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ARTICLE

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Histamine facilitates consolidation of fear extinction

Juliana Sartori Bonini[1,2], Weber Cláudio Da Silva[1,3], Clarice Kras Borges Da Silveira[1], Cristiano André Köhler[1], Iván Izquierdo[1,3] and Martín Cammarota[1,3]

[1] *Centro de Memória, Instituto do Cérebro, Pontifícia Universidade Católica do Rio Grande do Sul, Porto Alegre, RS, Brasil*
[2] *Departamento de Farmácia, Universidade Estadual do Centro-Oeste/UNICENTRO, Guarapuava, PR, Brasil*
[3] *Instituto Nacional de Neurociência Translacional, Conselho Nacional de Desenvolvimento Científico e Tecnológico, Brasil*

Abstract

Non-reinforced retrieval induces memory extinction, a phenomenon characterized by a decrease in the intensity of the learned response. This attribute has been used to develop extinction-based therapies to treat anxiety and post-traumatic stress disorders. Histamine modulates memory and anxiety but its role on fear extinction has not yet been evaluated. Therefore, using male Wistar rats, we determined the effect of the intra-hippocampal administration of different histaminergic agents on the extinction of step-down inhibitory avoidance (IA), a form of aversive learning. We found that intra-CA1 infusion of histamine immediately after non-reinforced retrieval facilitated consolidation of IA extinction in a dose-dependent manner. This facilitation was mimicked by the histamine *N*-methyltransferase inhibitor SKF91488 and the H_2 receptor agonist dimaprit, reversed by the H_2 receptor antagonist ranitidine, and unaffected by the H_1 antagonist pyrilamine, the H_3 antagonist thioperamide and the antagonist at the NMDA receptor (NMDAR) polyamine-binding site ifenprodil. Neither the H_1 agonist 2-2-pyridylethylamine nor the NMDAR polyamine-binding site agonist spermidine affected the consolidation of extinction while the H_3 receptor agonist imetit hampered it. Extinction induced the phosphorylation of ERK1 in dorsal CA1 while intra-CA1 infusion of the MEK inhibitor U0126 blocked extinction of the avoidance response. The extinction-induced phosphorylation of ERK1 was enhanced by histamine and dimaprit and blocked by ranitidine administered to dorsal CA1 after non-reinforced retrieval. Taken together, our data indicate that the hippocampal histaminergic system modulates the consolidation of fear extinction through a mechanism involving the H_2-dependent activation of ERK signalling.

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Introduction

Non-reinforced expression of conditioned fear leads to extinction of the learned response. This property has been used to develop extinction-based therapies to treat anxiety disorders. Nevertheless, although these therapies are effective, relapse following their apparent success is frequent (Rowe & Craske, 1998). Since extinction results from a new learning that inhibits the original memory (Pavlov, 1928), it is possible that treatments that facilitate memory formation also improve the therapeutic effectiveness of extinction (Davis *et al.* 2006).

Histamine is synthesized by neurons in the tuberomammilary nucleus which ramify throughout the brain. Of the four histamine receptors identified so far only H_1, H_2 and H_3 subtypes are expressed in the brain. H_1 and H_2 receptors potentiate excitatory inputs while H_3 receptors down-regulate histamine synthesis and release as well as release of other neurotransmitters (Haas & Panula, 2003; Panula *et al.* 1989). Histamine controls the sleep–wake cycle and nociception and modulates the activity of the hippocampus, a brain region essential for aversive memory processing (Brown *et al.* 2001). In fact, hippocampal histamine receptors are involved in fear memory consolidation and control anxiety (Da Silva *et al.* 2006;

Address for correspondence: Professor M. Cammarota, Laboratório de Neuroquímica e Neurofisiologia da Memória, Centro de Memória, Instituto do Cérebro, Pontifícia Universidade Católica de Rio Grande do Sul (PUCRS), Av. Ipiranga 6690, Andar 2, Porto Alegre, RS 90610-000, Brasil.
Tel.: +55 51 3320-3403 *Fax*: +55 51 3320-3312
Email: mcammaro@terra.com.br *or* martin.cammarota@pucrs.br

Passani *et al.* 2007; Zarrindast *et al.* 2002). However, the role of histamine in the consolidation of extinction memory has not yet been analysed.

