{-4}$], and a significant interaction of alprazolam group × day [$F(8,88) = 4.86$, $p<0.02$]. Multiple comparisons of the alprazolam groups to the vehicle group at individual time points showed in the 1 mg·kg^{-1} alprazolam group significant reductions of AI (two-tailed *t* tests) on days 5, 7, 8, and 9 after conditioning.

Effects in the AN experiment

Saccharin intake during the conditioning trial Eight animals from ten drank saccharin solution during the conditioning trial after 1 mg·kg^{-1} alprazolam and eight of ten animals in the vehicle group (Table 8). One-factor ANOVA of the saccharin intake of these animals during the conditioning trial revealed a significant effect of preconditioning treatment [$F(1,14) = 10.2$, $p<0.01$]. Compared to the vehicle-treated group, intake was decreased significantly in the alprazolam group. Also, when intake was expressed relative to body weight, one-factor ANOVA showed a significant effect of treatment group [$F(1,14) = 10.8$, $p<0.005$], with a reduction of intake in the alprazolam group compared to the vehicle-treated group.

Acquisition of AN A single unpunished saccharin exposure resulted in an AN (Fig. 4b). The day 1 data indicate that one exposure to saccharin alone resulted in an AI that was significantly attenuated compared to that of the saccharin-naïve group that had never experienced saccharin before the first preference test. Thus, the day 1 AI of the vehicle group

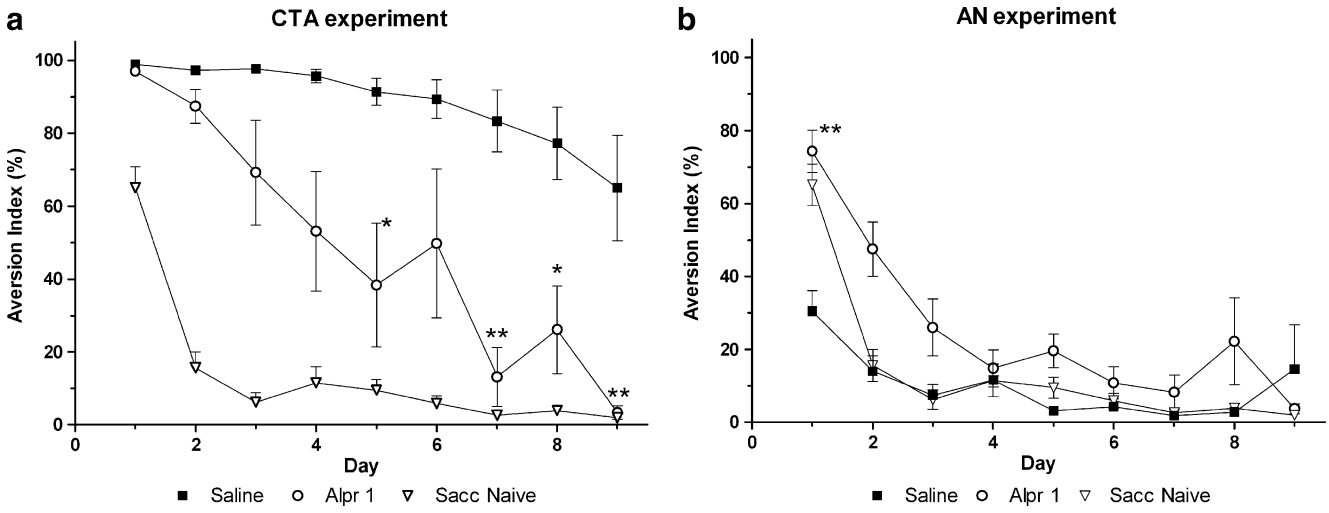

Fig. 4 **a** Acquisition of a CTA under the influence of alprazolam (1 mg·kg^{-1}) results in a CTA that shows faster extinction. **b** Acquisition of AN is inhibited by treatment with alprazolam (1 mg·kg^{-1}) before saccharin exposure. The graph shows the daily aversion indices of groups of eight to ten mice that received the indicated dose of alprazolam or vehicle per os before drinking saccharin in the conditioning trial, as well as the results of the saccharin-naïve control group. *$p<0.05$, **$p<0.01$ vs vehicle group (*t* tests, two-tailed)

was significantly less than that of the saccharin-naïve group ($p<0.001$, two-tailed t test).

