

The hypocretin/orexin system mediates the extinction of fear memories

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Abstract

Anxiety disorders are often associated with an inability to extinguish learned fear responses. The hypocretin/orexin system is involved in the regulation of emotional states and could also participate in the consolidation and extinction of aversive memories. Using hypocretin receptor-1 and hypocretin receptor-2 antagonists, hypocretin-1 and hypocretin-2 peptides, and hypocretin receptor-1 knockout mice, we investigated the role of the hypocretin system in cue- and context-dependent fear conditioning and extinction. Hypocretins were crucial for the consolidation of fear conditioning, and this effect was mainly observed in memories with a high emotional component. Notably, after the acquisition of fear memory, hypocretin receptor-1 blockade facilitated fear extinction while hypocretin-1 administration impaired this extinction process. The extinction-facilitating effects of the hypocretin receptor-1 antagonist SB334867 were associated with increased expression of cFos in the basolateral amygdala and the infralimbic cortex. Intra-amygdala, but neither intra-infralimbic prefrontal cortex nor intra-dorsohippocampal infusion of SB334867 enhanced fear extinction. These results reveal a key role for hypocretins in the extinction of aversive memories and suggest that hypocretin receptor-1 blockade could represent a novel therapeutic target for the treatment of diseases associated with inappropriate retention of fear such as posttraumatic stress disorder and phobias.

Keywords: fear, memory, extinction, hypocretin, mice, amygdala

Introduction

The formation of aversive memories after stressful situations is an adaptive phenomenon necessary for individuals to survive. However, deregulation of acquired fear, revealed by enhanced conditioning or resistance to fear extinction, is associated with anxiety disorders, such as posttraumatic stress disorder (PTSD) and phobias (Holmes and Singewald, 2013). The knowledge of the neural substrate underlying conditioning and extinction of fear is particularly relevant for the identification of novel potential treatments for these pathologies (Tronson *et al*, 2012). Human and animals develop robust fear of environmental contexts or cues paired with aversive events. In rodent models, animals learn to associate an aversive unconditioned stimulus (US) such as a brief footshock with a neutral conditioned stimulus (CS), typically a chamber or a tone. In these models, extinction learning is usually slower than fear learning and usually requires multiple nonreinforced expositions to the fear associated cues or context (Myers and Davis, 2007).

Hypocretin-1/orexin-A and hypocretin-2/orexin-B are lateral hypothalamic neuropeptides that project throughout the brain (de Lecea *et al*, 1998; Sakurai *et al*, 1998) and bind two different G-protein coupled receptors (Hcrtr-1 and Hcrtr-2) (de Lecea *et al*, 1998; Sakurai *et al*, 1998). Hypocretin-1 binds to both receptors with similar affinity, whereas hypocretin-2 binds to Hcrtr-2 with 10-fold greater affinity than to Hcrtr-1. The hypocretin system modulates diverse physiological functions including sleep/arousal, energy homeostasis and reward processing (Li *et al*, 2014). A role for hypocretins in emotional regulation has been recently proposed, consistent with the existence of hypocretin neuronal projections to several limbic areas (Johnson *et al*, 2012). In agreement, clinical research showed a reduced amygdala activity during aversive conditioning in human narcolepsy (Ponz *et al*, 2010), a condition associated

with a loss of hypocretin neurons (Peyron *et al*, 2000). Moreover, human subjects with panic anxiety have elevated hypocretin levels in the cerebrospinal fluid (Johnson *et al*, 2010), and activation of hypocretin-synthesizing neurons is necessary for developing a panic-prone state in rodent models (Johnson *et al*, 2010). In agreement, the dual hypocretin receptor antagonist almorexant reduced fear-conditioned startle reactions in the rat (Steiner *et al*, 2012).

The aim of this study was to investigate the involvement of the hypocretin system in the consolidation and extinction of aversive memories. We also evaluated the brain regions mediating hypocretin effects in fear extinction by using cFos expression. Finally, we infused the Hcrtr-1 antagonist SB334867 into the amygdala, infralimbic prefrontal cortex (IL) and the dorsal hippocampus to confirm the involvement of specific brain areas in this extinction process.

Methods and Materials

Animals

Experiments were performed in male C57BL/6J mice (Charles River, France), 129S1/SvImJ mice (Jackson Laboratories, USA) and Hcrtr-1 knockout (KO) mice and their wild-type (WT) littermates (Jackson Laboratories, USA) (8-12 week-old). KO mice were backcrossed for at least 7 generations to C57BL/6J mice, and genotyped as described previously (Flores *et al.*, 2014). Mice were housed four per cage in a temperature- ($21^{\circ}\text{C} \pm 1^{\circ}\text{C}$) and humidity-controlled ($55\% \pm 10\%$) room with a 12h light/dark cycle (light between 08:00 and 20:00). Food and water were available *ad libitum*. All animal procedures were conducted in accordance with the guidelines of the European Communities Directive 86/609/EEC regulating animal research and approved by the local ethical committee (CEAA-PRBB) and the statement of compliance with standards for use of laboratory animals by foreign institutions (#A5388-01 approved by the National Institutes of Health). All experiments were performed under blind conditions.

