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NMDA Partial Agonist Reverses Blocking of Extinction of Aversive Memory by $GABA_A$ Agonist in the Amygdala

Irit Akirav*[,1]

[1]*Department of Neurobiology, Weizmann Institute of Science, Rehovot, Israel*

The ability to extinguish aversive memories is of significant clinical interest. The amygdala plays an important role in emotional conditioning and its experimental extinction. It has been suggested that γ-aminobutyric acid (GABA) agonists retard extinction and that consolidation of extinction involves *N*-methyl-D-aspartate receptor (NMDAR)-mediated plasticity. The aim was to further explore the interaction between GABA and NMDA in the amygdala in consolidation of experimental extinction in the rat. To that end conditioned taste aversion (CTA) was used. In CTA, the amygdala has been reported to subserve both acquisition and extinction. The $GABA_A$ receptor agonist, muscimol, administered into the amygdala immediately after the first extinction session, caused lasting disruption of extinction of CTA for at least 2 weeks. However, the administration of $GABA_A$ receptor antagonists had no effect on extinction kinetics. Microinfusing the partial NMDA agonist D-cycloserine together with or after muscimol infusion reversed the blocking effects of muscimol. These findings could bear relevance to the potential involvement of extinction abnormalities in behavioral disorders, and their amelioration.

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INTRODUCTION

Experimental extinction is the decline in the frequency or intensity of the conditioned response following the withdrawal of reinforcement, and assumed to reflect relearning rather than unlearning (Berman and Dudai, 2001; Bouton, 1994; Myers and Davis, 2002; Rescorla, 1996). The ability to extinguish intense aversive memories is of significant clinical interest, in particularly concerning phobias, panic disorder, and post-traumatic stress disorder (PTSD) (Charney *et al*, 1993; Davis and Myers, 2002; Fyer, 1998; Gorman *et al*, 2000). Converging evidence indicates that the amygdala plays an important role in emotional conditioning and its experimental extinction (Davis, 1992; Falls *et al*, 1992; LeDoux, 1993; Lu *et al*, 2001; Maren, 1999; McGaugh *et al*, 1993; Nader *et al*, 2000).

Recent work on the neural basis of extinction indicates that γ-aminobutyric acid (GABA) and glutamate are critically involved (Davis *et al*, 2003; Pereira *et al*, 1989; Santini *et al*, 2001; Walker *et al*, 2002). Extinction is considered inhibitory learning that competes with excitatory conditioning. In this process, GABA may inhibit brain areas involved in fear and aversive learning (eg, the amygdala), and glutamate acting at *N*-methyl-D-aspartate (NMDA) receptors may play a role in the neural plasticity that permits this GABA-mediated inhibition to be exerted appropriately (Myers and Davis, 2002). It has been suggested that GABA agonists may retard extinction and that experimental extinction requires NMDA-mediated plasticity (Davis *et al*, 2003; Falls *et al*, 1992; Lee and Kim, 1998; Santini *et al*, 2001). This view is compatible with evidence that $GABA_A$ receptor agonists interrupt, whereas NMDA receptor agonists facilitate extinction of fear memories (Davis *et al*, 2003; Pereira *et al*, 1989; Santini *et al*, 2001; Walker *et al*, 2002).

This study was undertaken to further explore the interaction between GABA and NMDA in the amygdala in consolidation of experimental extinction, by using conditioned taste aversion (CTA). In CTA rats learn to avoid a taste if the first encounter with that taste is followed by malaise (Bures *et al*, 1988; Garcia *et al*, 1955). CTA acquisition and the resulting memory is robust and long lasting. Nevertheless, CTA memory can be extinguished following retrieval in the absence of reinforcer. Both acquisition and extinction of CTA are subserved by the amygdala (Bahar *et al*, 2004; Yamamoto *et al*, 1994).

Thus, the aim of this study was to examine whether modulation of $GABA_A$ receptors in the basolateral amygdala (BLA), can disrupt normal extinction of CTA, and whether NMDA receptor agonist can reverse this disruption.

*Correspondence: Dr I Akirav, Department of Neurobiology, Weizmann Institute of Science, Rehovot 76100, Israel, Tel: +972 8 9344418, Fax: +972 8 9344131, E-mail: Irit.Akirav@weizmann.ac.il
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MATERIALS AND METHODS

Animals

Male Wistar rats (~60 days old, 250–300 g) were caged individually at 22±2°C under 12 h light/dark cycles. Water and food were available *ad libitum* unless otherwise indicated.

All animal experiments were conducted according to the institutional care and use committee, which are in complete accordance with the NIH guidelines for care and use of laboratory animals.

Drugs

Muscimol (Mus; $GABA_A$ and $GABA_c$ receptor agonist; 0.05 μg/μl), bicuculline methiodide (Bicc; $GABA_A$ receptor antagonist; 0.005 or 0.05 μg/μl), gabazine ($GABA_A$ receptor antagonist; 6 or 12 ng/μl) and D-cycloserine (DCS, a partial agonist acts at the strychinine-insensitive glycine-recognition site of the NMDA receptor complex; 20 μg/μl), were from Sigma (St Louis, MO, USA). Drugs were dissolved in physiological saline, which was also used as control.