Here, we determined the effect of the intra-CA1 infusion of different histaminergic agents on the extinction of step-down inhibitory avoidance (IA), a well-established model for studying fear memory in the rat. Because histamine modulates ERK1/2 signalling (Passani *et al.* 2007), and these kinases are necessary for extinction (Herry *et al.* 2006), we also analysed the possible interplay between histamine and ERK activation.

Materials and methods

Subjects, surgery and infusion procedures

Male Wistar rats, aged 3 months (280–300 g) were used. They were housed at 22–24 °C and maintained on a 12-h light/dark cycle (lights on 07:00 hours) with free access to food and water. Rats were bilaterally implanted with 27-gauge stainless-steel cannulas in the CA1 region of the dorsal hippocampus under ketamine/xylazine anaesthesia. Stereotaxic coordinates were 4.0 mm posterior to bregma, 3.0 mm lateral to the midline, and 1.8 mm ventral to the skull surface (Paxinos & Watson, 1986). Infusions (1 μl/side) were carried out using an infusion pump. Placement of the cannulas was verified post-mortem: 2–4 h after the last behavioural test, 1 μl of a 4% Methylene Blue solution was infused as described above and the extension of the dye 30 min thereafter taken as an indication of the diffusion of the drug previously injected. Only data from animals with correct implants were analysed. All experimental procedures followed the guidelines of the USA National Institutes of Health Guide for the Care and Use of Laboratory Animals (DHEW Publications, NIH 80–23) and were approved by the Animal Care and Use Committees of the Pontifical Catholic University of Rio Grande do Sul.

IA training and extinction

The training apparatus was a 50 × 25 × 25 cm poly-(methyl methacrylate) box with a 5-cm high, 8-cm wide and 25-cm long platform on the left end of a grid of bronze bars. During the training session the animals were placed on the platform and when they stepped down onto the grid, received a 2-s, 0.8-mA scrambled footshock. To extinguish the avoidance response, rats were submitted to one non-reinforced IA test session 24 h or 72 h post-training. To achieve this, the animals were placed on the training box platform until they stepped down onto the grid. No footshock was given and the animals were allowed to explore the apparatus for 10 min. During this time, they stepped up onto the platform and down again several times. Retention was assessed in a second non-reinforced test session 24 h or 72 h later.

Open-field and plus-maze tests

To analyse their locomotor and exploratory activities, animals were placed in a 50 × 50 × 39 cm open-field arena with the floor divided into 12 equal squares. Line crossings and rearings were measured over a 5-min period. To evaluate their anxiety state, rats were exposed to an elevated plus-maze exactly as described by Pellow *et al.* (1985). The total number of entries into the four arms and the number of entries and time spent into the open arms were recorded over a 5-min session.

Immunoblot

Animals were killed by decapitation and the CA1 region of the dorsal hippocampus homogenized in ice-chilled buffer [20 mM Tris–HCl (pH 7.4), 0.32 M sucrose, 1 mM EDTA, 1 mM EGTA, 1 mM PMSF, 10 μg/ml aprotinin, 15 μg/ml leupeptin, 10 μg/ml bacitracin, 10 μg/ml pepstatin, 15 μg/ml trypsin inhibitor, 50 mM NaF, and 1 mM sodium orthovanadate]. Equal amounts of protein were fractionated by SDS–PAGE and electrotransferred to PVDF membranes. Blots were blocked in 100 mm Tris–HCl (pH 7.5), containing 0.9% NaCl and 0.1% Tween-20 and incubated overnight with anti-ERK1/2 or anti-phosphoERK1/2 antibodies. Immunoreactivity was quantified using the West-Pico enhanced chemiluminescence kit (Pierce, USA) and the ImageQuant RT-ECL system (GE, USA).

