Spermidine-induced improvement of reconsolidation of memory involves calcium-dependent protein kinase in rats

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In this study, we determined whether the calcium-dependent protein kinase (PKC) signaling pathway is involved in the improvement of fear memory reconsolidation induced by the intrahippocampal administration of spermidine in rats. Male Wistar rats were fear conditioned using a 0.4-mA footshock as an unconditioned stimulus. Twenty-four hours after training, animals were re-exposed to the apparatus in the absence of shock (reactivation session). Immediately after the reactivation session, spermidine (2–200 pmol/site), the PKC inhibitor 3-[1-(dimethylamino-propyl)indol-3-yl]-4-(indol-3-yl) maleimide hydrochloride (GF 109203X, 0.3–30 pg/site), the antagonist of the polyamine-binding site at the NMDA receptor, arcaine (0.2–200 pmol/site), or the PKC activator phorbol 12-myristate 13-acetate (PMA, 0.02–2 nmol/site) was injected. While the post-reactivation administration of spermidine (20 and 200 pmol/site) and PMA (2 nmol/site) improved memory reconsolidation, GF 109203X (1, 10, and 30 pg/site) and arcaine (200 pmol/site) impaired it. GF 109203X (0.3 pg/site) impaired memory reconsolidation in the presence of spermidine (200 pmol/site). PMA (0.2 nmol/site) prevented the arcaine (200 pmol/site)-induced impairment of memory reconsolidation. Anisomycin (2 μg/site) also impaired memory reconsolidation in the presence of spermidine (200 pmol/site). Drugs had no effect when they were administered in the absence of reactivation. These results suggest that the spermidine-induced enhancement of memory reconsolidation involves PKC activation.

[Supplemental material is available for this article.]

In 1968, Schneider and Sherman showed that an immediate electroconvulsive shock (0.5 sec after a reactivating footshock) impairs the recovery of a reactivated memory (Schneider and Sherman 1968). In the same year, Misani et al. (1968) suggested that consolidated memories become labile and susceptible to modulation when they are reactivated; that is, when they are evoked, they must undergo a new stabilization process to persist (Nader et al. 2000; Sara 2000; de la Fuente et al. 2011; Graff et al. 2014). This new stabilization process has been termed “reconsolidation” (Przybyslawski et al. 1999).

N-methyl-D-aspartate glutamatergic receptors (NMDAR), particularly those containing the GluN2B subunit, play an important role in reconsolidation (Wang et al. 2009). In fact, the antagonist of NMDA receptors that contain the GluN2B subunit, ifenprodil, prevents consolidated and stable auditory fear memories from returning to a labile state (Ben Mamou et al. 2006). Moreover, NMDA receptor antagonists, such as MK-801 and AP5, impair fear memory reconsolidation, whereas the agonist D-cycloserine enhances it in mice and rats (Ben Mamou et al. 2006; Lee et al. 2006; Flint et al. 2013; Merlo et al. 2014). It has been shown that spermidine, which is an endogenous polyamine that binds to the interface between the GluN1 and GluN2B subunits of the NMDA receptor (Mony et al. 2011), improves the reconsolidation of the fear conditioning memory (Ribeiro et al. 2013). Accordingly, the antagonist of this polyamine-binding site at the NMDAr, arcaine, impairs reconsolidation, which suggests a putative physiological role for polyamines in this process (Ribeiro et al. 2013).

Current behavioral and neurochemical evidence suggests that the facilitatory effect of spermidine on memory depends on nitric oxide synthase (NOS) activity because the administration of N(G)-nitro L-arginine methyl ester (L-NAME), which is a non-specific inhibitor of NOS, prevents the facilitatory effects of spermidine on the inhibitory avoidance task (Guerra et al. 2006). In addition, Guerra et al. (2011, 2012) showed that protein kinase A (PKA) and PKC inhibitors prevent the facilitatory effect of spermidine on the memory of an inhibitory avoidance task. Accordingly, they also prevented the spermidine-induced phosphorylation of PKC, PKA, and CREB in the hippocampus (Guerra et al. 2011, 2012). Therefore, the effect of spermidine on memory consolidation seems to involve a sequence of biochemical events that are triggered by NMDA receptors, followed by the activation of NOS and the sequential activation of PKC and PKA/CREB signaling in the hippocampus of rats. However, no study has addressed...
of contextual fear memories. (Bonini et al. 2007). Considering these studies, we hypothesized that the facilitatory effects of SPD on memory reconsolidation involve PKC. Therefore, in the current study, we investigated whether the intrahippocampal infusion of SPD and arcaine alters memory reconsolidation in rats and whether protein synthesis and PKC are involved in the polyamine-induced improvement of fear memory reconsolidation.