Behavioral Procedures

In CTA (Bures *et al*, 1988, 1998; Revusky and Garcia, 1970) organisms learn to avoid a novel taste if its ingestion is followed by transient toxicity. Unless indicated otherwise, saccharin (0.1% w/v) was used as the unfamiliar taste, and LiCl (0.15 M, 2% body weight, intraperitoneally (i.p.)) as the malaise-inducing agent. Rats water deprived for 24 h had access to tap water for 30 min from two pipettes containing 10 ml of water each. In all, 85–90% of the animals approached the pipettes within the 30 min. The experimenter held the pipettes in proximity to the mouth of animals that did not approach the pipettes, until they have started to drink. On the next day, rats were pretrained for 3 days to get their daily water ration once a day for 10 min from a pipette containing 10 ml of water. Stable 10-min water intakes were established by 2 days.

On day 5, they were allowed to drink saccharin instead of water for 10 min. At 40 min after the offset of the drinking period they were injected with LiCl i.p. At 3 days after training, rats were presented with three water pipettes and three saccharine pipettes, each containing 5 ml, to test their preference. This test session is defined as extinction training since animals are exposed to nonreinforced presentations of a conditioned stimulus (CS; eg, saccahrine) that had previously been paired with a fear-inducing unconditioned stimulus (US; eg, LiCl). Under these conditions, the conditioned rats preferred water to saccharin at a ratio of 9:1 in a multiple choice test situation (Berman *et al*, 1998). Multiple-bottle tests are more sensitive than one-bottle tests to obtain an aversion index. The conditioned aversion is presented as an aversion index, defined as (milliliters of water/(milliliters of water + milliliters of saccharin) × 100), consumed in the test; hence, 50 indicates equal preference. There was no significant difference between the groups in their total liquid intake during the tests (see Table 1).

Table 1 Total Fluid Consumption of the Different Experimental Groups on the First Session of Extinction

Figure #	Group	Mean±SEM
2a	Sal BLA	8.58±0.74
	Mus Test1	9.2±0.58
	Mus Test2	8.92±0.81
2b	Sal BLA	11.3±0.72
	Mus BLA	12.1±0.42
2c	Sal BLA-Sal i.p.	14.2±0.5
	Mus BLA-Sal i.p.	13.8±0.41
2d	Sal BLA	9.9±0.44
	Mus BLA	9.08±0.82
3	Sal BLA	13.1±0.51
	Bicc BLA	12.8±0.58
	Gabazine 6 ng	9±0.4
	Gabazine 12 ng	8.1±0.8
4a	Tets1-Sal	11.6±0.67
	Test1-Mus	9.1±0.44
	Test1-Mus+DCS	12±0.72
	Test1 Mus−Test2 DCS	9.7±0.33
4b	Cond Sal	10±0.49
	Cond DCS	13.9±0.66
	Test1 DCS	11.1±0.81

Data is presented as means±SEM.

Surgery and Drug Administration

Rats were anesthetized with 4.8 ml/kg Equithesin (2.12% w/v $MgSO_4$, 10% v/v ethanol, 39.1% v/v propylene glycol, 0.98% w/v sodium pentobarbital, and 4.2% w/v chloral hydrate), restrained in a stereotactic apparatus (David Kopf Instruments, Tujunga, CA, USA), and implanted bilaterally with stainless-steel guide cannula (23 gauge, thin wall) aimed to the basolateral amygdala (BLA; anteroposterior, −3 mm relative to bregma; lateral, ±5 mm; ventral, −7.6 mm). The cannulae were positioned in place with acrylic dental cement and secured by two skull screws. A stylus was placed in the guide cannula to prevent clogging. Animals were allowed 1 week to recuperate before being subjected to experimental manipulations. The stylus was removed from the guide cannula, and a 28-gauge injection cannula, extending 1.0 mm from the tip of the guide cannula, was inserted. The infusion cannula was connected via PE20 tubing to a Hamilton micro syringe driven by a micro infusion pump (CMA/100; Carnegie Medicin, Stockholm, Sweden). Microinfusion was performed bilaterally in a 1 μl volume per hemisphere delivered over 2 min. The infusion cannula was left in position before withdrawal for an additional 1 min to minimize dragging of the injected liquid along the injection tract.

The time course of microinfusion was as follows:

Figure 2a: Microinfusion into the BLA immediately following the test on test day 1 or 2.

Figures 2b, d and 3: Microinfusion into the BLA immediately following the test on test day 1.

Figure 2c: Microinfusion into the BLA immediately following exposure to saccharine (on conditioning day).

Figure 4a: Saline or muscimol or muscimol + DCS into the BLA immediately following the test on test day 1 and DCS into the BLA immediately following the test on test day 2.