Alprazolam (1 mg·kg^{-1}) inhibited the acquisition of AN (Fig. 4b). The effect of alprazolam was analyzed by one-factor ANOVA (CDZ group as factor) of the day 1 AI data of animals that had received saline after saccharin access. This showed a highly significant effect of alprazolam group [$F(1,14) = 29.3$, $p<10^{-4}$].

In these groups, with repeated saccharin exposure in daily preference tests, the neophobia attenuated further. Two-factor ANOVA of the AIs over all testing days showed a significant effect of day [$F(8,112) = 16.2$, $p<10^{-7}$], significant effects of alprazolam group [$F(1,14) = 12.5$, $p<0.01$], and the interaction of alprazolam group × day [$F(8,112) = 4.87$, $p<0.002$]. This reflects the steeper curve in the alprazolam group due to their lack of AN on day 1.

Discussion

The present experiments investigated the effects of anxiolytic benzodiazepines on acquisition of CTA and its subsequent extinction and on acquisition of AN. CTA is considered a form of classical fear conditioning (Bures et al. 1998; Yamamoto 2007) in that after pairing with the aversive LiCl US, the saccharin taste stimulus becomes a CS that elicits aversive reactions such as escape and avoidance. In contrast, acquisition of AN is a form of appetitive or safety learning (Ramirez-Lugo et al. 2006) in that after one experience of the sweet taste, without aversive consequences, the animal shows AN compared to animals without such experience, reflected in the increased preference for the saccharin solution.

The main findings were demonstrated by both CDZ and alprazolam. First, the effect on CTA acquisition was revealed as faster extinction of taste aversion rather than as lower AI in the first preference test. Second, the effect of the lowest doses was to selectively alter CTA without affecting AN. Finally, at higher doses, the acquisition of both CTA and AN was impaired. These results are compared with previous studies of CTA and other forms of fear conditioning. Possible neuroanatomical substrates are considered, as is relevance for benzodiazepine clinical anxiolytic actions.

Table 8 Saccharin intake in the AN conditioning trial of mice that received 1 mg·kg^{-1} of alprazolam or vehicle before access to saccharin

Treatment group	Saccharin intake	
	ml	ml·10g^{-1} body weight
Vehicle	0.99±0.14	0.42±0.06
1 mg·kg^{-1} alprazolam	0.48±0.06*	0.20±0.03*

Values are shown as mean ± SEM

*$p<0.01$ vs vehicle group (one-way ANOVA)

There are, to our knowledge, no previous studies of the effect of pre-conditioning benzodiazepine administration on acquisition of CTA evoked by LiCl pairing. However, pentobarbital, which possesses anxiolytic-like activity in other test, did not alter the AI in the first two bottle tests when administered before a sucrose–LiCl conditioning trial in rats (Concannon and Freda 1980). This resembles the effect of benzodiazepines observed here. Further preference tests, to allow extinction to be followed, were not performed.

The main other type of aversive conditioning in which the effects of benzodiazepines on acquisition have been studied is passive avoidance and a variant on it in which the readout is less sensitive to motor effects (Sanger and Joly 1985). Only few studies have utilized classical fear conditioning to investigate this question. Fanselow and Helmstetter (1988) found that pre-training midazolam administration attenuated conditioning as shown by subsequent freezing to the context associated with shock. The hippocampus seems to be one site of action for this effect, since immediate post-conditioning application of midazolam to the dorsal hippocampus reduced subsequent context- or cue-induced freezing (Gafford et al. 2005). Passive avoidance studies are in substantial agreement in showing that pre-conditioning administration of an anxiolytic benzodiazepine impairs acquisition of the avoidance response in rats and mice (Anglade et al. 1994; Broekkamp et al. 1984; Cryan et al. 2004; Jensen et al. 1979; Nagatani and Yamamoto 1991; Oishi et al. 1972; Patel et al. 1979). The same is true in a variation of the test where effects on motor activity were also determined (Sanger and Joly 1985). The amygdala is one brain region where inhibitory effects of benzodiazepines on passive avoidance acquisition are mediated, since midazolam injected into the amygdala before training impairs acquisition of passive avoidance (Dickinson-Anson and McGaugh 1993). Interestingly, in a four-plate test–retest procedure, mice injected with low-dose diazepam prior to conditioning displayed similar behavior (reduction in number of punished passages on retest trial) as vehicle-treated mice (Ripoll et al. 2005), indicating a passive avoidance acquisition. However, further retests as follow-up for extinction were not performed.