Results

Post-reactivation intrahippocampal spermidine improves reconsolidation

Figure 1 shows the effect of the bilateral intrahippocampal injection of spermidine (2–200 pmol/site) immediately after reactivation on the reconsolidation of fear conditioning. Statistical analysis (one-way ANOVA) of freezing scores at testing revealed a significant effect of the drug ($F_{(3,14)} = 3.78$, $\omega = 0.56$, $P = 0.035$). Post hoc analysis (SNK) revealed that spermidine at the doses of 20 and 200 pmol/site increased freezing scores. These results suggest that the spermidine facilitates memory reconsolidation. As expected, statistical analysis (one-way ANOVA) of the freezing scores during the reactivation session revealed no difference between the groups ($F_{(3,14)} = 0.04$, $P = 0.98$, reactivation data in a Supplemental Table S1), which indicates that the animals' behavior was similar between groups before drug administration.

GF 109203X impairs memory reconsolidation and prevents spermidine-induced improvement of memory

Figure 2A shows the effect of the bilateral intrahippocampal injection of the PKC inhibitor 3-[1(dimethylaminopropyl)in-
dol-3-yl]-4-(indol-3-yl) maleimide hydrochloride (GF 109203X) immediately post-reactivation at doses of 0.3, 1, 10, or 30 pg/site on the reconsolidation of fear conditioning. Statistical analysis (one-way ANOVA) of the freezing scores revealed a significant effect of drug treatment ($F_{(4,17)} = 9.21$, $\omega = 0.77$, $P = 0.0004$). Post hoc analysis (SNK) revealed that GF 109203X at the doses of 1, 10, and 30 pg/site decreased freezing scores. These results suggest that the intrahippocampal administration of the selective PKC inhibitor impairs memory reconsolidation. As expected, statistical analysis (one-way ANOVA) of the reactivation session freezing scores revealed no difference among the groups ($F_{(4,17)} = 0.22$, $P = 0.94$, reactivation data in a Supplemental Table S1), which indicates that the animals' behavior was similar between groups before drug administration.

Post-reactivation intrahippocampal administration of arcaine impairs reconsolidation of contextual fear memories

Figure 3 shows the effect of the bilateral intrahippocampal injection of arcaine (0.2–200 pmol/site) immediately post-reactivation on fear conditioning reconsolidation. Statistical analysis (one-way ANOVA) of freezing scores revealed a significant effect of drug treatment ($F_{(4,24)} = 2.94$, $\omega = 0.45$, $P = 0.041$). Post hoc analysis (SNK) revealed that arcaine at a dose of 200 pmol/site decreased freezing scores. These results suggest that intrahippocampal administration of arcaine impairs memory reconsolidation. As expected, statistical analysis (one-way ANOVA) of the reactivation session freezing scores revealed no difference among groups ($F_{(4,24)} = 0.41$, $P = 0.79$, reactivation data in a Supplemental Table S1).

Phorbol 12-myristate 13-acetate improves reconsolidation and prevents arcaine-induced impairment of reconsolidation

Figure 4A shows the effect of the bilateral intrahippocampal injection of the PKC activator phorbol 12-myristate 13-acetate (PMA) immediately post-reactivation at doses of 0.02–2 nmol/site on the reconsolidation of fear conditioning.
The effects of spermidine, GF 109203X, arcaine, PMA, and anisomycin are specific for reconsolidation of contextual fear memories

Figure 6 shows the effect of the bilateral intrahippocampal injection of spermidine (200 pmol/site), GF 109203X (30 pg/site), arcaine (200 pmol/site), PMA (2 nmol/site), or anisomycin (20 μg/site) 24 h after training in the absence of a reactivation session on the reconsolidation of fear conditioning. Statistical analysis (one-way ANOVA) of the freezing scores revealed that spermidine, GF 109203X, arcaine, PMA, and anisomycin did not alter contextual fear conditioning in the absence of reactivation ($F_{(5,23)} = 0.66, P = 0.65$).