Figure 4b: Saline or DCS into the BLA immediately following exposure to saccharine (on conditioning day) or DCS into BLA immediately following the test on test day 1.

Histology

At the completion of the behavioral experiments animals were anesthetized and microinfused into the BLA with 1 μl of Indian ink. Cannula location was examined under a light microscope following Nissl staining. Only data from animals with correct cannula placements (ie, within the lateral, basal and accessory basal nuclei) were included in the analyses. Figure 1a shows schematic drawing of BLA cannulae placements (coronal view at position −2.80, −3.14, −3.30 and −3.60 mm posterior to bregma). Solid black circles indicate the locations in a subset of animals (not all animals are shown in light of the number of rats involved in the experiments).

Animals that were microinfused with muscimol to the BLA or not infused (sham) ($n=4$ each) were anesthetized with 4.8 ml/kg Equithesin and perfused transcardially with 200 ml of saline, followed by 200 ml of 4% paraformaldehyde (PFA), pH 7.4. The brains were removed, postfixed in 4% PFA for 24 h, and then immersed in 25% sucrose in PBS until they sank. Coronal sections (20 μm) were cut on a freezing microtome and mounted on gelatin-coated glass slide and stained with creysl violet. Slides were examined under a light microscope. Figure 1b shows photomicrograph illustrating typical cannula tracks in BLA of representative brain section for muscimol microinfusion and sham (about 3.14 mm posterior to bregma). The drug infusion did not notably damage the BLA compared with the sham.

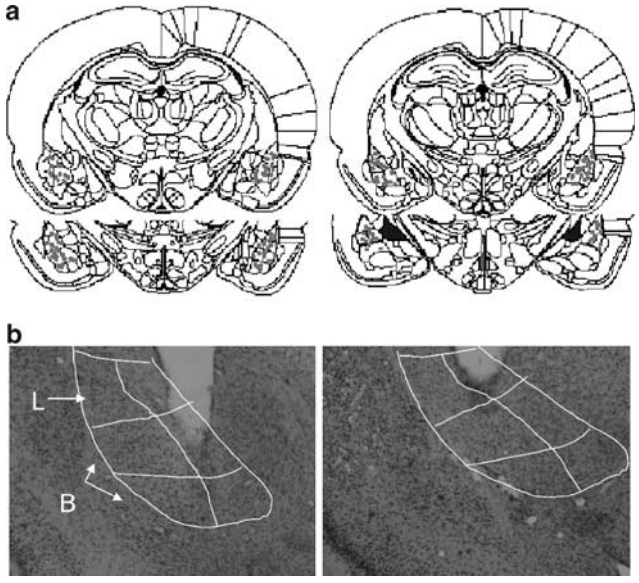

Figure 1 (a) Schematic drawings of BLA cannula placements. Shown is a coronal view at position 2.80, 3.14, 3.30 and 3.60 mm posterior to bregma. Solid black circles indicate the location of cannulae. (b) Photomicrograph illustrating typical cannula tracks in BLA of a representative brain section for muscimol microinfusion and sham (about 3.14 mm posterior to bregma; L: lateral, B: basolateral).

Statistics

Differences among the groups were determined using ANOVA. All *post hoc* comparisons were made by the least significant difference multiple comparison test (LSD).

RESULTS

Extinction of CTA was not Observed in the BLA Following $GABA_A$ Agonist Microinfusion

Animals microinfused with muscimol into the BLA immediately following the test on test day 1 (Mus Test1, $n=10$) showed marked aversion to saccharine for at least a week, compared with the saline microinfused animals (Sal BLA, $n=8$; Figure 2a). Another group was microinfused with muscimol into the BLA immediately following the test on test day 2 (Mus Test2, $n=7$). Repeated ANOVA revealed a significant difference between the groups ($F_{(2,22)}=22.182$, $P<0.001$). *Post hoc* comparison unveiled a significant difference between the saline and the Mus Test1 group throughout the experiment ($P<0.05$), except for the first test day (before drug infusion). The Mus Test2 group was significantly different from the Mus Test1 group on test days 2, 6 and 7 ($P<0.05$), and from the saline group on test day 6 ($P<0.05$).

Analyzing the aversion curve in the Mus Test2 group throughout the days revealed a significant reduction in aversion index ($F_{(1,6)}=4.956$, $P=0.036$). Comparing aversion for the Mus Test2 group between test day 2 and 7 also showed a significant reduction in aversion ($t_{(6)}=3.1$, $P=0.021$). Thus, although muscimol microinfused following test day 2 shifted the aversion curve up compared with the saline group, it did not significantly impair extinction.

To determine how long the effect of a single microinfusion of muscimol lasts, animals were tested for two weeks for their aversion to saccharine. Animals microinfused with muscimol into the BLA immediately following the test on test day 1 (Mus BLA, $n=10$) showed marked aversion to saccharine for at least two weeks, compared with the saline microinfused animals (Sal BLA, $n=8$; Figure 2b). Repeated ANOVA revealed a significant difference between the groups ($F_{(1,16)}=17.66$, $P<0.001$).