Drugs

The Hcrtr-1 antagonist SB334867 (3, 5 and 10mg/kg) (Tocris) was dissolved in 1% (wt/vol) 2-hydroxypropyl- β -cyclodextrin (Sigma) and 10% dimethyl sulfoxide (vol/vol) in distilled water. The Hcrtr-2 antagonist TCSOX229 (5, 10 and 15mg/kg) (Tocris) was dissolved in physiological saline. Both hypocretin antagonists were administered by intraperitoneal route (ip) in a volume of 5ml/kg body weight. The SB334867 and TCSOX229 doses were based on previous studies and did not modify spontaneous locomotor activity (Plaza-Zabala *et al.*, 2010 and 2012). Hypocretin-1 and hypocretin-2 (Sigma) were dissolved in physiological saline and a volume of 1 μ l was administered

by intracerebroventricular (icv) route at 0.75nmol/ μ l and 2.5nmol/ μ l, respectively. These doses were based on previous studies (Plaza-Zabala *et al*, 2012; Boutrel *et al*, 2005). For intra-cerebral microinjections, SB334867 (10nmol/side) was dissolved in a 25:75 sterile saline solution:dimethyl sulfoxide vehicle. This dose was based on preceding reports (Hollander *et al*, 2008; Narita *et al*, 2006). Ketamine hydrochloride (100mg/kg) (Imalgène® 1000, Merial) and xylazine hydrochloride (20mg/kg) (Ronpum®, Sigma) were mixed and dissolved in ethanol (5%) and distilled water (95%). This anesthetic mixture was administered ip in a volume of 10ml/kg body weight.

Behavioral experiments

Contextual fear conditioning and extinction. Mice were fear conditioned in a shuttle chamber (LE918, Panlab, Barcelona) surrounded by a larger sound-attenuating cabinet which had a ventilation fan to provide background noise. A speaker attached to the wall of the chamber delivered the tone stimuli. The shuttle chamber floor was constructed of parallel stainless-steel bars and connected to a scrambled shock generator (LE10026, Panlab, Barcelona). Before each trial, the chamber was cleaned with 70% ethanol and then with water to remove olfactory cues. On the training day, mice were placed in the conditioning chamber for 150sec before the exposure to an US (0.6mA footshock for 2sec) and then remained in the chamber for 30sec. Different experimental designs were used to evaluate the consolidation and the extinction of contextual fear memories. To study the consolidation process, pharmacological treatments were administered immediately after the training session. Testing was performed 24h after training by returning mice to the conditioning chamber and measuring the freezing behavior for 3min. Freezing, a rodent natural response to fear (LeDoux, 1993), was defined as the

absence of all movement except for that necessary for breathing and was scored manually. To evaluate the extinction of contextual fear memories, no pharmacological treatments were administered after the training session to preserve fear memory formation. Extinction trials (E1-E5) were performed at 24h, 48h, 72h, 96h and 120h after the training by scoring freezing behavior for 3min in the conditioning chamber. Pharmacological treatments were administered immediately after each extinction session.

Cued fear conditioning and extinction. A different group of mice underwent a cued fear conditioning (FC). On the training day, each mouse was placed in the conditioning chamber previously described for 120sec before the onset of three cue tones (3kHz, 90dB, 8sec long, 2sec between tones) each one co-terminating with the delivery of one footshock (0.6mA, 2sec), and then remained in the chamber for 30sec. This cue tone exposure did not induce freezing behavior by itself (Figure S1). To study the consolidation process, pharmacological treatments were administered immediately after the training trial and testing was performed 24h after training by placing mice in a different context (a transparent Plexiglas cylinder) for 6min. The first 3min mice were habituated to the new context and during the following 3min animals were re-exposed to the CS (18 cue tones, 8sec long, 2sec between tones). Freezing behavior was scored during the first 2min of cue re-exposure. To evaluate the extinction of cued fear memories, mice were placed in the transparent cylinder at 24h, 48h, and 72h after the footshock (extinction sessions E1-E4), and freezing behavior was evaluated as previously described. Pharmacological treatments were administered immediately after each extinction trial.

Novel object-recognition test. See Supplementary Materials and Methods.

Locomotor activity, Hot-plate test, and Light-dark box. Locomotion, nociception and anxiety-like behavior were evaluated in Hcrtr-1 KO mice. See Supplementary Materials and Methods.

Elevated plus-maze and Open-field test. Locomotion and anxiety-like behavior were evaluated in mice 24h after ip injections of SB334867 (3 and 5mg/kg), TCSOX229 (10 and 15mg/kg), hypocretin-1 (0.75nmol/ μ , icv) or respective vehicles. The following experimental sequence was performed: locomotor activity boxes 24h after injection, elevated plus-maze (15min later), and open-field test (30min later). See Supplementary Materials and Methods.