Discussion

The main findings of the current study were that while the i.h. injection of spermidine improved memory reconsolidation, arcaine impaired it. While phorbol 12-myristate 13-acetate prevented the arcaine-induced impairment of reconsolidation, GF 109203X treatment (PBS or anisomycin) versus treatment (PBS or spermidine) interaction ($F_{(1,16)} = 29.29, \omega = 0.65, P < 0.001$). Post hoc F tests for simple effects revealed a significant effect of pretreatment (PBS or anisomycin) only in the presence of spermidine ($F_{(1,16)} = 48.30, \omega = 0.90, P < 0.0001$) and a significant effect of treatment (PBS or spermidine) if pretreatment was PBS ($F_{(1,16)} = 7.76, \omega = 0.63, P < 0.05$). These results indicate that spermidine increases freezing scores per se and that it has a permissive role for the impairing effect of anisomycin. These results suggest that anisomycin impairs fear conditioning reconsolidation in the presence of spermidine. Statistical analysis (two-way ANOVA) of the reactivation freezing scores revealed no difference among groups in this experiment ($F_{(1,16)} = 0.006, P = 0.93$, reactivation data in a Suplemental Table S1).

Anisomycin impairs reconsolidation and prevents spermidine-induced improvement of reconsolidation

Figure 5A shows the effect of bilateral intrahippocampal injection of anisomycin (0.2, 2, 20 pg/site) immediately post-reactivation on the reconsolidation of fear conditioning. Statistical analysis (one-way ANOVA) of the test freezing scores revealed a significant effect of drug ($F_{(3,16)} = 21.63, \omega = 0.86, P = 0.0001$). Post hoc analysis (SNK) revealed that PMA (2 nmol/site) increased freezing scores. These results suggest that the intrahippocampal administration of PMA facilitates memory reconsolidation. As expected, statistical analysis (one-way ANOVA) of the reactivation session freezing scores revealed no difference among groups ($F_{(3,16)} = 0.66, P = 0.58$, reactivation data in a Supplemental Table S1).

Figure 4B shows the effect of the bilateral intrahippocampal injection of PMA at a noneffective dose (0.2 nmol/site, immediately post-reactivation) on the impairment of reconsolidation induced by arcaine (200 pmol/site, 5-min post-reactivation). Statistical analysis (two-way ANOVA) revealed a significant pretreatment (PBS or PMA) versus treatment (PBS or arcaine) interaction ($F_{(1,28)} = 6.11, \omega = 0.41, P = 0.019$). Post hoc F test for simple effect revealed a significant effect of treatment (PBS or arcaine) only if the animals were pretreated with PBS ($F_{(1,28)} = 11.56, \omega = 0.63, P < 0.01$). These results indicate that PMA prevents the arcaine-induced impairment of fear conditioning reconsolidation. Statistical analysis (two-way ANOVA) of the reactivation freezing scores also revealed no difference among groups in this experiment ($F_{(1,28)} = 0.24, P = 0.62$, reactivation data in a Supplemental Table S1).

The main findings of the current study were that while the i.h. injection of spermidine improved memory reconsolidation, arcaine impaired it. While phorbol 12-myristate 13-acetate prevented the arcaine-induced impairment of reconsolidation, GF 109203X

Statistical analysis (one-way ANOVA) of the test freezing scores revealed a significant effect of drug ($F_{(3,16)} = 11.16, \omega = 0.77, P = 0.0003$). Post hoc analysis (SNK) revealed that anisomycin (20 μg/site) decreases freezing scores. These results suggest that intrahippocampal anisomycin impairs memory reconsolidation. As expected, statistical analysis (one-way ANOVA) of reactivation session freezing scores revealed no difference among groups ($F_{(3,16)} = 0.33, P = 0.80$, reactivation data in a Supplemental Table S1).

Anisomycin impairs reconsolidation and prevents spermidine-induced improvement of reconsolidation

Figure 5B shows the effect of the bilateral intrahippocampal injection of anisomycin at a noneffective dose (0.2 μg/site, immediately post-reactivation) on the spermidine-induced (200 pmol/site, 5-min post-reactivation) improvement of reconsolidation. Statistical analysis (two-way ANOVA) revealed a significant pre-
PKC family members. Based on their receptor function, and gene expression (Nishizuka 1995) regulates neuronal activity at different levels, including neurotransmitter release, neurotransmitter synaptic interactions, and gene expression.