In order to verify that microinfusion of muscimol itself did not induce CTA, animals were microinfused with muscimol into the BLA immediately after exposure to saccharine, and received i.p. injection of saline instead of LiCl (Mus BLA-Sal i.p., $n=7$). Control animals were microinfused with saline into the BLA immediately after exposure to saccharine and also received i.p. injection of saline (Sal BLA-Sal i.p., $n=7$). Both groups did not show marked aversion to saccharine when tested for saccharine

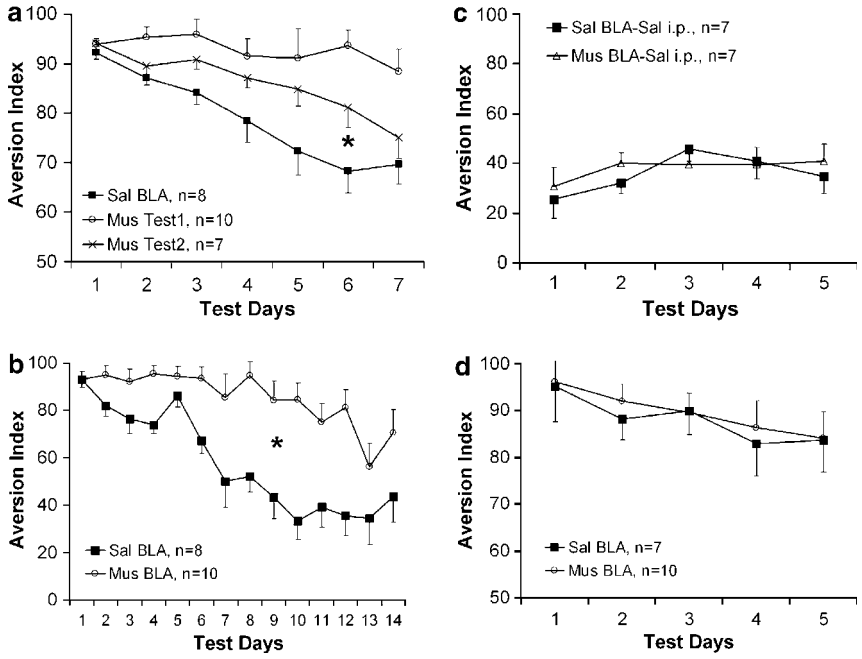

Figure 2 (a) Muscimol following the first extinction session blocks extinction of CTA in the BLA. Animals microinfused with muscimol into the BLA immediately following the test on test day 1 (Mus Test1) showed marked aversion to saccharine for at least a week, compared with the saline microinfused animals (Sal BLA). Microinfusing muscimol into the BLA immediately following the test on test day 2 (Mus Test2) shifted the aversion curve up but did not significantly impair extinction (*, $P<0.05$). (b) Long-lasting blocking effects of muscimol on CTA extinction. Rats injected i.p. with LiCl 40 min after consuming an unfamiliar solution of saccharine (Sal BLA) displayed high aversion to saccharine that gradually extinguished with repeated testing. Animals microinfused with muscimol into the BLA immediately following the test on day 1 (Mus BLA) showed marked aversion to saccharine for almost two weeks compared with the saline microinfused animals (Sal BLA) (*, $P<0.001$). (c) Muscimol itself does not induce CTA. Animals were microinfused with saline (Sal BLA-Sal i.p.) or muscimol (Mus BLA-Sal i.p.) into the BLA immediately following exposure to saccharine (on conditioning day) and received i.p. injection of saline (instead of LiCl). Both groups did not show marked aversion to saccharine when tested 3 days after conditioning and there was no significant difference between them. (d) Muscimol does not have lingering aversive effects on taste preference. Animals that were microinfused with saline (Sal BLA) or muscimol (Mus BLA) into the BLA following the test on day 1 and showed marked aversion to saccharine (not shown), were re-conditioned to a new taste (glycine) 1 week following their first conditioning to saccharine. Although the Mus BLA group showed marked aversion to saccharine with repeated testing, they were able to acquire new aversion to the new taste (glycine) and to show extinction with repeated testing. There was no significant difference between the Mus BLA and the Sal BLA groups in their aversion to glycine.

preference, with no significant difference between the groups (Figure 2c; $F_{(1,12)}<1$, NS).

Furthermore, to establish that the muscimol effect on CTA extinction was not due to long-lasting impairment in BLA function that is required for CTA training and testing, rats that were microinfused with saline (Sal BLA, $n=7$) or muscimol (Mus BLA, $n=10$) into the BLA following the test on test day 1 and displayed marked aversion to saccharine, were reconditioned to a new taste (glycine 1% w/v) 1 week after their first conditioning to saccharine. Although the Mus BLA group showed marked aversion to saccharine with repeated testing (as described in Figure 2a), the rats were able to acquire new aversion to the new taste (Figure 2d), and to show reduced aversion to glycine with repeated testing. ANOVA unveiled a significant reduction in aversion to glycine with repeated testing in both groups (Sal BLA: $F_{(1,6)}=10.948$, $P<0.05$; Mus BLA: $F_{(1,9)}=5.036$, $P=0.05$). There was no significant difference between the Mus BLA and the Sal BLA groups in their aversion to glycine $F_{(1,15)}<1$, NS.