Stereotaxic surgery and infusion procedure

Mice were implanted with unilateral cannulae in the lateral ventricle for icv administration of hypocretin-1 and hypocretin-2 to evaluate the role of these peptides in fear extinction. In a different group of mice, bilateral cannulae were implanted in the amygdala, the IL or the hippocampus and SB334867 microinjections were performed in these specific brain regions during contextual fear extinction. See Supplementary Materials and Methods.

Immunofluorescence studies

See Supplementary Materials and Methods.

Statistical analysis

One-way analysis of variance (ANOVA) was used to analyze consolidation of fear memory experiments and the activation of hypocretin neurons during fear extinction. Two-way ANOVA of repeated measures was used to analyze fear extinction behavioral

data and cFos expression during fear extinction under SB334867 treatment. Subsequent *post-hoc* comparison (Fisher's LSD) was employed when required. All data were analyzed using Statistica (StatSoft) software. The level of significance was $p < 0.05$ in all experiments.

Results

Hcrtr-1 and Hcrtr-2 contribute differently in the consolidation of fear memories

To evaluate the possible role of the hypocretinergic system in the consolidation of fear memories, we pharmacologically blocked Hcrtr-1 and Hcrtr-2 activity in contextual and cued FC paradigms. Mice were treated with selective Hcrtr-1 and Hcrtr-2 antagonists immediately after the FC session, and 24h later animals were tested for conditioned freezing behavior, a general reaction to perceived threat (Fanselow, 1980) (Figure 1a). Systemic administration of the selective Hcrtr-1 antagonist SB334867 (3, 5, and 10mg/kg, ip) significantly decreased the evoked freezing behavior when compared with the vehicle-treated group in both contextual ($F_{3,32}=21.19$, $p<0.001$) and cued ($F_{3,22}=10.04$, $p<0.001$) fear tests, at all doses used ($p<0.01$) as revealed by *post-hoc* comparison (Figure 1b,c). Hcrtr-1 KO mice also displayed significant reduced levels of freezing during contextual ($p<0.05$) and cued ($p<0.01$) fear tests, confirming a role for Hcrtr-1 in fear memory formation (Figure 1d,e). WT and Hcrtr-1 KO mice did not show differences in pain perception, anxiety-related behavior, and locomotor activity (Figure S2). In contrast, systemic injection of the selective Hcrtr-2 antagonist TCSOX229 (5, 10 and 15mg/kg, ip) shortened the freezing time during contextual fear test ($F_{3,45}=4.62$, $p<0.001$) at the doses of 10 and 15mg/kg ($p<0.01$) (Figure 1f), but did not interfere with cued fear consolidation ($F_{3,19}=1.07$, NS) (Figure 1g). These results suggest that Hcrtr-2 is selectively involved in contextual fear memory consolidation, in agreement with recent findings supporting a different role for Hcrtr-1 and Hcrtr-2 in the formation of contextual and cued fear memories (Soya *et al*, 2013). Interestingly, SB334567 and TCSOX229 administration 4h after the FC trial (Figure 1h) did not affect context- or cued-conditioned freezing response (Figure 1i,j,k), indicating that Hcrtr-1 and Hcrtr-2

signaling is recruited immediately after contextual and cued FC during the consolidation of fear memories.

To further clarify the specific involvement of Hcrtr-1 and Hcrtr-2 in the consolidation of emotional memories we used the novel object-recognition paradigm (Figure 1l), which reflects declarative memory with a low emotional component. In contrast to FC, systemic administration of SB334867 immediately after training did not modify memory consolidation ($F_{3,40}=1.94$, NS) (Figure 1m), although a tendency to decrease the discrimination index was observed at the higher dose (10mg/kg). In agreement, Hcrtr-1 KO mice exhibited normal memory performance in the novel-object recognition test (Figure 1n). TCSOX229 did not modify the discrimination index in this behavioral paradigm (Figure 1o). Total object exploration time was not altered in any of the different experiments performed (Figure S3). These results suggest that hypocretins could be mainly involved in the consolidation of highly emotional memories.