The currently reported spermidine-induced improvement of memory reconsolidation (Fig. 1A) is in agreement with Ribeiro et al. (2013), who showed that the systemic injection of spermidine facilitates the reconsolidation of fear conditioning memory (Ribeiro et al. 2013). Moreover, it implies the existence of polyaminesensitive modulation in the dorsal hippocampus in the reconsolidation of fear conditioning memories, a view that is fully corroborated by the finding that i.h. arcaine, a polyaminergic antagonist, disrupts memory reconsolidation (Fig. 3). Accordingly, pharmacological, functional imaging, and lesioning studies indicate an important role for the dorsal hippocampus in the reconsolidation of contextual fear conditioning (Boccia et al. 2010; Jobim et al. 2012; Lee et al. 2013; De Jaeger et al. 2014). These findings may also be interpreted as additional evidence supporting a role for hippocampal NMDA receptors, particularly those containing the GluN2 subunit, in memory reconsolidation (Pedreira et al. 2002; Lee et al. 2006; Ribeiro et al. 2013), as far as the agonist and the antagonist of the polyamine-binding site at the NMDA improved and disrupted memory reconsolidation, respectively.

The PKC family of Ser/Thr kinases (Nishizuka 1995) regulates neuronal activity at different levels, including neurotransmitter release, neurotransmitter receptor function, and gene expression (Ben-Ari et al. 1992; Meberg et al. 1993; Mack et al. 1998; Manseau et al. 1998; Kleschevnikov and Routtenberg 2001). There are at least 10 genes coding for PKC family members. Based on their structure and sensitivity to Ca2+ and diacylglycerol (DAG), these isoforms have been classified as conventional (α, β, and γ), novel (δ, ε, η, θ, and μ), and atypical PKCs (ζ and λ), which are structurally homologous, but can be regulated independently of calcium (Parekh et al. 2000; Weeber et al. 2000; Ohno and Nishizuka 2002). All PKC isoforms contain a site between the regulatory and catalytic domain that is susceptible to enzymatic proteolysis. High levels of cytoplasmic calcium can activate proteases that cleave PKC, releasing a cytoplasmic isoform of PKC called protein kinase Mζ (PKMζ) (Kishimoto et al. 1983). This fragment is constitutively active because it loses its regulatory domain. Increased PKMζ expression leads to persistent activation of PKC in hippocampal neurons. In this context, Sacktor (2008) suggested that PKCζ is particularly important for the maintenance of fear memories, though Volk et al. (2013) showed that PKM-ζ is not required for the induction or maintenance of long-term potentiation (LTP) in the CA1 region of the hippocampus. Lee et al. (2013) demonstrated that Prkze-null mice did not show deficits in learning or memory in tests of cued fear conditioning, novel object recognition, object location recognition, conditioned place preference (CPP) for cocaine, or motor learning, suggesting that the absence of PKMζ does not impair learning and memory in mice.

Bonini et al. (2007) showed that the post-retrieval injection of a PKC inhibitor into the CA1 region of the dorsal hippocampus disrupts further retention of spatial memory. Moreover, the amnesia induced by the post-retrieval injection of a PKC inhibitor is not caused by a delayed inhibitory effect on retrieval, but due to inhibition of a process that stabilizes the retrieved trace (Bonini et al. 2007). Crespo et al. (2012) also showed that the activation of PKCζ and PKMζ is necessary for the retrieval and reconsolidation of drug memory. Analogously, we showed that the deleterious effect of GF 109203X, an inhibitor of PKC, depends on the injection of spermidine. Accordingly, the deleterious effect of arcaine on memory reconsolidation was fully prevented by a noneffective dose of the PKC activator PMA, indicating that the improving effects of spermidine on memory reconsolidation involve the activation of PKC in the hippocampus.
Spermidine activates protein kinase C

The finding that anisomycin disrupts memory reconsolidation in the presence of spermidine suggests the involvement of protein synthesis in the currently described effects of spermidine. These results are in agreement with previous studies that showed that consolidation and reconsolidation require protein synthesis (Nader et al. 2000; Sol Fustiñana et al. 2014). Indeed, the injection of protein synthesis inhibitors before or after training or after reactivation impairs long-term memory (Nader et al. 2000; Lopez et al. 2015). Nevertheless, anisomycin can have other important effects on neurobiological function, including the induction of immediate early genes (Edwards and Mahadevan 1992; Rudy et al. 2006; Gold 2008; Radulovic and Tronson 2008), alterations in synaptic release (Canal et al. 2007), and profound suppression of neural activity (Sharma et al. 2012; Greenberg et al. 2014). Therefore, we cannot rule out that other effects of anisomycin unrelated to protein synthesis inhibition interfere in the spermidine-induced improvement of memory reconsolidation.