$GABA_A$ Antagonists do not Affect CTA Extinction Kinetics

Since the $GABA_A$ receptor agonist impaired CTA extinction, the possibility that $GABA_A$ antagonists might facilitate extinction, was investigated. Animals microinfused with bicuculline (Bicc BLA, $n=9$) or two doses of gabazine (Gabazine 6 ng, $n=11$ or Gabazine 12 ng, $n=7$) into the BLA immediately following the test on test day 1 were not significantly different from the saline group (Sal BLA, $n=9$; Figure 3; $F_{(3,32)}<1$, NS). Thus, the antagonists groups showed gradual extinction of CTA as the control group. The dose of bicuculline used here (0.005 μg/μl) seems appropriate since pilot studies demonstrated that its infusion into the amygdala facilitated CTA acquisition. Moreover, a higher dose of bicuculline (0.05 μg/μl) was found to induce non-specific sensory and motor effects (data not shown).

A Partial NMDA Agonist Reverses the Blocking Effects of Muscimol on CTA Extinction

The partial NMDA agonist DCS was found to facilitate extinction of conditioned fear (Davis *et al*, 2003; Ledgerwood *et al*, 2003, 2004; Walker *et al*, 2002), thus, here its role in CTA extinction was examined. Toward that end, animals were microinfused with both muscimol and DCS into the BLA immediately following the test on day 1 (Test 1-Mus + DCS, $n=10$). Another group was microinfused with muscimol into the BLA immediately after the test on day 1 and with DCS immediately following the test on day 2 (Test1 Mus-Test2 DCS, $n=12$). These groups were

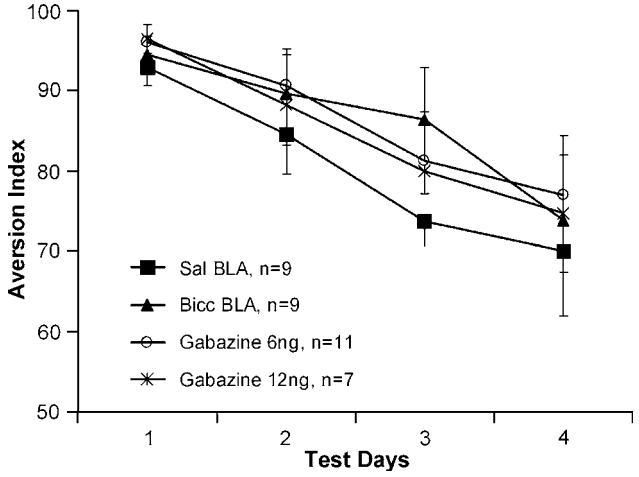

Figure 3 $GABA_A$ antagonists (bicuculline and gabazine) do not affect CTA extinction kinetics. Animals microinfused with bicuculline (Bicc BLA) or gabazine (Gabazine 6 or 12 ng) into the BLA immediately following the test on day 1 were not significantly different from the control group (Sal BLA).

compared to animals microinfused with muscimol into the BLA immediately after the test on day 1, with no DCS microinfusion (Test1 Mus, $n=10$). Repeated ANOVA unveiled a significant difference in aversion between the groups ($F_{(2,29)}=5.28$, $P<0.005$; Figure 4a). *Post hoc* comparisons revealed a significant difference between the Test1 Mus group and the Test1-Mus + DCS group ($P=0.004$) but not between the Test1 Mus and the Test1 Mus-Test2 DCS group. Thus, when DCS was microinfused following the first extinction session it reversed the blocking effects of muscimol on extinction. Additional ANOVA showed a significant difference between the Test1 Mus-Test2 DCS group to the Test1-Mus + DCS group on day 5 ($F_{(2,29)}=11.805$, $P<0.001$). Thus, when DCS was microinfused following the second extinction session, it partially recovered extinction.

As seen in Figure 4b, animals microinfused with DCS immediately following exposure to saccharine on conditioning day (Cond DCS, $n=9$) or immediately following test 1 (Test1 DCS, $n=8$) were not significantly different from control animals (Cond Sal, $n=8$) in their aversion to saccharine $F_{(2,21)}<1$, NS). Thus, the effects cannot be attributed to DCS-induced neurotoxicity. Furthermore, DCS did not have any effect of its own when administered after the first extinction session.