Benzodiazepines increase food and fluid intake apparently by enhancing taste palatability (Berridge and Treit 1986; Cooper 1989; Mathiasen and Mirza 2005), potentially modulating the strength of the conditioned response (Domjan and Gillan 1976; Reilly and Bornovalova 2005). At high doses, benzodiazepines can also cause sedation. It must therefore be considered whether learning-unrelated effects, such as direct effects on fluid

intake or sedative actions, can account for the present results. For several reasons, we consider it unlikely that these results are due to sedation. First, the AI is a ratio determined by two responses, drinking water and drinking saccharin. As these responses require similar effort, there is no reason why sedation would preferentially affect one or the other. Thus, the AI is insensitive to sedation. Secondly, despite dypsogenic effects with lower doses of CDZ, but not with higher doses or with alprazolam, we observed similar initial CTA magnitude and similarly accelerated extinction. Thus, altered fluid intake during the conditioning trial is not a major determinant of the benzodiazepine effects on CTA. Likewise, a reduction of the acquisition of AN by a dose of CDZ (12 mg/kg) that did not alter the initial saccharin intake shows that altered fluid intake during the conditioning trial is not responsible for the effects on acquisition of AN.

Interestingly, in the present studies, the benzodiazepines at the lowest doses altered CTA acquisition without affecting acquisition of AN. Thus, the substrates for CTA and AN learning seem to be different. In support of this conclusion, acquisition of AN, but not of CTA, is blocked by anesthesia, hypothermia, or electroconvulsive shock applied shortly after the novel taste exposure (Bures et al. 1998; Buresova and Bures 1980). This suggested that different gustatory short-term memories (GSTMs) in different circuits have different stabilities, the GSTM involved in CTA learning being more resistant to these disturbances, which could have survival value in promoting avoidance of poisons. Studies with anti-muscarinic agents also distinguish between CTA and AN. For example, formation of AN is more sensitive to muscarinic blockade of the insular cortex than formation of CTA (Gutierrez et al. 2003). Similarly, low systemic doses of scopolamine impaired the acquisition of AN without affecting CTA (Kelly et al. 2005). Further differentiating between CTA and AN, antagonism of glutamate NMDA-type receptors in the nucleus accumbens disrupted CTA, but not AN, memory formation (Ramirez-Lugo et al. 2006).

Considering candidate brain regions for the effects reported here, benzodiazepines produce their actions by positive modulation of the action of GABA at GABA-A receptors, which are widespread in the brain. Thus, the overall effect of a systemic application may result from the sum of diverse actions. The gustatory cortex is one region where enhanced GABA-A action inhibits the formation of a CTA: local application of the GABA-A agonist, muscimol, into the gustatory cortex inhibited CTA, whereas similar application into the dorsal hippocampus had the opposite effect (Stone et al. 2005). Certainly, GABA-A receptors are abundant in the parabrachial nucleus, amygdala, and insular cortex, which are important for the acquisition of CTA (Yamamoto 2007), but the effects of specifically modulating GABA-A activity in these regions on CTA formation have not yet been examined. Furthermore, while particular GABA-A receptor subunits play different roles in sedation, cognition, or anxiety (for review, see Atack, 2003), it is not yet known whether activation of specific GABA-A receptor subunits can account for the observed effects on CTA and AN learning. A further open question is whether strain differences in the presently observed effects occur and whether these differences may relate to strain variations in affinity and regional density of GABA-A receptors (Robertson 1979; Chapouthier et al. 1991; Hode et al. 2000) as well as in other benzodiazepine-mediated behavioral effects (Crabbe et al. 1998; Crawley et al. 1997; Griebel et al. 2000).