Hcrtr-1 blockade facilitates the extinction of fear memories

To assess the involvement of hypocretins in contextual and cued fear extinction, mice underwent a single FC training trial and did not receive treatment to preserve fear memory formation. Freezing response was scored 24h later in a first extinction session (E1). Animals were assigned to a treatment group (balanced for the freezing levels) and treated with hypocretin receptor agonists or antagonists immediately after the first extinction session and daily after each ensuing extinction trial (Figure 2a). Daily administration of SB334867 (3 and 5mg/kg, ip) significantly accelerated the context extinction process (treatment effect: $F_{2,37}=4.61$, $p<0.05$; interaction treatment x day: $F_{8,148}=3.03$, $p<0.01$) (Figure 2b). *Post-hoc* analyses showed a significant effect of SB334867 at the doses of 3mg/kg ($p<0.05$ at E2, $p<0.01$ at E3-E5) and 5mg/kg ($p<0.05$

at E2-E5). Consistently, the icv administration of hypocretin-1 (0.75nmol/ μ l) after each extinction session impaired the contextual extinction process (treatment effect: $F_{1,12}=24.12$, $p<0.001$; interaction treatment x day: $F_{4,48}=27.73$, $p<0.001$) (Figure 2d). *Post-hoc* comparisons revealed significant differences between vehicle- and hypocretin-1-treated groups from E3 to E5 ($p<0.05$ at E3, $p<0.01$ at E4-E5). In contrast, the administration of TCSOX229 (Figure 2c) (10 and 15mg/kg, ip) and hypocretin-2 (2.5nmol/ μ l) (Figure 2e) after each extinction trial did not modify contextual memory extinction (interaction treatment x day: $F_{8,80}=0.51$, NS, and $F_{4,36}=0.32$, NS, respectively). Similar results were obtained in the cued fear extinction paradigm. Thus, SB334867 (3 and 5mg/kg, ip) administration after each extinction trial enhanced cued fear extinction (treatment effect: $F_{2,20}=4.13$, $p<0.05$; interaction treatment x day: $F_{6,60}=3.84$, $p<0.01$) (Figure 2f). Subsequent *post-hoc* analysis revealed significant differences between vehicle- and SB334867-treated animals only at E2 (3 and 5mg/kg: $p<0.01$), probably due to the fast extinction exhibited by the vehicle group in these experimental conditions. In agreement, daily administration of hypocretin-1 (0.75nmol/ μ l, icv) delayed extinction of freezing behavior (treatment effect: $F_{1,13}=22.01$, $p<0.001$; interaction treatment x day: $F_{3,39}=5.99$, $p<0.01$) (Figure 2h), and significant differences were revealed from E2 to E4 ($p<0.05$ at E2; $p<0.01$ at E3-E4). On the contrary, administration of either TCSOX229 (10 and 15mg/kg, ip) or hypocretin-2 (2.5nmol/ μ l, icv) did not modify cued fear extinction (interaction treatment x day: $F_{6,63}=0.50$, NS and $F_{3,36}=0.78$, NS, respectively) (Figure 2g,i). Locomotor activity and anxiety-like responses were not modified in mice 24h after the acute administration of SB334867 (3 and 5mg/kg, ip), TCSOX229 (5 and 10mg/kg, ip), and hypocretin-1 (0.75nmol/ μ l, icv) (Figure S4), suggesting that the changes observed

in freezing behavior by hypocretin compounds were not due to unspecific effects on locomotion and anxiety.

To further study the role of Hcrtr-1 in fear extinction, we tested the extinction-facilitating effects of SB334867 in the 129S1/SvImJ inbred mouse strain, a well-established mouse model of impaired extinction (Hefner *et al*, 2008). Administration of SB334867 (5mg/kg, ip) after each extinction trial rescued contextual (treatment effect: $F_{1,11}=46.98$, $p<0.001$; interaction treatment x day: $F_{4,44}=8.04$, $p<0.01$) (Figure 2j), as well as cued fear extinction in these mice (treatment effect: $F_{1,22}=9.97$, $p<0.01$; interaction treatment x day: $F_{3,66}=7.09$, $p<0.001$) (Figure 2k). *Post-hoc* comparisons revealed significant differences between vehicle and SB334867 groups from E2 to E5 in contextual extinction ($p<0.01$) and from E3 to E4 in cued extinction ($p<0.05$), confirming the extinction-enhancing effects of Hcrtr-1 blockade.

We next studied the possible activation of hypocretin neurons in the lateral hypothalamus (LH) and the dorsomedial hypothalamus and perifornical area (DMH/PFA) during the contextual fear extinction process by using double-label immunofluorescence of cFos with hypocretin-1. C57BL/6J mice were exposed to fear extinction training, and brains removed and perfused at different time points of the extinction process: naïve mice (animals exposed to the chamber, but not to shock), 2h after the extinction session 2 (E2) and 2h after the last extinction trial (E5) (Figure 2l). The percentage of hypocretin neurons expressing cFos was increased during the extinction process in the LH ($F_{2,15}=9.38$, $p<0.01$) (Figure 2m) and the DMH/PFA ($F_{2,15}=7.90$, $p<0.01$) (Figure 2n,o). *Post-hoc* analysis showed significant differences in comparison with basal conditions at E2 ($p<0.05$) and E5 ($p<0.01$) time points, suggesting that hypocretin neurons are recruited during fear extinction to counteract this process.