Hirose et al. (2015) recently showed that polyanamines, particularly spermidine, can permeate NMDA receptors. Therefore, it is possible that a certain amount of extracellular spermidine may reach the intracellular milieu and modulate polyamine-sensitive mechanisms, such as protein synthesis (Nishimura et al. 2009; Igarashi and Kashiwagi 2010). In this context, it has been suggested that endogenous polyanamines may facilitate the action of antibiotics that interfere with protein synthesis, such as streptomycin (Nastri et al. 1993). Accordingly, Goldenberg and Algranati (1981) showed that streptomycin inhibits protein synthesis in polyamine-supplemented cells, whereas bacteria starved for polyanimes were less sensitive to the action of the antibiotic (Goldemberg and Algranati 1981). Although the structures of streptomycin and anisomycin differ markedly, polyanimes may facilitate the anisomycin-induced inhibition of protein synthesis. This would explain not only why anisomycin (by inhibiting protein synthesis) decreases the facilitatory effect of spermidine on memory but also why spermidine clearly facilitates the memory impairment induced by anisomycin. However, further studies are necessary to determine whether polyanimes facilitate the inhibitory effect of anisomycin on protein synthesis in brain cells.

In summary, this study showed that the intrahippocampal injection of GF 109203X impairs memory reconsolidation in the presence of spermidine and that the injection of PMA prevents the arcaine-induced impairment of memory reconsolidation. These findings suggest that ligands of the polyamine binding site at the NMDA receptor modulate memory reconsolidation by PKC-mediated mechanisms.

Materials and Methods

Animals

Experimentally naive adult male Wistar rats (260–320 g, n = 210), from the animal house of the Federal University of Santa Maria were used. The animals were housed four to a cage on a 12-h day/night cycle (lights on at 7:00 a.m.) at a temperature of 21°C with water and standard laboratory chow (Guabi) ad libitum. All experimental procedures were conducted in accordance with the policies on the use of animals and humans in neuroscience research, revised and approved by the Society for Neuroscience Research in January 1995 and with the institutional and national regulations for animal research (process 068/2011).

Surgery

Rats were anesthetized with ketamine (80 mg/kg) and xylazine (8 mg/kg, i.p.) and were implanted with two 27-gauge guide cannulae placed 1-mm above the CA1 region of the dorsal hippocampus at the following coordinates: A = 4.0 mm; L = 3.0 mm; and V = 2.0 mm (Paxinos and Watson 1986). Placement of injections was histologically verified, as described elsewhere (Rubin et al. 1997). A total of 222 animals were subjected to stereotaxic surgery. Twelve animals (6%) were excluded due to incorrect cannula placement. Only data from the animals with correct cannula placement were analyzed.

Drugs

N-(3-aminopropyl)-1,4-butanediamine trihydrochloride (spermidine; Sigma), 3-[1(dimethylaminopropyl) indol-3-yl]-4-[indol-3-yl] maleimide hydrochloride (GF 109203X; Sigma), 1,4-diguani- dinobutane sulfite (arcaine; Pfaltz & Bauer), phorbol 12-myristate 13-acetate (PMA; Sigma) or anisomycin (Sigma) were used. Spermidine, arcaine, and GF 109203X were dissolved in 50-mM phosphate-buffered saline solution (PBS; pH 7.4). Anisomycin was dissolved in 1 M HCl and diluted with PBS to the concentration of 50 mg/mL. The pH of the solution was adjusted to 7.4 with 0.15 M NaOH and the concentration adjusted to 40 mg/mL with PBS. PMA was dissolved in DMSO and diluted to the final concentration of 0.05% DMSO (v/v) with PBS. Drugs were bilaterally injected into the hippocampus (i.h., 0.5 µL/brain hemisphere for 1 min). The injections were performed using an infusion pump and a 30-gauge needle fitted into the guide cannula. The tip of the infusion needle protruded 1.0-mm beyond that of the guide cannula into the CA1 region in the dorsal hippocampus. The needles were left in place for additional 60 sec to minimize backflow. Doses were selected based on previous studies (Guerra et al. 2012) and pilot experiments.