Drugs and antibodies

Drugs were from Sigma-Aldrich (USA), Tocris Cookson Ltd (UK) or Promega (USA). Antibodies were from Cell Signaling (USA). Drugs were dissolved in saline or DMSO and stored at −20 °C. Before use aliquots were diluted to working concentration with vehicle (0.1% DMSO in saline; pH 7.2). The doses used were based on pilot experiments and on studies showing their effect on behavioural and physiological variables (Alvarez & Ruarte, 2002; Baldi *et al.* 2005; Di Carlo *et al.* 2000; Duch *et al.* 1978; Giovannini *et al.* 2003; Knoche *et al.* 2003; Malmberg-Aiello *et al.* 1997).

Statistical analyses

Data were analysed by Student's *t* test or one-way ANOVA followed by Dunnett's test.

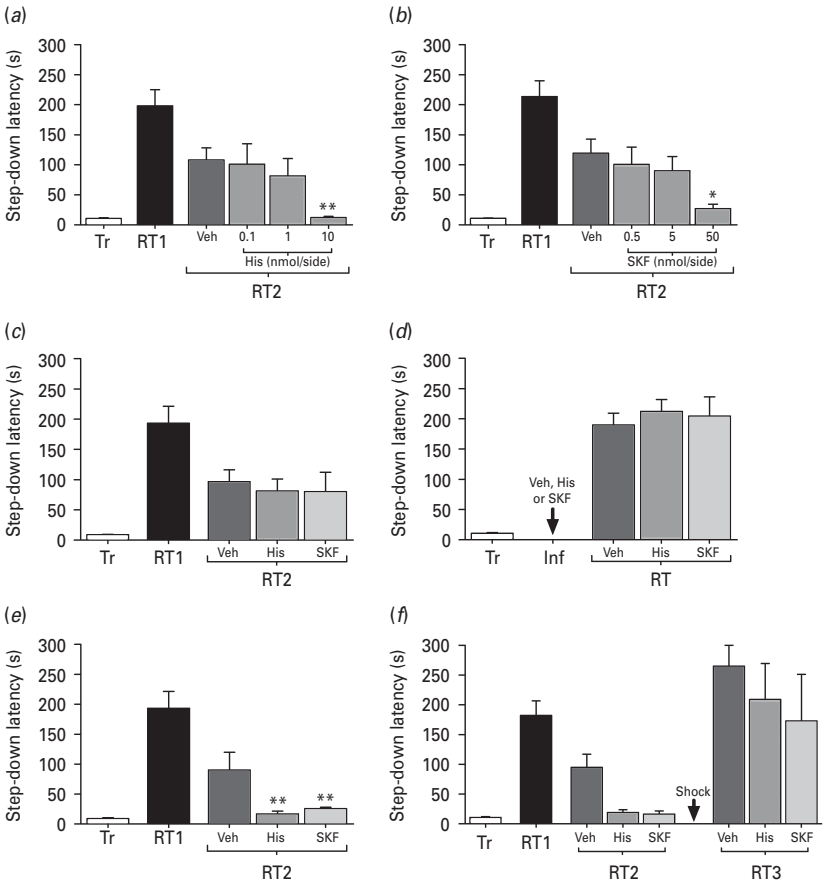

Fig. 1. Histamine and SKF91488 facilitate extinction. (*a*) Rats with infusion cannulas implanted in the CA1 region were trained (Tr) in inhibitory avoidance (IA) and 24 h later submitted to a non-reinforced test session (RT1). Immediately thereafter, animals received infusions of vehicle (Veh) or histamine (His). Retention was evaluated 24 h after RT1 (RT2). (*b*) Animals were treated as in (*a*) but received SKF91488 (SKF) instead of histamine. (*c*) Animals were treated as in (*a*) but received His (10 nmol/side) or SKF91488 (50 nmol/side) 90 min after the non-reinforced test session. (*d*) IA-trained rats received infusions of Veh, His (10 nmol/side) or SKF (50 nmol/side) 24 h post-training (Inf). Retention was evaluated 24 h later. (*e*) Animals were treated as in (*a*) and received infusions of His (10 nmol/side) or SKF (50 nmol/side) immediately after the non-reinforced test session. Retention was evaluated 72 h after RT1 (RT2). (*f*) Animals were treated as in (*a*) and received infusions of His (10 nmol/side) or SKF (50 nmol/side) immediately after RT1. Retention was evaluated 24 h later (RT2). Immediately after stepping-down onto the grid during RT2, rats received a footshock identical to that received during the training session. Retention was re-evaluated 24 h later (RT3). Bars represent mean (±S.E.M.); $n=16$–25 per group; * $p<0.05$ and ** $p<0.01$ in Dunnett's test after ANOVA.