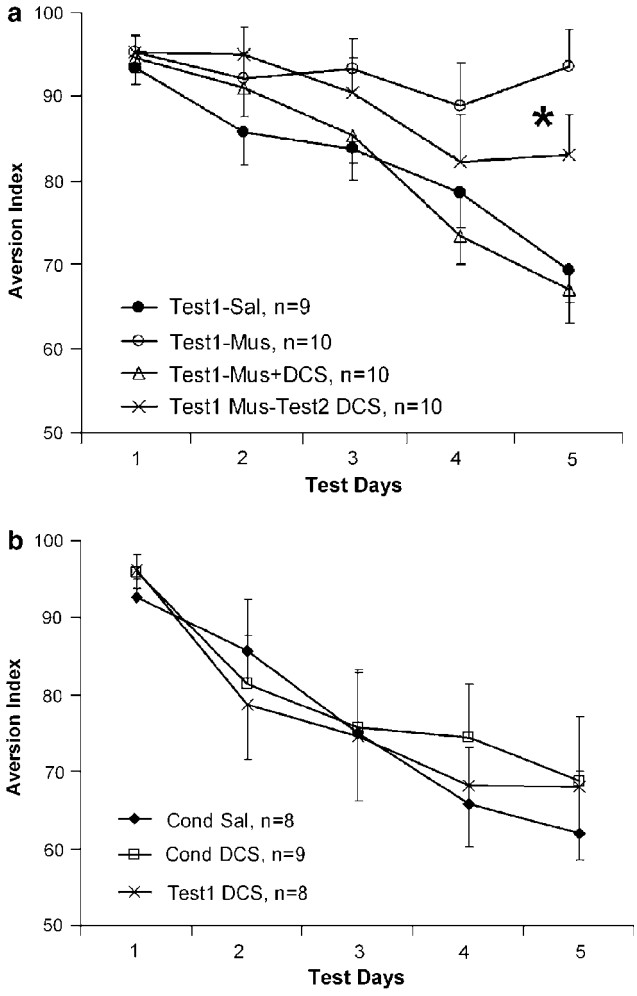

Figure 4 (a) DCS reverses the blocking effects of muscimol on CTA extinction. Animals microinfused with muscimol into the BLA immediately following the test on day 1 (Test1-Mus) showed marked aversion to saccharine that did not reduce with repeated testing. In contrast, animals microinfused with both muscimol and DCS showed marked aversion that extinguished with repeated testing (see Results for details) (*, $P<0.01$). (b) DCS does not have lingering aversive effects on taste preference. Animals microinfused with saline (Cond Sal) or DCS (Cond DCS) immediately following exposure to saccharine on conditioning day or microinfused with DCS immediately following the test on day 1 (Test1 DCS) were not significantly different in their aversion to saccharine. Thus, DCS effects are probably not due to neurotoxicity or other nonspecific influences.

DISCUSSION

The central finding of this study is that the $GABA_A$ receptor agonist, muscimol, microinfused into the amygdala immediately following a nonreinforced extinction trial, disrupts subsequent extinction of CTA for at least 2 weeks. Other studies, using contextual fear conditioning and inhibitory avoidance, have also shown a long-lasting effect on extinction following a single microinfusion (Szapiro *et al*, 2003; Vianna *et al*, 2003). It has been suggested that the conditions under which the first CS-no US association is perceived by the animals appear to be much more critical for the establishment of extinction than those prevalent at subsequent retention tests (Vianna *et al*, 2003).

The long-lasting effect of the inhibitor on CTA extinction did not result from permanent tissue damage since the agonist did not affect the ability to acquire and extinguish new CTA to a new taste. Furthermore, the lasting effect of muscimol on extinction did not generalize to other tastes. Importantly, muscimol infused following the second nonreinforced test shifted the extinction curve up, but did not significantly impair extinction.

The cannulae were implanted into the BLA. As we used a large volume of infusion (1 μl), the possibility of injection spread to other structures, especially the central amygdala nucleus, cannot be completely ruled out. However, recent pharmacological and neuroanatomical studies indicate that

the BLA, but not the central amygdala nucleus, is involved in CTA extinction (Bahar *et al*, 2004; Mickley *et al*, 2004), suggesting that the effects reported here were primarily located in the BLA.

$GABA_A$ in Amygdala and CTA Extinction

Evidence for the involvement of $GABA_A$ receptors in amygdala plasticity is particularly interesting due to the role of the amygdala in emotional processes and the role of the $GABA_A$ receptors in anxiety states. The BLA contains a high density of GABA neurons (Nitecka and Ben-Ari, 1987) and electrophysiological studies have suggested the existence of inhibitory intra-amygdaloid connections (Le Gal La Salle, 1976). It is also endowed with a highly divergent system of intrinsic glutamatergic connections. However, BLA projection cells have unusually low firing rates and this contradiction is explained by the presence of strong inhibitory weight in the BLA (Pare *et al*, 2003).