It is possible that interference with the acquisition of conditioned fear is relevant for the therapeutic action of benzodiazepines. This view is consistent with the conclusion (Davidson 2004) that these compounds are very effective in generalized anxiety disorder (GAD), but not in posttraumatic stress disorder (PTSD). In the case of GAD where no specific precipitator of anxiety is apparent, it is possible that the fear conditioning elicited by all the small everyday exposures to mildly aversive stimuli is too strong. By blocking this acquisition, benzodiazepines would exert a beneficial effect. The fact that during benzodiazepine treatment the beneficial effect on anxiety scores improves with treatment duration (Rickels et al. 1993) is consistent with an effect to reduce the accumulation of anxiety, allowing that already present to extinguish, rather than with blocking the expression of anxiety, which would be expected to be more immediate. Indeed, laboratory studies in humans, presumably most relevant to the clinical situation, do indicate that benzodiazepines are more effective in reducing acquisition of conditioned fear than its expression. For example, it was recently shown that diazepam suppressed the acquisition of cue fear conditioning (Scaife et al. 2005, 2007). However, at doses that suppressed the acquisition of conditioned fear, diazepam did not reduce its expression (Scaife et al. 2005). In substantial agreement is a similar study (Baas et al. 2002) in which a cue was made aversive by verbal instruction that it may signal shocks. Enhancement of acoustic startle in the presence of this cue was not reduced by diazepam, whereas in contrast, potentiation of the startle response by darkness, an unconditioned effect, was inhibited by diazepam. This emphasizes that reduction of unconditioned fear, clearly observable in animal tests such as the elevated plus maze, light–dark box, open field (Bourin and Hascoet 2003; Crawley 1985; Hogg 1996; Prut and Belzung 2003), and light-enhanced acoustic startle response (de Jongh et al. 2002), is an important action of benzodiazepines contributing to their overall clinical effect. Recent studies in man also indicate that expression of context-conditioned fear, compared to cue-conditioned fear, may be more sensitive to

benzodiazepine anxiolytics (Grillon et al. 2006). Animal studies provide conflicting evidence about whether benzodiazepines inhibit the expression of conditioned fear (Davis 1990). There are good examples of both positive (Beck and Fibiger 1995; Stanhope and Dourish 1996) and negative effects (Sanger and Joly 1985; Stein and Berger 1969). A detailed review of the evidence is beyond the scope of the present article and would have to consider species, the specific drugs and doses, cue or context conditioning, and the nature of the readout, passive avoidance, conditioned freezing, fear-potentiated startle, or conditioned emotional response.

In the case of PTSD, the traumatic events responsible for the anxiety have occurred before treatment. Therefore, it could be imagined that in PTSD, the conditioned fear acquired during the traumatic event has strongly generalized to innocuous interoceptive and environmental stimuli and that recovery is a process of extinction. Consistent with such a view, benzodiazepines seem to have a similar effect on PTSD in man and on extinction in animals. Thus, they delay extinction of CTA when given before extinction trials (Kelly, unpublished), produce only state-dependent extinction of conditioned freezing (Bouton et al. 1990), and in the treatment of PTSD appear to delay recovery (Davidson 2004; Gelpin et al. 1996; Mellman et al. 2002). In addition to the above-suggested mechanism of PTSD, other mechanisms such as sensitization by associative and non-associative mechanisms (Burriss et al. 2007; Iwamoto et al. 2007; Rau et al. 2005; Siegmund and Wotjak 2006) and changes in hippocampal volume (Villarreal et al. 2002; Wignall et al. 2004) may certainly also contribute to the prolonged persistence of symptoms.

In summary, the present studies show that two anxiolytic benzodiazepines, CDZ and alprazolam, affected the acquisition of CTA by causing a CTA that presented faster extinction. This effect showed some selectivity compared to a similar non-aversive conditioning, AN, in that the lowest doses of both drugs selectively altered CTA without affecting AN. Evidence has been discussed to support the conclusion that selective impairment of aversive learning by benzodiazepines is of relevance for their clinical anxiolytic actions.

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