Results

To analyse the role of the hippocampal histaminergic system in the consolidation of fear extinction, rats received IA training and 24 h later were submitted to a 10-min non-reinforced test session to extinguish the avoidance response. At different times after this session, the animals received bilateral intra-CA1 infusions of vehicle, histamine (0.1–10 nmol/side) or SKF91488 (0.5–50 nmol/side), a histamine *N*-methyltransferase inhibitor which blocks histamine inactivation (Malmberg-Aiello *et al.* 1997). Extinction was assessed 24 h later. When administered immediately after non-reinforced retrieval, histamine (Fig. 1*a*; $F_{3,78}=3.97$, $p<0.05$; $q=3.02$ for 10 nmol/side) and SKF91488 (Fig. 1*b*; $F_{3,96}=3.22$, $p<0.05$; $q=2.98$ for 50 nmol/side) facilitated the consolidation of extinction in a dose-dependent manner. Histamine and SKF91488 had no effect on the consolidation of extinction when administered 90 min after the non-reinforced test (Fig. 1*c*) and did not affect IA memory when administered 24 h post-training in the absence of a non-reinforced retention test session (Fig. 1*d*). The effect of histamine and SKF91488 on the consolidation of extinction was also observed when retention was assessed 72 h after the non-reinforced test (Fig. 1*e*).

Intra-CA1 administration of histamine and SKF91488 immediately after non-reinforced retrieval did not affect reacquisition of IA memory upon retraining (Fig. 1*f*; $F_{2,44}=0.56$, $p=0.57$).

H_1, H_2 and H_3 receptors are expressed in the hippocampus. Moreover, histamine binds to the polyamine site of the *N*-methyl-D-aspartate receptor (NMDAR; Haas & Panula, 2003). Since activation of different histamine receptor subtypes induces opposite physiological responses (Baldi *et al.* 2005) and polyamines modulate fear conditioning (Camera *et al.* 2007), we studied the specific participation of H_1, H_2 and H_3 receptors and of the NMDAR polyamine site in extinction.

When administered to dorsal CA1 immediately after non-reinforced retrieval the H_2 agonist dimaprit (10 nmol/side), but not the H_1 agonist 2-2-pyridylethylamine (2-PEA; 10 nmol/side; Braga *et al.* 1996) or the agonist at NMDAR polyamine-binding site, spermidine (10 nmol/side; Berlese *et al.* 2005), mimicked the facilitation induced by histamine (Fig. 2). Concurring with the putative role of H_3 receptors as negative regulators of histamine synthesis and release, the H_3 receptor agonist imetit (10 nmol/side) hampered the consolidation of extinction memory, suggesting that endogenous histamine modulates this process, possibly through activation of hippocampal H_2 receptors ($F_{4,95}=9.39$, $p<0.001$; $q=2.70$ for dimaprit and $q=3.39$ for imetit). In order to analyse this hypothesis, we studied the effect of specific antagonists of histamine receptor subtypes on extinction. When administered to dorsal CA1 immediately after non-reinforced retrieval, the H_2 antagonist ranitidine (50 nmol/side), but not the H_1 antagonist pyrilamine (50 nmol/side), the H_3 antagonist thioperamide (50 nmol/side) or the NMDAR polyamine-binding site antagonist ifenprodil (50 nmol/side; Sotres-Bayon *et al.* 2009) blocked extinction (Fig. 3*a*; $F_{4,95}=3.23$, $p<0.05$; $q=2.83$ for ranitidine). Co-infusion of ranitidine, but not pyrilamine, thioperamide or ifenprodil, abolished the facilitation induced by histamine (Fig. 3*b*; $F_{4,95}=19.45$, $p<0.001$; $q=7.37$ for ranitidine). Ranitidine, dimaprit and imetit did not affect IA memory when administered to dorsal CA1 24 h post-training in the absence of a non-reinforced retention test session (Fig. 3*c*). None of these compounds, nor histamine or SKF91488, modified the animals' performance in the open field or in the elevated plus-maze when administered to dorsal CA1 24 h prior to the respective behavioural sessions (Table 1).