Several studies support the central role GABA neurotransmission has in extinction, however, there are different reports regarding the role of GABA in extinction (Harris and Westbrook, 1998; Marsicano *et al*, 2002; McGaugh *et al*, 1990; Pereira *et al*, 1988, 1989; Shumyatsky *et al*, 2002). Systemic administration of the $GABA_A$ antagonist picrotoxin after the extinction of inhibitory avoidance learning enhanced extinction retention during testing (McGaugh *et al*, 1990), and the $GABA_A$-positive allosteric modulator diazepam impaired extinction retention when administered before extinction in a shuttle avoidance task (Pereira *et al*, 1989). Recently, Azad *et al* (2004) have shown that cannabinoid type 1 receptors reduce GABAergic synaptic transmission in the amygdala and by that facilitate extinction of aversive memories.

However, McCabe *et al* (2004) showed that benzodiazepine agonists administered to mice following training significantly facilitated extinction on a food reinforced lever-press procedure. Additionally, Shumyatsky *et al* (2002) found that gastrin-releasing peptide (GRP) receptor-deficient mice showed decreased inhibition of principal neurons through the GABAergic interneurons in the amygdala and showed impaired extinction of long-term fear memory. More recently, Chhatwal *et al* (2005) showed that gephyrin mRNA and protein levels in the BLA significantly increased after fear extinction training, suggesting that the modulation of gephyrin and $GABA_A$ receptor expression in the BLA may play a role in the experience-dependent plasticity underlying extinction.

Overall, this data suggests that manipulation of GABA transmission may have very different effects depending on whether it is administered prior or after extinction training or before retention test, and what is the behavioral paradigm used.

The Importance of GABA in Expression and Consolidation of Extinction

It has been suggested that GABA-mediated inhibition is involved in the expression of extinction and not only in the plasticity of extinction (Davis and Myers, 2002). In view of this point, Harris and Westbrook (1998) demonstrated that systemic injection of the beta-carboline FG 7142 (a $GABA_A$ inverse agonist) before extinction training attenuated both the acquisition and the expression of fear memory extinction. However, here muscimol was infused immediately following the first extinction session, hence, during the time window of stabilization of the memory trace (ie, consolidation). By this, most likely that muscimol affected extinction plasticity and avoided possible influence on the expression of extinction. It seems that manipulation of GABA may affect different memory processes (ie, acquisition, consolidation or retrieval) or affect the expression of extinction depending on the time of drug administration.

The Lasting Effect of Muscimol on CTA Extinction

The crucial moment for the beginning of extinction is the first time the animals perceive that the US no longer follows the CS (ie, in the first test; Pavlov, 1927; Rescorla, 2001). Hence, this study corroborates with other extinction experiments suggesting that drugs given at the time of the first of a series of retrieval tests are able to affect extinction in a long-lasting manner (Falls *et al*, 1992; Lu *et al*, 2001; Szapiro *et al*, 2002; Vianna *et al*, 2001, 2003). For example, administering the protein synthesis inhibitor anisomycin into the insular cortex immediately after the first extinction session blocked CTA (Berman and Dudai, 2001). Intrahippocampal administering of a high dose of muscimol impaired whereas the GABAergic antagonist picrotoxin enhanced extinction of fear conditioning when administered after an extinction session (McGaugh *et al*, 1990; Corcoran and Maren, 2001). Moreover, it has been suggested that the molecular requirements of extinction play a role only at the time of the initiation of extinction and shortly thereafter, later they are no longer needed (Cammarota *et al*, 2004). In support of that, in this study muscimol infused to the BLA following the second extinction session did not significantly impair extinction whereas infusion following the first extinction session resulted in long-term impairment.

It is intriguing why extinction was not just delayed, but actually cancelled over almost 2 weeks of testing in the muscimol-treated animals. Most studies did not extend their observations beyond the second test, thus it can-not be determined whether there was any subsequent spontaneous recovery in those animals (but see: Berman and Dudai, 2001; Santini *et al*, 2001; Vianna *et al*, 2001). Importantly, there are other studies that also found a lasting blockade of extinction following a single drug treatment (Vianna *et al*, 2003; Szapiro *et al*, 2003). Vianna *et al* (2003) have shown that intrahippocampal anisomycin or inhibitors of gene transcription before the first extinction session blocked extinction of inhibitory avoidance for several days following the paharmacological manipulation. Szapiro *et al* (2003) reported that post-training intrahippocampal infusion of NMDA antagonist or protein kinase inhibitor blocked extinction of inhibitory avoidance for the subsequent 4 days of testing. They suggested that when the onset of extinction is prevented in the first test session, it usually becomes detectable only many (ie, >5) trials later (Konorski, 1948). Thus, retrieval of the CS-US association overcomes the CS-no US association after the first test, making extinction learning and consolidation more difficult. More to the point, it has been shown that the onset of

detectable extinction varies over experiments even using the same animal or the same task (Izquierdo *et al*, 1999). Taken together, it remains to be investigated what determines weather a single infusion, at the critical time of extinction acquisition or consolidation, will have a long-term effect or not. This may be dependent on the type of learning examined, the time of infusion, the drug infused and the brain region involved.

The muscimol dose administered here did not have adverse effects, which might have influenced performance in this task; it did not induce CTA when LiCl was not injected and did not disturb subsequent acquisition and extinction of a new taste (glycine). Thus, its effects cannot be attributed to neurotoxicity or general impairment.