The extinction-facilitating effects of Hcrtr-1 blockade are associated with an increased activity of the infralimbic prefrontal cortex and the basolateral amygdala

To identify the brain areas responsible for the extinction-enhancing effect of Hcrtr-1 blockade we analyzed the possible activation of the main brain regions involved in the extinction circuit of fear memories by using cFos immunofluorescence. Thus, cFos expression in prelimbic prefrontal cortex (PL), IL, dorsal hippocampus and basolateral amygdala (BLA) was evaluated at different time points of the contextual extinction process (no shock, E2 and E5) in mice treated with vehicle or SB334867 (5mg/kg, ip) immediately after each extinction trial (Figure 3a). PL showed a significant activation during contextual extinction at both E2 and E5 time points that was not modified by SB334867 treatment (day effect: $F_{2,36}=38.63$, $p<0.001$; interaction day x treatment: $F_{2,36}=2.18$, NS) (Figure 3b). Similarly, dorsal hippocampus exhibited a significant increase in cFos expression during E2 and E5, which remained unaffected by SB334867 treatment (day effect: $F_{2,36}=5.09$, $p<0.01$; interaction day x treatment: $F_{2,36}=0.04$, NS) (Figure 3d). An increase in cFos expression was also observed in IL at E2 and E5 compared to the naïve group (day effect: $F_{2,36}=22.65$, $p<0.001$; interaction day x treatment: $F_{2,36}=3.83$, $p<0.05$). Notably, SB334867 treatment induced a higher activation of this brain region at E2 ($p<0.01$) in comparison with vehicle-treated animals (Figure 3c,f). A similar effect was also observed in the BLA. This nucleus was significantly activated during extinction compared with control mice (day effect: $F_{2,36}=19.13$, $p<0.001$; interaction day x treatment: $F_{2,36}=4.01$, $p<0.05$) (Figure 3e,g), and SB334867 treatment enhanced cFos expression after the E2 extinction trial ($p<0.01$). SB334867 did not modify cFos expression by itself in control no shock animals (Figure 3b,c,d,e). The blockade of Hcrtr-1 could have a more prominent effect at the beginning

of the extinction process in agreement with the increase of cFos in IL and BLA on E2. Thus, a single injection of SB334867 (5mg/kg, ip, after the first extinction session) (Figure S5) accelerated fear extinction on E2 and E3, although no significant differences were observed following E4 and E5. As a whole, these results show that the extinction-facilitating effects of Hcrtr-1 blockade are associated with an increased activity of the IL and the BLA, which are key regions involved in the extinction of aversive memories.

Intra-amygdala infusion of SB334867 facilitates fear extinction

We next evaluated the possible direct participation of Hcrtr-1 in specific brain regions mediating the extinction of aversive memories. For this purpose, mice were bilaterally implanted with cannulae into the BLA, the IL or the dorsal hippocampus and received intra-structure SB334867 microinjections (10nmol/0.5 μ l/side) after each contextual extinction session. Blockade of Hcrtr-1 into the amygdala significantly accelerated context-induced fear extinction (treatment effect: $F_{1,28}=5.94$, $p<0.05$; interaction day x treatment: $F_{4,112}=2.46$, $p<0.05$) (Figure 4a). In contrast, SB334867 injections into the IL and the dorsal hippocampus had no effect in extinction of aversive memories (interaction day x treatment: $F_{4,88}=0.94$, NS and $F_{4,80}=0.89$, NS, respectively) (Figures 4d,g). Figures 4b,e,h show the location of the injection sites, and Figures 4c,f,i are representative images of bilateral cannulae positions. These results suggest that the acceleration of fear extinction induced by SB334867 is partially mediated by Hcrtr-1 into the amygdala, while is independent of the activation of Hcrtr-1 in both the IL and the dorsal hippocampus.

Discussion

Our data show that hypocretins play a crucial role in the consolidation of fear memories, while these neuropeptides are not involved in memories with a low emotional component. Notably, after the acquisition of fear, the Hcrtr-1 antagonist SB334867 facilitates extinction of aversive memories and increases the activity of relevant brain areas related to the extinction process, such as the BLA and the IL. Direct infusion of SB334867 into the amygdala also enhances fear extinction.

Pharmacological blockade with low doses of SB334867 immediately after the footshock, or genetic deletion of Hcrtr-1, impaired freezing responses in both cued and contextual FC paradigms. Consistent with previous reports, Hcrtr-1 KO mice showed similar pain perception, locomotor activity and anxiety-like behavior than WT animals (Soya *et al*, 2013; Scott *et al*, 2011), suggesting that the differences between the two genotypes in fear memory formation are not due to alterations in sensory-motor abilities of the KO mice. In contrast, the administration of the Hcrtr-2 antagonist TCSOX229 only impaired contextual FC. These data show a different role for Hcrtr-1 and Hcrtr-2 in the consolidation of cued and contextual fear memories, in agreement with recent data by using KO mice (Soya *et al*, 2013). The different location of these receptors could explain this divergent response. Thus, Hcrtr-2 is highly expressed in the hippocampus (Johnson *et al*, 2012), which plays a critical role in the consolidation of contextual fear memories (Maren *et al*, 2013), but its expression in amygdala is minimal which is involved in both processes (Maren *et al*, 2013; Flavell and Lee, 2012). Hcrtr-1 is located in both the amygdala and the hippocampus (Johnson *et al*, 2012). Interestingly, Hcrtr-1 and Hcrtr-2 antagonists did not modify freezing behavior when administered 4h after the FC trial demonstrating that hypocretin signaling is necessary for the early consolidation of aversive memories. In contrast, recent studies in rats showed a specific