Histamine regulates ERK signalling (Passani *et al.* 2007). Thus, we investigated the modulation of ERK phosphorylation by histamine during the consolidation of extinction. Intra-CA1 infusion of the MEK1/2 inhibitor U0126 (2 pmol/side) immediately after non-reinforced retrieval blocked extinction (Fig. 4*a*; $t_{38}=3.38$, $p<0.01$), which was accompanied by an increase in the phosphorylation state of ERK1, peaking 120 min after retrieval (Fig. 4*b*; $F_{3,20}=8.24$, $p<0.001$; $q=4.25$ for 120 min). This increase was enhanced further by histamine and dimaprit and reduced by ranitidine administered to dorsal CA1 immediately after non-reinforced retrieval (Fig. 4*c*; $F_{3,20}=22.64$, $p<0.001$; $q=2.59$ for histamine, $q=4.83$ for dimaprit, $q=2.97$ for ranitidine). Ranitidine did not affect basal ERK1/2 phosphorylation (Fig. 4*d*).

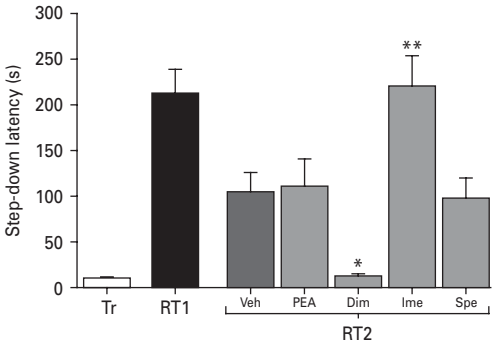

Fig. 2. Dimaprit mimics the facilitatory effect of histamine. Rats with infusion cannulas implanted in the CA1 region were trained (Tr) in inhibitory avoidance and 24 h later submitted to a non-reinforced test session (RT1). Immediately thereafter, animals received infusions of vehicle (Veh), 2-2-pyridylethylamine (PEA), dimaprit (Dim), imetit (Ime) or spermidine (Spe). Retention was evaluated 24 h after RT1 (RT2). Bars represent mean (± S.E.M.); $n=20$ per group; * $p<0.05$ and ** $p<0.01$ in Dunnett's test after ANOVA.

Discussion

The behavioural outcome of histamine administration is not easy to predict because different histamine receptor subtypes induce opposite biochemical effects. Indeed, reports regarding the role of histamine on learning are controversial. While earlier work indicates that histamine improves memory, recent findings suggest that it hinders or has no effect on retention (Alvarez *et al.* 2001; De Almeida & Izquierdo, 1986; Eidi *et al.* 2003; Klapdor *et al.* 1994). Thus, to avoid the drawbacks associated with the use of multiple-trial learning paradigms and systemic administration of drugs, which make it difficult to interpret experiments regarding the role of histamine in memory formation, we evaluated its involvement in the consolidation of extinction memory by using a single-trial extinction protocol and stereotaxically