Conditioning apparatus

Contextual fear conditioning, reactivation, and testing took place in a fear conditioning chamber (30 × 25 × 25 cm) located in a well-lit room. The front wall and ceiling of the chamber were made of clear acrylic plastic, whereas the lateral and rear walls were made of opaque plastic. The floor of the chamber consisted of 32 stainless steel rods (3-mm diameter) spaced 1-cm apart and wired to a shock generator. The chamber was cleaned with 30% ethyl alcohol before and after each rat occupied it.

Behavioral procedure

Contextual fear conditioning

In the conditioning trial, each animal was subjected to a single fear conditioning training session, as described by Rubin et al. (2004) with some modifications. In brief, the rat was placed in

Figure 6. Spermidine, phorbol 12-myristate 13-acetate (PMA), arcaine, GF109203X, and anisomycin are specific for reconsolidation of contextual fear memories. Rats received an intrahippocampal administration of PBS, spermidine (SPD, 200 pmol/site), GF109203X (30 pg/site), arcaine (ARC, 200 pmol/site), PMA (2 nmol/site), anisomycin (ANI, 20 µg/site) 24 h after training in the absence of reactivation session, and were tested for memory reconsolidation 1 d later. Data are means ± SEM percentage of freezing in the testing session (n = 4–5 animals in each group).
Experiment 4

This experiment was designed to investigate the effect of PMA, a PKC activator, on memory reconsolidation to determine whether PMA prevents the arcaine-induced impairment of memory reconsolidation. Animals were trained in the fear conditioning apparatus as described above. Immediately after the reactivation session, the animals were injected (i.h.) with PBS or PMA (0.02, 0.2 or 2 nmol/site), and 24 h later, they were tested in the fear conditioning apparatus where their freezing responses were scored as described above.

Once it was determined that PMA at a dose of 0.2 nmol/site did not alter the reconsolidation of memory, the effect of PMA on the arcaine-induced impairment of memory reconsolidation was determined. The animals were trained in the fear conditioning apparatus as described above. Immediately after the reactivation session, the animals were injected with PBS or PMA (0.2 nmol/site), and 5 min later, they were injected with PBS or arcaine (200 pmol/site). Twenty-four hours after reactivation, the animals were tested in the fear conditioning apparatus and their freezing responses were scored.

Experiment 5

This experiment was designed to investigate the involvement of protein synthesis in the effect of spermidine on memory reconsolidation.

Animals were trained in the fear conditioning apparatus as described above. Immediately after the reactivation session, the animals were injected (i.h.) with PBS or a protein synthesis inhibitor, anisomycin (0.2, 2, or 20 μg/site), and 24 h later, they were tested in the fear conditioning apparatus where their freezing responses were scored as described above.

Once it was determined that anisomycin at a dose of 0.2 μg/site did not alter memory reconsolidation, the effect of anisomycin on the spermidine-induced improvement of memory reconsolidation was determined. The animals were trained in the fear conditioning apparatus as described above. Immediately after the reactivation session, the animals were injected with PBS or anisomycin (0.2 μg/site, i.h.), and 5 min later, they were injected with PBS or SPD (200 pmol/site, i.h.). Twenty-four hours after reactivation, the animals were tested in the fear conditioning apparatus and their freezing responses were scored.

Experiment 6

Control experiments without the memory reactivation session were performed to evaluate whether the effects of spermidine, GF 109203X, arcaine, PMA, or anisomycin are specific for the reconsolidation of contextual fear memories. Animals were trained in the fear conditioning apparatus but without the memory reactivation session 24 h later. The animals were injected (i.h.) with PBS, spermidine (200 pmol/site), GF 109203X (30 pg/site), arcaine (200 pmol/site), PMA (2 nmol/site) or anisomycin (20 μg/site) 24 h after training, and 24 h later, they were tested in the fear conditioning apparatus and had their freezing responses scored.

Statistics

The data were analyzed by one or two-way analysis of variance (ANOVA), depending on the experimental design. Post hoc analyses were carried out by the Student–Newman–Keuls test and F test for simple effect when indicated. P < 0.05 was considered significant.

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