role for Hcrtr-1 in the acquisition phase of threat learning (Sears *et al*, 2013), as revealed by the reduction of freezing levels when SB334867 was injected by icv route before the auditory FC paradigm. Notably, we did not observe any modification in the consolidation of object-recognition memory due to Hcrtr-1 deletion or pharmacological blockade in contrast to the results of FC, suggesting that hypocretins are mainly involved in the consolidation of highly emotional memories. Consistent with this, the dual hypocretin receptor antagonist DORA-22 did not produce cognitive impairment in the object-recognition test in rats at doses that affected sleep (Uslaner *et al*, 2013), although contradictory results have been reported regarding the effects of hypocretin antagonists in spatial memory using the Morris water maze (Dietrich and Jenck, 2010; Akbari *et al*, 2006 and 2007). In agreement with a role for hypocretins in threat memories, clinical research has shown abnormal emotional learning in human narcolepsy (Ponz *et al*, 2010). Moreover, the dual hypocretin antagonist almorexant reduced fear-potentiated startle responses (Steiner *et al*, 2012), and prepro-hypocretin mRNA levels increased after context-induced FC in rats (Chen *et al*, 2014). The mechanisms by which Hcrtr-1 regulates the consolidation of fear memories need to be investigated in the future. Hypocretin-containing neurons are sensitive and can also regulate the activity of corticotrophin-releasing factor (CRF) (Winsky-Sommerer *et al.*, 2004), which is involved in the consolidation of aversive memories (Isogawa *et al.*, 2013). Therefore, the CRF system could be a possible mediator of this effect.

We next evaluated the possible involvement of hypocretins in the extinction of fear memories. For this purpose, Hcrtr-1 and Hcrtr-2 antagonists, and hypocretin-1 and -2 peptides were administered following the formation of aversive memories. In both cued and contextual FC paradigms, we found that Hcrtr-1 blockade with SB334867 strongly facilitated fear extinction consolidation while hypocretin-1 infusion impaired this

response. On the contrary, Hcrtr-2 blockade and hypocretin-2 infusion were ineffective suggesting that hypocretin-1 through Hcrtr-1 activation plays a specific and key role in the extinction of aversive memories. This new physiological function of the hypocretin system was confirmed in the 129S1/SvImJ mouse strain. These mice exhibit a profound impairment of fear extinction (Hefner *et al*, 2008; Camp *et al*, 2012) and are considered a valuable model for identifying extinction facilitating drugs (Whittle *et al*, 2010; Gunduz-Cinar *et al*, 2013). We show that Hcrtr-1 blockade rescues deficient extinction of fear memories in 129S1/SvImJ mice suggesting the potential usefulness of this pharmacological target in diseases characterized by inappropriate maintenance of aversive memories. Interestingly, we observed an activation of hypocretin neurons in both the LH and the DMH/PFA during the contextual fear extinction process in C57BL/6J animals as revealed by double-label immunofluorescence of cFos with hypocretin-1. Together with the behavioral data, these biochemical results indicate that hypocretin neurons are engaged to preserve fear during the normal physiological process of fear extinction. In addition, chronic hcrtr-1 blockade could be important to maintain the facilitation of fear memory extinction given that hypocretin neurons remain activated during the whole extinction process.

IL, hippocampus and amygdala are key structures involved in the neurobiological substrate underlying fear extinction (Herry *et al*, 2008; Milad and Quirk, 2012). Thus, IL and the hippocampus activate the inhibitory circuits of the amygdala during extinction learning (Herry *et al*, 2008). Several studies have examined the patterns of the activation of these brain regions during fear extinction by quantifying cFos and Zif268 immediately-early gene (IEG) expression. These studies conclude that impaired fear extinction is associated with reduced IEG expression in the cortico-amygdala circuit (Holmes and Singewald, 2013; Herry and Mons, 2004; Herry *et al*, 2010), while