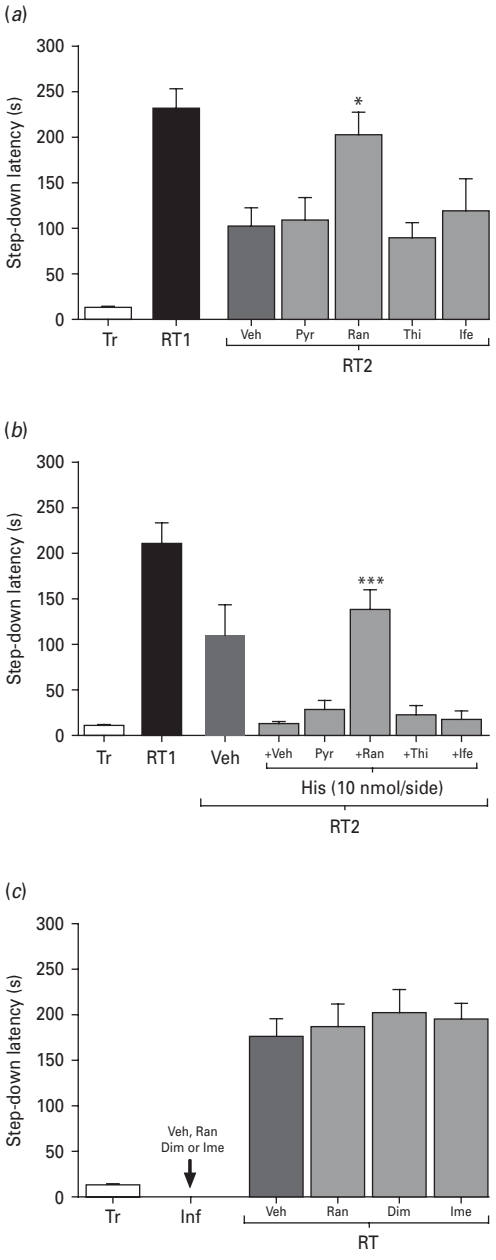

Fig. 3. The facilitatory effect of histamine is mediated by H_2 receptors. (*a*) Rats with infusion cannulas implanted in the CA1 region were trained (Tr) in inhibitory avoidance (IA) and 24 h later submitted to a non-reinforced test session (RT1). Immediately thereafter, animals received infusions of vehicle (Veh), pyrilamine (Pyr), ranitidine (Ran), thioperamide (Thi) or ifenprodil (Ife). Retention was evaluated 24 h after RT1 (RT2). (*b*) Animals were treated as in (*a*) but received Veh or Veh, Pyr, Ran, Thi or Ife plus histamine (His) (10 nmol/side). (*c*) IA-trained rats received intra-CA1 Veh, Ran, dimaprit (Dim) or imetit (Ime) 24 h post-training (Inf). Retention was evaluated 24 h thereafter. Bars represent mean (±S.E.M.); $n=20$ per group; * $p<0.05$ and *** $p<0.001$ in Dunnett's test after ANOVA.

localized microinfusions. We studied the histaminergic system in the hippocampus because this brain region is essential for fear extinction (Quirk & Mueller, 2008).

We found that when administered to dorsal CA1 after non-reinforced retrieval histamine facilitated extinction in a dose-dependent manner, an effect mimicked by increasing endogenous histamine levels through inhibition of histamine *N*-methyltransferase and by administration of the H_2 receptor agonist dimaprit. This facilitation was long-lasting, blocked by the H_2 receptor antagonist ranitidine, was contingent with the non-reinforced expression of IA memory and only occurred when histamine was infused immediately and not 90 min after the extinction session. This suggests that it was due to a time- and H_2-receptor-dependent enhancement of extinction memory consolidation and not to a delayed action on retrieval or on locomotor and exploratory activities. This claim is supported by findings showing that histamine did not affect the animals' performance in the elevated plus-maze and in the open-field tasks when administered 24 h prior to the respective behavioural sessions. Moreover, the fact that histamine enhanced the extinction-induced phosphorylation of ERK1 through a mechanism involving H_2 receptors, suggests that activation of the ERK cascade during the consolidation of extinction is modulated by this imidazolamine.