The fact that muscimol by itself did not induce CTA rules out the possibility that the resistance to extinction seen in muscimol microinfused animals results from enhanced conditioning. In other studies, muscimol infusion was found to have a behavioral effect of at least 60–80 min (Bast *et al*, 2001), which is a suitable time scale for the consolidation of extinction to be formed. Furthermore, the effects are probably not due to drug actions still present on the test days following drug administration. Studies on the involvement of the amygdala in fear conditioning suggest that local microinfusions into single brain areas do not interfere with learning merely on the basis of state dependency (Helmstetter and Bellgowan, 1994; Muller *et al*, 1997). In any case in this study, the drugs were administered following the liquid consumption to avoid drug influence on the animals' drinking.

$GABA_A$ and NMDA

Recent theories suggest that extinction is at least partially based on an active relearning process, in which the prolonged reexposure to the CS in the absence of the US triggers the formation of a new memory trace encoding the dissociation between the CS and the US (Myers and Davis, 2002; Quirk *et al*, 2000; Suzuki *et al*, 2004). This raised the interesting possibility that NMDA receptor agonists might facilitate extinction. There is evidence that the partial NMDA agonist DCS facilitates extinction of learned fear in rats and was suggested to have substantial clinical value in the treatment of anxiety disorders and to have a number of potential clinical benefits (Birk, 2004; Ledgerwood *et al*, 2005; Ressler *et al*, 2004). Specifically, in fear conditioning, it has been shown that systemic or intra-amygdala administration of DCS before extinction training facilitated extinction (Davis *et al*, 2003; Richardson *et al*, 2004; Walker *et al*, 2002). Here, we found that microinfusing DCS together with muscimol reversed the impairing effects of muscimol on CTA extinction. Thus, $GABA_A$ receptor agonist was found to impair CTA extinction and NMDA receptor agonist to reverse it.

Cellular Mechanisms Involved in Extinction

Myers and Davis (2002) have suggested that the consolidation of the plasticity associated with extinction depends not on GABA but on some other neurotransmitters (for example glutamate). Accordingly, extinction is associated with a strengthening of connections between sensory pathways transmitting conditioned stimulus (CS)-related information and a population of GABAergic cells mediating extinction performance. Hence, GABA release during extinction training would be expected to hinder extinction since the development of neural plasticity requires significant excitation of target cells (ie, membrane depolarization, activation of NMDA receptors, calcium entry, etc.), which GABA release would counteract. Thus it follows that GABA agonists could retard extinction if they are present during the *critical period of plasticity* (ie, acquisition or consolidation of extinction). Muscimol increases inhibition of neurons bearing the $GABA_A$ receptor, thus it increases the inhibition of neurons in the BLA that are subject to inhibition via $GABA_A$ receptors. Therefore, the long-term influence of a single muscimol microinfusion on extinction of CTA may possibly result from GABA inhibition of principal (possibly glutamatergic) neurons that changes the cells' response to the CS, and may result in long-term resistance to extinction. In other words, the presence of a GABAergic agonist during the critical time of plasticity may have the effect of a salient stimulus that strongly disrupts the consolidation of CS-no US association. This may result in a strong memory for the aversiveness of the taste, which is then markedly resistant to extinction. In any case, the evidence strongly suggests that extinction leads to plasticity-dependent alterations within the GABA circuit in the amygdala.

Potential Relevance to Post-Traumatic Stress

In the majority of people exposed to trauma, the re-experiencing, avoidance and hyper arousal responses extinguish over time. However, in a substantial minority, extinction fails and these persisting responses become the symptoms of PTSD. It has been suggested that PTSD is a disorder caused in part by the failure of extinction of predictable post-traumatic physiological and psychological reactions. Thus, deficits in extinction of conditioned fear have been proposed as a basis for the sustained anxiety responses seen in PTSD and treatment-resistant phobias (Charney *et al*, 1993; Rothbaum and Davis, 2003).

In clinical populations, a reduced ability to extinguish conditioned fear associations might contribute to the persistence of maladaptive fear and may reduce the effectiveness of therapeutic interventions that rely on extinction processes (eg, systemic desensitization, exposure, and imagery therapies). The effectiveness of these clinical approaches might be facilitated by pharmacological interventions that promote extinction (Walker *et al*, 2002). It has been suggested that the consolidation of extinction involves relearning that reinforces extinction memory through NMDA-mediated plasticity (Davis *et al*, 2003; Santini *et al*, 2001). We show here that the balance between GABA and NMDA may be a critical factor in the ability to extinguish aversive memories. This should be taken into consideration when using DCS or GABA-based agents to treat clinical fear.

Summary

In conclusion, these findings may have important clinical implications because they are beginning to identify key factors involved in the extinction of aversive experiences

that may be relevant to both further research aiming to understand the mechanisms of extinction impairment and to the development of new therapeutic drugs.

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