a complete extinction of conditioned fear is related to increased IEG levels in these brain areas (Herry and Mons, 2004). Several animal models characterized by resistance to extinction such as the 129S1/SvImJ mouse strain (Hefner *et al*, 2008), mice lacking dynorphin (Bilkei-Gorzo *et al*, 2012) or a poor-extinction subpopulation of C57BL/6J mice (Herry and Mons, 2004) showed IL hypoactivity, consistent with human studies showing a correlation between patients with PTSD and hypoactivation of the functionally analogous ventromedial prefrontal cortex region (Milad *et al*, 2009). Likewise, reduced activity of the BLA was found in the same animal models of impaired fear extinction (Hefner *et al*, 2008; Herry and Mons, 2004; Milad *et al*, 2009). Two different neuronal populations are activated in the BLA during fear conditioning and extinction, and a reduced cFos expression in this area could reflect a failure of the recruitment of extinction-related neurons (Herry *et al*, 2008). Notably, the contextual extinction-facilitating effects of SB334867 was associated with increased cFos expression in both the IL and the BLA, suggesting that the blockage of the hypocretin signaling increases the inhibitory control from the IL over the BLA during this process, and facilitates the activation of the subpopulation of BLA neurons involved in fear extinction. Consistent with this hypothesis, dietary zinc restriction rescued deficient extinction learning and normalized the cortico-amygdala hypoactivity observed in 129S1/SvImJ mice (Whittle *et al*, 2010). In the present study, SB334867 administration did not modify cFos expression in the PL and dorsal hippocampus. PL has an opposite role to IL and is mainly involved in the formation of aversive memories (Sierra-Mercado *et al*, 2011).

Hypocretin fibers (Peyron *et al*, 1998) and Hcrtr-1 (Hervieu *et al*, 2001; Marcus *et al*, 2001) show a moderate expression in the BLA, the IL and the hippocampus. We next evaluated a possible role for Hcrtr-1 in these brain areas in the contextual fear

extinction. SB334867 infusion in the BLA facilitated extinction of fear memory while this hypocretin antagonist did not produce any effect when administered in either the IL or the dorsal hippocampus. These results suggest a direct involvement of hypocretin activity within the amygdala. Recently, hypocretin signaling in the BLA has been shown to modulate anxious behavior (Arendt *et al*, 2014), emphasizing the significance of this nucleus in the mediation of hypocretin effects. On the other hand, the lack of effect of SB334867 infusion in the IL suggests that the increased activity of this nucleus associated with the extinction-facilitating effects of Hcrtr-1 blockade is an indirect effect. The paraventricular thalamic nucleus (PVT) could be one possible candidate to modulate this effect. Indeed, this brain area contains high density of Hcrtr-1 (Marcus *et al.*, 2001), which stimulation modulates fear and anxiety-like behaviors in rodents (Li *et al.*, 2010; Hsu *et al.*, 2014), and this nucleus has strong projections to the IL (Vertes and Hoover, 2008). Future experimental work will be necessary to elucidate the brain areas responsible for the disinhibition of the IL induced by SB334867.

In conclusion, our results reveal that hypocretins are selectively involved in the consolidation of highly emotional memories. We show for the first time a key role for the hypocretin system in the BLA in the extinction of fear memories. The possibility of pharmacologically improve extinction of this process is a major uncovered challenge for the treatment of diseases characterized by pathological fear memories such as PTSD and phobias. These results suggest that Hcrtr-1 blockage could be a promising therapeutical approach for the treatment of these diseases.

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Supplementary information is available at the *Neuropsychopharmacology* website

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Figure legends

Figure 1. Hcrtr-1 and Hcrtr-2 modulate differently context- and cue-induced fear memory consolidation. (a) Schematic representation of the experimental design for b-g behavioral tests. (b-g) Freezing levels scored during the (b,d,f) contextual and (c,e,g) cued memory test in mice treated with (b,c) SB334867 (3, 5 and 10mg/kg, ip) or vehicle (n = 6-14 mice per group) and with (f,g) TCSOX229 (5, 10 and 15mg/kg, ip) or vehicle (n = 6-13 mice per group) immediately after the FC trial, as well as in (d,e) Hcrtr-1 KO and WT mice (n = 7-9 mice per group). (h) Schematic representation of the experimental design for i-j behavioral tests. (i-k) Freezing levels scored during the (i,k) contextual or (j) cued memory test in mice (n = 6 mice per group) injected 4h after the FC session with (i,j) SB334867 (3, 5 and 10mg/kg, ip) or vehicle and with (k) TCSOX229 (5, 10 and 15mg/kg, ip) or vehicle. (l) Schematic representation of the experimental design for m-o behavioral tests. (m-o) Discrimination index obtained in the novel object-recognition paradigm by mice treated with (m) SB334867 (3, 5 and 10mg/kg, ip) or vehicle (n = 8-14 mice per group) and with (o) TCSOX229 (5, 10 and 15mg/kg, ip) or vehicle (n = 8-14 mice per group) immediately after the training, as well as in (n) Hcrtr-1 KO and WT mice (n = 8 mice per group). Data are expressed as mean \pm SEM. ★p<0.05, ★★p<0.01 compared with control group. FC, fear conditioning; VEH, vehicle; SB, SB334867; TCS, TCSOX229; WT, wild-type mice; KO, Hcrtr-1 knockout mice.