Our data concur with reports showing the requirement of hippocampal H_2 receptors for consolidation of IA long-term memory (Da Silva *et al.* 2006) and are in partial agreement with others indicating that intra-CA3 infusions of H_2 and H_3 agonists improve contextual fear memory (Passani *et al.* 2007), supporting the hypothesis that consolidation of fear memory and of fear extinction share some basic biochemical mechanisms within the hippocampus. In particular, ERK activity is essential for both processes (Izquierdo *et al.* 2006; Maren & Quirk, 2004). It was postulated that ERK1/2 coordinate responses to extracellular signals during memory consolidation (Sweatt, 2001) and, based on findings showing that ERK signalling is triggered by memory retrieval in CA1 neurons only when anticipation of a fearful experience is not fulfilled, it was proposed that these kinases are essential for coupling error prediction to fear attenuation during extinction (Huh *et al.* 2009). Although the absence of a behavioural phenotype in ERK1 null mice suggested that ERK2 is the ERK isoform responsible for memory formation (Selcher *et al.* 2001), a recent report regarding extinction in ERK1 mutant mice indicates that pharmacological modulation of this kinase in the

Table 1. Histamine, SKF91488, dimaprit, ranitidine and imetit do not affect locomotor and exploratory activities or anxiety state. Histamine, SKF91488, dimaprit, ranitidine or imetit were infused into dorsal CA1 24 h before submitting rats to open-field or plus-maze sessions. Data are expressed as mean (±S.E.M.); $n=10$ per group

	Vehicle	Histamine (10 nmol/side)	SKF91488 (50 nmol/side)	Dimaprit (10 nmol/side)	Ranitidine (50 nmol/side)	Imetit (10 nmol/side
Rearings	18.05±1.37	14.50±2.92	18.40±2.11	17.63±2.81	14.13±2.86	14.25±3.02
Crossings	62.44±4.32	64.20±12.93	68.70±8.51	61.98±9.11	62.38±7.94	63.63±7.57
Time in open arms (s)	115.4±9.8	114.9±11.5	116.2±18.9	123.8±15.2	127.1±26.8	119.8±17.8
Entries in open arms	6.29±0.47	5.94±1.09	7.00±0.73	7.23±1.25	7.11±1.45	7.80±1.00
Total entries	12.63±0.69	11.78±0.91	14.88±1.53	13.81±2.43	13.67±2.22	14.80±1.26

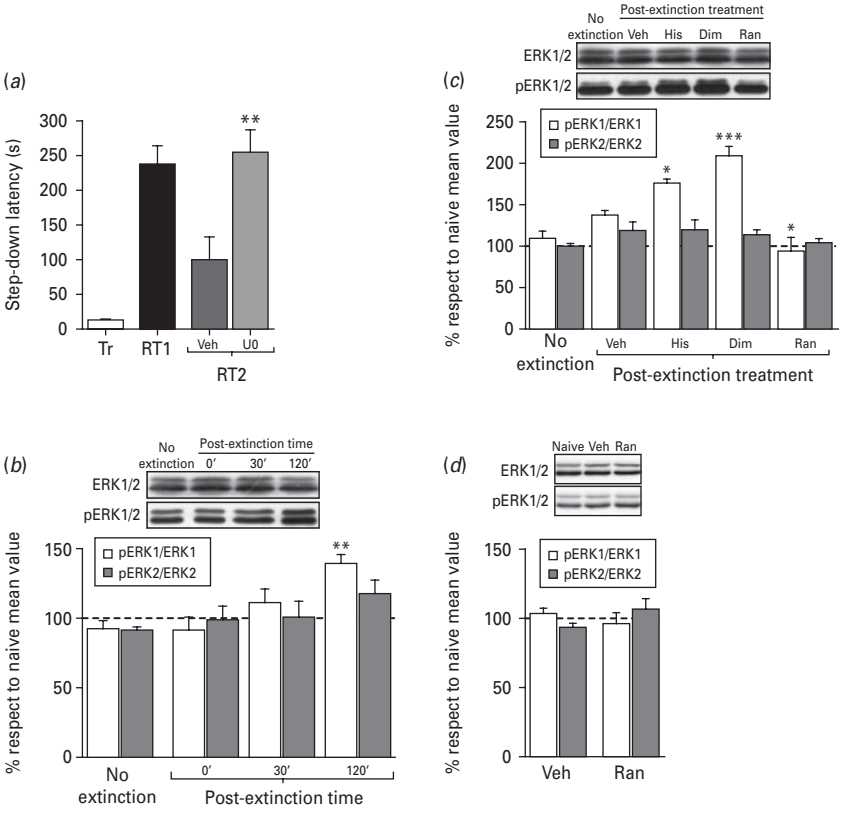

Fig. 4. Extinction increases ERK1 phosphorylation in a H_2 receptor-dependent manner. (*a*) Rats with infusion cannulas implanted in the CA1 region were trained (Tr) in inhibitory avoidance (IA) and 24 h later submitted to a non-reinforced test session (RT1). Immediately thereafter, animals received infusions of vehicle (Veh) or U0126 (U0). Retention was evaluated 24 h after RT1 (RT2). Bars represent mean (±S.E.M.); $n=20$ per group; ** $p<0.01$ in Student's t test. (*b*) IA-trained animals were tested as in (*a*) but immediately, 30 min or 120 min thereafter were killed by decapitation, and total homogenates from dorsal CA1 immunoblotted with antibodies against ERK1/2 or ERK1/2 when phosphorylated at Thr^{202}/Tyr^{204} (pERK1/2). IA-trained rats not submitted to the extinction protocol (no extinction) were used as controls. Data are expressed as mean (±S.E.M.). ** $p<0.01$ in Dunnett's test after ANOVA; $n=6$ per group. (*c*) Animals were treated as in (*b*) but immediately after the extinction session received intra-CA1 Veh, histamine (His; 10 nmol/side), dimaprit (Dim; 10 nmol/side) or ranitidine (Ran; 50 nmol/side) and were killed 120 min later. Data are expressed as mean (±S.E.M.). * $p<0.05$ and *** $p<0.001$ in Dunnett's test after ANOVA; $n=6$ per group. (*d*) Rats with infusion cannulas implanted in the CA1 region received infusions of vehicle (Veh) or ranitidine (Ran; 50 nmol/side) and 120 min thereafter were killed by decapitation. Total homogenates from dorsal CA1 were immunoblotted as in (*b*) and (*c*). Data are expressed as mean (±S.E.M.); $n=4$ per group.

hippocampus is a therapeutic option for the treatment of anxiety and depression (Tronson *et al.* 2008). Indeed, although typically considered equivalent, ERK1 and ERK2 have different signalling properties (Frémin *et al.* 2009; Krens *et al.* 2008; Lefloch *et al.* 2008). Thus, ERK1 controls ERK2 by acting as a partial agonist for MEK binding, and recent work suggests that while the catalytic action of ERK1 is predominantly cytoplasmatic, ERK2 phosphorylates both cytoplasmatic and nuclear targets (Marchi *et al.* 2008; Vantaggiato *et al.* 2006). Future research about the extinction-induced phosphorylation of proteins will help to resolve this question.

We previously reported that the full NMDAR antagonist AP5 blocks the consolidation of extinction memory (Cammarota *et al.* 2005). However, we did not find any effect of the NR2B-containing NMDAR antagonist ifenprodil on this process. This discrepancy could be explained by the different properties conferred on the NMDAR by NR2A and NR2B subunits. For example, NR2A- but not NR2B-containing receptors are necessary for long-term depression and potentiation (Morishita *et al.* 2007; Zhang *et al.* 2009), and NR2A but not NR2B is essential for NMDAR-dependent dendritic protein synthesis (Tran *et al.* 2007).

Anti-histamines are frequently prescribed to treat asthma and rhinitis and, although they cause undesirable behavioural side-effects, their consumption is rapidly growing (Banthin & Miller, 2006; Bender, 2005; Halpert *et al.* 2002; Lieberman, 2009; Van Ruitenbeek *et al.* 2008). It is known that histamine acts as a danger response signal modulating fear and that lesion of the tuberomammilary nucleus reduces anxiety (Frisch *et al.* 1998). However, the potential use of histaminergic agents to treat the behavioural and cognitive disorders at the root of anxiety and post-traumatic stress disorder has barely been studied. In this context, our finding that histamine facilitates the consolidation of fear extinction through a mechanism involving the H_2 receptor-dependent phosphorylation of ERK1 in the hippocampus is relevant.

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Statement of Interest

None.

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