Figure 2. Hypocretin transmission is involved in the extinction of fear memories through Hcrtr-1 signaling. (a) Schematic representation of the experimental design for b-k tests. (b-i) Time course of the freezing levels scored during (b-e) contextual and (f-i) cued extinction trials in mice treated immediately after each extinction session with (b,f)

SB334867 (3 and 5mg/kg, ip) or vehicle (n = 7-14 mice per group), (c,g) TCSOX229 (10 and 15mg/kg, ip) or vehicle (n = 7-8 mice per group), (d,h) hypocretin-1 (0.75nmol/ μ l, icv) or vehicle (n = 6-8 mice per group), and (e,i) hypocretin-2 (2.5nmol/ μ l, icv) or vehicle (n = 6-7 per group). (j,k) Time course of the freezing levels scored during (j) contextual and (k) cued extinction trials in 129S1/SvImJ mice treated with SB334867 (5mg/kg, ip) or vehicle (n = 7-13 mice per group) immediately after each extinction session. (l) Schematic representation of the experimental design for m-o biochemical experiments. (m,n) Percentage of hypocretin cells expressing cFos in the (m) LH and the (n) DMH/PFA of mice exposed to the context without receiving any foot shock and mice subjected to FC and subsequent extinction until E2 and E5 (n = 5-7 mice per group). (o) Representative images of the DMH/PFA obtained via fluorescence microscopy after direct double labeling combining rabbit polyclonal antiserum to cFos with mouse monoclonal antibody to hypocretin-1. The scale bars represent 40 μ m in general images and 10 μ m in magnified images. Data are expressed as mean \pm SEM. $\star p < 0.05$, $\star \star p < 0.01$ compared with vehicle group; $\# p < 0.05$, $\#\# p < 0.01$ compared with no shock group. FC, fear conditioning; E1-E5, extinction trials 1-5; VEH, vehicle; SB, SB334867; TCS, TCSOX229; HCRT-1, hypocretin-1; HCRT-2, hypocretin-2; S1, 129S1/SvImJ mice; LH, lateral hypothalamus; DMH/PFA, dorsomedial hypothalamus and perifornical area.

Figure 3. The extinction-facilitating effects of Hcrtr-1 blockade are associated with an increased activity of the infralimbic prefrontal cortex and the basolateral amygdala. (a) Schematic representation of the experimental design for B-G biochemical experiments. (b-e) Number of neurons expressing cFos in the (b) PL, (c) IL, (d) dHPC and (e) BLA of mice exposed to the context without receiving footshock and mice subjected to FC

and subsequent extinction until E2 and E5, treated with SB334867 (5mg/kg, ip) or vehicle immediately after each extinction session (n = 5-8 mice per group). (f,g) Schematic representation of the anatomic location of (f) IL (bregma +1.54 mm) and (g) BLA (bregma -1.58 mm) pictures adapted from Paxinos and Franklin's stereotaxic atlas (Paxinos and Franklin, 2001), and representative images of both regions obtained via fluorescence microscopy after direct labeling with rabbit polyclonal antiserum to cFos. Only no shock and E2 groups are represented. Quantified areas appear delimited by a discontinuous line. Data are expressed as mean \pm SEM. ★★p<0.01 compared with vehicle group; #p<0.05, □□ ##p<0.01 compared with no-shock group. FC, fear conditioning; E1-E5, extinction trials 1-5; VEH, vehicle; SB, SB334867; TCS, TCSOX229; PL, prelimbic prefrontal cortex; IL, infralimbic prefrontal cortex; dHPC, dorsal hippocampus; BLA, basolateral amygdala.

Figure 4. Intra-amygdala infusion of SB334867 facilitates fear extinction. Mice were bilaterally cannulated in the BLA, the IL and the dHPC and after recovery were subjected to contextual fear conditioning and subsequent extinction procedure. (a,d,g) Time course of the freezing levels scored during contextual extinction trials in mice receiving bilateral SB334867 (10nmol/side) microinfusions in the (a) BLA (n = 15 mice per group), the (d) IL (n = 11-13 mice per group) or the (g) dHPC (n = 9-13 mice per group) immediately after each extinction session. (b,e,h) Location of the vehicle (○) and SB334867 (●) injection sites for each of the mice included in the (b) intra-BLA and the (h) intra-dHPC (between bregma -1.34 and -1.94 mm), and the (d) intra-IL (between bregma +1.78 and +1.42). (c,f,i) Representative coronal sections of mouse brains stained with cresyl violet showing the bilateral (c) intra-BLA, (f) intra-IL and (i) intra-dHPC cannulae placements. Data are expressed as mean \pm SEM. ★p<0.05 compared

with vehicle group. E1-E5, extinction trials 1-5; VEH, vehicle; SB, SB334867; BLA, basolateral amygdala; IL, infralimbic prefrontal cortex; dHPC, dorsal hippocampus.